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Active wheat gluten films obtained by thermoplastic processing

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ABSTRACT

Active films based on glycerol-plasticized wheat gluten protein containing different thyme oil concentrations (0–15 wt.%) were prepared by a thermoplastic process involving relatively high temperature and pressure. A complete thermal, structural, mechanical, antimicrobial and antioxidant characterization of all formulations was carried out. Antimicrobial activity tests showed that neat thyme oil presented a meaningful antimicrobial activity. The addition of thyme essential oil to formulations based on gluten protein allowed to prepare biodegradable and edible films with increased *in vitro* antioxidant and antimicrobial properties for the most concentrated samples. However, increasing essential oil concentration led to a continuous decrease in the tensile and storage modulus but to an increase in the deformability of the films. Only films containing 10 and 15 wt.% thyme oil showed reduced moisture sorption with respect to the control film (0% thyme oil), while the water vapor permeability and total soluble matter were not markedly affected by its addition. These changes were attributed to the interference of the oil in the formation of the protein molecular network, and were corroborated by the analysis of the films microstructures by SEM.

1. Introduction

Microbial growth and oxidation reactions occurring on food surface are two of the main causes of deterioration and loss of fresh and processed food products (Türe, Gällstedt, & Hedenqvist, 2012). Direct application of antimicrobial and/or antioxidant substances on the food surface to limit the undesirable microorganisms and oxidative reactions may result in the inactivation or evaporation of active agents and rapid migration into the bulk of foods (Quintavalla & Vicini, 2002; Türe et al., 2012). A proposed way of overcoming this problem and providing a sustainable packaging is to incorporate the antimicrobial agent into bio-based edible packaging materials (Cha & Chinnan, 2004; Türe et al., 2012).

Protein based materials have been explored as potential packaging materials because of their good barrier properties against oxygen and aroma compounds (Cuq, Gontard, & Guilbert, 1998; Türe et al., 2012). Among them, wheat gluten has been taken into account due to its interesting viscoelastic properties, ability to cross-link upon heating, low water solubility, low cost and availability as a co-product of the wheat starch industry (Zubeldía, Ansorena, & Marcovich, 2015). Moreover, wheat gluten films can be obtained by thermoplastic processing, which consists of mixing proteins and plasticizer by a combination of heat and shear (Hernandez-Izquierdo & Krochta, 2008) followed by an additional stage involving further thermo-mechanical treatments (e.g. compression molding) (Pommet, Redl, Guilbrt, & Morel, 2005; Sun, Song, & Zheng, 2008), which, from economical and environmental viewpoints, is the most viable way to produce rigid gluten-based materials since it is fast and requires no solvent (Gällstedt, Mattozzi, Johansson, & Hedenqvist, 2004; Jansens, Lagrain, Rombouts, Smet, & Delcour, 2011).

Film packaging can improve food storage, mainly as a result of their ability to act as barriers to water, preventing dehydration, and to oxygen and light, reducing lipid oxidation. Furthermore, a variety of compounds, including organic acids, enzymes and spices have been proposed for active food packaging (Martínez, Partal, García-Morales, Guerrero & Gallegos, 2013; Tharanathan, 2003). Essential oils (EO's) extracted from plants are rich sources of biologically active compounds such as terpenoids and phenolic that also have antimicrobial and antioxidant properties (Burt, 2004; Lamber, Skandamis, Coote, & Nychas, 2001). In particular, the antioxidant activity and antimicrobial properties of thyme essential oil have been demonstrated and attributed to its major components, thymol and carvacrol (Kuorwel, Cran, Sonneveld, Miltz, & Bigger, 2011).

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Despite the great potential of essential oils, their use in food preservation remains limited mainly due to their intense aroma and possible changes in the organoleptic properties of the food. The use of edible films to carry essential oils could minimize the required doses by the encapsulation effect in the polymer matrix, which limits their volatilization and controls the compounds release, reducing the negative impact of these ingredients. However, only a few studies have been reported on the development of antimicrobial and antioxidant biodegradable films using thermoplastic processes such as compression molding (Dawson, Carl, Acton, & Han, 2002), probably because of the inactivation of the incorporated active agents due to the high temperature and pressure associated with the process (Del Nobile et al., 2009).

The present work was performed in order to find an antimicrobial-antioxidant/material system that would respond to the compression molding; i.e. a material that would remain antimicrobial and antioxidant while having sufficient mechanical integrity. Thus, results related with the characterization of films obtained by intensive mixing followed by compression molding based on wheat gluten plasticized with glycerol and thyme essential oil are presented.

2. Materials and methods

2.1. Materials

Wheat gluten, food grade, protein content 77.8% (dry matter) according to the manufacturer and moisture content of 9.08 ± 0.10% (Zubeldía et al., 2015) provided by Dietetics Los Pinos (Mar del Plata, Argentina); glycerol, technical grade (DEM Chemicals, Mar del Plata, Argentina) and thyme essential oil (TO), obtained from the plant Thymus vulgaris (Las Boticarias, Buenos Aires, Argentina) were used to produce the films.

