Carbohydrate Polymers 101 (2014) 582–591

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Characterization of κ-carrageenan films incorporated plant essential oils with improved antimicrobial activity

Saeedeh Shojaee-Aliabadi[a], Hedayat Hosseini[a], Mohammad Amin Mohammadifar[a], Abdorreza Mohammadi[a], Mehran Ghasemlou[b], Seyede Marzieh Hosseini[a], Ramin Khaksar[a,*]

[a] Department of Food Science and Technology, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Sciences and Food Technology, Shaheed Beheshti University of Medical Sciences, Tehran, Iran
[b] School of Packaging, Michigan State University, East Lansing, MI 48824, USA

ARTICLE INFO

Article history:
Received 31 July 2013
Received in revised form 31 August 2013
Accepted 19 September 2013
Available online 29 September 2013

Keywords:
Kappa-carrageenan
Edible film
Zataria multiflora Boiss
Mentha pulegium
Antimicrobial activity
Vapor phase

ABSTRACT

Antioxidant and antimicrobial kappa-carrageenan-based films containing different concentrations of *Zataria multiflora Boiss* (ZEO) and *Mentha pulegium* (MEO) essential oils were developed, and their water vapor permeability (WVP), optical, microstructure, antioxidant and antimicrobial properties were characterized. ZEO and MEO decreased the WVP of the emulsified films; for example, 3% ZEO reduced WVP by around 80%. Increasing the content of ZEO or MEO from 1% to 3% (v/v) increased values for elongation at break from 37.43% to 44.74% and from 36.09% to 41.25% respectively. Carrageenan-composite films were less resistant to breakage, more flexible and more opaque with lower gloss. These properties were related to the film's microstructure as analyzed by atomic force microscopy and scanning electron microscopy. ZEO affected the antioxidant properties of the films more markedly than MEO, e.g., ZEO containing films showed DPPH radical scavenging of 80.6% which were two-fold higher than those having MEO. The films' antimicrobial activities were increased by incorporating essential oils, particularly ZEO, which were more effective against the bacteria in the direct-contact method than a vapor phase. *S. aureus* was found to be the most sensitive bacterium to either ZEO or MEO, followed by *B. cereus* and *E. coli*. A highest inhibition zone of 544.05 mm^2 was observed for *S. aureus* around the films incorporated with 3% (v/v) ZEO. The total inhibitory zone of 3% (v/v) MEO formulated films was 20.43 for *S. typhimurium* and 10.15 mm^2 for *P. aeruginosa*. These results revealed that ZEO and MEO have good potential to be incorporated into κ-carrageenan to make antimicrobial and antioxidant edible films for food applications.

1. Introduction

In the last few years, numerous research efforts have aimed to improve alternative ways of packaging to protect food products from environmental conditions. One of the current research trends is the replacement of synthetic polymers with natural compounds. This is closely connected with growing consumer demand for high-quality and long-shelf-life products and increased awareness of environmental issues. The development of bioactive edible coatings containing biodegradable polymers combined with natural antimicrobial and/or antioxidant compounds appears to be an interesting strategy for maintaining food quality and providing extra protection against oxidative agents and minimizing post-packaging pathogen contamination (Campos, Gerschenson, & Flores, 2011).

Carrageenans are natural, water-soluble hydrocolloids composed of a linear chain of sulphated galactans and extracted from certain species of red seaweed. They are classified according to the number and position of a sulfated ester on 3,6-anhydro-D-galactose residues. Carrageenans have high potential as a film-forming material. In one study, Park (1996) reported that Kappa-carrageenan is able to produce a clear film with excellent mechanical and structural properties with a tensile strength higher than those of ι and γ-carrageenan films. However they are poor water vapor barrier like other hydrocolloid-based films.

A number of hydrophobic compounds, such as lipids, are frequently incorporated into hydrocolloid-based films as depressors of water vapor permeability (WVP). The incorporation of plant essential oils into these films represents an interesting alternative to lipids (Benavides, Villalobos-Carvajal, & Reyes, 2012; Ojagh, Rezaei, Razavi, & Hosseini, 2010). Furthermore, their potential health benefits, as well as their strong antioxidant/antimicrobial

* Corresponding author at: Food Science and Technology Department, Faculty of Nutrition and Food Science, Shahid Beheshti University, P.O. Box 19395-4741, Tehran, Iran. Tel.:/Fax: +98 21 22376480.
E-mail address: r.khaksar@sbmu.ac.ir (R. Khaksar).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.09.070

properties, make them possible substitutes for synthetic antioxidant/antimicrobial agents to achieve oxidative and microbial stability, as well as safer food products (Bakkali, Averbeck, Averbeck, & Idaomar, 2008).

Zataria multiflora Boiss (ZEO) and *Mentha pulegium* (MEO), which are locally called Avishan-shirazi and Pune, respectively, are aromatic and medicinal plants belonging to Labiatae family that are commonly used as herbal teas and flavoring agents in Iran. Various functionalities of ZEO and MEO, the result of their high content of phenolic oxygenated monoterpens, are extensively discussed in the literature (Kamkar, Javan, Asadi, & Kamalinejad, 2010; Saei-Dehkordi, Tajik, Moradi, & Khalighi-Sigaroodi, 2010).

Recently, several reports on the antimicrobial activity of various essential oils incorporated into edible films using direct application (direct contact between microorganisms and antimicrobial agents) were published (Broumand, Emam-Djomeh, Hamedi, & Razavi, 2011; Sánchez-González, González-Martínez, Chiralt, & Cháfer, 2010). However, some authors reported that the vapor phase of essential oils (no direct contact between the essential oil and the medium surface) exhibits good inhibitive power against foodborne pathogens and spoilage bacteria, and is even more effective than direct application; this in turn can reduce the organoleptic alteration induced by essential oils (Lopez, Sanchez, Batlle, & Nerin, 2005; Tyagi & Malik, 2011). To the best of our knowledge, no comparative study has been conducted for comparing the antibacterial effectiveness of hydrocolloid-based films containing essential oils in the vapor phase vs. direct contact. Moreover, there are few reported data on the characteristics of carrageenan-essential oil composite films. Therefore, this study aims to develop a composite edible film based on carrageenan and two essential oils – ZEO and MEO – and to assess the film's antimicrobial effect in both the vapor phase and direct contact, as well as its antioxidant activity. Furthermore, some characteristics such as WVP, mechanical, optical properties and surface morphology were analyzed to examine potential applications of these films as food-packaging material.

2. Materials and methods

2.1. Materials

κ-Carrageenan (Rico Co., Philippines), Essential oils (ZEO and MEO, supplied by Barij Company, Kashan, Iran), Tween 80 and glycerol (Fluka, Sigma Aldrich, St. Louis, MO, USA), were used to prepare film-forming dispersions (FFD). Mueller-Hinton agar (MHA) and Mueller-Hinton Broth (MHB) were bought from Merck Co. (Darmstadt, Germany). Folin–Ciocalteu reagent, sodium carbonate, standard gallic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were of analytical grade.

2.2. Bacterial strains

Staphylococcus aureus ATCC 25923; *Bacillus cereus* PTCC 1154, *Escherichia coli* ATCC 25922; *Pseudomonas aeruginosa* ATCC 27853; *Salmonella typhimurium* ATCC 14028 were provided by the Iranian Research Organization for Science and Technology (Tehran, Iran). Stock cultures of the studied bacteria were grown in MHB at 30 °C for 24 h before the tests.

