Carbohydrate Polymer Technologies and Applications 5 (2023) 100307

Contents lists available at ScienceDirect

Carbohydrate Polymer Technologies and Applications

journal homepage: www.sciencedirect.com/journal/carbohydrate-polymer-technologies-and-applications

Citric acid, chitosan and oregano essential oil impact on physical and antimicrobial properties of cassava starch films

Maury S. Hernández [a,b], Leandro N. Ludueña [c], Silvia K. Flores [a,b,*]

[a] Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Intendente Guiraldes 2620, 1428 Ciudad Autónoma de Buenos Aires, Argentina
[b] Instituto de Tecnología de Alimentos y Procesos Químicos (ITAPROQ), CONICET - Universidad de Buenos Aires, Buenos Aires, Argentina
[c] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Instituto de Investigaciones en Ciencia y Tecnología de Materiales (INTEMA), Grupo de Materiales Compuestos Termoplásticos (CoMP), Facultad de Ingeniería, Universidad Nacional de Mar del Plata, Av. Colón 10850 (B7606BVZ) Mar del Plata, Argentina

ARTICLE INFO

Keywords:
Active edible films
Response surface methodology
Optimization
Physical properties
Antimicrobial action

ABSTRACT

The research deals with the development of antimicrobial edible films based on cassava starch/glycerol, added with oregano essential oil and prepared by casting. The effect of citric acid and chitosan content on the film's physical properties was studied using a response surface methodology. It was generally found that citric acid acted as a plasticizer while chitosan reinforced the film matrix, depending on the amount involved. One film formulation was selected for its high stress at break (σ_b, 15±1 MPa) and low solubility in water (SW, 12.8±0.3 %). In the second step, the influence of citric acid or oregano oil presence on the properties of the biopolymer matrix was investigated. The thermal stability, chemical interactions, water vapor permeability (WVP), microstructure and antimicrobial activity of the optimized film were compared with control systems formulated with starch/glycerol/chitosan, starch/glycerol/chitosan/citric acid or starch/glycerol/chitosan/oregano oil. The citric acid addition increased σ_b, WVP and thermal resistance while oregano oil improved the WVP but produced irregularities in the microstructure of the starch/glycerol/chitosan matrix. Moreover, the presence of oregano oil generated a very efficient antimicrobial film against external contamination by *Zygosaccharomyces bailii*. This research confirms that developed films are a relevant alternative for the production of biodegradable active packaging.

1. Introduction

The production of petroleum-based stuff has been increasing continuously reaching 390 Million tons in 2021 (Plastics Europe Report, 2022), while primary plastic waste accumulation in the environment, including soils, water ending and coasts, is expected to grow to 25 Million tons by 2025. In this context, there is growing concern about environmental contamination caused by plastic packaging that promotes a shift towards more sustainable and biodegradable materials production (Meereboer et al., 2020).

A promising alternative to reduce the pollution caused by synthetic polymers is the research and development of edible and biodegradable matrices based on renewable sources that are abundant in nature, have low production costs and can be useful for multiple proposes including hydrogels for drug delivery (Nasalapure et al., 2021), micro-nanoparticles for encapsulation of active compounds (Alzate et al., 2020) and food packaging (Atta et al., 2021). Mentioned materials include natural biopolymers such as polysaccharides (cellulose and derivatives, starch, pectin, galactomannans, carrageenans, chitosan, pullulan, alginate, etc.), proteins (soy protein, collagen, casein, whey, etc.) and their blends. Among them, starch, the major energy reservoir in plants, is one the most studied biopolymers being an auspicious alternative because of it is fully biodegradable, low cost, and widely available (García et al., 2022). However, the use of starch materials for food packaging has been restricted due to its hydrophilic characteristic, poor moisture barrier and its high water sensitivity. Several strategies have been proposed to overcome these drawbacks: hydrophobic substance addition, a mixture of starch with other hydrocolloids, composite

Abbreviations: RSM, Response Surface Methodology; RH, Relative humidity; WVP, Water Vapor Permeability; TGA, Thermogravimetric Analysis; DTGA, Derivate Thermogravimetric Analysis; FTIR, Fourier Transform Infrared Spectroscopy; ESEM, Environmental Scanning Electron Microscopy; CFU, Colony Forming Units.

* Corresponding author.
E-mail address: sflores@di.fcen.uba.ar (S.K. Flores).

https://doi.org/10.1016/j.carpta.2023.100307

Available online 2 March 2023

networks, chemical modification, etc. (Azeredo & Waldron, 2016; Li et al., 2019; Wu et al., 2019). Particularly, blends of starch with other bio-based polymers have been produced to form mixed materials with improved physical properties (Herniou et al., 2019; Shahbazi et al., 2018). In previous work, starch edible films were improved in relation to mechanical resistance when hydroxypropyl methylcellulose or xanthan gum were incorporated into film formulation elaborated by casting method (Basch, et al., 2013; Arismendi et al., 2013). However, other characteristics such as solubility in water or water vapour permeability (WVP) still required to be improved.

Another interesting combination is the mixture of starch and chitosan. Chitosan is a polysaccharide that is mainly obtained by the value addition of wastes from crustacean shells. Chitosan has an excellent film-forming capacity, selective permeability (CO_2 and O_2), good mechanical properties, as well as proven antimicrobial activity (Florez et al., 2022; Sun et al., 2017). Vásconez et al. (2009) observed an important improvement in solubility and WVP when chitosan was incorporated into starch films. On the contrary, Zheng et al. (2019) studied the physicochemical performance of casted chitosan-acorn starch films and reported that tensile strength increased but WVP decreased with the amount of added starch until a maximum was reached. Such diverse results clearly evidence the dependency of film properties on several factors such as the proportion of biopolymers, additives presence, the filmmaking process, etc. Moreover, it is known that the performance of chitosan is dependent on its molecular weight and its degree of deacetylation, while starch filmmaking effectiveness depends on botanic origin, amylose/amylopectin ratio, molecular weight, chain length distribution, etc. Therefore, it is not possible to predict the properties of starch/chitosan-based films from the previous reports. Consequently, systematic research is needed to complete the information related to this kind of film.

On the other hand, edible films offer the possibility to incorporate functional agents such as antimicrobials, antioxidants, etc. in their structure to control their release and, in turn, act as a barrier to external factors of deterioration (Bhargava et al., 2020). In this regard, active and ecological packaging systems can be created to attend to the increasing demand of consumers for healthier, fresh and innocuous foods (Chawla et al., 2021). The use of edible films and coatings containing antimicrobials is a useful tool to protect food against pathogens and spoilage flora to extend the shelf-life. The most common natural antimicrobials used are organic acids, chitosan, plant extracts and their essential oils (Campos et al., 2011; Valencia et al., 2018).

Oregano essential oil is a natural food additive, considered as GRAS (generally recognized as safe) according to FDA, has a hydrophobic nature and has important antioxidant and antimicrobial properties. Moreover, the use of oregano oil for the constitution of active edible films could promote improvements in mechanical, barrier and solubility film properties. Moreover, carvacrol, one of the main compounds of oregano oil, was proposed in previous work (Alzate et al., 2017) to develop effective antimicrobial active edible films to protect foods.

It is important to remark that focused research on the combined addition of natural antimicrobials in edible film formulation and the impact on its properties are limited. We hypothesize that the incorporation of citric acid, chitosan and oregano essential oil improves the physical properties and the antimicrobial action of cassava starch-based edible films. In addition, the magnitude of such effects depends on the levels and characteristics of the components involved. To the best of our knowledge, there are no reports that study at the same time the combined effect of chitosan, citric acid and oregano essential oil on the physicochemical and antimicrobial properties of starch edible films. Thus, it is relevant to study the unique advantages that this king of packaging material could offer. Therefore, this research aimed to develop antimicrobial edible films by studying the effect of the addition of citric acid and chitosan on the physical and mechanical properties of cassava starch/glycerol films added with oregano essential oil, using a Response Surface Methodology. Moreover, the optimization of the film formulation, requiring maximum tensile stress and minimum solubility in water, and a further analysis of the influence of citric acid or oregano oil on microstructure, physical properties and antimicrobial action were also aimed.

2. Materials and methods

2.1. Materials

Food grade cassava starch (Bernesa S.A., Argentina. Purity: 92–98 g/100 g. Amylose content, 19 g/100 g with molecular weights of 68×10^6 g/mol (amylopectin) and 0.8×10^6 g/mol (amylose) (Alzate et al., 2017), chitosan (Parafarm, Argentina. Purity: 90.8 g/100 g, deacetylation degree 95 %, Mw 334 kD), glycerol (BioPack, Argentina. Purity $\geq$ 99.5 g/100 g), citric acid (Cicarelli, Argentina. Purity: 99.5 g/100 g), oregano essential oil (Euma S.A.I.C.I.yF., Argentina. Used as was provided, without further processing). Other reagents were analytical grade and were used without further purification.

2.2. Preparation of edible films

In the first study, the films were formulated by modifying the percentages of citric acid and chitosan according to a Composite Central Design experimental scheme (Table 1). Nine formulations were tested, including two replicates of the central point (levels of citric acid and chitosan coded as 0 in Table 1). All formulations are described in Table 2. The steps followed for film production, shown in **SM1**, were those previously suggested by Alzate et al. (2017) with modifications.

Briefly, chitosan solutions were prepared in 0.5 % v/v glacial acetic acid (Anedra, Argentina), based on Composite Central Design requirements (Table 2). Cassava starch aqueous suspensions (2 g/50 g slurry) were also prepared, stirred (600 rpm) and heated (2.0 ± 0.2 °C min^{-1}) on a heating magnetic stirrer (ARE Velp Scientifica, Italy) until 85 ± 5 °C to reach starch gelatinization. Afterward, the corresponding chitosan solution was incorporated into the cassava starch slurry and stirred until a homogeneous mixture was observed. Then, glycerol was added under stirring (600 rpm). Later, citric acid was added according to Composite Central Design (Table 2), continuing with stirring (600 rpm) at 40 ± 2 °C for 30 min. Finally, oregano essential oil was added and an emulsion was generated using a homogenizer device (T25 digital, Ultra Turrax®, IKA, Germany) at 13,500 rpm for 3 minutes. The final starch, glycerol and oregano oil concentrations were 2 g/100 g slurry, 0.41 g/100 g slurry and 1 mL/100 g slurry respectively for all formulations. The mixtures were cast in silicone molds (Silikomart, Italy) and dried in a hot air oven (Lab-Line Instruments Inc., USA) at 1.0 ± 0.5 m s^{-1} and 40 ± 5 °C for 16 h. Once constituted, all films were separated from the molds and stabilized at 25 ± 1 °C in an atmosphere of 57 ± 2 % RH generated inside a vacuum desiccator (Nalgene, USA) containing a saturated solution of NaBr (BioPack, Argentina) for 7 days previous testing.

The film composition was optimized by analysis of the Composite Central Design (see 2.5 section) and the recommended system included 0.13 g citric acid/100 g slurry and 0.38 g chitosan/100 g slurry.

In the second study, three control systems were prepared to analyze the effect of individual citric acid or oregano oil incorporation on film

Table 1
Independent variables (citric acid, chitosan) and their coded and decoded levels for the Composite Central Design.

