

Antioxidant Migration Studies in Chitosan Films Incorporated with Plant Extracts

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ABSTRACT: The aim of this work was to develop an active biopolymer based on chitosan by incorporating natural antioxidants. Five essential oils (ginger, rosemary, sage, tea tree and thyme) and six hydro-alcoholic extracts (from ginger, green and black tea, kenaf leaves, rosemary and sage plants) were tested. Migration assays were carried out to evaluate the films' activity, and total phenolic content and antioxidant activity were monitored in the simulant during storage. Interaction between natural compounds and polymeric matrix was evaluated by FTIR spectroscopy. The diffusion of the phenolic compounds was not detected in the films incorporated with hydro-alcoholic extracts (HAEs), indicating their entrapment in the chitosan. Migration was observed in the films with essential oils (EOs), and biobased films incorporated with ginger, sage or rosemary essential oils, presented the highest diffusion and antioxidant activity in the simulant, highlighting their functionality and potential to be used as food active packaging material.

KEYWORDS: Active packaging, antioxidant activity, biopolymers, chitosan, phenolic content

1 INTRODUCTION

The most frequently used natural antioxidants are associated with secondary plant metabolites such as vitamins C and E (ascorbic acid and tocopherol, respectively) and numerous plant phytochemicals (such as phenolic acids, flavonoids, terpenes, carotenoids, volatile oils, among others) [1, 2]. Phenolic compounds are a large group of molecules (more than 8000 have been reported) with known antioxidant activity, which are widely dispersed in plants and are common in diets worldwide [1, 2]. The antioxidant activities of phenolic compounds have been extensively analyzed [1, 3]; they can participate in protection against the harmful effects of ROS (reactive oxygen species) and exhibit a wide range of biological effects. Therefore, these compounds have remarkable potential to be used as food preservatives either by direct incorporation as additives [4, 5] or into polymeric matrices as active packaging systems [5–10].

Oxidation is a naturally occurring process in biological systems, and in many cases is considered undesirable in food matrices, leading to their deterioration and resulting in the limitation of the product shelf life [11]. Active packaging with antioxidant properties is a novel technology approach to improve the preservation of food products, and the use of natural antioxidants as active compounds is driven by the trend of customer preference for more natural (less synthetic) products [12, 13].

Chitosan (poly- β -(1,4)-2-amino-2-deoxy-D-glucose) is a natural biopolymer obtained from the deacetylation of poly chitin (N-acetyl-D-glucosamine) found in exoskeletons of crustaceans and insects and on the cell walls of fungi and microorganisms [14]. This polysaccharide, the second most abundant in nature, is derived from renewable resources and due to its biodegradability, biocompatibility, ability to form membranes, gels, fibers, particles and resistant flexible films, and its nontoxicity, has potential to be used in food-grade films [15]. Moreover, biopolymers, including chitosan, and their applications, have shown an exponential growth in the past decades as a response to consumer demand for more environmentally friendly products [16].

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Supplementation of the biopolymers by incorporation of antioxidant compounds is being considered as a strategy to improve the bioactivity of the materials [17]. In fact, there are some studies and reviews on the antioxidant activity of biobased films incorporated with natural antioxidants (e.g., [6, 8, 18]). Yet, the existing studies use pure compounds or natural antioxidants, individually, and only a few studies compare more than two antioxidants. The reviews on the subject are on antioxidant release systems, the various methods for incorporating antioxidant compounds into the package, the issues to be considered in packaging design, and the methods used to determine the oxidation protection effect of antioxidant active films. But, there are no relevant studies that compare the antioxidant activity of different active biobased films (namely those based on chitosan). In a previous work, Souza *et al.* [19] compared chitosan films incorporated with several natural antioxidants in terms of their physical properties. Among the tested extracts, black tea and green tea hydro-alcoholic extracts (HAEs) and sage, thyme and rosemary essential oils (EOs) were underlined as the most favorable due to the improved mechanical properties of the resulting films. However, in order to rank the different active films for the food packaging industry, it is also mandatory to evaluate the migration potential of the antioxidants to the food matrices or their barrier effect when incorporated into the biobased films. Thus, the aim of this work was to test the migration of antioxidant agents incorporated into the chitosan films, namely, five essential oils and six hydro-alcoholic extracts, into a fat food simulant.

2 MATERIAL AND METHODS

2.1 Materials

Chitosan with high molecular weight (31–37 kDa) and with a deacetylation degree of 75% was used as polymeric matrix and purchased from Sigma-Aldrich (Iceland). Active compounds were purchased in a local market: five different food-grade essential oils, used as purchased (Biover, Belgium), namely, Ginger EO (*Zingiber officinale* Roscoe); Rosemary EO (*Rosmarinus officinalis* L. ct. camphor); Sage EO (*Salvia lavandulifolia* Vahl); Tea Tree EO (*Melaleuca alternifolia* [Maiden & Betche] Cheel); and Thyme EO (*Thymus zygis* Loefl. ex L. ct. linolol); and six different HAEs prepared from dried plants, namely, Black and Green Tea (*Camellia sinensis* [L.] Kuntze) from the Azores Islands (Gorreana, Portugal); Rosemary (*Rosmarinus officinalis* L.); Ginger (*Zingiber officinale* Roscoe); Sage (*Salvia officinalis* L.); and Kenaf leaves (*Hibiscus cannabinus* L.) variety Everglades 41, harvested in September 2005 before

flowering (14th–17th October), from kenaf pilot fields in Caparica (latitude 38°40'03"N, longitude 9°12'8"W, altitude of 50 m), Portugal.