2.3. Preparation of films

κ-Carrageenan-based films were prepared by the method of Park (1996) with some modifications. Preliminary experiments were conducted to determine the appropriate concentration of plasticizer (glycerol) for preparing films. Results showed that filmogenic solutions containing 50% (w/w) glycerol (based on carrageenan weight) were easily removed from the plate.

Film solutions were prepared by dissolving κ-carrageenan (1%, w/v) in distilled water under magnetic stirring for 15 min at 82 °C. Following the addition of glycerol at constant concentration (50%, w/w based on κ-carrageenan weight), stirring was continued for a further 25 min at 82 °C. The emulsions were obtained by adding ZEO and MEO to the carrageenan solution to reach final concentrations of 1, 2 and 3% (v/v) and Tween 80 as an emulsifier in quantities proportional to the essential oils (0.1, 0.2 and 0.3%, v/v, respectively). FFDs without any essential oils were also prepared for later comparison. Homogenization was carried out using a rotor-stator homogenizer (IKA T25-Digital Ultra Turrax, Staufen, Germany) at 13,500 rpm for 3 min at 80 °C, and the emulsions were cooled to 65 °C to remove any air bubbles produced during homogenization.

The FFDs were cast on the center of a rimmed circular area (177 cm^2) of clean and leveled glass plates, then dried at 30 °C for 30 h. Casting and drying were carried out at 30 °C, which is below the helix melting point reported for carrageenan polymer (Karbowiak, Debeaufort, Champion, & Voilley, 2006). Dried films were peeled off the casting surfaces and stored inside desiccators at 25 °C and 53% relative humidity (RH) until evaluation. Saturated magnesium nitrate solution was used to meet required RH.

2.4. Determination of physical properties of films

2.4.1. Thickness

Film thickness was determined using a manual digital micrometer (Mituto, Tokyo, Japan) to the nearest 0.001 mm. Reported values were average of at least 10 random locations for each film sheet.

2.4.2. Moisture content

The films' moisture content was determined by drying in an oven at 110 °C until a constant weight was reached (dry sample weight) (Ghasemlou et al., 2013). Three replications of each film treatment were used for calculating the moisture content.

2.4.3. Film solubility in water

The films' water solubility was determined in triplicate according to method of Ojagh et al. (2010). Briefly, pre-weighed film samples (1 cm × 3 cm) were immersed under constant agitation in 50 ml of distilled water for 6 h at 25 °C. After filtration, undissolved film was dried at 110 °C to constant weight. The initial dry weight was determined by drying at 110 °C to constant weight. The water solubility (%) of the film was calculated according to the equation $WS(\%) = ((W_0 - W_f)/W_0) \times 100$, where W_0 is the initial weight of the film expressed as dry matter and W_f is the weight of the desiccated undissolved film.

2.5. Optical properties

The gloss of the films was measured at incidence angles of 60°, according to the ASTM ID: D523-08 using a flat-surface gloss meter (Multi.Gloss 268, Minolta, Germany). Prior to optical measurements, films were conditioned in desiccators at 25 °C and 53% RH. Gloss measurements were performed on the side of the film in contact with air during drying and over a black matte standard plate; six replicates were taken per formulation. Results were expressed as gloss units, relative to a highly polished surface of black glass with a standard value near 100.

The lightness (L), redness (a) and yellowness (b) color system was used to evaluate the color of films by a colorimeter (Minolta CR 300 Series, Minolta Camera Co., Ltd., Osaka, Japan). The measurements were taken on white standard backgrounds ($L^* = 93.49$, $a^* = -0.25$ and $b^* = -0.09$). All measurements were performed in

triplicate. Total color difference (ΔE) and whiteness index (WI) were calculated using following equations:

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

$$\mathrm{WI} = 100 - \sqrt{(100 - L)^2 + a^2 + b^2} \quad (2)$$

where L^*, a^* and b^* are the color parameter values of the standard and L, a and b are the color parameter values of the sample.

The opacity of the film specimens was evaluated by measuring the absorbance at 600 nm using a spectrophotometer (Shimadzu UV-VIS 1601, Japan) according to the method of Gómez-Estaca, Giménez, Montero, and Gómez-Guillén (2009). An empty test cell was used as the reference. Opacity was calculated using the following equation:

$$\mathrm{Op} = \frac{\mathrm{Abs}_{600}}{x} \quad (3)$$

where Abs_{600} is the value of absorbance at 600 nm and x is the film thickness (mm). According to this equation, low values of Op demonstrate higher transparency. Three replicates of each film were tested.

2.6. Water vapor permeability (WVP)

The film samples' WVP was determined at 25 °C and 75% RH gradient by gravimetric method (ASTM ID: E96/E96M-12). The test films (0.00287 m^2 film area) were used to seal cups containing anhydrous calcium chloride (0% RH, assay cup) or nothing (control cup). The cups were placed inside desiccators maintained at 75% RH with a sodium-chloride-saturated solution (Merck, Darmstadt, Germany). The difference in RH corresponds to a driving force of 1753.55 Pa, expressed as water vapor partial pressure. Weight gain of the test cups over a 7 days period was recorded periodically to the nearest 0.0001 g. The slope of the weight gain vs. time plot was divided by exposed film area to obtain the water vapor transmission rate according to Eq. (4):

$$\mathrm{WVTR} = \frac{\text{slope}}{\text{film area}} = \frac{\Delta m}{A\Delta t} \quad (4)$$

After the permeation tests, this was multiplied by the thickness of the film and divided by the pressure difference between the inner and outer surfaces to obtain the WVP as calculated by Eq. (5). All tests were performed in triplicate.

$$\mathrm{WVP} = \frac{\Delta m}{A\Delta t}\frac{X}{\Delta p}. \quad (5)$$

2.7. Mechanical properties

The film samples' mechanical properties, including tensile strength (MPa) and elongation at break (%), were measured at 25 °C with a Testometric Machine M350-10CT (Testometric Co., Ltd., Rochdale, Lancs., UK) according to ASTM ID: D882-12. All of the tested film strips (1.5 cm × 10 cm) were equilibrated at 25 °C and 53% RH in desiccators containing $Mg(NO_3)_2$ saturated solutions for 48 h prior to testing. Equilibrated film strips were fixed between the grips with an initial separation of 50 mm, and the cross-head speed was set at 50 mm/min. Tensile strength was determined by dividing the peak load by the cross-sectional area of the initial film specimen. Elongation was calculated by percent change in the length of the specimen from the original distance between the grips (50 mm). A total of six replicates for each formulation were tested.

2.8. Scanning electron microscopy (SEM)

The microstructure of the dried films' cross-sections was observed by using scanning electron microscopy (Oxford Instruments INCA Penta FET × X3, Oxfordshire, UK). Film samples were cryofractured by immersion in liquid nitrogen, then mounted on the specimen holder with double-sided adhesive tape. After gold coating using a BAL-TEC SCD 005 sputter coater (BAL-TEC AG, Balzers, Liechtenstein), the images were captured using an accelerating voltage of 20 kV.