Coded Levels	Decoded Levels citric acid (g/100 g slurry)	chitosan (g/100 g slurry)
-1.41	0.07	0.19
-1	0.13	0.22
0	0.27	0.30
1	0.41	0.38
1.41	0.47	0.41

Table 2
Measured responses for Composite Central Design.

Run	citric acid (g/100 g slurry)	Chitosan (g/100 g slurry)	ε_b (%)	σ_b (MPa)	YM (MPa)	SW (%)	YI	CIE Lab L*	a*	b*
R1	0.27	0.19	64 ± 5^{b}	4.8 ± 0.5$^{a, b}$	139 ± 12$^{a, b}$	40.4 ± 0.7^{g}	20 ± 1^{a}	86.6 ± 0.3^{e,f}	-1.21 ± 0.04^{b}	9.8 ± 0.6^{a}
R2	0.41	0.38	7.4 ± 0.6^{a}	10.2 ± 0.9$^{c, d}$	327 ± 38^{c}	35.8 ± 0.8^{f}	29 ± 2^{d,e}	85.1 ± 0.5^{a,b}	-1.0 ± 0.1^{e}	14.9 ± 0.9^{e,f}
R3	0.41	0.22	111 ± 13^{c}	6.3 ± 0.6^{b}	107 ± 11^{a}	33 ± 1^{e,f}	19.4 ± 0.9^{a}	86.4 ± 0.2^{d,e}	-1.21 ± 0.03^{b}	9.8 ± 0.4^{a}
R4	0.27	0.30	4.3 ± 0.2^{a}	10 ± 1$^{c, d}$	456 ± 53^{e}	32 ± 4^{d,e}	27.4 ± 0.9^{c}	85.3 ± 0.2^{b,c}	-1.07 ± 0.07^{c}	13.9 ± 0.4^{c,d}
R5	0.27	0.41	14 ± 1^{a}	11 ± 1^{d}	373± 25$^{c, d}$	29.1 ± 0.3^{d}	31 ± 2^{e}	85.5 ± 0.4^{b,c}	-0.97 ± 0.09^{d,e}	15.3 ± 0.6^{f}
R6	0.47	0.30	67 ± 9^{b}	6.2 ± 0.9^{b}	193 ± 8^{b}	41 ± 4^{g}	29 ± 1^{d,e}	84.6 ± 0.3^{a}	-1.06 ± 0.05^{c,d}	14.8 ± 0.5^{e,f}
R7	0.13	0.38	6.7 ± 0.8^{a}	15 ± 1^{e}	569 ± 24^{f}	12.8 ± 0.3^{a}	33 ± 2^{f}	86.2 ± 0.4^{d}	-1.1 ± 0.1$^{c,d, e}$	17 ± 1^{g}
R8	0.27	0.30	7.6 ± 0.4^{a}	10 ± 1$^{c, d}$	461 ± 23^{e}	35 ± 3^{e,f}	28.3 ± 0.9^{c,d}	85.5 ± 0.3^{c}	-1.06 ± 0.04^{c,d}	14.4 ± 0.4^{d,e}
R9	0.07	0.30	3.1 ± 0.2^{a}	10 ± 1$^{c, d}$	675 ± 63^{g}	17 ± 2^{b}	28 ± 2^{c}	87.0 ± 0.4^{f}	-1.39 ± 0.06^{a}	14 ± 1^{d,e}
R10	0.13	0.22	1.1 ± 0.1^{a}	4.4 ± 0.5^{a}	295 ± 33^{c}	24.7 ± 0.5^{c}	23 ± 1^{b}	86.9 ± 0.4^{f}	-1.25 ± 0.04^{b}	11.8 ± 0.7^{b}
R11	0.27	0.30	2.1 ± 0.1^{a}	8.5 ± 0.5^{c}	410 ± 20$^{d, e}$	36 ± 3^{e,f}	26 ± 2^{c,d}	85.4 ± 0.2^{b,c}	-1.1 ± 0.1^{c}	13.2 ± 0.9^{c,d}

ε_b: Strain at break. σ_b: Stress at break. YM: Young Modulus. SW: Solubility in Water. YI: Yellowness Index. L*, a*, b*: CIE Lab parameters.
Values followed by the same letter do not differ significantly (α: 0.05).

properties: C1 (2.0/0.41/0.0/0.38/1.0); C2 (2.0/0.41/0.13/0.38/0.0) and C3 (2.0/0.41/0.0/0.38/0.0) where numbers in brackets indicate the cassava starch/glycerol/citric acid/chitosan/oregano oil content expressed as g/100 g slurry or mL/g slurry for oregano oil. The preparation of the control systems was carried out according to the procedure described above. The properties of control films were compared with those corresponding to the optimized film.

2.3. Physical properties of edible films

The determination of the mechanical properties of the films was conducted according to Arismendi et al. (2013). Force (N) curves were obtained as a function of the clamp displacement (mm). The maximum stress at break (σ_b), the deformation at break (ε_b) and Young's modulus were calculated individually for each specimen.

The solubility in water and the water vapor permeability (WVP) were determined following the technique used by Alzate et al. (2017).

The color evaluation was performed using a CM-700d/600d photocolorimeter (Minolta, Japan) according to the procedure reported by Alzate et al. (2017) with minor changes. The CIE Lab L* (luminosity), a* (green-red), b* (blue-yellow) and Yellow Index (YI) parameters were reported.

The thermogravimetric Analysis (TGA) was carried out with a TA Instruments TGA HI-Res^{TM}500. Samples were heated at a constant rate of 10 °C/min from room temperature to 600 °C under nitrogen atmosphere. Derivative thermogravimetric analysis (DTGA) was performed to identify the temperatures for the maximum thermal degradation rates of the components.

The chemical structure and interactions were studied using Fourier Transform Infrared Spectroscopy (FTIR). Samples were analyzed on a Nicolet 8700 FTIR spectrophotometer equipped with an Attenuated Total Reflectance accessory in the spectral range from 4000 to 575 cm^{-1}.

The structural characteristics were determined by Environmental Scanning Electron Microscopy (ESEM) (XL-30 ESEM, Philips, The Netherlands). The surface of the dried samples was coated with gold and examined. To observe the cross-section, films were frozen in liquid N_2 and cryo-fractured. All images were obtained with an acceleration voltage of 20 kV and magnifications up to 200X.

2.4. Effectiveness of edible films as barriers to yeast contamination

The antimicrobial action of films was evaluated using the yeast *Zygosaccharomyces bailii* (ATCC MYA4549), spoilage microorganism resistant to acidic media and antimicrobials (Osimani et al., 2022), according to Alzate et al. (2017). The inoculum was prepared in Sabouraud broth (Biokar Diagnostics, France) at 25 °C for 24 h. To study the protective behavior of the films when applied to high water activity (a_w) and acidic products, Sabouraud agar (Biokar Diagnostics, France) of a_w 0.980 and pH 4.5 was formulated. Film disks of 1 cm diameter were placed on the surface of the formulated agar. Then, a 10 μL culture of *Z. bailii* with 6×10^5 CFU/mL was seeded on the surface of the film disks. The samples were incubated at 25 °C. Sampling was carried out at selected times by taking two disks of each formulation and suspending each one in 1 mL of peptone water (Biokar Diagnostics, France). Serial dilutions were then prepared in peptone water to cell enumeration (log CFU/g film) by surface agar plate count and incubation at 25 °C for 5 days. All tests were performed at least in duplicate for each formulation.

2.5. Experimental design and statistical analysis

The RSM has been extensively used to design, improve and optimize processes and formulations. It is possible to model and analyze how the responses of interest are influenced by several quantitative factors. Despite the analysis and conclusions being constrained to the selected intervals of the independent variables and it is not possible the extrapolation beyond that range, the SRM made it possible to optimize the responses and determine the values of the optimal factors (Ashrit et al., 2022). In the present research, the Statgraphics Centurion XV software version 6.0 (StatSoft Inc., USA) was used for the experimental design and regression analysis of the data obtained (Arismendi et al., 2013). To define the formulations of the films a Composite Central Design with two factors, five levels and three central points was used. The two selected independent variables were citric acid and chitosan (Table 1).

Dependent variables were fitted to the following second-degree polynomial function:

$$\psi = B_0 + B_1\chi_1 + B_2\chi_2 + B_{11}\chi_2^2 + B_{22}\chi_2^2 + B_{12}\chi_1\chi_2 \quad (1)$$

where ψ is the generic dependent variable; B_0 is the value of fitted response at the central point of the design, i.e. ($\chi_1 = 0$ and $\chi_2 = 0$); B_1 and B_2 are the linear regression coefficients; B_{11} and B_{22} are the quadratic regression coefficients; and B_{12} is the cross-product regression coefficient; χ_1 and χ_2 are the independent variables.

The analysis of variance (ANOVA) was performed and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The quality of the fitted equation was expressed by the coefficient of determination (R^2), lack of fit and F-test values. The three-dimensional response surfaces were plotted based on Eq. 1, and the optimal levels of chitosan and citric acid were selected using the desirability function procedure (Barros Neto et al., 2003).

In addition, one-way ANOVA (Statgraphics Centurion XV) with a significance level (α) of 0.05, followed by Fisher's least significant difference (LSD) *post-test*, was performed to identify significant differences among samples.

3. Results and discussion

3.1. Composite central design analysis

The evaluated responses were fitted to a second-order polynomial (Eq. 1) which reasonably represents the influence of citric acid and chitosan on film properties, in the region where the levels of the factors were selected. In such a quadratic model, the citric acid and chitosan