2.2 Chemicals

All chemicals used were of analytical reagent grade. Acetic acid glacial, glycerol, gallic acid, and Tween 80 (polyethylene glycol sorbitan monolaurate) were purchased from Alfa Aesar (Germany), while Folin-Ciocalteu reagent, sodium carbonate anhydrous, sodium hydroxide, sodium nitrite, aluminum chloride and 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Panreac (Spain). Ethanol absolute, 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium peroxodisulfate, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and (+)-catechin hydrate (> 96%) were supplied by Sigma-Aldrich (Germany). All the water used was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.3 Hydro-Alcoholic Extracts (HAEs)

Hydro-alcoholic extracts were obtained using solvent extraction assisted by ultrasonic force according to Turkmen *et al.* [20] and Pascoal *et al.* [4] with modifications. To enhance the superficial contact and maximize the phenolic content in the solvent media, prior to the extraction, all plants were ground into powder using an electric blender (ProfiCook model KSW 1021, Germany). To obtain each plant extract, 5 g of powder was weighed in an analytical balance (Mettler Toledo model AB204, Switzerland) and mixed in 50 mL of ethanol 50% (v/v) using an Ultra-Turrax® disperser (IKA model T18, Germany). The mixture was kept refrigerated in the dark at 7 °C ± 2 °C for 24 h and then submitted to ultrasonication for 30 min/50 Hz in an ultrasonic bath (Selecta, Barcelona, Spain) at room temperature (20 °C ± 2 °C). The system was centrifuged in conical tubes of 80 mL for 30 min at 4 °C with 10000 × g measured in the bottom of the tube using an angle rotor (6 × 80/85 mL, NR12165, Sigma, Germany) and a refrigerated centrifuge (Sigma model 4K15, Germany), the supernatant was removed, and the extraction was repeated once more. The combined supernatants from both extractions were filtered through Whatman n° 4 filter paper, and the volume was corrected to 100 mL with ethanol 50% followed by concentration by rotary evaporator (Büchi Rotavapor model R-200, Switzerland) at 40 °C (Büchi heating bath model B-490, Switzerland) until a final volume of 25 mL (4 times reduction) was reached and, to avoid degradation, the extracts were stored at –18 °C until analysis and use.

2.4 EO and HAE Characterization

The HAEs and EOs used in the films were characterized by means of their total phenolic content (TPC) and antioxidant activity. Total phenolic content was determined according to the Folin-Ciocalteu method [21] with slight modifications. Briefly, 1 mL of diluted HAE or EO was mixed with 3 mL of Milli-Q water and 0.25 mL of Folin-Ciocalteu reagent. The mixture was incubated for 5 min at room temperature previous to the addition of 0.75 mL of sodium carbonate solution 5% (w/v), and then it was agitated and stored in a dark chamber for 60 min. The mixture absorbance was measured at 760 nm using a UV-Vis spectrophotometer (SPEKOL 1500, Analytik Jena, Germany). A calibration curve was constructed using gallic acid solutions from 0–120 mg.L⁻¹. Total phenolic content is expressed in mg gallic acid equivalent (GAE).mL⁻¹ of EO or g dried plant (in the case of HAE).

Antioxidant activity was measured by two different methods: DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay [22] and ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assays [23].

The ability to scavenge free radicals by the EOs and HAEs tested was measured by DPPH assay. An aliquot, or a diluted aliquot, of 1 mL of each natural antioxidant was mixed with 3 mL of 60 µmol.L⁻¹ DPPH ethanolic solution. The mixture was kept in the dark for 20 min and the absorbance was read at 517 nm using a UV-VIS spectrophotometer (SPEKOL 1500, Analytik Jena, Germany). DPPH, in its radical form, is purple and has a maximum absorbance at 517 nm. Upon the activity of antioxidant, the compound is reduced (losing the purple color) and its absorption decreases. The results were calculated in two forms: by the percentage of inhibition using Equation 1 and as the Trolox equivalent antioxidant capacity (TEAC) using a calibration curve.

Scavenging(%)

$$= \frac{(\text{Initial absorbance} - \text{Sample absorbance})}{\text{Initial absorbance}} \times 100 \quad (1)$$

ABTS assay was also carried out. Briefly, to generate the ABTS^{•+} cation equal parts of ABTS solution (7 Mm.L⁻¹) and potassium persulfate solution (2.45 Mm.L⁻¹) were mixed and incubated for 16 h at room temperature in the dark. Then, ABTS^{•+} cations generated were added to ethanol 80% until an absorbance of 0.7 ± 0.05 nm at 734 nm was reached. This was the ABTS^{•+} working solution used in the assay. An aliquot or a diluted aliquot of 0.5 mL of each natural antioxidant (EO or HAE) was added to 3.5 mL of the resulting ABTS^{•+} working solution, mixed and incubated for 6 min in the dark and the final absorbance was measured in a

UV-Vis spectrophotometer (SPEKOL 1500, Analytik Jena, Germany) at 734 nm. A calibrating curve was constructed using standard solutions of Trolox. The results were expressed as Trolox equivalents (µmol Trolox.mL⁻¹ EOs or g dry plant).