2.9. Atomic force microscopy (AFM)

The surface morphology of the films previously equilibrated at 53% RH was studied by atomic force microscopy (Dualscope/Rasterscope C26, DME, Denmark) with a 200 μm × 200 μm scan size and a 6 μm vertical range. A sharpened cantilever was positioned over the sample, and 80 μm × 80 μm images were obtained. Three images of different zones were captured per formulation and analyzed offline with Dualscope/Rasterscope SPM software (Version 2.1.1.2) to transform them into a three-dimensional image and to calculate the roughness values. Two statistical parameters related to sample roughness were considered: Sa (average of the absolute value of the height deviations from a mean surface), and Sq (root-mean-square average of height deviations taken from the mean data plane).

2.10. Estimation of total phenolic content

The total phenolic content of the films were estimated according to the method of Siripatrawan and Harte (2010) involving the Folin–Ciocalteu reagent and gallic acid as standard, with some modifications. Briefly, 25 mg of each film sample was dissolved in 5 ml of distilled water, then extract solution (0.1 ml), distilled water (7 ml), and Folin–Ciocalteu reagent (0.5 ml) were mixed and kept at room temperature for 8 min, after which 1.5 ml sodium carbonate (2%, w/v) and water were added to obtain a final volume of 10 ml. The mixture was stirred thoroughly and allowed to stand for 2 h at room temperature prior to an absorbance reading at 765 nm in a spectrophotometer (Shimadzu UV-VIS 1601, Japan). The results were expressed as mg gallic acid equivalents (GAE) per gram of dried film according to the following equation:

$$T = \frac{CV}{M} \quad (6)$$

where T is total content of phenolic compounds (milligrams per gram dried film, in GAE), C is the concentration of gallic acid obtained from the calibration curve (milligrams per milliliter), V is the volume of film extract (milliliters) and M is the weight of dried film (grams).

2.11. DPPH radical-scavenging activity

The films' DPPH radical-scavenging activity was determined using the method of Brand-Williams, Cuvelier, and Berset (1995) on the basis of bleaching of the bluish-red or purple color solution of DPPH as a reagent. Briefly, 25 mg of each film sample was dissolved in 5 ml of distilled water, then 0.1 ml of film extract solution was added to 3.9 ml of the DPPH solution (0.1 mM methanol solution). The mixture was incubated for 60 min in darkness at ambient temperature. The absorbance was read against pure methanol at

517 nm, and the percentage of DPPH radical-scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging activity}\,(\%) = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}\right) \times 100 \qquad (7)$$

where A_{blank} is the absorbance of the control, and A_{sample} is the absorbance of the test compound.

2.12. Evaluation of films' antimicrobial activity

2.12.1. Disk diffusion method

The antimicrobial activity of the films was qualitatively evaluated following an agar diffusion assay. The antimicrobial films were aseptically cut into 6 mm-diameter discs and placed on plates containing MHA. The medium had been previously seeded with 100 μl of an overnight broth culture containing approximately 10^8 CFU/ml of the test bacteria. The plates were incubated at 30 °C for 24 h. The diameters of the zones free of bacterial growth were measured using a caliper to the nearest 0.02 mm. The whole zone area was calculated, then subtracted from the film disk area; this difference in area was reported as the "zone of inhibition" (Ojagh et al., 2010). The tests were carried out in triplicate for each formulation.

2.12.2. Disk volatilization method

The disk volatilization method was used to examine the films' antimicrobial activities in vapor phase according to Lopez et al. (2005). Briefly, the MHA medium was seeded with 100 μl of an overnight broth culture containing approximately 10^8 CFU/ml of the test bacteria. The antimicrobial films were aseptically cut into 6 mm diameter discs and laid on the inside surface of the upper lid, with no direct contact between the discs and the bacteria strains. The plate was then sealed using parafilm to prevent leakage of essential oil vapor, then incubated at 30 °C for 24 h. The diameters of zones free of bacterial growth were measured in millimeters, and the whole zone area was reported as the "zone of inhibition". The tests were carried out in triplicate for each formulation.

2.13. Statistical analysis

The experiments were factorial with a completely randomized design. The statistical analysis of the data was performed through an analysis of variance (ANOVA) using SPSS statistical software version 16 (SPSS Inc., Chicago, IL, USA). Duncan's multiple range test was used to determine any significant differences among the treatments at a 95% confidence level.

3. Results and discussion

3.1. Film formulation

Preliminary studies were performed to determine the appropriate plasticizer and essential oil concentration for the film formulation. The films prepared without plastisizer were brittle, and cracked on the plates during drying. Thus it was necessary to use glycerol as a plasticizer. The addition of glycerol at 50% (w/w, film dry weight basis) yielded films with good flexibility, but at higher ratios of glycerol in the formulation, films were sticky and wet. Therefore, a concentration of 50% glycerol was selected for the present study. It was found that films formulated with the studied essential oils at concentrations lower than 1% (v/v) did not show antimicrobial activities, and those containing more than 3% gave the samples an increasingly strong aroma.

3.2. Physical properties of films

Table 1 shows the effects of incorporating essential oils on the physical properties of carrageenan-based films. There was no significant difference among the thicknesses of emulsified films with exception of films containing ZEO at 3%, which had significantly ($p < 0.05$) higher thickness value (62 μm).

The moisture content was 20.15% for the samples without essential oils; this decreased significantly ($p < 0.05$) to between 16.09% and 14.96% for the films containing 3% ZEO and MEO, respectively, which is attributed to an increase in the films' hydrophobicity.

The solubility of the emulsified films decreased similarly. Lower solubility of MEO-containing films compared to ZEO-containing ones, despite MEO's lower hydrophobicity, may be explained by an increase in interaction between the hydroxyl groups of carrageenan chains and MEO components, leading to a decrease in the availability of hydroxyl groups and thus reducing polysaccharide-water interactions. This in turn would result in a decrease in the films' solubility, which is beneficial when product integrity and water resistance are desired. These results were similar to those of Ghasemlou, Khodaiyan, Oromiehie, and Yarm (2011), whose kefiran films tended to become more hydrophobic with an increase in oleic-acid content.

3.3. Water vapor permeability (WVP)

Table 1 shows the WVP values for the film samples tested. The value for the control films (0%, w/w essential oil) was $2.384\,\text{g s}^{-1}\,\text{m}^{-1}\,\text{Pa}^{-1} \times 10^{-10}$, which fell significantly ($p < 0.05$) to 0.692 and $0.945\,\text{g s}^{-1}\,\text{m}^{-1}\,\text{Pa}^{-1} \times 10^{-10}$ for the films containing 1% ZEO and MEO, respectively. As expected, the WVP values tended to decrease more as higher concentrations of essential oils were incorporated. It is well known that the presence of lipids within an FFD reduces the hydrophilic–hydrophobic ratio of the film matrix and causes discontinuities in the hydrophilic phase, thus increasing the tortuosity factor for mass transfer in the continuous matrix; this in turn enhances the film's water-barrier properties (Pérez-Gago & Krochta, 2001). Additionally, it has been reported that essential oils can reduce the compactness of the film structure, thus increasing WVP (Bonilla, Atarés, Vargas, & Chiralt, 2012; Hosseini, Razavi, & Mousavi, 2009).

In the present study, although the mechanical properties of films deteriorated when essential oils were added (Table 1), SEM showed a homogeneous and compact structure of emulsified films even at the highest concentrations. These results indicated that the hydrophobicity of MEO and ZEO overcame the loss of structure compactness, thus reducing the films' WVP. A significant difference ($p < 0.05$) was observed after the incorporation of 3% ZEO ($0.425\,\text{g s}^{-1}\,\text{m}^{-1}\,\text{Pa}^{-1} \times 10^{-10}$), which was then much lower than that for films emulsified with MEO. The effectiveness of ZEO in reducing WVP can be partially explained by the more hydrophobic nature of the main components of this essential oil (thymol, carvacrol and γ-terpinene), which are less polar than those of MEO (pulegone, menthone and piperitone) (Kamkar et al., 2010; Saei-Dehkordi et al., 2010).