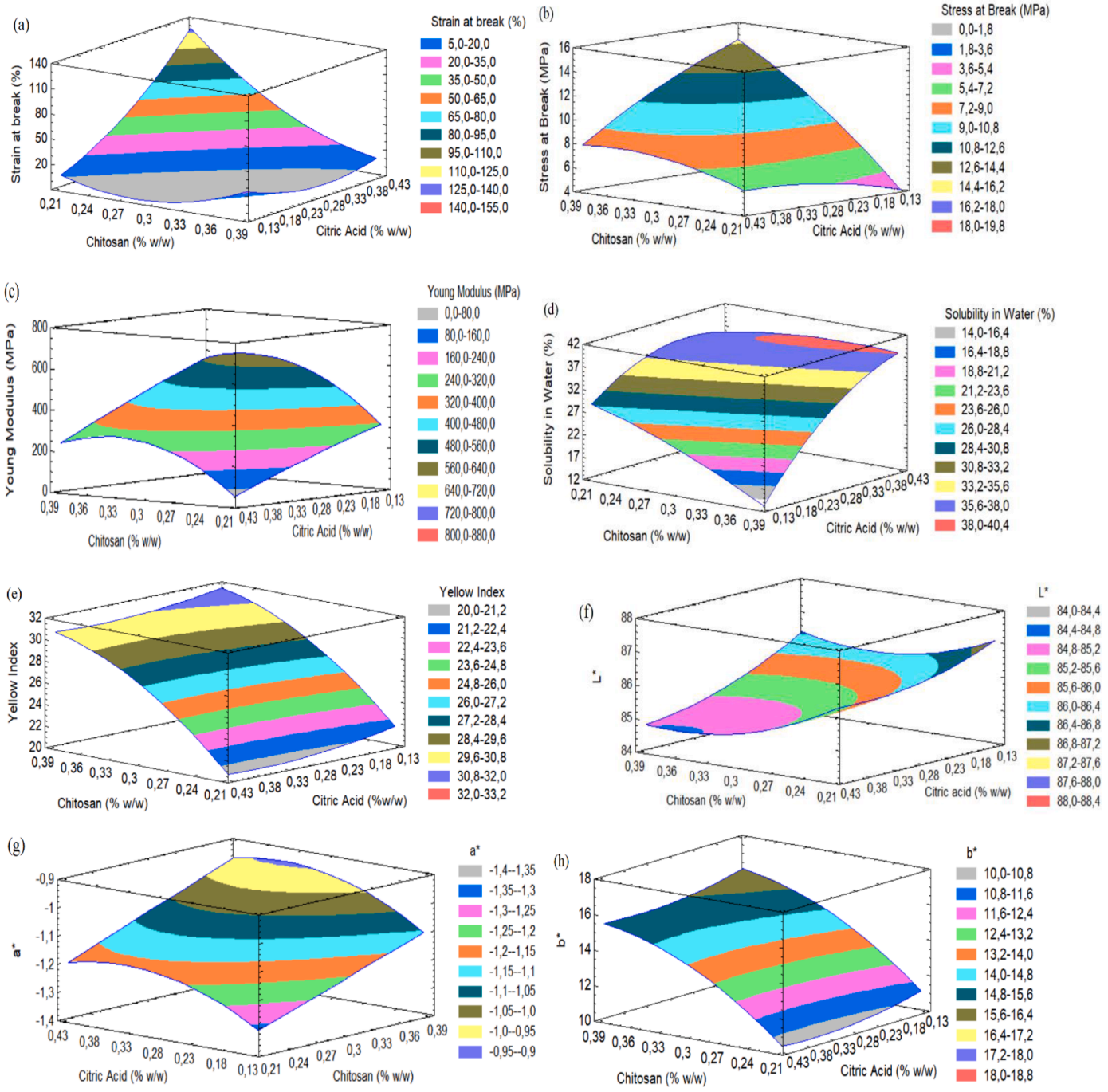

Fig. 1. Response surfaces for: (a) Strain at break; (b) Stress at break (MPa), (c) Young Modulus (MPa), (d) Solubility in Water (%), (e) Yellowness Index, (f) L*, (g) a*, (h) b*.

variables are expressed in the common units of concentration and the corresponding coefficients acquired the convenient units to allow the calculation of response.

The measured responses obtained for mechanical properties strain at break (ε_b, %), stress at break (σ_b, MPa), Young Modulus (MPa), solubility in water (%), Yellowness Index and color parameters (L*, a*, b*) are summarized in Table 2. Additionally, graphical presentation of curves from tensile assays and videos of the characteristics of mechanical testing can be seen in **SM2, SM3** and **SM4**. The corresponding response surfaces are shown in Fig. 1. The coefficients of the prediction equation and the adequacy of the regression are reported in Table 3. All the responses were satisfactorily fitted with the proposed polynomial equation with R^2 values between 0.8783 - 0.9837, adequate F_{reg} values and a non-significant lack of fit ($p>0.0604$), which indicated that the model suitably predicted the responses obtained.

3.1.1. Mechanical properties

According to the second-degree polynomial selected model that describes the strain at break (ε_b) response (Table 3), the linear coefficients for chitosan (negative) and citric acid (positive), as well as both square coefficients (positives) were highly significant ($p<0.01$), suggesting there is a combination of chitosan and citric acid that minimizes the strain value. It was also observed that chitosan coefficients were higher than ones for citric acid, indicating the biopolymer exerted a dominant effect on ε_b. As the interaction coefficient, B_{12}, resulted negative and significant ($p<0.01$), an antagonistic interaction could be stated. Fig. 1a shows that increasing ε_b (%) values were obtained as the amount of chitosan was reduced below 0.30 % w/w and the level of citric acid increased from 0.27 % w/w. For such levels of chitosan, the positive influence of citric acid on the strain was very significant. Accordingly, a higher ε_b value was observed for R3 (0.41 citric acid/0.22 chitosan) than R10 (0.13 citric acid and 0.22 chitosan), tending Run 10 to the lowest ε_b value (Table 2). Contrarily, for high levels of chitosan, the effect of citric acid on strain was reduced. Following the quadratic model, the inferior strains were registered for higher contents of chitosan. However, for systems with high chitosan and low citric acid (< 0.27 % w/w) contents, the strain showed a slight increase (R7, 0.13 citric acid/0.38 chitosan, in comparison with R10, 0.13 citric acid and 0.22 chitosan).

Regarding stress at break (σ_b), Table 3 shows that the main significant ($p<0.05$) coefficient was the linear and positive one for chitosan, exhibiting a main linear dependence of the stress with the amount of chitosan used in film formulation. However, it is observed in Fig. 1b that when citric acid content was low (<0.27 % w/w) the positive effect of chitosan on σ_b was more evident. Interestingly, the stress was slightly increased as citric acid rose for formulations with inferior levels of chitosan (R10 vs R3, Table 2). Wu et al. (2009) reported that cross-linking bonds between citric acid and biopolymers could create, increasing the film resistance to tensile forces.

Analysis of the Young Modulus response (Table 3), revealed that the linear coefficients (positive) for chitosan and (negative) for citric acid were highly significant ($p<0.01$), being the magnitude of the chitosan coefficient higher than citric acid one. The negative quadratic coefficient for chitosan was also significant ($p<0.05$), predicting a maximum Young Modulus value, which was observed for intermedia level of chitosan and low levels of citric acid (Run 9, 0.07 citric acid/0.30 chitosan, Table 2). Fig. 1c also denotes that as citric acid decreased, the Young Modulus increased, for any chitosan level.

According to statistical analysis, the effects of citric acid and chitosan on mechanical properties depend on the levels used each. In general, citric acid exerted a plasticizing effect on mechanical properties, probably by interrupting interactions between biopolymer chains, while chitosan reinforced the whole polysaccharide matrix. Probably, a more resistant network could be generated due to chitosan and starch chain interactions through hydrogen bonds or electrostatic forces. The low deformability could be related to the impossibility of polymeric chains to slide for maintaining ductile behavior (Chillo et al., 2008; Sun et al., 2017; Wu et al., 2019). However, those dependencies are not linear since significant curvatures or interactions were detected. It is important to highlight that this analysis allows us to understand the impact of film formulation, in terms of citric acid and chitosan levels, on the mechanical resistance to decide which could be the most convenient combination of additives to obtain a starch/glycerol based film with high resistance to act as an active packaging material with antimicrobial functionalities, in agreement with the objectives of the present research.

3.1.2. Solubility in water

The linear positive coefficient for citric acid was highly significant ($p<0.01$) while quadratic and interaction terms were less influential, showing that solubility increased as citric acid increased, with a slight curvature. Fig. 1d shows that solubility increased for high citric acid amounts. Especially, at high chitosan levels, the effect of citric acid was more relevant in determining a broader variation of solubility in comparison with results for films with low chitosan content. It can be seen in Table 2 that R6 (0.47 citric acid/0.30 chitosan) and R1 (0.27 citric acid/0.19 chitosan), showed the highest values. As was previously mentioned, citric acid mainly acted as a plasticizer at high chitosan levels, which could also increase the solubility because more hydrophilic groups were exposed to water as a solvent (Ghanbarzadeh et al., 2011; Wu et al., 2019).

Table 3
Coefficients of second-degree polynomial function for different variables and ANOVA analysis.

Coefficient	ε_b (%)	σ_b (MPa)	YM (MPa)	SW (%)	YI	L*	a*	b*
B_0	120.79	-22.66	-1258.54	29.62	-4.18	93.87	-1.78	-2.86
Linear								
B_1	-1172.13***	138.47**	11346.30***	-36.76*	174.38***	-40.41***	1.09***	96.55***
B_2	549.96***	52.45*	-369.71***	60.39***	-17.19	-7.43***	2.16***	-11.51
Square								
B_{11}	2653.60***	-98.44	-16289.20**	-157.57	-201.43	64.61**	0.01	-113.81
B_{22}	690.15***	-22.59	-463.39	-188.09*	20.23	13.56	-3.31**	15.73
Interaction								
B_{12}	-2504.81***	-160.23*	-1236.63	331.69*	7.02	-14.52	0.44	0.23
Lack of fit (P)	0.1017	0.1849	0.1683	0.1961	0.2166	0.0878	0.0604	0.2537
F_{reg}	60.2301	8.5488	22.1100	10.2941	10.4488	11.9736	7.2188	10.8104
R^2	0.9837	0.8968	0.9567	0.9115	0.9127	0.9229	0.8783	0.9153

1: chitosan, 2: citric acid
* Significant at $p<0.1$
** Significant at $p<0.05$
*** Significant at $p<0.01$
ε_b: Strain at break. σ_b: Stress at break. YM: Young Modulus. SW: Solubility in Water. YI: Yellowness Index. L*, a*, b*: CIE Lab parameter

3.1.3. Color evaluation

The quadratic prediction model of L* indicated very significant linear negative coefficients for chitosan and citric acid ($p<0.01$, Table 3). Therefore, an increase of these components trended to decrease L*, denoting a less luminous film was obtained. Besides, a square positive coefficient was also significant for chitosan ($p<0.05$), suggesting a slight curvature of the response. Accordingly, there was a chitosan level that minimized the L* value (around 0.47 citric acid/0.30 chitosan (R6), Table 2). In addition, Fig. 1f denotes that parameter L* acquired the highest values with citric acid and chitosan decrements. As was previously mentioned, chitosan addition increased the film darkening and, as a consequence the L* reduction.

Regression analysis showed that only the linear and positive coefficient for chitosan was vastly significant ($p<0.01$, Table 3), specifying that Yellowness Index and b* values increased as a function of chitosan content being the model curvature not significant **(**Figs. 1e and 1h**)**. This trend was attributed to the light brown original color of chitosan whereby its addition to formulation promoted a yellow component increase in the film color (Chillo et al., 2008). Contrarily, the different citric acid levels did not affect the Yellowness and b* significantly. Accordingly, R7 (0.13 citric acid/0.38 chitosan, Table 2) and R5 (0.27 citric acid/0.41 chitosan, Table 2**)** tended to maximize Yellowness and b* results.

Regarding a* parameter, films showed negative values (greenness) from -0.97 to -1.25, Table 2). A linear (positive) coefficient was highly significant for chitosan and citric acid ($p<0.01$), and a square (negative) coefficient was significant for citric acid ($p<0.05$). Fig. 1g illustrates that a* was increased (less negative) as chitosan content was higher. In addition, the lowest greenness (highest a*) trended to be reached for intermedia citric acid levels and high chitosan amount (R5, Table 2).