2.5 Film Preparation

Chitosan films were casted according to Souza *et al.* [19]. Film-forming dispersion (FFD) was prepared by dissolving 1.5% (w/v) of chitosan in 1% (v/v) of glacial acetic acid solution in ultrapure water with constant agitation using a magnetic stir plate during a 24 hour period at room temperature. Glycerol, the plasticizer, was incorporated into the proportion of 30% (w/w chitosan) and the system was agitated for 5 min to complete homogenization. On treatments where EOs were added, Tween 80 at a level of 0.2% w/v of essential oil was used as emulsifier [24]. The EO or HAE was incorporated into the system at the level of 1% (v/v FFD) and homogenized on a magnetic stir plate for an additional 5 min at room temperature. Degasification was performed with an ultrasonic bath for 5 min. Films were casted in a glass mold (18×25 cm) using 140 mL of the resulting solutions and dried for 72 h at room temperature. Chitosan films without incorporation of active compounds were used as control. After drying, films were peeled and stored protected from light until evaluation. Three repetitions were done for each EO and HAE tested, as well as with pristine chitosan films.

2.6 Sample Characterization

2.6.1 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The FTIR spectra of biobased films were from 4000 to 650 cm⁻¹, resolution of 1 cm⁻¹ in ATR mode using a Spectrum Two FTIR spectrometer (PerkinElmer, USA) according to Siripatrawan and Harte [25].

2.6.2 Migration Assay

Migration assay was carried out according to López-de-Dicastillo *et al.* [26]. The release of the active compounds from polymeric matrix was determined by the specific migration into 95% ethanol solution (fatty food simulant) at 37 °C ± 2 °C during 10 days. Square samples of 2.4 cm² of each treatment were placed inside amber vials and 4 mL of the simulant was added, achieving an area-to-volume ratio of 6 dm².L⁻¹. The average thickness of the films produced (61 µm) did not statistically change with the incorporation of

the natural antioxidant extracts [14]. Therefore, the antioxidant properties of each film can be expressed based on sample surface area instead of sample weight. Periodically, total phenol content present in the simulant media and its antioxidant activity were determined by Folin-Ciocalteu method and DPPH/ABTS assays, respectively, as described above in Section 2.4.

Complementary diffusion coefficients were also calculated from the plot of $M_{F,t}/M_{P,0}$ versus $t^{0.5}$ using initial migration data according to the model based on the Fick's second law described in Equation 2 [27]:

$$\frac{M_{F,t}}{M_{P,0}} = \frac{2}{L_p} \left(\frac{Dt}{\pi} \right)^{0.5} \quad (2)$$

where $M_{F,t}$ (mg AGE) is the amount of migrant in the food (simulant) at time t , $M_{P,0}$ (mg AGE) is the initial amount of migrant in the packaging film, D ($\text{cm}^2 \cdot \text{s}^{-1}$) is the diffusion coefficient of migrant in the packaging film and L_p (cm) is the thickness of the packaging film.

2.7 Statistical Analysis

For each active compound used, three samples were analyzed and the results were expressed as mean values \pm standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test with $\alpha = 0.05$. Statistical Analysis System (SAS), version 9.2, was used to treat the data.

3 RESULTS AND DISCUSSION

3.1 EO and HAE Characterization

Both EO and HAE incorporated into the chitosan film-forming solution demonstrated high content in total polyphenols with remarkable antioxidant activity (Table 1).

A good correlation was found between HAEs' antioxidant activity and HAEs' TPC, i.e., hydro-alcoholic extracts with highest TPC (green and black tea and rosemary HAEs) were also the ones that presented the highest antioxidant activity, and the ones with the lowest TPC (kenaf and ginger HAEs) also presented the lowest antioxidant activity. A comparison of the findings with published data is difficult due to the different types of extraction methods used [28]. Kenaf, ginger and green and black tea HAEs showed smaller TPC than what was reported [4, 20, 29–32]. In opposition, rosemary and sage HAEs were higher than those reported in the literature for similar extraction processes [2]. But differences can be attributed to the different time of contact, the different energy/temperature employed or by the use of a solvent to which polyphenols have higher affinity (e.g., methanol) [20, 29, 31].

The antioxidant activity of the EOs was correlated with their TPC for ginger and thyme, but not for the remaining EOs studied. Tea tree was the richest in phenolic content but the poorest in terms of antioxidant activity. Rosemary EOs showed a low TPC in

Table 1 Total phenolic content and antioxidant activity of essential oils and hydro-alcoholic extracts incorporated into chitosan films.