3.4. Mechanical properties

Tensile strength (TS) and elongation at break (EB) are useful characteristics in predicting the ability of edible films to maintain their integrity when used as food packaging (Ghasemlou, Khodaiyan, & Oromiehie, 2011). Table 1 shows the effects of incorporating different essential oil types and concentrations on films' mechanical properties of films. Films without essential oils exhibited a TS value of 26.291 MPa, which was within the range found by Park (1996) (22–32 MPa) for films based on κ-carrageenan.

Table 1
Physical, WVP and mechanical properties of carrageenan films formulated with different concentration of ZEO and MEO.[a]

Film	Thickness (μm)	Moisture content (%)	Solubility in water (%)	WVP ($g\,s^{-1}\,m^{-1}\,Pa^{-1} \times 10^{-10}$)	Elongation at break (%)	Tensile strength (MPa)
Control	31.33 ± 1.15a	20.15 ± 2.53a	26.32 ± 1.03a	2.383 ± 0.04a	36.46 ± 1.04e	26.29 ± 2.93a
ZEO1	37.44 ± 1.53c	20.85 ± 1.06a	25.88 ± 0.75a	0.692 ± 0.10c	37.43 ± 1.56d	15.86 ± 3.14c
ZEO2	41.12 ± 2.89d	18.23 ± 1.37b	22.06 ± 0.49b	0.577 ± 0.09d	39.75 ± 4.19c	12.75 ± 1.54d
ZEO3	62.33 ± 3.79f	16.09 ± 1.92c	17.98 ± 1.66d	0.425 ± 0.07f	44.74 ± 5.91a	10.34 ± 2.90f
MEO1	32.56 ± 1.52b	19.20 ± 1.23b	23.46 ± 1.23b	0.945 ± 0.08b	36.09 ± 3.45e	25.81 ± 4.54a
MEO2	38.18 ± 5.69c	17.11 ± 0.91c	20.87 ± 0.94c	0.665 ± 0.05c	37.67 ± 2.60d	22.22 ± 1.61b
MEO3	48.67 ± 4.51e	14.96 ± 1.84d	16.04 ± 1.35e	0.496 ± 0.03e	41.25 ± 6.25b	17.17 ± 3.29c

[a] Data reported are average values and standard deviations. Values within each column with different letters are significantly different ($p < 0.05$).

The presence of essential oil droplets in the film matrix affected both the TS and EB of the emulsified films. Regardless of the type of essential oil, the incorporation of oil content reduced the TS of emulsified films, while it increased the EB significantly ($p < 0.05$); the resulting films were softer and less resistant to breakage, but more stretchable, depending on the essential oils concentration. The greater the oil content, the greater the effect; for example, as essential oil content increased from 1% to 3%, TS values were reduced in the films prepared with ZEO and MEO by a factor of 2.5 and 1.5, respectively. This effect may be linked to the ability of carrageenan to interact with other components such as essential oils because of its strong electrolyte property (Karbowiak et al., 2006), which may cause a decrease in intermolecular polymer interactions that are stronger than those between less polar essential oil molecules and polar polymer molecules. This effect consequently would weaken the molecular organization of the carrageenan network and lead to a reduction in the TS.

In our study, this effect was more pronounced when ZEO was added to the carrageenan films.

Considering the fact that the interactions between polymer and phenolic compounds depends on the characteristics of the phenolic compounds and the individual phenolic constituents (Kroll & Rawel, 2001), the effectiveness of ZEO in reducing TS is probably linked to its lower capacity for interaction with the carrageenan matrix, relative to that of MEO.

The elongation responses of the two studied essential-oil composite films showed similar trends, with a significant increase ($p < 0.05$) compared to the control film as the concentrations of ZEO and MEO increased. The presence of essential oil appears to have some plasticizing effect on carrageenan-based films that alters the balance of interaction forces in the polysaccharide network, with a consequent loss of film cohesion that facilitates chain mobility during film stretching (Atarés, De Jesús, Talens, & Chiralt, 2010). The decreasing TS and increasing EB are common results of essential oil incorporation, which have been broadly discussed in research on other biopolymer films (Atarés et al., 2010; Benavides et al., 2012).

3.5. Optical properties

Films' optical properties directly affect consumers' tendency to accept edible films as food packaging. Table 2 shows the gloss values of the films measured at incidence-angle values of 60°. According to ASTM ID: D523-08 this geometry is appropriate for results between 10 and 70, which included most our results. Gloss is linked to surface roughness: generally, the rougher the surface, the lower the gloss (Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2009). Films incorporating ZEO were less glossy than the control sample. Glossiness decreased as the oil concentration increased for ZEO-containing films, whereas the loss of glossiness for MEO-containing films was only significant for films with 3% MEO ($p < 0.05$).

These effects can be attributed to the greater roughness of emulsified films, which decreases the specular reflectance, and thus the gloss. This roughness may be a result of the coalescence and creaming of the essential oil droplets during film drying, which lead to surface discontinuities (Bonilla et al., 2012; Sánchez-González et al., 2009). It has been previously reported that the stability of initial emulsion, which in turn greatly depends on particle size and lipid–polymer interactions, is a key factor in preventing oil-droplet migration during drying (Fabra, Pérez-Masiá, Talens, & Chiralt, 2011). In the case of MEO-containing films, it could be assumed that the greater lipid–polymer interactions caused a lower migration during drying, resulting in higher gloss.

Film color is an important factor in product acceptability to consumers. Table 2 shows the L, a and b values, total color difference (ΔE), whiteness index (WI) and opacity values of pure and composite essential oil-containing, carrageenan-based films. Essential oil-free film appeared clear and transparent without any tint. MEO, which is itself colorless, did not significantly ($p > 0.05$) change the color parameters of composite films. Films with ZEO showed a higher b value and ΔE and lower L and WI values than those with MEO; ZEO gave the films a darker appearance, with a light yellowish tint. The b value was markedly affected by the addition and amount of ZEO. The more ZEO that was incorporated, the higher the b value, and, consequently, the lower the WI. This can be linked to the phenolic compounds of essential oils, which may absorb low-wavelength light. Similar effects on color parameters and gloss of adding oil to the film matrix has been previously observed by Benavides et al. (2012).

The opacity difference of films containing ZEO compared to the control follows the same trend as brightness and WI (Table 2). Film containing 3% ZEO was the most opaque, probably due to an increase in light-scattering induced by oil droplets in the film network. Light-scattering phenomena mainly depend on the content and particle size of the dispersed phase; the more droplets, the greater the intensity of the light-scattering, and the lower the transparency (Sánchez-González et al., 2009). Nevertheless, all the obtained emulsified films, even at highest concentration of essential oil, were not much colored, and their transparency and gloss were suitable for food packaging.

3.6. Film microstructure

A microstructural study of the structural arrangement of the different components in dried film matrices contributes to a better knowledge of water-vapor transmission mechanisms and mechanical and optical properties (Fabra, Talens, & Chiralt, 2009).