3.1.4. Optimization of film formulation

To select a film formulation with high mechanical resistance and low SW, which are convenient characteristics of active food packaging, an optimization technique was performed where the σ_b and the SW were required to be maximum and minimum, respectively. According to Desirability Function Procedure applied, R7 (0.13 citric acid/0.38 chitosan) meets the announced criteria simultaneously (σ_b: 15±1 MPa and solubility in water: 12.8±0.3 %) with a very appropriate Desirability value of 0.9136. The stress results are in agreement with those reported by Chillo et al. (2008) for edible films based on cassava starch/glycerol/chitosan (1-20 MPa) but without antimicrobial agents. Moreover, the optimized σ_b represents an improvement in comparison with typical strength values of plane starch films plasticized with glycerol and containing essential oil (2.6-5.2 MPa) (Li et al., 2018) which is known to reduce the mechanical resistance of films. Regarding SW, the present research found a lower value than those reported by Vázcones et al. (2009), 25 %, and Li et al. (2018), 18 %, being more convenient for films destined to act as a packaging material. Moreover, the recommended film was further studied to analyze the influence of the citric acid and/or oregano essential oil addition on the thermal and mechanical properties, chemical structure (FTIR), solubility in water, WVP, color parameters, microstructure and antimicrobial activity, as discussed in the following section.

3.2. Analysis of citric acid and oregano essential oil addition on properties of films

3.2.1. Thermal analysis

The thermal stability of films was studied through thermogravimetric analysis (Fig. 2). The first weight loss, from the initial

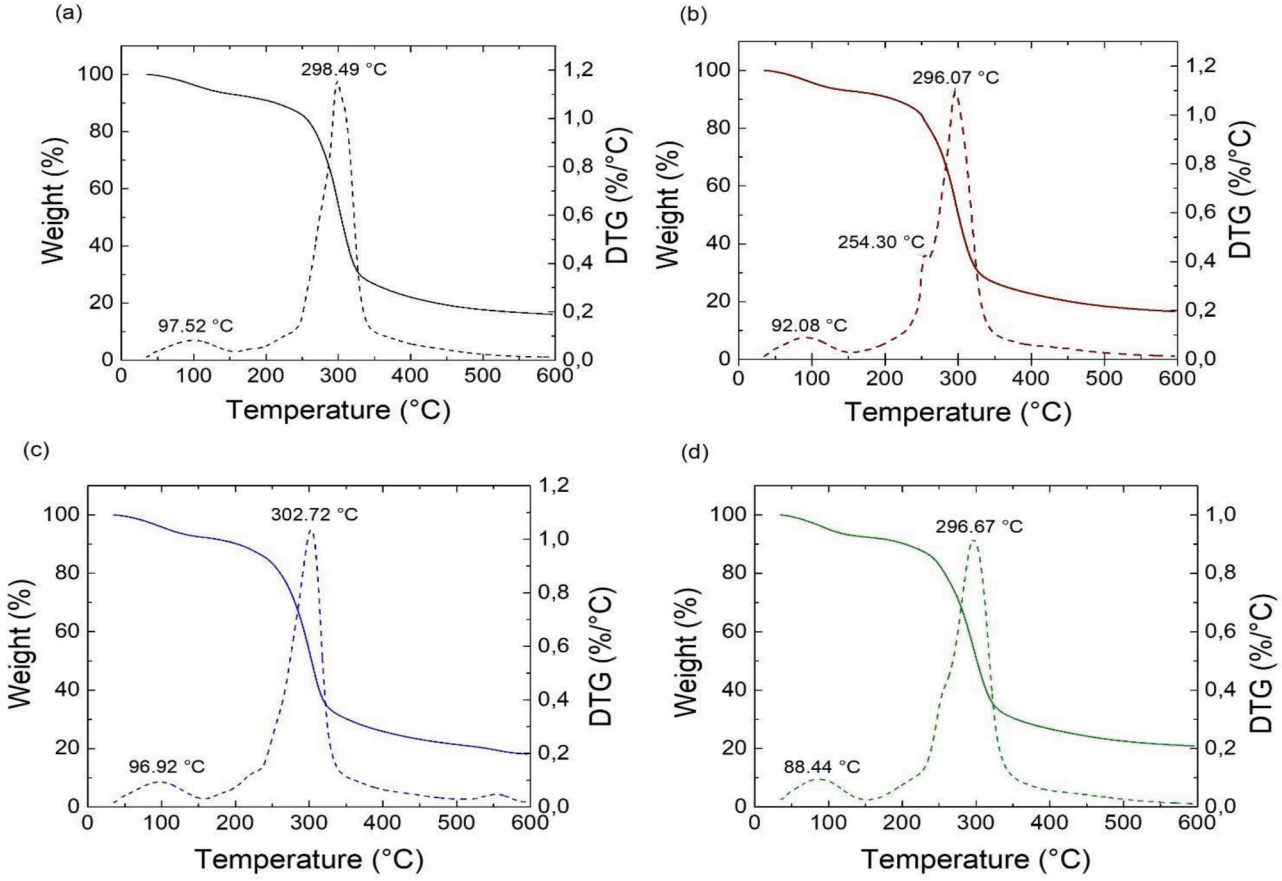

Fig. 2. TGA and DTG curves of films: (a) citric acid 0.13/ chitosan 0.38/ oregano oil 1.0; (b) citric acid 0.0 / chitosan 0.38 / oregano oil 1.0; (c) citric acid 0.13/ chitosan 0.38 / oregano oil 0.0; (d) citric acid 0.0 / chitosan 0.38 /oregano oil 0.0.

temperature to approximately 88-98 °C, could be mainly attributed to the removal of moisture. In addition, it was reported that the loss of minor volatile compounds present in oregano oil could occur continuously from room temperature until the boiling point of other components (~ 232-237 °C for carvacrol and thymol) was reached (Pelissari et al., 2009). A second weight loss, ~254 °C, was observed for C1 (citric acid 0.0/chitosan 0.38/oregano oil 1.0) films (Fig. 2b) and it is probably associated with the oregano oil components linked to the biopolymer matrix. The main weight loss occurs between 255 °C and above 400 °C for all samples. It was related to the decomposition of the $–CH_2OH$ group and the plasticization of starch, as well as, the decomposition of glycerol (~290 °C). Finally, the total degradation of the chitosan and cassava starch cyclic structures arises at 600 °C (Wu et al., 2019; Lozano et al., 2018). It is interesting to remark that C2 (citric acid 0.13/ chitosan 0.38 / oregano oil 0) films showed the main weight loss at the highest temperature (302.7 °C), showing a slight improvement in resistance to thermal degradation.

3.2.2. *Fourier transform infrared spectroscopy (FTIR)*

FTIR spectra of the tested samples are presented in Fig. 3. For comparison reasons, two extra films based on individual biopolymers (cassava starch/glycerol and chitosan/glycerol), oregano oil and citric acid spectra were incorporated.

In general, it was observed that the FTIR spectra profile of analyzed films (R7 and controls, Fig. 3 a), b), c) and d)) are similar showing typical vibrations bands of biopolymers based on starch and chitosan. This indicates that new covalent bonds were not formed or detected (González et al., 2021) by the addition of citric acid or oregano oil. However, shifts in several bands were identified which are representative of physical interactions among film components. The FTIR spectra of all films and oregano oil present a broad band located in the region of 3600-3100 cm^{-1} which is mainly due to the superposition of the stretching vibrations of O-H and N-H groups participating in intra and intermolecular H bonds (O-H 3500-3250 cm^{-1} and N-H 3400-3250 cm^{-1}, respectively). In addition, it was previously reported (Mathew

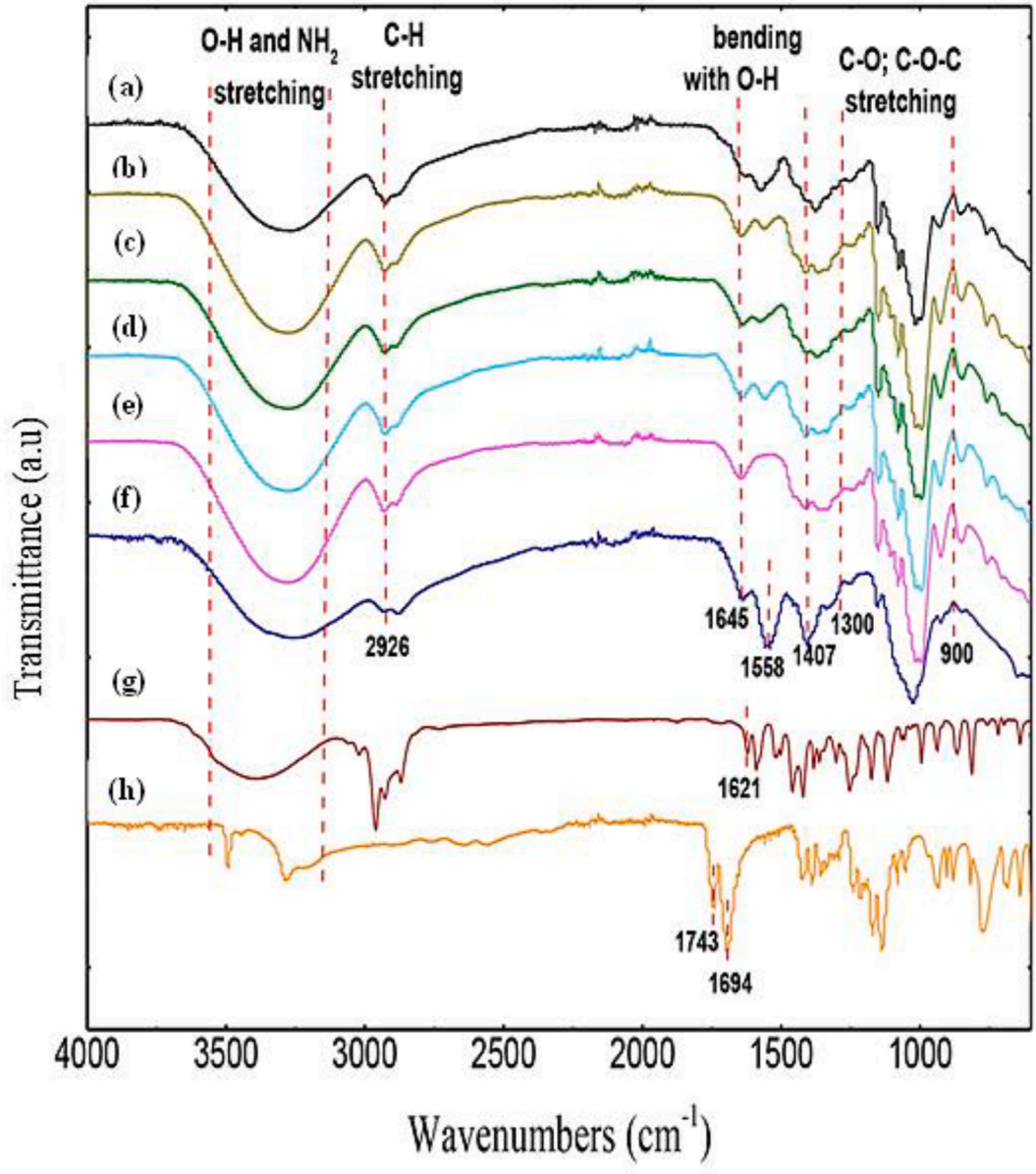

Fig. 3. . FTIR spectra of films based on starch/glycerol and added as follows: (a) film R7, citric acid 0.13/chitosan 0.38/oregano oil 1.0; (b) film C1, citric acid 0.0/chitosan 0.38/oregano oil 1.0; (c) film C2, citric acid 0.13/chitosan 0.38/oregano oil 0.0; (d) film C3, citric acid 0.0/chitosan 0.38/oregano oil 0.0. Films based on individual biopolymers (e) cassava starch/glycerol, (f) chitosan/glycerol, and individual components (g) oregano oil and (h) citric acid spectra were incorporated for comparison reasons.