2.2. Preparation of films

Wheat gluten (WG) powder was mixed with glycerol (20 wt.% referred to the total mass) and with the appropriate amounts of thyme essential oil, leading to films containing 0–15 wt.% of the active component in a laboratory intensive mixer operated at 50 rpm and 80 °C during 5 min. The paste was then removed from the mixer, cut manually into pellets and formed into films by pressing them at 100 °C and 100 kg/m^2 for 10 min in a hydraulic heated press (Zubeldía et al., 2015). The visual appearance of the films was completely homogenous.

2.3. Extraction and quantification of thymol and carvacrol by HPLC

The average concentration of thymol and carvacrol in the wheat gluten films was determined by means of the method described by (Torres, Romero, Macan, Guarda, & Galotto, 2014) with some modifications. Extraction was performed by a shaker holding 0.5 gr of each film in flasks filled with 20 mL of methanol at room temperature for 24 h in dark conditions. Afterwards, these flasks were sonicated in an ultrasound chamber (PS-30A, RoHs, China) during 20 min at room temperature; methanol was recovered and filtered to be analyzed by HPLC. Quantification of thymol and carvacrol was determined by High Performance Liquid Chromatography (HPLC, Agilent 1200 series, Santa Clara, CA, USA) equipped with a high pressure pump, automatic injector and a UV–visible diode array detector. The analytical column was a Kromasil 100-5C18 (Eka Chemical, Stockholm, Sweden). The mobile phase was an acetonitrile: distilled water (40:60 v/v) mixture with a flow rate of 1.0 mL/min and an injection volume of 10 μL. The detection of thymol and carvacrol was performed with a wavelength of 274 nm. The extracts and standard compounds were analyzed under the same conditions.

2.4. Characterization techniques

Antimicrobial activity was evaluated by the agar diffusion method, determining the sensitivity of the native microflora of lettuce (Ponce, Aguero, Roura, Del valle, & Moreira, 2008) and broccoli (Moreira, Ponce, Ansorena, & Roura, 2011), and pure cultures of *Escherichia coli* (ATCC 25922), *Listeria innocua* (CIP 8011), *Pseudomona aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 25923) to thyme oil and active WG films as described in Ponce, Fritz, del Valle, and Roura (2003) and Ramos et al. (2012). WG film disks without incorporation of TO and distilled water were used as control. Native microflora of lettuce and broccoli were prepared according to Ponce et al. (2003).

The microbial growth without seeding with bacterial culture was evaluated using unsterilized WG films discs and placing them on BHI agar plate. Samples were incubated at 37 °C for one week.

The antioxidant activity and the Total Phenolic (TP) content were evaluated by using hydrophilic extracts obtained according to Shivashankara, Isobe, Al-Haq, Takenaka, and Shiina (2004). The antioxidant capacity was analyzed using the stable radical 2,2-diphenil-1-picrylhydrazyl (DPPH) as previously reported by Brand-Williams, Cuvelier, and Berset (1995). Trolox was used as the standard of the measurement and the antioxidant activity was reported in mg Trolox/g. TP was determined spectrophotometrically using the Folin-Ciocalteu reagent (FCR) as described in Singleton and Rossi (1965). Results were expressed as mg gallic acid equivalents (GAE)/g.

Tensile and Dynamic Mechanical tests, Water vapor permeability (WVP), Total soluble matter (DRY and WET methods) and Equilibrium Moisture Content measurements, as well as Scanning Electron Microscopy (cryo-fractured film cross-section) and Statistical analysis were performed as described in Zubeldía et al. (2015).

Thermogravimetric (TGA) measurements were carried out on a Shimadzu TGA- 50 thermogravimetric analyzer. Thermal degradation was performed under air atmosphere, using heating ramps of 10 °C min^{-1} in the temperature range of 25–750 °C and samples of 4–10 mg.

3. Results and discussion

3.1. Antimicrobial and antioxidant properties

Total phenolic content, antioxidant capacity and thymol and carvacrol content in thyme essential oil are listed in Table 1. Some

Table 1
Thyme essential oil characterization (thymol and carvacrol concentration, total phenolic content and antioxidant capacity).

Pure thyme oil characterization	
Compounds	(% of TO)[a]
Thymol	65.8
Carvacrol	6.1
Others	28.1
Total phenolic content and antioxidant capacity	
TP (mg GAE/g)	74.54 ± 2.2
DPPH (mg trolox/g)	10.97 ± 0.4

TP and DPPH reported values were measured in triplicate and correspond to the mean ± standard deviation.

[a] Expressed as percentage of the total peak area of the chromatograms (referred to the total thyme oil concentration).

variances between our results and others were seen, as Jouki, Mortazavi, Yazdi, and Koocheki (2014) reports 46.42% and 12.42% for thymol and carvacrol content, respectively. However these authors mentioned also that these variances could be attributed to differences in herbal species and their ecotypes, and other environmental parameters.