Fig. 1 shows cross-sectional micrographs of the carrageenan-based films, which show notable differences as a function of the film composition. While a smooth and continuous microstructure was observed for the pure κ-carrageenan film, emulsified films (Fig. 1) showed discontinuities associated with the essential oil droplets embedded in the polymer matrix.

At lower concentrations, emulsified films tended to show a smaller oil-droplet size. However, a coarser microstructure was observed in the films that incorporated the highest concentration

Table 2
Optical properties of carrageenan films formulated with different concentration of ZEO and MEO.[a]

Film	Gloss	*L*	*a*	*b*	Δ*E*	WI	Op
Control	46 ± 2.15a	88.41 ± 1.04a	−0.27 ± 0.07d	0.86 ± 1.15d	5.26 ± 0.23d	88.27 ± 0.52a	0.81 ± 0.02f
ZEO1	19 ± 2.31d	81.59 ± 0.92b	−0.47 ± 0.06c	3.45 ± 0.17c	12.08 ± 0.91c	81.58 ± 1.19d	3.17 ± 0.35c
ZEO2	16 ± 1.78e	78.43 ± 0.74c	−0.87 ± 0.12a	6.68 ± 0.78b	16.68 ± 0.48b	77.19 ± 2.57e	5.25 ± 0.56b
ZEO3	10 ± 0.94f	70.50 ± 2.34d	−0.51 ± 1.02b	9.09 ± 1.64a	23.62 ± 0.56a	70.23 ± 0.91f	6.31 ± 0.74a
MEO1	43 ± 3.38b	88.48 ± 0.89a	−0.28 ± 0.08d	0.86 ± 0.14d	5.22 ± 0.13d	88.21 ± 0.14a	1.04 ± 0.16f
MEO2	42 ± 2.76bc	87.56 ± 1.28a	−0.29 ± 0.06d	0.84 ± 0.24d	5.80 ± 0.11d	85.58 ± 1.73b	1.32 ± 0.07e
MEO3	40 ± 2.47c	87.22 ± 0.38a	−0.29 ± 0.03d	0.87 ± 0.17d	6.04 ± 0.08d	81.48 ± 1.10c	1.75 ± 0.89d

[a] Data reported are average values and standard deviations. Values within each column with different letters are significantly different.

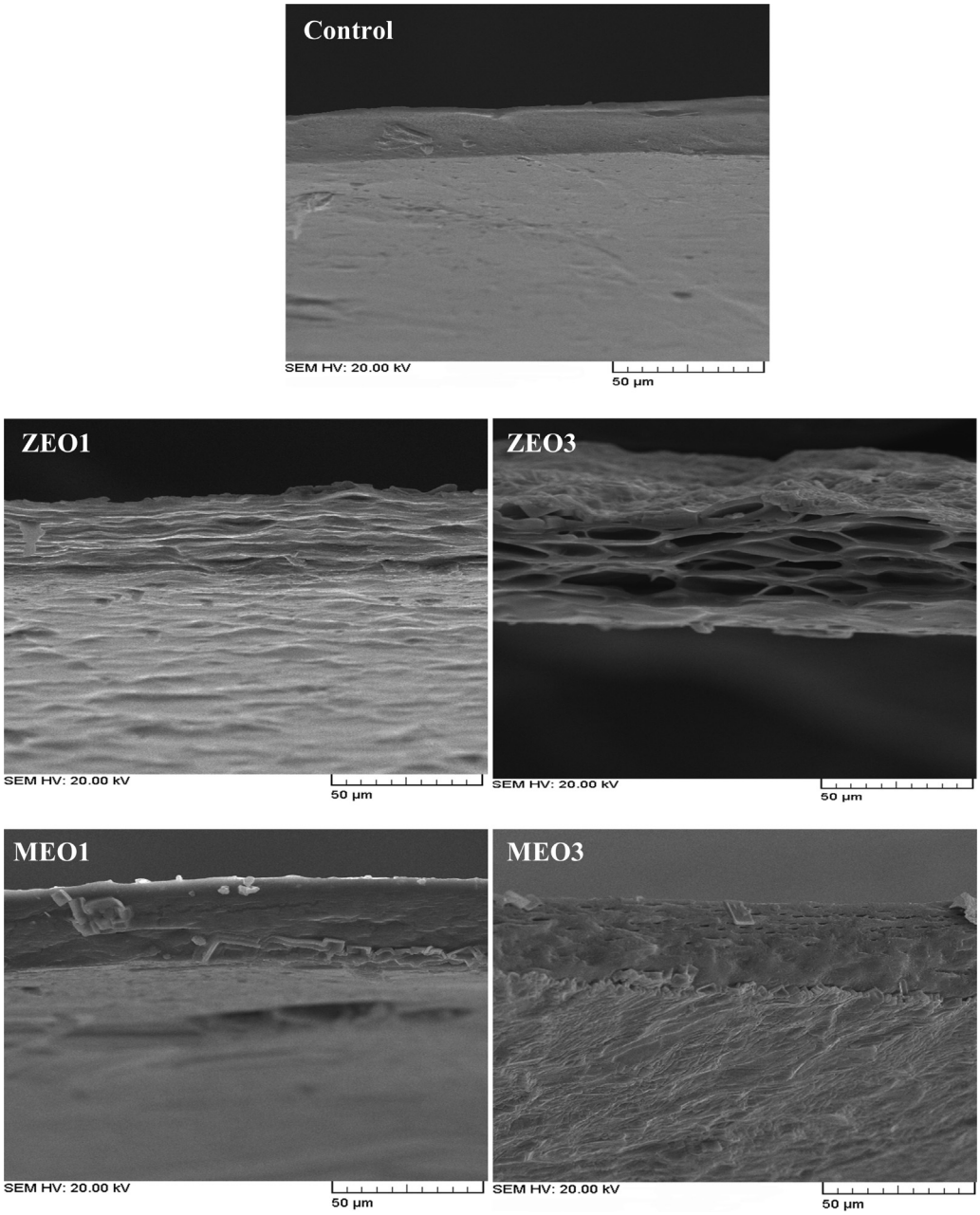

Fig. 1. Scanning electron micrographs of cross-section of different carrageenan films.

of essential oils, due to the fact that the higher lipid content favors the flocculation rate (Sánchez-González et al., 2009). On the basis of SEM micrographs, it appears that a homogenization process could produce oil droplets that were equal in size. However, throughout the drying period, the oil droplets became slightly enlarged, probably due to the deformation forces occurring during the structural arrangement of the carrageenan chain. ZEO-containing films showed extensive discontinuities compared to MEO. This difference is probably due to the different behavior of oils during homogenization and drying, which is