et al., 2006) that -NH_3^+ in chitosan (dissolved in acidic media) can establish numerous H bonds with electronegative atoms of starch (-OH, C-O-C), helping to improve the compatibility between biopolymers. This band showed a minimum at 3283.9, 3281 and 3284.1 cm^{-1} in C3 (citric acid 0.0/chitosan 0.38 oregano oil 0.0), C2 (citric acid 0.13/ chitosan 0.38 / oregano oil 0.0) and C1 (citric acid 0.0/ chitosan 0.38 / oregano oil 1.0) films, respectively, while it was shifted to 3267.7 cm^{-1} in R7 (citric acid 0.13/ chitosan 0.38/ oregano oil 1.0) system. Such red shifting in frequency is a measure of the increase in strength of the H bond (Silverstein et al., 1991) after the simultaneous addition of citric acid and oregano oil in the film matrix (R7). According to Wu et al. (2019), these intermolecular interactions could be due to the formation of hydrogen bonds between -OH groups of cassava starch and chitosan. In addition, other proton donor groups (-COOH, -OH, -NH_2, -$CONH_2$) and proton acceptor atoms (O and N) in water, glycerol, citric acid and oregano oil could be participated promoting the shifting of the O-H stretching band in R7. The magnitude of the shifting was 2.9, -0.2 and 16.2 cm^{-1} for C2, C1 and R7 respectively, in comparison with O-H stretching band in C3 (starch, chitosan and glycerol but without citric acid and oregano oil). To estimate the percentage of hydrogen bonding in films, it was considered that the broad band around 3279 cm^{-1} is the result of the contribution of several absorption events, including free (~3500 cm^{-1}), intra (~3450 cm^{-1}) and intermolecular (~3180 cm^{-1}) H-O stretching (Janardhnan & Sain, 2011; Zagar & Grdadolnik, 2003). Regarding the absorbance intensity at those frequencies, the calculated percentages of free, intra e inter OH were 22%, 35.5% and 42.5% respectively for R7 while average values for C1, C2 and C3 were 20.7%, 35.6% and 43.7% (Guo et al., 2010; Liu et al., 2020) (**SM5**). It is possible to observe that intra and inter O-H stretching prevailed in all films. However, these interactions were more strength in R7 (lower wavenumber of the O-H band) than in C1, C2 and C3.

Another common band observed was the symmetric and asymmetric C-H bond stretching. Furthermore, a band at 1645 cm^{-1} assignable to the H-O-H bending of the water in films (water bound to the structure) was observed for all the films but it shifted at a lower wavenumber, 1631–1635 cm^{-1}, and showed lower intensity, in R7 (cassava starch/ glycerol/chitosan with citric acid and oregano oil) and C2 (cassava starch/glycerol/chitosan with citric acid) films, probably due to a high number of the molecular interactions among film-forming components instead water-cassava starch/chitosan interactions. According to Kumar et al. (2018), when water molecules are strongly interacting through H bonding, their bending vibration requires relatively higher energy to undergo excitation. Therefore, a red shifting of this band suggested that the extent of such interaction between acceptor groups with the bound water present in R7 and C2 systems decreased.

The band observed at 1652 cm^{-1} in chitosan/glycerol film, can be associated with C=O stretching (Amide I) due to the chitin residues present in the films. This mode was superimposed to those of O-H bending of water (~1645 cm^{-1}). Another evident characteristic peak in chitosan/glycerol sample is the N-H bending (Amide II) at 1557 cm^{-1} and, similarly, it was observed in C3 (citric acid 0.0/ chitosan 0.38/ oregano oil 0.0) (1557 cm^{-1}). However, this bending was shifted to 1575, 1564 and 1573 cm^{-1} in C1 (citric acid 0.0 /chitosan 0.38/oregano oil 1.0), C2 (citric acid 0.13/chitosan 0.38/oregano oil 0.0) and R7 (citric acid 0.13/chitosan 0.38/oregano oil 1.0), respectively. This result indicates the interaction of the hydroxyl groups of cassava starch, citric acid or oregano oil with the amide groups of chitosan (Pelissari et al., 2009).

Finally, several authors Berti et al. (2021), indicated that in the FTIR spectroscopy of polysaccharides, the peaks located in the region between 1300 - 900 cm^{-1} are mainly owing to the C-C and C-O stretching in the pyranose ring (centered at 1015 cm^{-1}) and to the C-O-C stretching of the glycosidic bond located at 1151 cm^{-1}. It is interesting to note that the peaks at 1013 cm^{-1} and 994 cm^{-1} in C3 (citric acid 0/ chitosan 0.38/oregano oil 0.0), C2 (citric acid 0.13/chitosan 0.38/oregano oil 0.0) and C1(citric acid 0/chitosan 0.38/oregano oil 1.0) were displaced to 1017 cm^{-1} and 997 cm^{-1} in R7 (citric acid 0.13/ chitosan 0.38/oregano oil 1.0) suggesting a diminishing physical interaction between -OH from starch or chitosan and C-O-C group of anhydroglucose ring due to a new biopolymer-citric acid or biopolymer-oregano oil interactions (Ma et al., 2008).

3.2.3. Mechanical properties

The curves from tensile assays are shown in **SM2**. It can be seen in Table 4 that C3 (citric acid 0.0/chitosan 0.38/ oregano oil 0.0) films presented σ_b and YM values of 14.6±0.5 MPa and 763±65 MPa, respectively. As was established in FTIR analysis, chitosan and cassava starch are compatible biopolymers for films constitution, since interchain H bonds could be established between –NH_2 and –OH groups, promoting a continuous and resistant matrix (Vásconez et al., 2009)

On the other hand, the oregano oil addition (C1, citric acid 0.0/ chitosan 0.38/oregano oil 1.0) showed a non-significant ($p>0.05$) tendency to reduce such parameters. The amount of oregano oil added (1 g/ 100 g slurry) seemed to be not enough to affect the mechanical resistance developed in C3 (citric acid 0.0/chitosan 0.38/oregano oil 0.0). Therefore, films C1 (citric acid 0.0/chitosan 0.38/ oregano oil 1.0) and C3 (citric acid 0.0/chitosan 0.38/oregano oil 0.0) exhibited the lowest σ_b values, in comparison with R7 (citric acid 0.13/ chitosan 0.38/ oregano oil 1.0) and C2 (citric acid 0.13/chitosan 0.38/oregano oil 0.0). On the contrary, citric acid incorporation (C2, citric acid 0.13/chitosan 0.38/oregano oil 0.0 films) rendered the most resistant to tensile forces and stiffest films. It could be postulated that more physical and/or chemical intermolecular junction points among biopolymers were promoted, which reinforced mechanical response in citric acid 0.13/chitosan 0.38/oregano oil 0.0 films. Such results could be associated with a decrease in pH level that induces hydrolysis of cassava starch or chitosan glycosidic bonds during slurry preparation, rendering shorter and linear fragments of biopolymers and, as a consequence, a stiffer film matrix (Shi et al., 2007). In addition, several authors have previously reported a cross-linker action of citric acid under controlled conditions (citric acid level, temperature and reaction time, polysaccharide matrix, solvent used, etc.) by forming di-ester bindings (Ma et al., 2008; Wu et al., 2019). However, after adding citric acid into cassava starch/glycerol/chitosan films, the esterification between citric acid and either starch or chitosan, could not be corroborated by FTIR analysis due to the absence of a new peak around 1720 cm^{-1} (C3, citric acid 0.0/chitosan 0.38/oregano oil 0.0 vs C2, citric acid 0.13/chitosan 0.38/oregano oil 0.0 films). The σ_b value for R7 (citric acid 0.13/chitosan 0.38/ oregano oil 1.0) film, showed an intermediate behavior between C2 (citric acid 0.13/chitosan 0.38 /oregano oil 0.0) and C1 (citric acid 0/chitosan 0.38/oregano oil 1.0) films, because both components (citric acid and oregano oil) counteract their individual effects (Pelissari et al., 2009). However, YM showed the lowest value for R7 (citric acid 0.13/chitosan 0.38/ oregano oil 1.0), suggesting that material with a reduced solid character was obtained after the combined addition of citric acid and oregano oil.

Regarding the extensibility of films, C1 (citric acid 0.0/chitosan 0.38/oregano oil 1.0) exposed the highest ε_b, showing the important plasticizer action of oregano oil (Pelissari et al., 2009). In agreement with the reinforced effect observed for citric acid addition, the ε_b was lower for C2 (citric acid 0.13/ chitosan 0.38/oregano oil 0.0) and R7 (citric acid 0.13/chitosan 0.38/oregano oil 1.0) films in comparison with C1 (citric acid 0.0/chitosan 0.38 /oregano oil 1.0) and C3 (citric acid 0.0/chitosan 0.38/oregano oil 0.0). It is interesting to mention that C1 (citric acid 0.0/chitosan 0.38/oregano oil 1.0), R7 (citric acid 0.13/chitosan 0.38/oregano oil 1.0) and Run 2 (citric acid 0.41/chitosan 0.38/ oregano oil 1.0, Table 2) films, had similar formulation except for citric acid content. As was previously discussed, the incorporation of citric acid at a level of 0.13 g/100 g slurry, increased σ_b (C1, citric acid 0.0/chitosan 0.38/oregano oil 1.0) vs R7 (citric acid 0.13/ chitosan 0.38/ oregano oil 1.0). On the contrary, the addition of 0.41 g/100 g slurry citric acid (Run 2), rendered films with lower σ_b and YM but

Table 4
Measured responses for optimized edible film and controls.