Sample	Total phenolic content (mg gallic acid/g dry plant)	DPPH assay ($\mu\text{molTrolox/g dry plant}$)	ABTS assay ($\mu\text{molTrolox/g dry plant}$)
Green tea hydro alcoholic extract	68 ± 1^a	904 ± 36^a	1636 ± 77^a
Black tea hydro alcoholic extract	30 ± 1^b	592 ± 32^b	920 ± 20^b
Kenaf hydro alcoholic extract	11.8 ± 0.2^d	160 ± 3^e	147 ± 2^e
Ginger hydro alcoholic extract	7.4 ± 0.02^d	43.6 ± 0.8^f	199 ± 0^e
Rosemary hydro alcoholic extract	33 ± 0^b	476 ± 14^c	508 ± 30^c
Sage hydro alcoholic extract	20.8 ± 0.4^c	306 ± 8^d	347 ± 8^d
Sample	Total phenolic content (mg gallic acid/mL)	DPPH assay ($\mu\text{molTrolox/mL}$)	ABTS assay ($\mu\text{molTrolox/mL}$)
Ginger essential oil	16 ± 1^b	2.83 ± 0.06^a	10.0 ± 0.3^a
Rosemary essential oil	8.9 ± 0.8^c	2.98 ± 0.09^a	14.8 ± 0.1^a
Sage essential oil	7.0 ± 0.8^{cd}	0.98 ± 0.02^b	11.9 ± 0.2^a
Tea tree essential oil	23 ± 1^a	0.30 ± 0.08^d	3.9 ± 0.2^b
Thyme essential oil	6.4 ± 0.3^d	0.72 ± 0.02^c	4.4 ± 0.1^b

a-f: Different superscripts within the same column, within essential oils or hydro alcoholic extracts, indicate significant differences among samples ($p < 0.05$).

comparison to tea tree and ginger, but its antioxidant activity was the highest. Sage EO presented a similar TPC to rosemary EO, which was correlated with the antioxidant activity measured through the DPPH assay. Yet, sage EO results by the ABTS assay showed a significant high antioxidant activity. This can be explained by its different chemical composition, since the antioxidant activity depends not only on the concentration of phenolic compounds but also its phenolic profile [29]. Results in essential oil TPC and antioxidant activity presented in this study show differences to those presented in the literature. This can be due to several factors, such as geographical location, season, environmental conditions, nutritional status of the plants, the solvent used to dilute the EOs, among other factors [33].

3.2 Migration Assay

Only chitosan films incorporated with EO showed migration into fatty simulant media after 10 days of storage at $37 \text{ }^{\circ}\text{C} \pm 2 \text{ }^{\circ}\text{C}$. HAE extracts did not diffuse toward simulant, thus no phenolic content or antioxidant activity was detected by Folin-Ciocalteu method and DPPH/ABTS assays, respectively. Apparently, the compounds present in HAE extracts had a stronger affinity with the polymeric matrix than with the simulant used (fatty food simulant), resulting in lower solubility and undetectable diffusion by the methods used. Good entrapment of active compounds within polymer matrix reduces its release to simulant [34]. According to a previous study [19], films incorporated with HAEs exhibited higher solubility in water than chitosan incorporated with EOs, and a lower swelling degree, suggesting lower free volume between knots

to swell with ethanol, thus lower relaxation of the matrix and release of active compounds. The incorporation of only 1% (v/v) of HAE in the chitosan and its hydrophilic character might also explain the results obtained. Perhaps novel formulations with higher content of hydro-alcoholic extracts may result in an observed diffusion to the simulant, as reported previously by López-de-Dicastillo *et al.* [26], who incorporated more than 5% of green tea extracts in ethylene-vinyl alcohol copolymer (EVOH) films.

The good interaction between HAE and chitosan polymer is an advantage since the polyphenols of the extracts incorporated can also protect the thin film produced against oxidation [17]. Furthermore, as the final purpose of the material is to be used as active food packaging, when in direct contact with foods (complex matrices with either hydrophobic and hydrophilic parts), a different behavior is expected, with potential diffusion of the active compounds toward the products packaged resulting in gradual preservation throughout their shelf life. Also, the polyphenols entrapped in the chitosan chains can improve the barrier properties of the material, thus retarding possible contact of the food packaged with water, oxygen or light since the bioactive compounds represent a shield and a reaction media prior to contact with food.

Chitosan films incorporated with EOs exhibited an “exponential growth to a maximum” type migration profile (Figure 1), reaching equilibrium during the first 48 hours of the assay, as previously reported by several works in the literature [26,35]. A proportional release was observed in TPC with the TPC present in the crude EOs: a higher release of TPC to the simulant media was observed for the EOs that showed the highest TPC, ginger and tea tree; and films incorporated