Table 2 shows the susceptibility of two native microflora and four pure cultures to thyme oil. Native microflora of lettuce and broccoli and Gram-positive bacteria strains (*L. innocua* and *S. aureus*) were extremely sensitive to thyme oil showing inhibition halos larger than 20 mm. As regards Gram-negative bacteria (*E. coli* and *P. aeruginosa*), these strains resulted very sensitive to thyme oil. The antimicrobial activity of TO has been attributed to the terpenes (carvacrol and thymol) contained in this essential oil. According to Burt (2004), carvacrol and thymol disintegrate the outer membrane of Gram (−) bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to adenosine triphosphate. Carvacrol dissolves in the phospholipid bilayer of Gram-positive bacteria and causes expansion and destabilization of the membrane leading to cell death (Ultee, Kets, & Smid, 1999). Numerous studies investigating the action of essential oil against different indicator microorganism agree that essential oils are most effective against Gram-positive bacteria than against Gram-negative (Burt, 2004; Pelissari, Grossmann, Yamashita, & Pineda, 2009). Burt (2004) suggests that this behavior may be related to the presence of an additional external membrane surrounding the cell wall in Gram-negative bacteria, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering.

The amount of thymol and carvacrol present in formulations before and after processing is indicated in Table 3. Results show that around 73.2–80.4% of the initial thymol content and around 72.9–75.4% of the initial carvacrol content remained in the wheat gluten films after processing (average losses of 23.2% and 25.8% for thymol and carvacrol, respectively). Similar results were obtained by Ramos, Jiménez, Peltzer, and Garrigós (2014) when adding thymol to PLA films obtained by compression molding. They found that approximately 30% of the initial thymol was lost during processing by evaporation or degradation. Similar looses were reported by Hwang et al. (2013) when incorporating resveratrol and α-tocopherol to PLA/starch blends films obtained by compression molding. On the other hand, Rupika, Bigger, Sonneveld, Cran, and Miltz (2007) studied the effect of plastic films containing mixtures of thymol and carvacrol prepared by extrusion blow-molding. These authors reported losses after processing of 65.4% and 77.6% for thymol and carvacrol, respectively. In the present work the losses were much smaller than in the work of Rupika et al. (2007) because of the lower temperatures involved in our process compared to extrusion.

The antimicrobial properties of the WG films enriched with TO were evaluated by measuring the size of the "clear" inhibition zone around the tested films. Table 4 shows inhibition zones ranging from 7.5 to 8.3 mm in the presence of Gram (−) bacteria and 6.5–11.1 mm in the case of Gram (+) bacteria. Examples of clear inhibition zones around the active films (B, C), in comparison with the no inhibition of the control film (A) are shown in Fig. 1. Inhibition was increased with increasing concentration of essential oil. As expected, the films containing the highest oil content (15%) presented the greatest zone of inhibition ($p < 0.05$). The results indicated that a concentration of at least 10 wt.% TO was necessary to obtain a significant and clear inhibition zone in the case of Gram-positive bacteria and only films containing 15 wt.% TO showed significant antimicrobial activity against both types of bacteria and native microflora of the two selected vegetables. As in the case of pure thyme oil, WG films with TO were significantly more effective against Gram (+) bacteria than against Gram (−) bacteria.

Several studies tested the antibacterial activity of edible films incorporated with different natural substances (e.g. Sivarooban, Hettiarachchy, & Johnson, 2008), but fewer focused on films incorporated with thyme essential oil. Moreover, the addition of thyme oil to films obtained by compression molding was scarcely studied. Jouki et al. (2014) added thyme essential oil to quince seed mucilage films obtained by casting and found that films containing 2% oil presented antimicrobial activity against all tested microorganisms. During extrusion or compression molding of antimicrobial films, the temperature and mechanical energy input, such as shearing forces, must be carefully considered (Kuorwel et al., 2011). As discussed before, high processing temperatures, result in considerable losses of volatile antimicrobial agents (Suppakul, Sonneveld, & Bigger, 2011), which was consistent with the studies of Ramos et al. (2014), Hwang et al. (2013) and Türe et al. (2012). Türe et al. (2012) incorporating potassium sorbate (10–15%) to wheat gluten films obtained by compression molding found that a concentration of at least 10 wt.% PS was necessary to inhibit all tested microorganisms. Ramos et al., (2014) added thymol (4–8 wt.%) and modified montmorillonite to poly lactic acid (PLA) to produce nano-biocomposite films obtained by compression molding. They found that a concentration of 8% of thymol was necessary to achieve antimicrobial action because approximately 30% of the initially thymol was lost during processing by evaporation or degradation. Similar results were reported by Hwang et al. (2013) when incorporating resveratrol and α-tocopherol to PLA/starch blends films obtained by compression molding.