Film	ε_b (%)	σ_b (MPa)	YM (MPa)	SW (%)	WVP(g/m s Pa) x 10^{-9}	YI	CIE Lab L*	a*	b*
R7: Citric Acid 0.13/ Chitosan 0.38/Oregano Oil 1.0									
	6.1 ± 0.2^{a}	18 ± 3^{b}	610 ± 59^{a}	17 ± 2^{a}	1.39 ± 0.09^{b}	32.1 ± 1.3^{b}	85.3 ± 0.4^{b}	-0.63 ± 0.06^{c}	16.4 ± 0.7^{b}
C1: Citric Acid 0.0/Chitosan 0.38 /Oregano Oil 1.0									
	10.6 ± 0.6^{d}	13.3 ± 0.9^{a}	704 ± 61^{b}	18 ± 1^{a}	0.81 ± 0.04^{a}	45.1 ± 0.9^{c}	81.8 ± 0.5^{a}	-1.01 ± 0.08^{b}	23.2 ± 1.4^{c}
C2: Citric Acid 0.13 /Chitosan 0.38 /Oregano Oil 0.0									
	7.2 ± 0.8^{b}	29 ± 2^{c}	1024 ± 121^{c}	18.3 ± 0.8^{a}	$1.1 \pm 0.2^{a,b}$	23.5 ± 2.2^{a}	87.4 ± 0.7^{c}	-1.6 ± 0.1^{a}	12 ± 1^{a}
C3: Citric Acid 0.0/Chitosan 0.38 /Oregano Oil 0.0									
	8.7 ± 0.8^{c}	14.6 ± 0.5^{a}	763 ± 65^{b}	26 ± 2^{b}	$1.1 \pm 0.1^{a,b}$	23 ± 1^{a}	85.7 ± 0.6^{b}	-1.70 ± 0.07^{a}	12.1 ± 0.7^{a}

ε_b: Strain at break. σ_b: Stress at break. YM: Young Modulus. SW: Solubility in Water. WVP: Water Vapor Permeability. YI: Yellowness Index. L*, a*, b*: CIE Lab parameters. Values followed by the same letter do not differ significantly (α: 0.05).

higher ε_b (R7, citric acid 0.13/chitosan 0.38/oregano oil 1.0 vs. Run 2, citric acid 0.41/chitosan 0.38/ oregano oil 1.0,), suggesting that there was a critical citric acid amount that could reinforce the film matrix, and that exceeding this level, the citric acid may act as a plasticizer agent (Wu et al., 2019).

It must be highlighted that the materials herein developed (Table 4) showed high mechanical resistance, comparable with those of traditional commodities used to obtain films or trays for food packaging. It was reported that low density polyethylene trays presented σ_b around 6-17 MPa and YM around 300-500 MPa, while high density polyethylene or polypropylene had strengths of 21-40 MPa and YM ranging from 600 to 1500 MPa (Eriksen et al., 2019; Yam, 2009). In addition, the obtained cassava starch/chitosan films introduce the advantage of incorporating antimicrobial compounds, converting films into active packaging materials.

3.2.4. Solubility in water and water vapor permeability (WVP)

The solubility in water (Table 4**)** was significantly ($p<0.05$) reduced, around 31.6%, by addition of citric acid and/or oregano oil (C1, citric acid 0.0/chitosan 0.38/oregano oil 1.0; C2, citric acid 0.13/chitosan 0.38 /oregano oil 0.0 and R7, citric acid 0.13/chitosan 0.38/oregano oil 1.0 vs. C3, citric acid 0.0/chitosan 0.38/oregano oil 0.0). This result could denote that citric acid reduces the solubility of the film because promote a stiffer film matrix as was previously mentioned. Therefore, it was observed that better conditions for biopolymer interactions or increased hydrophobic character were obtained after citric acid and oregano oil incorporation, either in a single or combined way. Regarding R7 (citric acid 0.13/chitosan 0.38/oregano oil 1.0), it was not observed an additive effect due to the simultaneous presence of oregano oil and citric acid in the reduction of solubility in water.

On the other hand, the WVP of the films increased significantly ($p<0.05$) with the incorporation of citric acid and oregano oil (Table 4). The highest value of WVP was obtained for R7 (citric acid 0.13/chitosan 0.38/oregano oil 1.0) film probably due to the increment of interaction with water vapor and advocating the permeability. On the contrary, C1 films were the ones with the better barrier to water vapor. The oregano oil addition could reduce the matrix hydrophilic character, avoiding the mobility of water vapor molecules through the material. However, adding citric acid only (C2 films, citric acid 0.13/chitosan 0.38 /oregano oil 0.0) did not modify this parameter in comparison with C3 system (citric acid 0.0/chitosan 0.38/oregano oil 0.0). Lower values of WVP in C1 (citric acid 0.0/chitosan 0.38/oregano oil 1.0), C2 (citric acid 0.13/chitosan 0.38 /oregano oil 0.0) and C3 (citric acid 0.0/chitosan 0.38/oregano oil 0.0) films could be associated to raised interactions attributable to H bonding among chitosan, cassava starch, and individual citric acid or oregano oil. The WVP values agree with those reported by Pelissari et al. (2009) in cassava starch/chitosan films adding oregano oil.

Additionally, the contact angle of films was determined (**SM6**). It was observed that all films have hydrophilic surfaces (angle $< 65°$). The lowest angle was for C2, citric acid 0.13/chitosan 0.38 /oregano oil 0.0, ($4.7° \pm 0.3°$), suggesting that more polar (hydrophilic) sites could appear on surface of this film. Such phenomenon could explain, in part, the high WVP value observed since water vapor molecules may be faster absorbed on surface. On the contrary, higher angle values were obtained for other films (15.8°-25°).

3.2.5. Color evaluation

Films C2 (citric acid 0.13/chitosan 0.38 /oregano oil 0.0) and C3 (citric acid 0.0/chitosan 0.38/oregano oil 0.0) showed higher values of L* (Table 4) but lower values of a*, b* and Yellowness Index. The addition of citric acid and oregano oil in base-film formulation (Film R7: citric acid 0.13/chitosan 0.38/oregano oil 1.0) reduced significantly L* (85.3) and increased a*, b* and Yellowness Index. Similar behavior was exhibited by parameters of Film C1(citric acid 0.0/chitosan 0.38 /oregano oil 1.0): the lowest L* value and the highest values of b* and Yellowness Index. The own color of the oregano oil, produced more browned matrices and, accordingly, less luminous (L*) and greenness (a*) materials (Suput et al., 2016).

3.2.6. ESEM

ESEM images of surfaces and cross-sections of all films can be seen in Fig. 4. The microstructure of C3 (citric acid 0.0/chitosan 0.38/oregano oil 0.0) shows the compatibility of cassava starch and chitosan biopolymers, plasticized with glycerol: uniform surface with the presence of particles (Fig. 4**g**), while the cross-section displays the level of integration of both macromolecules resulting in a compact material (Mathew et al., 2006) (Fig. 4h).

In the case of C2 (citric acid 0.13/chitosan 0.38/oregano oil 0.0), it was also homogeneous and continuous on the surface (Fig. 4e and the cross-section displays a denser and more compact matrix (Fig. 4f) than C3 (citric acid 0.0/chitosan 0.38/oregano oil 0.0) films. The citric acid addition improved the compatibility between cassava starch and chitosan (C3 film: citric acid 0.0/chitosan 0.38/oregano oil 0.0) and this result could explain, in part, the high stiffness and resistance to dissolution in water material, as was previously described. On the contrary, the oregano oil addition (C1 film: citric acid 0.0/chitosan 0.38/oregano oil 1.0), introduced a new phase that was evenly distributed through the film matrix, without evident flocculation but, at the same time, rendering a more irregular microstructure. Roughness in C1 (citric acid 0.0/chitosan 0.38/oregano oil 1.0), surface (Fig. 4c) may be related to the distribution of a hydrophobic compound like oregano oil (Cao & Song, 2019). In addition, the cross-section of C1 (citric acid 0.0/chitosan 0.38/oregano oil 1.0) (Fig. 4d) was less compact and showed the presence of pores (Shi et al., 2007). When both the citric acid and oregano oil were added (R7 film: citric acid 0.13/chitosan 0.38/oregano oil 1.0), it can be observed that the surface remained uneven but with a different structure (Fig. 4a) showing folds and absence of pores. A previous report (Dhumal et al., 2019) stated that the addition of carvacrol into sago starch/guar gum matrix produced roughness in the film surface. The

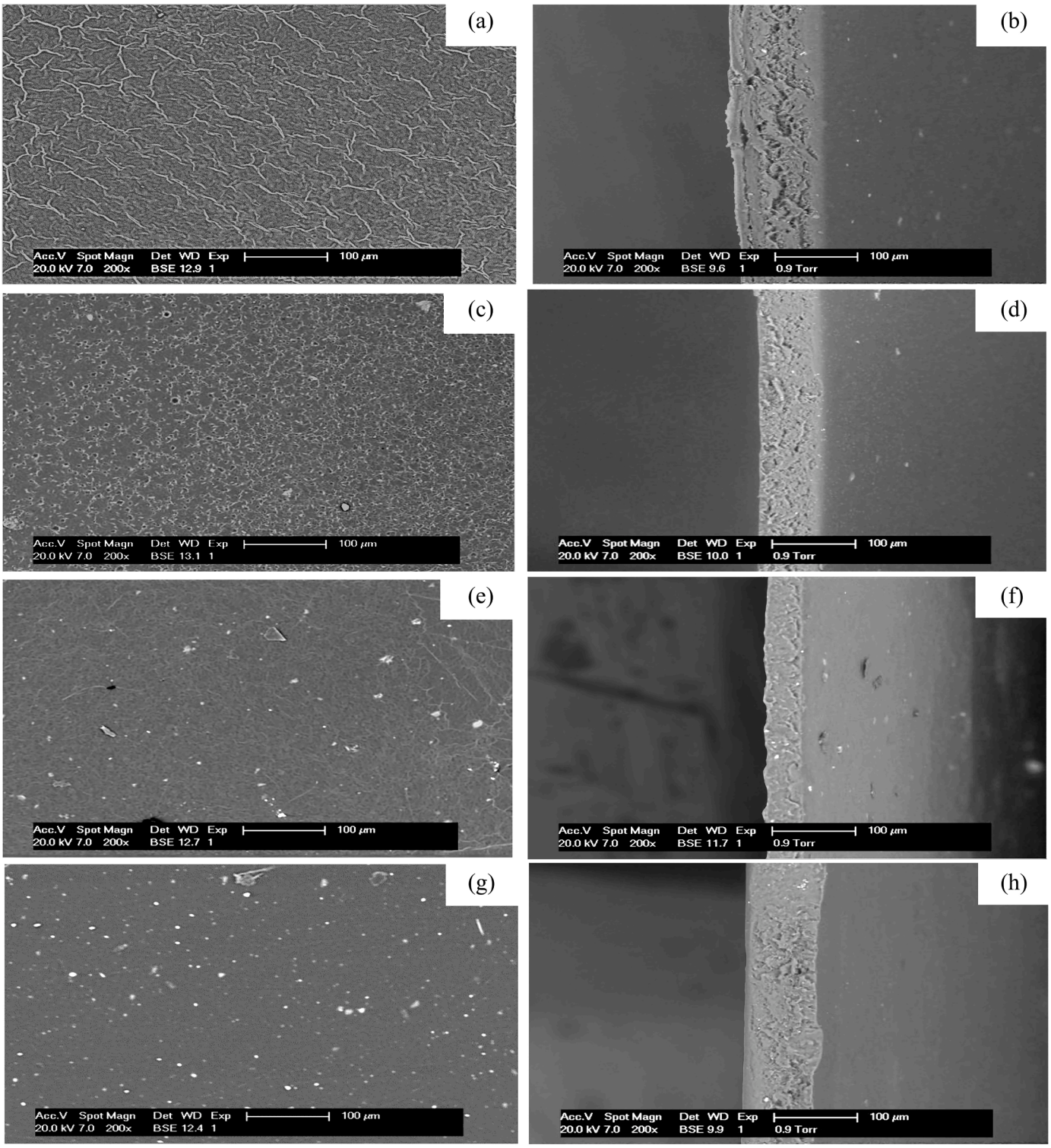

Fig. 4. ESEM micrographs of films. Surfaces: (a) R7, citric acid 0.13/chitosan 0.38/oregano oil 1.0; (c) C1, citric acid 0.0/chitosan 0.38/oregano oil 1.0; (d) C2, citric acid 0.13/chitosan 0.38/oregano oil 0.0; (e) C3, citric acid 0.0/ chitosan 0.38/oregano oil 0.0. Cross-sections: (b) R7, citric acid 0.13/chitosan 0.38/oregano oil 1.0; (e) C1, citric acid 0.0/chitosan 0.38/oregano oil 1.0; (g) C2, citric acid 0.13/chitosan 0.38/oregano oil 0.0; (h) C3, citric acid 0.0/ chitosan 0.38/oregano oil 0.0. Magnification: 200x.

porosity observed in the cross-section (Fig. 4b) due to oregano oil, might contribute to the lowest values of ε_b and Young Modulus (Section 3.2.3), suggesting that the elongation capacity is considerably affected by the structure and that a less elastic film was obtained (Wang et al., 2011). The following factors have a critical role in the final size, shape and distribution of the oil phase in films made from an emulsion: mechanical shear (intensity and time), the interaction between the film components, the rate of solvent evaporation and the corresponding increase of the viscosity of films dispersion, chemical composition and the interfacial tension between the different phases (Agarwal et al., 2020).