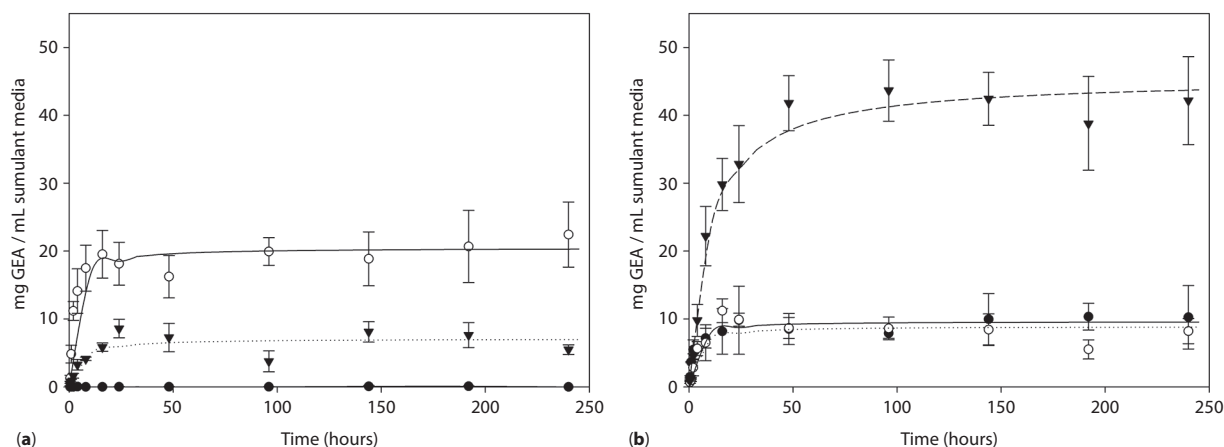


Figure 1 Total phenolic content of simulant media. Migration assay of chitosan films incorporated with different essential oils during 10 days storage at $37 \text{ }^{\circ}\text{C} \pm 2 \text{ }^{\circ}\text{C}$: (a) (—●—) Chitosan film; (···○···) Chitosan-Tea Tree essential oil film; (—▼—) Chitosan-Rosemary essential oil film; (b) (—●—) Chitosan-Sage essential oil film; (···○···) Chitosan-Thyme essential oil film; (—▼—) Chitosan-Ginger essential oil film.

with rosemary, sage and thyme EO, which showed a lower TPC, also released a lower amount of phenolic compounds to the simulant. All the films, except the chitosan-ginger EO film, showed a similar release ratio between maximum phenolic content diffused and the total incorporated into the chitosan (0.34–0.52, Table 2). Chitosan-ginger EO film showed the highest release ratio, 0.92 (Table 2). It is known that the diffusion process, characteristic in migrations of additives and contaminants from packaging materials, is governed by the models based on Fick's second law (Eq. 2), which have been extensively used to provide estimations of the diffusion coefficients based on experimental data [27, 36].

Calculated diffusion coefficients are correlated with the release ratio of active compounds (Table 2), since ginger EO showed the highest coefficient ($(5 \pm 1) \times 10^{-10}$ (cm².s⁻¹)), followed by sage EO ($(2.3 \pm 0.3) \times 10^{-10}$ (cm².s⁻¹)), thyme EO ($(1.5 \pm 0.4) \times 10^{-10}$ (cm².s⁻¹)), tea tree EO ($(1.1 \pm 0.3) \times 10^{-10}$ (cm².s⁻¹)) and rosemary EO ($(0.6 \pm 0.1) \times 10^{-10}$ (cm².s⁻¹)). The findings are in good agreement with the results reported by Ramos *et al.* [37] that calculated the diffusion coefficient of carvacrol and thymol from polypropylene films in different simulant media. The authors reported values of D in the same magnitude: 2×10^{-10} (cm².s⁻¹) and 1×10^{-10} (cm².s⁻¹) for carvacrol and thymol at ethanol 95%, respectively [37].

According to Jamshidian *et al.* [34], the fate of migration depends on several factors: (1) the characteristics of the polymer—its morphology, molecular weight, distribution, crystallinity, density, and orientation that may interfere with sizes, shapes and distribution of microcavities, therefore with diffusion path; (2) type of active compounds incorporated—its polarity, shape, chemical composition, solubility; (3) interaction polymer \times active compounds incorporated—plasticizing and anticizing effects. Considering that the amount of glycerol and EOs incorporated were constant, the difference in the migration profile can only

be explained by the type of active compounds incorporated, namely, its chemical composition, solubility and polarity. In the case of ginger EO, the higher release toward simulant media probably occurred due to a stronger solubility in ethanol 95%. The lipophilic character of the ginger EOs active compounds was also suggested in the review made by Brewer [29] on natural antioxidants.

Antioxidant activity of the compounds diffused is shown in Figures 2 and 3. For the DPPH assay and ABTS assay, the antioxidant activity of the simulant media reached a maximum during the first 48 hours of the studies, following the pattern of the phenolic release. The only exception to this behavior was chitosan incorporated with rosemary EO. In this film, the antioxidant activity increased along the migration assay, reaching a maximum only at 192 hours (although the equilibrium is not well defined in the ABTS assay). In this case, the compounds with antioxidant activity presented a lower rate of diffusion to the simulant media than what was observed for the other EO films. According to Brewer [29], solvents with medium polarity, such as ethanol 50%, are able to extract higher concentrations of compounds with antioxidant activity from rosemary EO than solvents with lower polarity, such as ethanol 95%, which was used in the migration assay, explaining the slow rate of diffusion of the active compounds to the simulant. However, the ethanolic solution obtained in the chitosan-rosemary EO migration study showed the highest antioxidant activity, which correlated with the high antioxidant activity shown by rosemary EO. Phenolic diterpenes (carnosic acid, carnosol, rosmannol, rosmadial, 12-methoxycarnosic acid, epi- and isorosmanol) and phenolic acids (rosmarinic and caffeic) are the constituents of rosemary EO with the highest antioxidant activity [29]. A good relation between antioxidant activity of the EOs and antioxidant activity of the migration solution were also observed for the remaining films incorporated with EOs. The higher

Table 2 Migration results in chitosan-based films.