In order to investigate the efficiency of TO as an antimicrobial agent in a more realistic situation, the microbial growth, without seeding with bacteria on the WG films was investigated visually (results not shown). Results reveal that after one week at 37 °C the surface of the film without TO was covered with microbes. Microbes were also present in the agar solution. In contrast, WG films (with TO concentration higher than 10%) showed no microbial growth. Evidently the residual TO left in the film after processing was sufficient to prevent microbial growth. This is a very promising result since a wheat gluten film in contact with an agar solution is basically a "worst case" in edible/non-edible packaging applications.

The antioxidant activity and total phenolic content of active WG films are shown in Table 5. Antioxidant activity was performed to

Table 2
Antimicrobial activity of thyme essential oil.

Diameter of inhibition zone (mm)						
	Native microflora		Gram − bacteria		Gram + bacteria	
	Lettuce	Broccoli	*E. coli*	*P. aeruginosa*	*L. innocua*	*S. aureus*
Water	ND	ND	ND	ND	ND	ND
Pure TO	39.6 ± 1.3	29.0 ± 0.9	16.4 ± 0.7	19.0 ± 0.9	25.0 ± 1.5	33.5 ± 1.8

Reported values were measured in triplicate and correspond to the mean ± standard deviation.
ND, not detected.

Table 3
Nominal and after processing concentrations of thymol and carvacrol in active wheat gluten films.

Sample	Nominal		After processing			
	Thymol concentration (wt.%)	Carvacrol concentration (wt.%)	Thymol		Carvacrol	
			Concentration (wt.%)	Retention (%)	Concentration (wt.%)	Retention (%)
1.4 wt.% TO	0.92	0.08	0.74 ± 0.05	80.4	0.06 ± 0.05	72.9
3.5 wt.% TO	2.30	0.21	1.79 ± 0.08	77.8	0.16 ± 0.02	75.1
10 wt.% TO	6.58	0.61	4.82 ± 0.1	73.2	0.46 ± 0.09	75.4
15 wt.% TO	9.87	0.91	6.81 ± 0.3	68.9	0.69 ± 0.06	74.9

Reported values were measured in triplicate and correspond to the mean ± standard deviation.

Table 4
Inhibition zone diameters yielded by WG film disks with various concentrations of TO.

Sample	Diameter of inhibition zone (mm)					
	Native microflora		Gram (−) bacteria		Gram (+) bacteria	
	Lettuce	Broccoli	*E. coli*	*P. aeruginosa*	*L. innocua*	*S. aureus*
Control	ND	ND	ND	ND	ND	ND
1.4 wt.% TO	ND	ND	ND	ND	ND	ND
3.5 wt.% TO	ND	ND	ND	ND	6.9 ± 0.3^{c}	6.5 ± 0.2^{c}
10 wt.% TO	7.6 ± 0.2^{b}	7.8 ± 0.3^{b}	7.9 ± 0.4^{b}	7.5 ± 0.4^{b}	8.6 ± 0.3^{b}	8.4 ± 0.2^{b}
15 wt.% TO	8.7 ± 0.3^{a}	12 ± 0.6^{a}	8.3 ± 0.4^{a}	8.2 ± 0.4^{a}	11.1 ± 0.7^{a}	9.2 ± 0.5^{a}

[a,b,c] Different letters in the same column indicate significant differences ($p < 0.05$).
Reported values correspond to the mean ± standard deviation. ND, not detected.

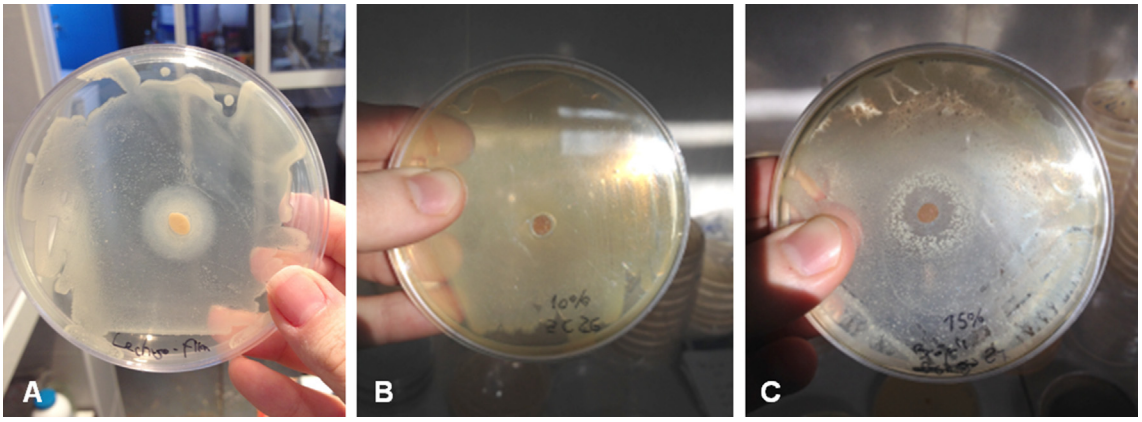

Fig. 1. Inhibition zones around the control and selected active films (6 mm diameter discs). **A:** Control film on agar seeded with lettuce microflora; **B:** 10% TO film on agar seeded with *E. coli*; **C:** 15% TO film on agar seeded with broccoli microflora.