3.2.7. Antimicrobial activity

The objective of the antimicrobial barrier test was to verify that the preservatives would be able to act to protect food. Particularly, yeast is a microorganism that can grow at low pH (Osimani et al., 2022), such as the food model proposed (pH 4.5 and a_w 0.98). Therefore, yeasts should not survive on the film surface. Fig. 5 shows the antimicrobial performance of studied films as a barrier against *Z. bailii* external contamination. It can be seen that R7 (citric acid 0.13/chitosan 0.38/oregano oil 1.0) and C1 (citric acid 0.0/chitosan 0.38/oregano oil 1.0) films had the highest efficiency to control the yeast growth, diminishing the *Z. bailii* count at least 2 Log cycles (none colony counted in a suspension of a film disk, ~0.022 g, in 1 mL peptone water) along 96 h storage at 25 °C, revealing the availability of the oregano oil to exert its important antimicrobial action. On the contrary, C2 (citric acid 0.13/chitosan 0.38 /oregano oil 0) and C3 (citric acid 0.0/chitosan 0.38 /oregano oil 0.0) films without oregano oil, showed 2 log cycles increase of yeast population from 48 h of storage. These results indicate that the antimicrobial active packaging based on cassava starch/chitosan containing oregano oil, could successfully control yeast external contamination on the surface of an acidic and high a_w semisolid food (Alzate et al., 2017). In addition, the presence of citric acid (R7: citric acid 0.13/chitosan 0.38/oregano oil 1.0 vs. C1: citric acid 0.0/chitosan 0.38/oregano oil 1.0 films), seems not to introduce an improvement from the antimicrobial protection point of view.

These results demonstrate that oregano essential oil added to film formulation was able to be in contact with yeast to kill it. Several mechanisms can be involved in the effective action of preservatives: the diffusion of antimicrobial from the film surface to the cell wall of yeast in enough amounts to reach the minimal inhibitory concentration (Alzate et al., 2017) and the proper adhesion of yeast onto the film surface. It was reported that yeast cells are characterized by a negative charge due to the presence of carboxyl, phosphoryl and hydroxyl groups. However, the overall cell wall charge could not be the unique determinant of cell adhesion (Kregiel et al., 2012). According to contact angle results (**SM6**), assayed films have a surface with hydrophilic character (polar or charged groups such as –OH, -COOH, $-COO^-$ and $-NH_3^+$). The final charge density on the film surface can modulate the ability of yeast to interact with the surface to be immobilized for enough time; allowing oregano oil to reach the microorganism surface by diffusion. In a previous research (Alzate et al., 2017), it was verified the mobility from biopolymer matrix and effectiveness of carvacrol, one of the major antimicrobial component of oregano oil, using the standardized diffusion in agar method which is commonly used for screening the potential antimicrobial and antifungal action of traditional and new active compounds.

4. Conclusions

In the present study, edible films based on cassava starch and glycerol were developed by the casting method, to which the natural antimicrobial agents citric acid, chitosan and oregano essential oil were combined in the same formulation for the first time. The effect of the addition of citric acid and chitosan on the physical properties of the starch/glycerol films added with oregano oil was investigated using a RSM. The results revealed that the effects of citric acid and chitosan on the mechanical properties depended on the amounts used in each case. In general, increasing the citric acid content increased the flexibility and water solubility of the films, while high chitosan content increased the tensile test resistance and yellowness. An optimization procedure was used to recommend a film formulation with high mechanical resistance and low solubility.

For a more detailed investigation of the influence of citric acid and oregano oil on the starch/glycerol/chitosan film properties, additional systems with or without citric acid or oregano oil were prepared and compared with the optimized film. The FTIR analysis showed that citric acid and oregano oil increased the strength of hydrogen bond interactions. Incorporation of citric acid into the film of plane cassava starch improved thermal resistance, fracture strength, and water solubility while promoting a high level of hydrophilic groups that increased the WVP and hydrophilicity of the film surface. On the other hand, the addition of oregano oil reduced the WVP but browned films and led to inhomogeneities in the microstructure. The presence of oregano oil was important to obtain a very effective antimicrobial active film against external yeast contamination, showing that the preservative was able to protect an acidic food model. In addition, the developed material showed mechanical properties comparable to those of conventional plastic products for packaging applications. Although further characterization tests are still pending (efficacy in real foods such as cheese or sausage, the antimicrobial barrier against foodborne pathogens and biodegradability), the obtained results are a relevant contribution to understanding the effect of the film formulation on the physicochemical and antimicrobial performance of films with potential application in active packaging for food protection.

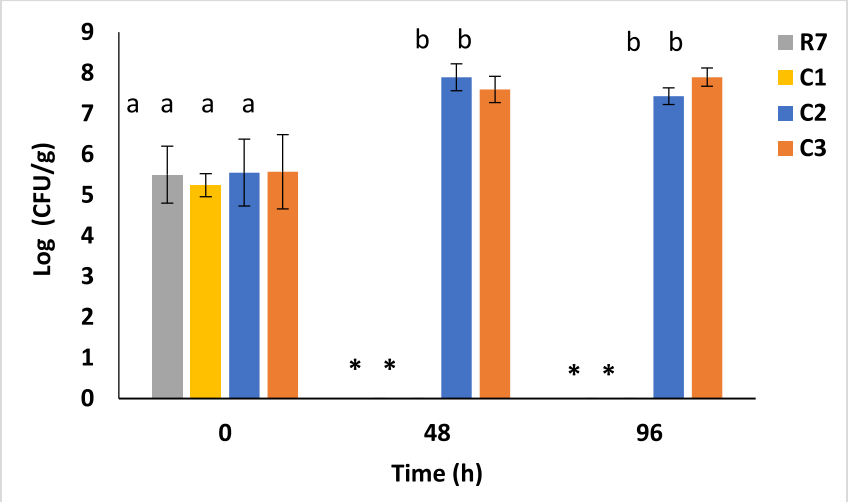

Fig. 5. Antimicrobial barrier against a *Z. bailii* external contamination during storage at 25 °C. *: none colony counted for R7 (citric acid 0.13/chitosan 0.38/oregano oil 1.0) and C1 (citric acid 0.0/chitosan 0.38/oregano oil 1.0) systems. C2 (citric acid 0.13/chitosan 0.38 /oregano oil 0) and C3 (citric acid 0.0/chitosan 0.38 /oregano oil 0.0). Bars with the same letter do not differ significantly (α: 0.05).

Declarations of Competing Interest

None.

Data availability

Data will be made available on request.

Acknowledgements

This study was funded by the University of Buenos Aires (UBACYT 2018-2020 20020170100092BA), the National Agency for Scientific and Technical Research (PICT-2019-1842 and PICT-2020-03137), CONICET (PIP-2021-2023) and the National University of Mar del Plata (15/G618-ING622/21). The authors thank Lic. Andrea Romano from the Argentine Miner Geological Service for assistance in ESEM image acquisition.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.carpta.2023.100307.

References

Agarwal, S., Hoque, M., Bandara, N., Pal, K., & Sarkar, P. (2020). Synthesis and characterization of tamarind kernel powder-based antimicrobial edible films loaded with geraniol. *Food Packaging and Shelf Life, 26*, Article 100562. https://doi.org/10.1016/j.fpsl.2020.100562

Alzate, P., Gerschenson, L., & Flores, S. (2020). Ultrasound application for production of nano-structured particles from esterified starches to retain potassium sorbate. *Carbohydrate Polymers, 247*, Article 116759. https://doi.org/10.1016/j.carbpol.2020.116759

Alzate, P., Miramont, S., Flores, S., & Gerschenson, L. (2017). Effect of the potassium sorbate and carvacrol addition on the properties and antimicrobial activity of tapioca starch–Hydroxypropyl methylcellulose edible films. *Starch-Stärke, 69*(5-6), Article 600261. https://doi.org/10.1002/star.201600261

Arismendi, C., Chillo, S., Conte, A., Del Nobile, M., Flores, S., & Gerschenson, L. (2013). Optimization of physical properties of xanthan gum/tapioca starch edible matrices containing potassium sorbate and evaluation of its antimicrobial effectiveness. *LWT-Food Science and Technology, 53*(1), 290–296. doi: 10.1016/j.lwt.2013.01.022.

Ashrit, P., Sadanandan, B., Kyathsandra Natraj, L., Shetty, K., Vaniyamparambath, V., & Raghu, A. (2022). A microplate-based Response Surface Methodology model for growth optimization and biofilm formation on polystyrene polymeric material in a *Candida albicans* and *Escherichia coli* co-culture. *Polymers Advanced Technologies, 33*(9), 2872–2885. doi: 10.1002/pat.5753.

Atta, O., Manan, S., Shahzad, A., Ul-Islam, M., Ullah, M., & Yang, G. (2021). Biobased materials for active food packaging: A review. *Food Hydrocolloids, 125*, Article 107419. https://doi.org/10.1016/j.foodhyd.2021.107419

Azeredo, H., & Waldron, K. (2016). Crosslinking in polysaccharide and protein films and coatings for food contact–A review. *Trends in Food Science & Technology, 52*, 109–122. https://doi.org/10.1016/j.tifs.2016.04.008

Barros Neto, B., Spacino, I., & Bruns, R (2003). *Como Fazer Experimentos. Pesquisa e Desenvolvimento na Ciência e na Indústria* (2nd ed.). Sao Paulo: Unicamp.