Film	Diffusion coefficient (cm ² /s)	Maximum total diffused/total incorporated into films	Maximum total antioxidant activity in the simulant/total antioxidant activity in the film
Chitosan_Tea tree essential oil film	$(1.1 \pm 0.3) \times 10^{-10}$	0.40	0.88
Chitosan_Rosemary essential oil film	$(0.6 \pm 0.1) \times 10^{-10}$	0.34	0.61
Chitosan_Sage essential oil film	$(2.3 \pm 0.3) \times 10^{-10}$	0.52	0.94
Chitosan_Thyme essential oil film	$(1.5 \pm 0.4) \times 10^{-10}$	0.41	0.84
Chitosan_Ginger essential oil film	$(5 \pm 1) \times 10^{-10}$	0.92	1.0

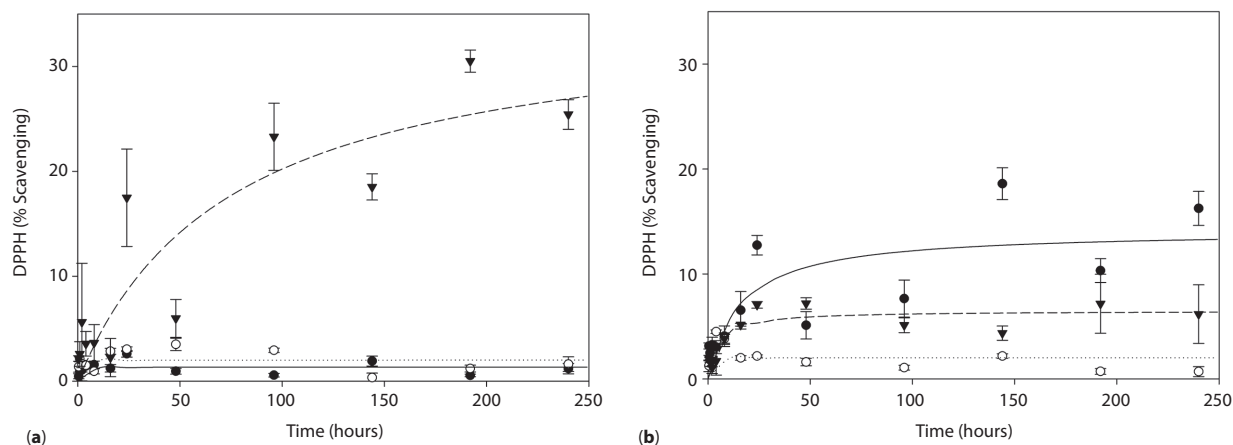


Figure 2 Antioxidant activity of simulant media expressed in DPPH by scavenging percentage. Migration assay of chitosan films incorporated with different essential oils during 10 days storage at $37\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$: (a) (—●—) Chitosan film; (···○···) Chitosan-Tea Tree essential oil film; (—▼—) Chitosan-Rosemary essential oil film; (b) (—●—) Chitosan-Sage essential oil film; (···○···) Chitosan-Thyme essential oil film; (—▼—) Chitosan-Ginger essential oil film.

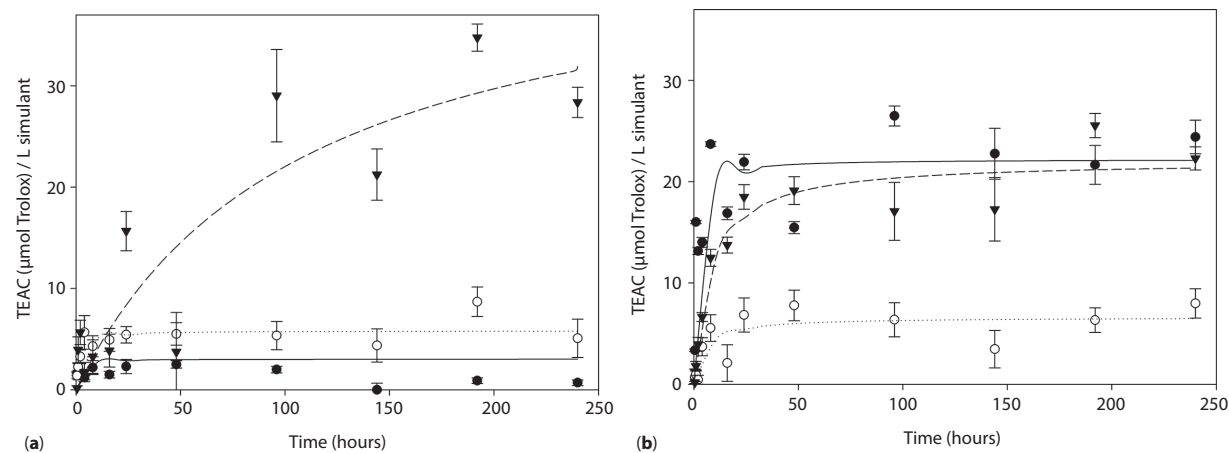


Figure 3 Antioxidant activity of simulant media expressed in Trolox equivalent (TEAC). Migration assay of chitosan films incorporated with different essential oils during 10 days storage at $37\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$: (a) (—●—) Chitosan film; (···○···) Chitosan-Tea Tree essential oil film; (—▼—) Chitosan-Rosemary essential oil film; (b) (—●—) Chitosan-Sage essential oil film; (···○···) Chitosan-Thyme essential oil film; (—▼—) Chitosan-Ginger essential oil film.