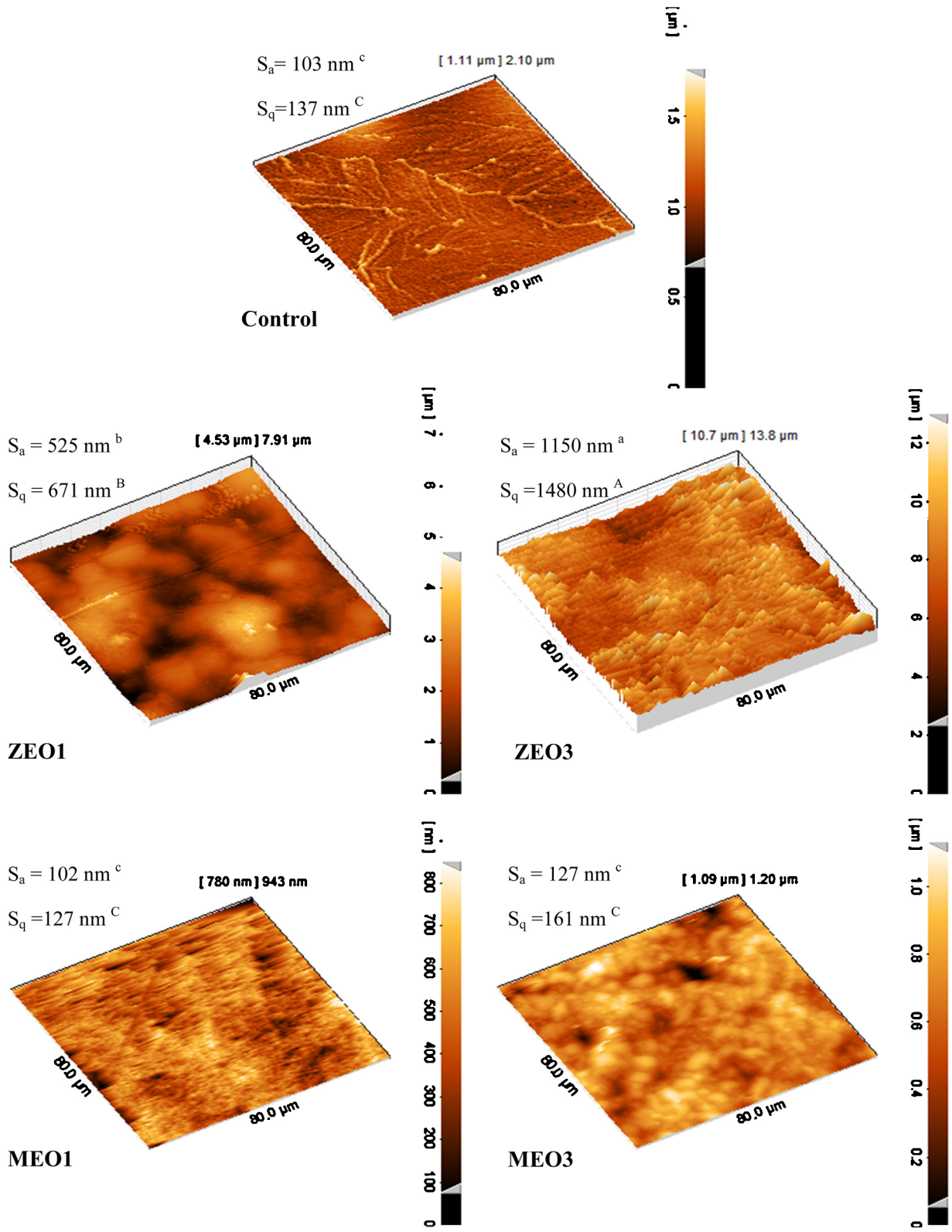

Fig. 2. Typical AFM image and Sa and Sq of carrageenan films (mean values with different superscript in small letters for Sa and in capital letters for Sq are significantly different ($p < 0.05$)).

determined by the oil type and the complex interactions between the oil and the polysaccharide, resulting in different dried-film structures (Atarés et al., 2010). It seems that the extent of the flocculation and coalescence occurring during the solvent evaporation for the films with ZEO was more pronounced than for those with MEO.

3.7. Surface morphology

The interruption in the κ-carrageenan matrix provoked by oil droplets, as observed by SEM, affected the topology of the film surface, which was analyzed through AFM. Fig. 2 shows the surface topographies of carrageenan-based films with and without

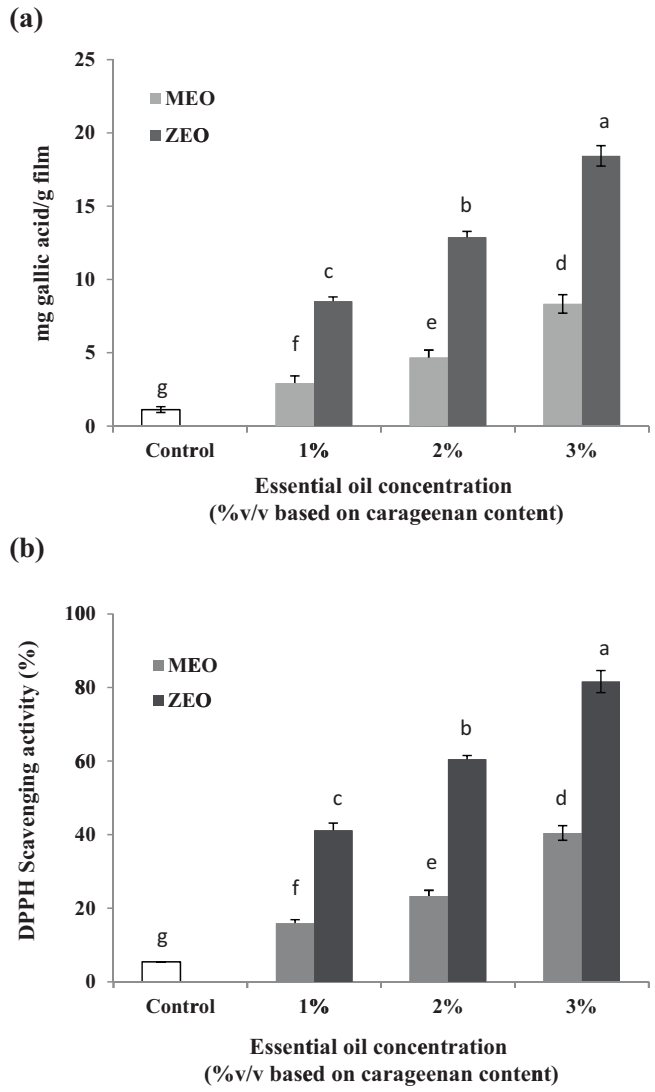

Fig. 3. Total phenol contents (a) and DPPH scavenging activities (b) of carrageenan films with ZEO and MEO.

essential oils. The Sa and Sq parameters presented similar trends for the two kinds of films. The oil-free film had a smooth surface, with Sa and Sq values of 103 and 137, respectively, which suggests that there were few contaminant microparticles. Roughness parameters did not change significantly ($p > 0.05$) when MEO was added. However, the space frequency of the irregularities was not the same as those of control films. The surface irregularities of the ZEO-containing films were more accentuated than those for films prepared with MEO, thus producing a rougher film surface. The roughness differences between the films containing ZEO and MEO, which were consistent with the film microstructure observed by SEM, may be related to the ability of MEO-containing emulsions to remain more stable against aggregation during drying, as suggested by the SEM and gloss analyses. The reliefs, as measured by SEM, tended to be greater with increases in oil content increased.

3.8. Total phenolic content and antioxidant activity

Fig. 3a shows total phenolic content (TPC) of carrageenan films incorporating ZEO and MEO. A low TPC was measured for pure-carrageenan film (1.12 mg gallic acid/g film). Both ZEO and MEO had a significant effect ($p < 0.05$) on the films' TPC. The highest TPC (18.43 mg gallic acid/g film) was observed in films with 3% of ZEO, whereas MEO showed little TPC.