Table 5
Antioxidant activity and total phenolic content of WG films enriched with TO.

Sample	DPPH (mg trolox/g)	TP (mg GAE/g)
Control	ND	ND
1.4 wt.% TO	ND	0.92 ± 0.09^{c}
3.5 wt.% TO	ND	2.47 ± 0.18^{c}
10 wt.% TO	1.511 ± 0.13^{b}	9.98 ± 0.3^{b}
15 wt.% TO	2.062 ± 0.13^{a}	13.28 ± 0.8^{a}

[a,b,c] Different letters in the same column indicate significant differences ($p < 0.05$).
Reported values correspond to the mean ± standard deviation. ND, not detected.

evaluate if the remaining TO in the WG matrix was enough to be considered an efficient antioxidant in these formulations. As expected, films showed lower antioxidant capacity than pure compound (Table 1) since the mass fraction of the essential oil in the films is relatively low. The antioxidant activity of wheat gluten-TO films significantly increased ($p < 0.05$) with increasing TO concentration, but only films containing more than 10% TO showed a significant antioxidant activity, as determined by the inhibition of the DPPH radical. Similar results were found for κ-carrageenan films incorporated with *Satureja hortensis* essential oil (Shojaee-Aliabadi et al., 2014) and chitosan films incorporated with *Zataria multiflora* essential oil (Moradi et al., 2012). Particularly, for edible films containing thyme essential oil, similar results were reported by Ruiz-Navajas, Viuda-Martos, Sendra, Perez-Alvarez, and Fernandez-Lopez (2013) and Jouki et al. (2014) for chitosan edible films and quince seed mucilage films, respectively.

The antioxidant power of essential oils, caused mainly by their phenolic compounds, has been reviewed by Shan, Cai, Sun, and Corke (2005) and Dimitrios (2006). The total phenolic content (TP), expressed as gallic acid equivalent was 74.54 mg GAE/g for pure thyme oil (Table 1) and ranged ($p < 0.05$) from 0.92 to 13.28 mg/g for films enriched with TO. The results showed that total phenolic content in the WG films was significantly increased ($p < 0.05$) with increasing thyme essential oil concentration and the highest value (13.28 mg GAE/g film) was for the film formulated with 15% TO. As Shojaee-Aliabadi et al. (2014) and Shan et al.,

(2005) mentioned, there was a linear correlation between TP content and antioxidant activity.

3.2. Mechanical and dynamic mechanical properties

Table 6 summarized the tensile properties of the different films. The incorporation of thyme essential oil affects the mechanical behavior of the films by decreasing the tensile modulus (**E**) and strength (σ_m) and increasing the elongation at break (ε_b; $p < 0.05$). In this case, the essential oil seems to be acting as a plasticizer, affecting in some way the interactions between macromolecular chains in the polymer matrix. Addition of lipid or oil in protein-based films may hinder polymer chain-to-chain interactions and provide flexible domains within the film (Tongnuanchan, Benjakul, & Prodpran, 2012). According to Arfat, Benjakul, Prodpran, and Sumpavapol (2014) the incorporation of essential oil into fish protein isolate/fish skin gelatin blend film could enhance the development of heterogeneous film matrix, leading to discontinuity of film network. Similar results are reported in literature by Türe et al. (2012), who incorporated potassium sorbate as antimicrobial agent for compression molded WG films and Arrieta, Peltzer, Garrigós, and Jiménez (2013) for sodium and calcium caseinate edible films modified with carvacrol, among others. This effect could be primarily explained by the replacement of polymers by the lipids in the film matrix: the interactions between polar polymer molecules are much stronger than those between non-polar lipid molecules and polar polymer (Atarés, De Jesús, Talens, & Chiralt, 2010). Nevertheless, the effect that produces the essential oil on the mechanical behavior is of potential value for food packaging applications as these films could find application in products requiring film flexibility (Giteru et al., 2015; Kavoosi, Dadfar, & Purfard, 2013).

Fig. 2 shows the results of DMA tests in torsion mode, over a temperature interval from −50 to 150 °C, carried out on active wheat gluten films. For the control sample (0% thyme oil), the storage modulus (G′) decreases with increasing temperature up to 130 °C, followed by a short temperature interval where G′ levels off (rubbery-like plateau). No evidence of further cross-linking during the test is found (G′ increasing with temperature), as was reported by other researchers (Martínez et al, 2013). Thyme essential oil addition also exerts a clear and pronounced influence on the general dynamical behavior: the sample containing 1.4% TO shows essentially the same behavior as control sample, with slightly higher G′ values in the whole range of temperature, while the opposite occurs with samples containing more than 3.5% TO film. Films containing higher amounts of TO have increasing temperature susceptibility, they soften at lower temperatures and thus, do not reach the rubbery-like plateau. According to Martinez et al (2013) for these protein-based systems, the plateau may be attributed to a situation that falls between a temporary entangled network and covalent cross-linking (Ross-Murphy, 1995), as hydrophobic interactions usually act not at a point on the chain as covalent cross-links do, but involve more extended "junction zones". In the present case, the films containing increasing concentrations of TO behave as if the number of sites available for interactions among chains were reduced or as if the cross-linking degree of these samples were lower than that of the control/low TO content ones.