the antioxidant activity of the Eos, the higher the antioxidant activity observed in the migration solution (as was observed for rosemary EO in the DPPH and ABTS assays and also for ginger and sage EOs in the ABTS assay); and the lower the antioxidant activity of the Eos, the lower the antioxidant activity observed in the migration solution (as was observed for thyme and tea tree EOs) (Figures 2 and 3). The exception was ginger EO films in the DPPH assay. In this case, ginger EO presented a high antioxidant activity (similar to the one presented by rosemary EO), but in the simulant media it scavenges only 5% of the DPPH radicals, much lower than the 25% observed for rosemary EO (Figure 2). Ginger antioxidant activity may be attributed to the presence of phenolic acids (caffeic and gallic) and

volatiles (e.g., α - and β -pinene, gingerol, p-cymene) [29]. Sage antioxidant activity is linked with some of the same compounds found in rosemary (rosmanol, rosmadial, carnosol, carnosic acid and rosmarinic acid) along with flavonoids [29], and also with α - and β -pinene and 1,8-cineole [38]. Concerning thyme, the antioxidant compounds associated with it are thymol (the most effective) and other volatiles such as carvacrol, α - and β -pinene, 1,8-cineole, p-cymene and α -thujene, the phenolic acid, rosmarinic acid, and flavonoids [29,38]. The phenolic compounds found in tea tree are terpinen-4-ol, α - and γ -terpine and 1,8-cineole, but only the last has antioxidant activity [39].

All the films, except the chitosan-rosemary EO film, in terms of antioxidant activity measured through the

ABTS assay, showed a similar result for the maximum diffused or total incorporated into the chitosan (0.84–1.00, Table 2). Chitosan-rosemary EO film showed the lowest ratio, 0.61 (Table 2). Yet, this lower diffusion of compounds with antioxidant activity can be due to the slower release of the phenols from the chitosan matrix due to the low polarity of the ethanol 95%, as explained before. It also should be mentioned that the phenolic compounds released to the simulant media remained stable and did not degrade during the migration test (10 days) and no significant loss of volatile antioxidants was observed during the drying

of the films (< 5%). In fact, the method used to produce the films was chosen to avoid/prevent those losses.

3.3 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The ATR-FTIR spectra of chitosan films incorporated with natural antioxidants from different sources is shown in Figure 4.

In general, all spectra were similar since the amount of active compounds incorporated (1%) was small and