A DPPH scavenging assay was used to determine the films' antioxidant activity. This method has become a general test method for measurement of free radical scavenging ability of films added with plant extracts. Being rapid, simple and independent of sample polarity, the DPPH assay is very convenient for the quick screening of many samples for radical scavenging activity (Krishnaiah, Sarbatly, & Nithyanandam, 2011). The control film showed slight antioxidant activity, probably because of its naturally occurring polyphenols (1.12 mg gallic acid/g film). The antioxidant activity of pure κ-carrageenan film was considerably improved by adding either ZEO or MEO. However, films with ZEO showed significantly ($p < 0.05$) higher free-radical-scavenging capacity than those with the same proportion of MEO, e.g., scavenging activity of films containing 3% ZEO were 80.6% compared to MEO ones (40.5%). These differences may be attributed to different major constituents of essential oils and the intensity of possible interactions of their phenolic compounds with carrageenan chains, which seems to be greater in the case of MEO. Increasing the essential oils content increased the films' antioxidant activities: for instance, the presence of 3% ZEO and MEO caused an increase in antioxidant power of more 15 and 12 times, respectively, compared to the control film. This confirms the effectiveness of these essential oils. According to Shan, Cai, Sun, and Corke (2005), the antioxidant activities of essential oils are associated with their phenolic content; our results were also in line with the TPC of the assessed essential oils. Similar results have been found by other authors for chitosan-based film containing green tea (Siripatrawan & Harte, 2010).

3.9. Antimicrobial activity

Table 3 shows the inhibitory effect of emulsified κ-carrageenan films against the five selected bacteria in direct contact and vapor phase. The carrageenan film with no essential oil did not prevent the growth of the studied pathogenic bacteria. In the case of direct contact test, the results showed that all composite films containing ZEO inhibited the growth of the five test bacteria, with exception of *P. aeruginosa*, which was not inhibited at the lowest essential oil concentration (1%). As expected, antimicrobial activity was stronger at the higher concentrations of ZEO. *S. aureus* was found to be the most sensitive bacteria to ZEO for all tested concentrations; *B. cereus* and *E. coli* ranked next, followed by *S. typhimurium* and *P. aeruginosa.* The minimum concentration of MEO at which films showed a clear zone of inhibition was 1% for *S. aureus* and *B. cereus* and 3% for the rest of the studied bacteria. Carrageenan films with 3% MEO presented a larger zone of inhibition for *S. aureus* and *B. cereus*, but gram-negative ones were more resistant.

The antimicrobial activity of ZEO and MEO has been attributed to their main constituents which contain the terpenes: thymol and carvacrol (ZEO) (Saei-Dehkordi et al., 2010) and pulegone (MEO) (Kamkar et al., 2010). The positive effect of adding essential oil on films' antimicrobial activities has been previously described for other edible films (Broumand et al., 2011; Hosseini et al., 2009; Shakeri, Shahidi, Beiraghi-Toosi, & Bahrami, 2011).

In the vapor-phase test, the inhibitory effects of composite films were determined by circular inhibition areas, rather than the clear inhibitory zones around the film cuts in the direct contact test. Carrageenan films with MEO were found not to be effective at reducing the microbial growth of the tested bacteria in vapor phase except for *S. aureus* and *B. cereus* (Table 3). In contrast, ZEO-containing films were able to form a clear zone of inhibition on all bacteria at all concentrations higher than 1%, except *P. aeruginosa*, which was sensitive only to the highest concentration. As with the

Table 3
Antimicrobial activities of different concentrations of ZEO and MEO incorporated in carrageenan films in overlay and vapor phase test.[a]

Film	Inhibition zone (mm^2)				
	S. aureus	*B. cereus*	*E. coli*	*S. typhimurium*	*P. aeruginosa*
Direct contact					
Control	0.00g	0.00g	0.00e	0.00e	0.00d
ZEO1	54.24 ± 2.36f	10.96 ± 0.97e	37.23 ± 3.46c	36.32 ± 1.68c	0.00d
ZEO2	151.36 ± 21.38c	125.60 ± 6.89c	113.04 ± 11.24b	50.24 ± 1.79b	35.77 ± 0.41b
ZEO3	544.05 ± 30.98a	462.38 ± 25.54a	351.62 ± 18.71a	255.12 ± 20.31a	172.70 ± 17.09a
MEO1	21.98 ± 4.12e	10.21 ± 1.26f	0.00e	0.00e	0.00d
MEO2	107.40 ± 7.60d	66.73 ± 7.9d	0.00e	0.00e	0.00d
MEO3	198.62 ± 20.61b	148.37 ± 15.69b	33.34 ± 5.61d	20.43 ± 0.418d	10.15 ± 1.07c
Vapor phase					
Control	0.00e	0.00e	0.00c	0.00c	0.00b
ZEO1	0.00e	0.00e	0.00c	0.00c	0.00b
ZEO2	95.45 ± 9.87c	99.10 ± 6.13b	65.58 ± 6.49b	38.46 ± 1.26b	0.00b
ZEO3	236.86 ± 14.67a	229.86 ± 11.64a	176.62 ± 10.32a	153.86 ± 15.46a	94.98 ± 7.55a
MEO1	0.00e	0.00e	0.00c	0.00c	0.00b
MEO2	78.90 ± 8.39d	63.86 ± 8.59d	0.00c	0.00c	0.00b
MEO3	133.61 ± 14.50b	79.50 ± 4.66c	0.00c	0.00c	0.00b

[a] Data reported are average values and standard deviations. Values within each column with different letters are significantly different ($p < 0.05$).

direct-contact test, the zone of inhibition due to oil vapors also rose as oil concentration increased.

Although there were similarity between the results of the direct-contact and vapor phase tests for all bacteria, the inhibitory effects induced by ZEO vapors were weaker than those induced by direct contact. Lopez et al. (2005) reported similar results, pointing out that basil and rosemary oil showed no inhibitory effects in vapor phase despite their effectiveness in direct contact.

Tyagi and Malik (2011) reported that essential oils in vapor phase inhibited the growth of most bacteria more than in direct contact. This is in contrast with our results. It seems that adding essential oil to a filter disk, rather than incorporating it in a film, may be a possible reason for this discrepancy. Entrapping the essential oil molecules in a film's polymer matrix may limit their diffusion and evaporation, thus reducing the oil's antimicrobial activities. In the direct-contact test, the film is directly exposed to the agar surface, which has high moisture content. Carrageenan is a hydrophilic polymer, because of its molecules' numerous hydroxyl groups; this lets it absorb water (Karbowiak et al., 2006). Interpenetration of water molecules into the film matrix results in swelling, thus gradually widening the meshes of polymer network. This leads in turn to more release of the essential oil component into the surroundings, thus resulting in higher antimicrobial activities compared to the vapor-phase test, where availability of water is limited.

4. Conclusion

Carrageenan-essential oil composite films with improved WVP were developed by emulsification; WVP was reduced the most by the incorporation of ZEO. The carrageenan films' resistance to breakage was reduced by incorporating essential oils due to the presence of discontinuities. The films incorporating essential oils showed good antioxidant properties; this effect was greatly improved when ZEO was added. Films containing ZEO showed significant antimicrobial effects even in vapor phase, which suggest an approach for using these films with no need to directly apply them to food, and thus decrease organoleptic alterations. The results of this study suggested that ZEO and MEO as natural antioxidants and antimicrobials have potential for use in carrageenan film while producing some changes in mechanical and optical properties. The films developed in this study will have applications in packaging a wide range of oxidative and microbial-sensitive food products.