These features are also reflected in than δ curves (Fig. 2B): the sample containing 1.4% thyme essential oil does not bring about any substantial changes in the thermo-mechanical response respect to the control sample, while the more concentrated samples (10–15% TO) evidenced a shift of the gel–glasslike transitions towards lower temperatures as the same time that the intensity (height) of the peaks increase, which indicates that the volume fraction of the material undergoing the transition increases with TO concentration, a behavior related to plasticizer effect (Pouplin, Redl, & Gontard, 1999).

Table 6
Tensile properties of active films.

Sample	E (MPa)	σ_m (MPa)	ε_b (%)
Control	88.9 ± 24.0^{a}	4.9 ± 1.0^{a}	28.2 ± 6.3^{a,b}
1.4 wt.% TO	30.0 ± 2.6^{b}	2.4 ± 0.5^{b}	22.7 ± 8.8^{b}
3.5 wt.% TO	20.5± 4.2^{c,b}	2.1 ± 0.4^{b}	35.2 ± 5.3^{b}
10 wt.% TO	17.7 ± 2.7^{c,b}	2.1 ± 0.5^{b}	48.5 ± 10.1^{a}
15 wt.% TO	7.8 ± 5.1^{c}	1.1 ± 0.2^{c}	54.1 ± 5.1^{a}

^{a,b,c} Different letters in the same column indicate significant differences ($p < 0.05$). Reported values correspond to the mean ± standard deviation.
E: tensile modulus; **σ_m:** tensile strength **ε_b:** elongation at break.

3.3. WVP, TSM and equilibrium moisture content

The affinity for water of the different samples is presented in Table 7. The WVP of the samples containing high amounts of essential oil (10 and 15%) are similar to that of the control films ($p < 0.05$), while the active specimens with low content of TO exhibit higher values. These results suggest that the oil interacts somehow with the protein/plasticizer, leading to film microstructures that depend on TO content, as was previously envisioned from the analysis of the mechanical and thermo-mechanical properties. Atarés et al. (2010) attributed similar findings to the interactions of oil components with some protein tails that could promote the decrease in the hydrophobic character of the active protein matrix. The difficulties in integrating the lipid in the hydrophilic network may cause matrix disruptions and create void spaces at the protein–lipid interface. As our films are prepared by a dry method (as opposite to emulsion casting), the homogenization process could be even less efficient and thus, these heterogeneities could become the factor controlling the water vapor transmission rate. Moreover, when the amount of incorporated oil is low, the lipid discontinuities that increase the tortuosity factor for transfer of water molecules are limited and could not compensate the disruptions introduced by TO into the active films. As water vapor transfer normally occurs through the hydrophilic portion of film network and thus, depends on hydrophilic/hydrophobic ratio of film constituent (Tongnuanchan et al., 2012), the situation reverses for higher TO concentrations reaching WVP values comparable to the control sample. Moreover, a slight increase in WVP was also reported in related works, for example in gelatin films with clove or bergamot essential oils added (Ahmad, Benjakul, Prodpran, & Agustini, 2012; Giménez. Gómez-Guillén, López-Caballero, Gómez-Estaca & Montero, 2012).

Concerning TSM results, it is noted that the total soluble matter of the control sample obtained from the WET method is higher than the corresponding to the DRY one, which indicates that during drying at 105 °C, gluten proteins further cross-linked. It is known that gluten proteins undergo disulfide bonding upon heating, which leads to the formation of a three-dimensional macromolecular network (Gällstedt et al., 2004; Pommet et al., 2005; Zubeldía et al., 2015). Moreover, the type and degree of protein reactions depend on the heating conditions and on the initial gluten powder moisture content (Sun et al., 2008; Zubeldía et al., 2015), among other factors. On the other hand, both WET and DRY TSM values are similar for the active films, which could indicate that the essential oil inhibits the additional cross-linking that indeed takes place during heating at 105 °C in the control sample, but also that these samples reached the larger cross-linking degree possible during processing/molding steps. Moreover, the DRY