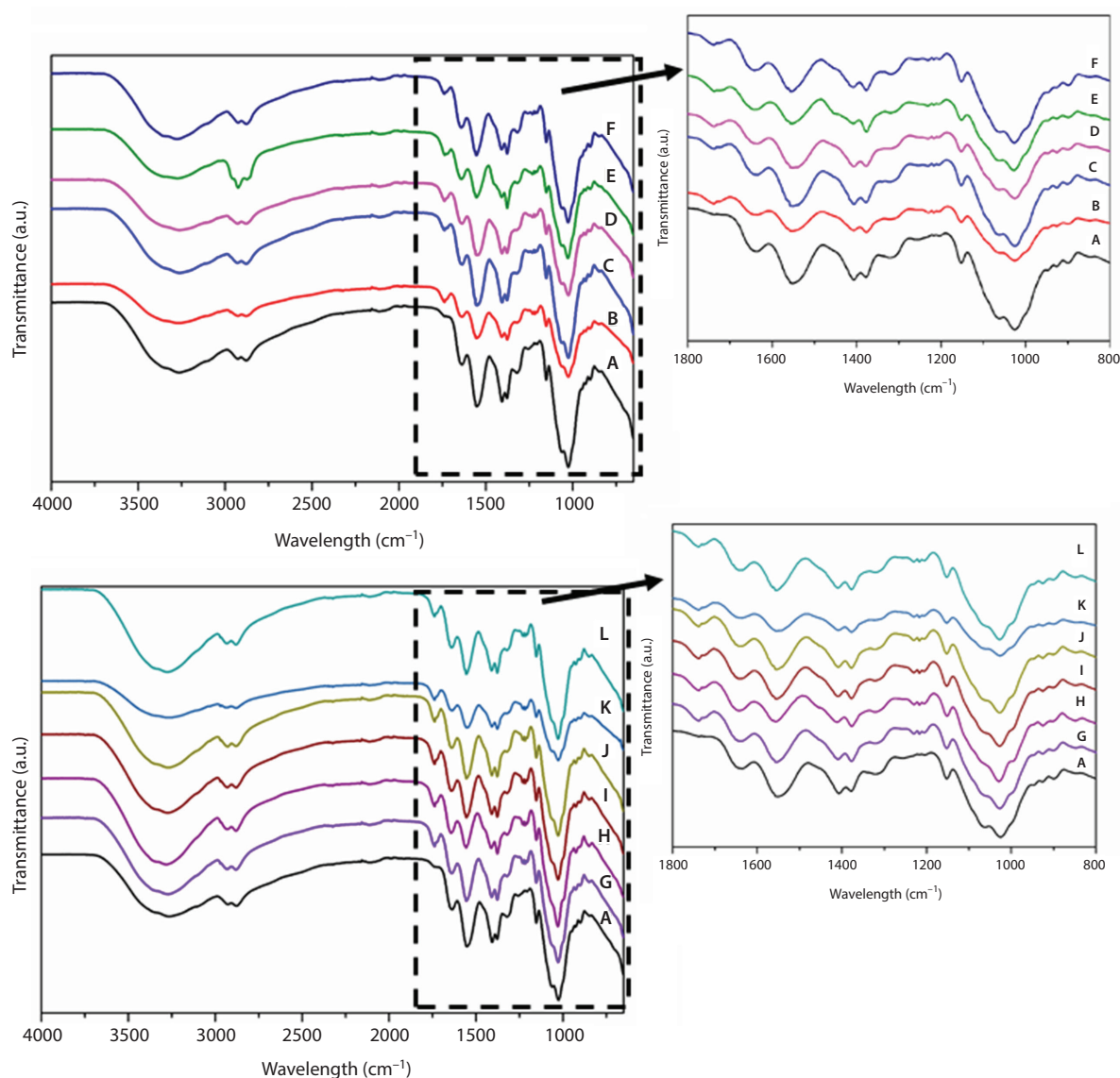


Figure 4 ATR-FTIR spectra of chitosan (Ch)-based films. (A) Neat chitosan film; (B) Chitosan-Tea Tree essential oil film; (C) Chitosan-Rosemary essential oil film; (D) Chitosan-Sage essential oil film; (E) Chitosan-Ginger essential oil film; (F) Chitosan-Thyme essential oil film; (G) Chitosan-Sage hydro-alcoholic extract film; (H) Chitosan-Kenaf hydro-alcoholic extract film; (I) Chitosan-Ginger hydro-alcoholic extract film; (J) Chitosan-Black Tea hydro-alcoholic extract film; (K) Chitosan-Green Tea hydro-alcoholic extract film; (L) Chitosan-Rosemary hydro-alcoholic extract film.

ATR mode was used. Infrared spectra of pristine chitosan films exhibited characteristic absorption bands at 3332 cm^{-1} (-OH asymmetric stretching); 3246 cm^{-1} (-NH asymmetric stretching); 2867 cm^{-1} (C-H bonding of $-\text{NHCOCH}_3$ metal group); 1635 cm^{-1} (amide I); 1550 cm^{-1} (vibration $-\text{NH}_2$ – amide II); 1337 cm^{-1} (skeleton vibration involving C-N stretching of amide III) and 1376-1404 cm^{-1} ($-\text{CH}_2$ bending) [40–42]. Also, the peaks at 922 cm^{-1} , 1022 cm^{-1} and 1047 cm^{-1} (skeletal vibrations involving the C–O stretching) and 1150 cm^{-1} (asymmetric stretching of the C–O–C bridge) are characteristic of the saccharide structure of chitosan [41].

The incorporation of natural antioxidants provoked changes in the absorption spectra between bands 1200 cm^{-1} and 1400 cm^{-1} due to the hydroxyl groups of phenolic compounds and the C–O and C=O vibrations [25, 41, 42]. Absorption at 1550 cm^{-1} (amide II) decreased, especially with the incorporation of ginger and tea tree EOs and kenaf and green tea HAEs, suggesting that chitosan amide group bonded with the polyphenols present in the EOs and HAEs tested [42].

The new peak at 1715–1730 cm^{-1} that was seen in all the spectra with OEs and HAEs, was attributed to an ester bond between carbonyl groups of phenolic acids and chitosan, as suggested by Silva-Weiss *et al.* [42] and Siripatrawan and Harte [25]. The absorption reduction observed between 3500 and 3000 cm^{-1} for some of the films incorporated with EOs or HAEs (namely green tea HAE and ginger, tea tree and sage EOs) confirms the linkage between phenolic compounds and chitosan, as these bands correspond to stretching of free hydroxyl and to asymmetric and symmetric stretching of the N–H bonds in amino group. This interaction between polyphenolic hydroxyl groups and chitosan amide groups results in improvements in mechanical and barrier properties [19, 25].

4 CONCLUSIONS

Chitosan films were successfully casted with incorporation of different natural compounds, either hydroalcoholic extracts or essential oils, resulting in homogeneous materials for further use in the food industry. The successful incorporation and interaction between the polymeric matrices and the phenolic compounds was observed through migration assays, especially for films incorporated with HAEs, where no diffusion was observed for all the extracts tested. The good interaction between phenolic compounds from HAEs and chitosan can protect the food packaged by other mechanisms, such as acting as an improved barrier against light, water and oxygen from the outer environment. On the other hand, biopolymers added with EO showed exponential diffusion growth, and the active

compounds present at the simulant kept its antioxidant activity. EOs showed a faster release toward fatty food simulant, highlighting their use for this type of product. Regarding the antioxidant potential verified in the simulant media, films incorporated with ginger, sage and rosemary EOs presented the best results.

In order to rank the different HAEs and EOs for further incorporation into chitosan for food packaging, further studies are demanded, namely the identification of the antimicrobial activity of the novel materials and their behavior when in direct contact with food matrices.

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