Basch, C., Jagus, R., & Flores, S. (2013). Physical and antimicrobial properties of tapioca starch-HPMC edible films incorporated with nisin and/or potassium sorbate. *Food and Bioprocess Technology, 6*, 2419–2428. https://doi.org/10.1007/s11947-012-0860-3

Bhargava, N., Sharanagat, V. S., Mor, R. S., & Kumar, K. (2020). Active and intelligent biodegradable packaging films using food and food waste-derived bioactive compounds: A review. *Trends in Food Science & Technology, 105*, 385–401. https://doi.org/10.1016/j.tifs.2020.09.015

Berti, S., Jagus, R., & Flores, S. (2021). Effect of rice bran addition on physical properties of antimicrobial biocomposite films based on starch. *Food and Bioprocess Technology, 14*(9), 1700–1711. https://doi.org/10.1007/s11947-021-02669-0

Campos, C., Gerschenson, L., & Flores, S. (2011). Development of edible films and coatings with antimicrobial activity. *Food and Bioprocess Technology, 4*, 849–875. https://doi.org/10.1007/s11947-010-0434-1

Cao, T., & Song, K. (2019). Effects of gum karaya addition on the characteristics of loquat seed starch films containing oregano essential oil. *Food Hydrocolloids, 97*, Article 105198. https://doi.org/10.1016/j.foodhyd.2019.105198

Chawla, R, Sivakumar, S., & Kaur, H. (2021). Antimicrobial edible films in food packaging: Current scenario and recent nanotechnological advancements-a review. *Carbohydrate Polymer Technologies and Applications, 2*, Article 100024. https://doi.org/10.1016/j.carpta.2020.100024

Chillo, S., Flores, S., Mastromatteo, M., Conte, A., Gerschenson, L., & Del Nobile, M. (2008). Influence of glycerol and chitosan on tapioca starch-based edible film properties. *Journal of Food Engineering, 88*(2), 159–168. https://doi.org/10.1016/j.jfoodeng.2008.02.002

Dhumal, C., Ahmed, J., Bandara, N., & Sarkar, P. (2019). Improvement of antimicrobial activity of sago starch/guar gum bi-phasic edible films by incorporating carvacrol and citral. *Food Packaging and Shelf Life, 21*, Article 100380. https://doi.org/10.1016/j.fpsl.2019.100380

Eriksen, M., Christiansen, J., Daugaard, A., & Astrup, T. (2019). Closing the loop for PET, PE and PP waste from households: Influence of material properties and product design for plastic recycling. *Waste Management, 96*, 75–85. https://doi.org/10.1016/j.wasman.2019.07.005

Florez, M., Guerra, E., Cazon, P., & Vazquez, M. (2022). Chitosan for food packaging: Recent advances in active and intelligent films. *Food Hydrocolloids, 124*, Article 107328. https://doi.org/10.1016/j.foodhyd.2021.107328

García, L., Cabrera, G., Soria, C., Castaño, J., Guadarrama, A., & Rodríguez, S. (2022). Progress in starch-based materials for food packaging applications. *Polysaccharides, 3*, 136–177. https://doi.org/10.3390/polysaccharides3010007

Ghanbarzadeh, B., Almasi, H., & Entezami, A. (2011). Improving the barrier and mechanical properties of corn starch-based edible films: Effect of citric acid and carboxymethyl cellulose. *Industrial Crops and Products, 33*(1), 229–235. https://doi.org/10.1016/j.indcrop.2010.10.016

González, L., Loubes, M., Bertotto, M., Baeza, R., & Tolaba, M. (2021). Flow behavior and syneresis of ball milled rice starch and their correlations with starch structure. *Carbohydrate Polymer Technologies and Applications, 2*, Article 100168. https://doi.org/10.1016/j.carpta.2021.100168

Guo, L., Sato, H., Hashimoto, T., & Ozaki, Y. (2010). FTIR study on hydrogen-bonding interactions in biodegradable polymer blends of Poly(3-hydroxybutyrate) and Poly(4-vinylphenol). *Macromolecules, 43*, 3897–3902. https://doi.org/10.1021/ma100307m

Herniou, C., Mendieta, J., & Gutiérrez, T. (2019). Characterization of biodegradable/non-compostable films made from cellulose acetate/corn starch blends processed under reactive extrusion conditions. *Food Hydrocolloids, 89*, 67–79. https://doi.org/10.1016/j.foodhyd.2018.10.024

Janardhnan, S., & Sain, M. (2011). Isolation of cellulose nanofibers: Effect of biotreatment on hydrogen bonding network in wood fibers. *International Journal of Polymer Science*, 1–6. https://doi.org/10.1155/2011/279610, 2011.

Kregiel, D., Berlowska, J., & Ambroziak, W. (2012). Adhesion of yeast cells to different porous supports, stability of cell-carrier systems and formation of volatile by-products. *World Journal of Microbiology and Biotechnology, 28*, 3399–3408. https://doi.org/10.1007/s11274-012-1151-x

Kumar, S, kumar, V., & Kumar, S. (2018). The influence of bound water on the FTIR characteristics of starch and starch nanocrystals obtained from selected natural sources. *Starch - Stärke.* , Article 1700026. https://doi.org/10.1002/star.201700026

Li, J., Ye, F., Lei, L., & Zhao, G. (2018). Combined effects of octenyl succination and oregano essential oil on sweet potato starch films with an emphasis on water resistance. *International Journal of Biological Macromolecules, 115*, 547–553. https://doi.org/10.1016/j.ijbiomac.2018.04.093

Li, K., Zhu, J., Guan, G., & Wu, H. (2019). Preparation of chitosan-sodium alginate films through layer-by-layer assembly and ferulic acid crosslinking: Film properties, characterization, and formation mechanism. *International Journal of Biological Macromolecules, 122*, 485–492. https://doi.org/10.1016/j.ijbiomac.2018.10.188

Liu, Y., Zhao, J., Peng, Y., Luo, J., Cao, L., & Liu, X. (2020). Comparative study on the properties of epoxy derived from aromatic and heteroaromatic compounds: The role of hydrogen bonding. *Industrial & Engineering Chemistry Research, 59*, 1914–1924. doi: 10.1021/acs.iecr.9b05904.

Lozano, J., Díaz, N., Velasco, C., Melo, J., Páramo, U., Paraguay, F., & Zapién, S. (2018). Chitosan-starch films with natural extracts: Physical, chemical, morphological and thermal properties. *Materials, 11*(1), 120. https://doi.org/10.3390/ma11010120

Ma, X., Jian, R., Chang, P., & Yu, J. (2008). Fabrication and characterization of citric acid-modified starch nanoparticles/plasticized-starch composites. *Biomacromolecules, 9*(11), 3314–3320. https://doi.org/10.1021/bm800987c

Mathew, S., Brahmakumar, M., & Abraham, T. (2006). Microstructural imaging and characterization of the mechanical, chemical, thermal, and swelling properties of starch–chitosan blend films. *Biopolymers: Original Research on Biomolecules, 82*(2), 176–187. https://doi.org/10.1002/bip.20480

Meereboer, K., Misra, M., & Mohanty, A. (2020). Review of recent advances in the biodegradability of polyhydroxyalkanoate (PHA) bioplastics and their composites. *Green Chemistry, 22*, 5519–5558. doi: 10.1039/d0gc01647k.

Nasalapure, A., Chalannavar, R., Kasai, D., Reddy, K., & Raghu, A. (2021). Novel polymeric hydrogel composites: Synthesis, physicochemical, mechanical and biocompatible properties. *Nano Express, 2*, Article 030003. https://doi.org/10.1088/2632-959X/ac11bf

Osimani, A., Garofalo, C., Harasym, J., & Aquilanti, L. (2022). Use of essential oils against foodborne spoilage yeasts: Advantages and drawbacks. *Current Opinion in Food Science, 45*, Article 100821. https://doi.org/10.1016/j.cofs.2022.100821

Pelissari, F., Grossmann, M., Yamashita, F., & Pineda, E. (2009). Antimicrobial, mechanical, and barrier properties of cassava starch– Chitosan films incorporated with oregano essential oil. *Journal of agricultural and food chemistry, 57*, 7499–7504. https://doi.org/10.1021/jf9002363

Plastics Europe Report (2022). Plastics – the Facts 2022. October 2022. https://plasticseurope.org/knowledge-hub/plastics-the-facts-2022/Last accessed 10 February 2023.

Shahbazi, M., Majzoobi, M., & Farahnaky, A. (2018). Physical modification of starch by high-pressure homogenization for improving functional properties of κ-carrageenan/starch blend film. *Food Hydrocolloids, 85*, 204–214. https://doi.org/10.1016/j.foodhyd.2018.07.017

Shi, R., Zhang, Z., Liu, Q., Han, Y., Zhang, L., Chen, D., & Tian, W. (2007). Characterization of citric acid/glycerol co-plasticized thermoplastic starch prepared

by melt blending. *Carbohydrate Polymers, 69*(4), 748–755. https://doi.org/10.1016/j.carbpol.2007.02.010

Silverstein, R., Bassler, G., & Morrill, T. (1991). *Spectrometric identification of organic compounds* (5th ed.). New York: Wiley.

Sun, L., Sun, J., Chen, L., Niu, P., Yang, X., & Guo, Y. (2017). Preparation and characterization of chitosan film incorporated with thinned young apple polyphenols as an active packaging material. *Carbohydrate Polymers, 163*, 81–91. https://doi.org/10.1016/j.carbpol.2017.01.016

Suput, D., Lazic, V., Pezo, L., Markov, S., Vastag, Z., Popovic, L., & Popovic, S. (2016). Characterization of starch edible films with different essential oils addition. *Polish Journal of Food and Nutrition Sciences, 66*(4), 277–285. https://doi.org/10.1515/pjfns-2016-0008

Valencia, C., Vargas, M., Atarés, L., & Chiralt, A. (2018). Thermoplastic cassava starch-chitosan bilayer films containing essential oils. *Food Hydrocolloids, 75*, 107–115. https://doi.org/10.1016/j.foodhyd.2017.09.008

Vásconez, M., Flores, S., Campos, C., Alvarado, J., & Gerschenson, L. (2009). Antimicrobial activity and physical properties of chitosan–tapioca starch based edible films and coatings. *Food Research International, 42*(7), 762–769. https://doi.org/10.1016/j.foodres.2009.02.026

Wang, L., Liu, F., Jiang, Y., Chai, Z., Li, P., Cheng, Y., & Leng, X. (2011). Synergistic antimicrobial activities of natural essential oils with chitosan films. *Journal of Agricultural and Food Chemistry, 59*(23), 12411–12419. https://doi.org/10.1021/jf203165k

Wu, H., Lei, Y., Lu, J., Zhu, R., Xiao, D., Jiao, C., & Li, S. (2019). Effect of citric acid induced crosslinking on the structure and properties of potato starch/chitosan composite films. *Food Hydrocolloids, 97*, Article 105208. https://doi.org/10.1016/j.foodhyd.2019.105208

Yam, K. (2009). *The Wiley encyclopedia of packaging technology* (3rd ed.). New York: John Wiley & Sons.

Zagar, E., & Grdadolnik, J. (2003). An infrared spectroscopic study of H-bond network in hyperbranched polyester polyol. *Journal of Molecular Structure, 658*, 143–152. https://doi.org/10.1016/S0022-2860(03)00286-2

Zheng, K., Xiao, S., Li, W., Wang, W., Chen, H., Yang, F., & Qin, C. (2019). Chitosan-acorn starch-eugenol edible film: Physico-chemical, barrier, antimicrobial, antioxidant and structural properties. *International Journal of Biological Macromolecules, 135*, 344–352. https://doi.org/10.1016/j.ijbiomac.2019.05.151