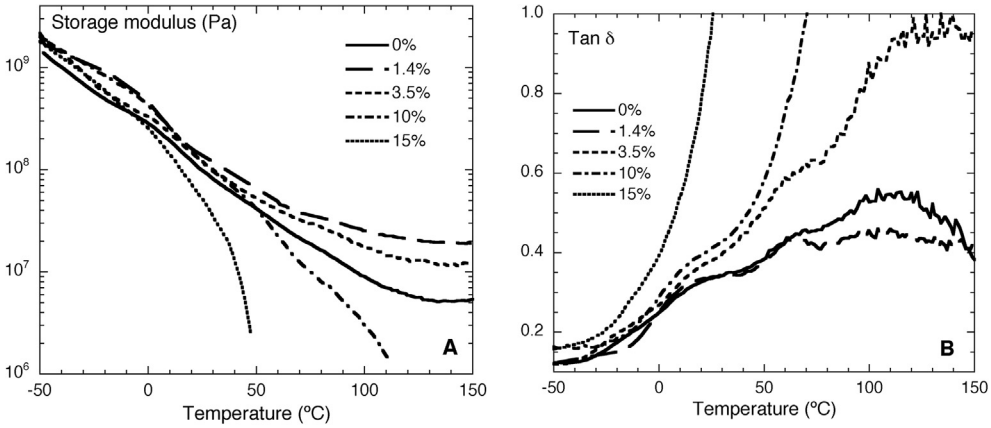

Fig. 2. Dynamic mechanical tests in torsion mode as a function of the temperature for wheat gluten active films. **A.** Storage modulus (G′) and **B.** tan δ curves.

Table 7
Water permeation values (WVP), Total soluble matter (TSM) and equilibrium moisture content (Meq) of WG active films.

Sample	WVP ($\times 10^{10}$) g/(Pa.s.m)	TSM DRY (%)	TSM WET (%)	Meq (%)
Control	1.75 ± 0.01^{b}	22.9 ± 0.20^{ab}	24.4 ± 0.20^{a}	63.4 ± 1.9^{b}
1.4 wt.% TO	2.62 ± 0.81^{a}	23.6 ± 0.20^{a}	23.7 ± 0.10^{ab}	64.4 ± 1.4^{ab}
3.5 wt.% TO	2.57 ± 0.56^{a}	23.7 ± 0.10^{a}	23.2 ± 0.10^{b}	68.6 ± 3.2^{a}
10 wt.% TO	1.50 ± 0.62^{b}	21.8 ± 0.17^{b}	22.0 ± 0.17^{c}	55.7 ± 1.4^{c}
15 wt.% TO	1.70 ± 0.51^{b}	21.6 ± 0.61^{b}	21.5 ± 0.74^{c}	55.4 ± 1.5^{c}

^{a,b,c} Different letters in the same column indicate significant differences ($p < 0.05$). Reported values correspond to the mean ± standard deviation.

values of the samples with low amounts of TO are higher than the corresponding to the control sample, while the opposite is noticed for the films containing 10–15% essential oil, which confirms that the microstructure of the films changes according to the TO concentration, probably by acting as a defect at low concentrations but inducing stronger interactions between protein chains at higher contents.

Regarding equilibrium moisture content, data presented in Table 7 revealed that there are no significant differences ($p < 0.05$) among control and active films with low concentrations of essential oil. However, the moisture absorption of the samples containing 10–15% TO is lower, reflecting the influence of the hydrophobic lipid component on film composition that leads to a reduction of the global hydrophilicity of the samples.

3.4. Thermal degradation

TGA thermograms presenting thermal degradation behavior of neat essential oil and WG films containing TO are illustrated in Fig. 3. Thyme essential oil degraded in two steps, leaving almost no residual waste after 140 °C. On the other hand, four main stages of weight loss were observed for all films, in temperature ranges that depend on the essential oil concentration. The weight loss in the lower temperature range (up to 210 °C) could be associated with the loss of moisture and low molecular weight components in the films, including glycerol and aroma compounds from the TO, as indicated in related papers (Arrieta et al, 2013; Tongnuanchan, Benjakul, & Prodpran, 2014). The films lost the 10% of their mass at lower temperature as the content of TO increases, which indicated that not all the oil was evaporated during processing steps. The other stages of weight loss were most likely associated with the degradation of the larger size or highly cross-linked protein fractions, although the temperature for loosing half of the initial mass decreases as the TO content increases. The presence of additives in protein-based film contributes to the decrease in the number of protein–protein bonds, resulting in lower thermal stability of the samples (Arrieta et al., 2013; Verbeek & Van den Berg, 2010).

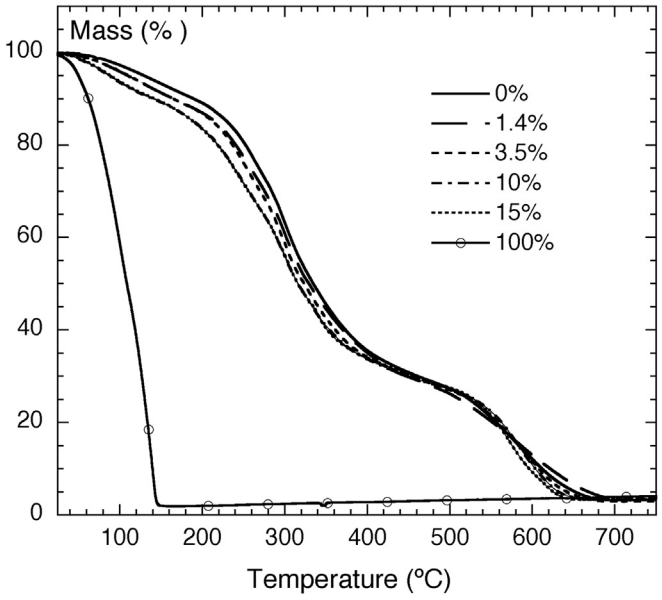

Fig. 3. Thermogravimetric curves of neat essential oil and wheat gluten active films.