References

ASTM Standard D523. (2008). *Standard test method for specular gloss*. West Conshohocken, PA: ASTM International. http://dx.doi.org/10.1520/D0523-08 www.astm.org

ASTM Standard E96/E96M. (2012). *Standard test methods for water vapor transmission of material*. Philadelphia, PA: ASTM, International. http://dx.doi.org/10.1520/E0096_E0096M-12 www.astm.org

ASTM Standard D882. (2012). *Standard test method for tensile properties of thin plastic sheeting*. Philadelphia, PA: ASTM International. http://dx.doi.org/10.1520/D0882-12 www.astm.org

Atarés, L., De Jesús, C., Talens, P., & Chiralt, A. (2010). Characterization of SPI-based edible films incorporated with cinnamon or ginger essential oils. *Journal of Food Engineering*, *99*(3), 384–391.

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils—A review. *Food and Chemical Toxicology*, *46*(2), 446–475.

Benavides, S., Villalobos-Carvajal, R., & Reyes, J. E. (2012). Physical, mechanical and antibacterial properties of alginate film: Effect of the crosslinking degree and oregano essential oil concentration. *Journal of Food Engineering*, *110*(2), 232–239.

Bonilla, J., Atarés, L., Vargas, M., & Chiralt, A. (2012). Effect of essential oils and homogenization conditions on properties of chitosan-based films. *Food Hydrocolloids*, *26*(1), 9–16.

Brand-Williams, W., Cuvelier, M., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology*, *28*(1), 25–30.

Broumand, A., Emam-Djomeh, Z., Hamedi, M., & Razavi, S. H. (2011). Antimicrobial, water vapour permeability, mechanical and thermal properties of casein based Zataraia multiflora Boiss. Extract containing film. *LWT-Food Science and Technology*, *44*(10), 2316–2323.

Campos, C. A., Gerschenson, L. N., & Flores, S. K. (2011). Development of edible films and coatings with antimicrobial activity. *Food and Bioprocess Technology*, *4*(6), 849–875.

Fabra, M. J., Pérez-Masiá, R., Talens, P., & Chiralt, A. (2011). Influence of the homogenization conditions and lipid self-association on properties of sodium caseinate based films containing oleic and stearic acids. *Food Hydrocolloids*, *25*(5), 1112–1121.

Fabra, M. J., Talens, P., & Chiralt, A. (2009). Microstructure and optical properties of sodium caseinate films containing oleic acid-beeswax mixtures. *Food Hydrocolloids*, *23*(3), 676–683.

Ghasemlou, M., Khodaiyan, F., Oromiehie, A., & Yarmand, M. S. (2011). Characterization of edible emulsified films with low affinity to water based on kefiran and oleic acid. *International Journal of Biological Macromolecules*, *49*(3), 378–384.

Ghasemlou, M., Khodaiyan, F., & Oromiehie, A. (2011). Physical, mechanical, barrier, and thermal properties of polyol-plasticized biodegradable edible film made from kefiran. *Carbohydrate Polymers*, *84*(1), 477–483.

Ghasemlou, M., Aliheidari, N., Fahmi, R., Shojaee-Aliabadi, S., Keshavarz, B., Cran, M. J., et al. (2013). Physical, mechanical and barrier properties of corn starch films incorporated with plant essential oils. *Carbohydrate Polymers*, *98*(1), 1117–1126.

Gómez-Estaca, J., Giménez, B., Montero, P., & Gómez-Guillén, M. C. (2009). Incorporation of antioxidant borage extract into edible films based on sole skin gelatin or a commercial fish gelatin. *Journal of Food Engineering*, *92*(1), 78–85.

Hosseini, M. H., Razavi, S. H., & Mousavi, M. A. (2009). Antimicrobial, physical and mechanical properties of chitosan-based films incorporated with thyme, clove and cinnamon essential oils. *Journal of Food Processing and Preservation*, *33*(6), 727–743.

Kamkar, A., Javan, A. J., Asadi, F., & Kamalinejad, M. (2010). The antioxidative effect of Iranian Mentha pulegium extracts and essential oil in sunflower oil. *Food and Chemical Toxicology*, *48*(7), 1796–1800.

Karbowiak, T., Debeaufort, F., Champion, D., & Voilley, A. (2006). Wetting properties at the surface of iota-carrageenan-based edible films. *Journal of Colloid and Interface Science*, *294*(2), 400–410.

Krishnaiah, D., Sarbatly, R., & Nithyanandam, R. (2011). A review of the antioxidant potential of medicinal plant species. *Food and Bioproducts Processing*, *893*(3), 217–233.

Kroll, J., & Rawel, H. (2001). Reactions of plant phenols with myoglobin: Influence of chemical structure of the phenolic compounds. *Journal of Food Science*, *66*(1), 48–58.

Lopez, P., Sanchez, C., Batlle, R., & Nerin, C. (2005). Solid-and vapor-phase antimicrobial activities of six essential oils: Susceptibility of selected foodborne bacterial and fungal strains. *Journal of Agricultural and Food Chemistry*, *53*(17), 6939–6946.

Ojagh, S. M., Rezaei, M., Razavi, S. H., & Hosseini, S. M. H. (2010). Development and evaluation of a novel biodegradable film made from chitosan and cinnamon essential oil with low affinity toward water. *Food Chemistry*, *122*(1), 161–166.

Park, H. (1996). Gas and mechanical barrier properties of carrageenan-based biopolymer films. *Food Science and Industry*, *29*, 47–53.

Pérez-Gago, M. B., & Krochta, J. M. (2001). Lipid particle size effect on water vapor permeability and mechanical properties of whey protein/beeswax emulsion films. *Journal of Agricultural and Food Chemistry*, *49*(2), 996–1002.

Saei-Dehkordi, S. S., Tajik, H., Moradi, M., & Khalighi-Sigaroodi, F. (2010). Chemical composition of essential oils in *Zataria multiflora* Boiss. from different parts of Iran and their radical scavenging and antimicrobial activity. *Food and Chemical Toxicology*, *48*(6), 1562–1567.

Sánchez-González, L., González-Martínez, C., Chiralt, A., & Cháfer, M. (2010). Physical and antimicrobial properties of chitosan-tea tree essential oil composite films. *Journal of Food Engineering*, *98*(4), 443–452.

Sánchez-González, L., Vargas, M., González-Martínez, C., Chiralt, A., & Cháfer, M. (2009). Characterization of edible films based on hydroxypropylmethylcellulose and tea tree essential oil. *Food Hydrocolloids*, *23*(8), 2102–2109.

Shakeri, M. S., Shahidi, F., Beiraghi-Toosi, S., & Bahrami, A. (2011). Antimicrobial activity of *Zataria multiflora* Boiss. essential oil incorporated with whey protein based films on pathogenic and probiotic bacteria. *International Journal of Food Science and Technology*, *46*(3), 549–554.

Shan, B., Cai, Y. Z., Sun, M., & Corke, H. (2005). Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *Journal of Agricultural and Food Chemistry*, *53*(20), 7749–7759.

Siripatrawan, U., & Harte, B. R. (2010). Physical properties and antioxidant activity of an active film from chitosan incorporated with green tea extract. *Food Hydrocolloids*, *24*(8), 770–775.

Tyagi, A. K., & Malik, A. (2011). Antimicrobial potential and chemical composition of Mentha piperita oil in liquid and vapour phase against food spoiling microorganisms? *Food Control*, *22*(11), 1707–1714.