3.5. Film microstructure

SEM micrographs of the freeze-fractured cross-section of active films are illustrated in Fig. 4. With the addition of TO, the film cross-section became rougher, clearly evidencing plasticization features as compared with the control film. Gluten powder prior to molding consists of particles with variable sizes. In the control sample

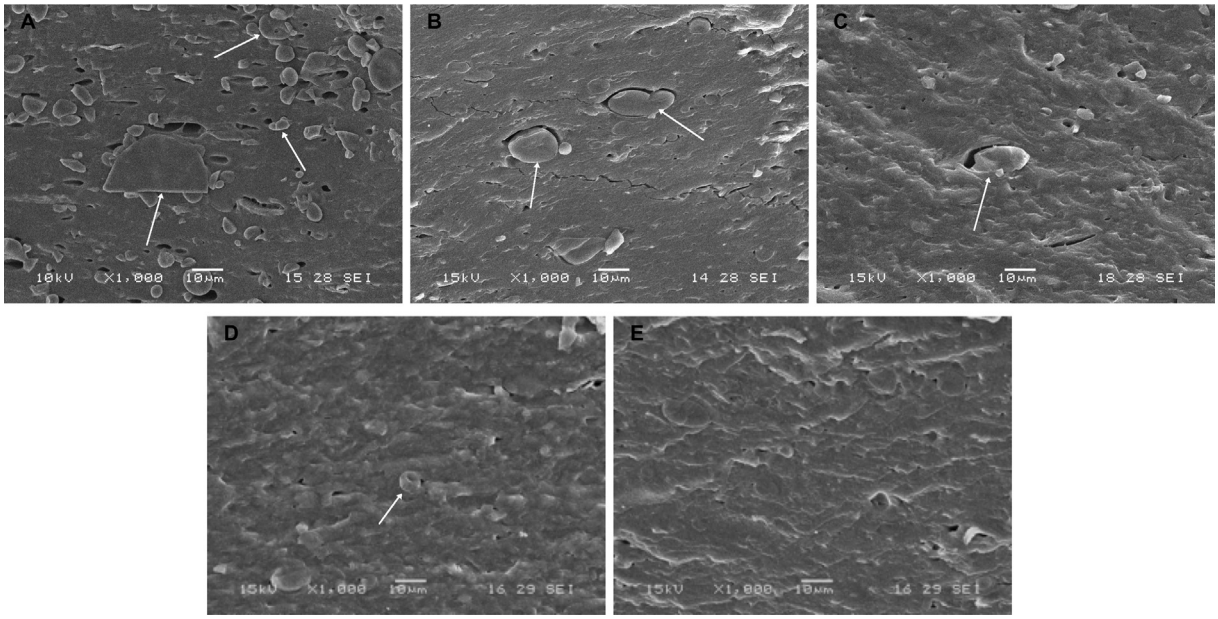

Fig. 4. SEM micrographs of the freeze-fractured cross-section of WG active films. **A:** control film; **B:** 1.4% TO; **C:** 3.5% TO; **D:** 10% TO; **E:** 15% TO.

particle-like structures are still present with voids in between (Fig. 4A). It seems likely that these particle-like structures and voids result from incomplete polymer flow during mixing/molding steps under the applied conditions, as indicated by Mangavel et al. (2004). The presence of particle-like structures decreased with increasing TO content, which can be related to the improved polymer flow under these conditions. Meanwhile, all active films exhibited discontinuities in the form of micro-pores or cavities, which could be attributed to the evaporation of TO during processing, as informed in related works (Broumand, Emam-Djomeh, Hamedi & Razavi, 2011).

4. Conclusions

It was shown that using thyme oil as the active agent it is possible to make antioxidant and antimicrobial WG films by thermoplastic processing. The addition of the oil leads to heterogeneous films containing hydrophobic discontinuities that reduce the mechanical performance in terms of strength and modulus, but enhance their flexibility. Samples containing lower amounts of TO presented larger WVP and equilibrium moisture content values than control sample, but showed the opposite behavior at higher concentrations. Thermogravimetric measurements confirmed that thyme oil was still present in all formulations after processing and that it would be able to be released from the films to foodstuff as active additive. Antimicrobial *in vitro* results indicated that at least 10 wt.% TO was necessary to obtain a significant and clear inhibition zone in the case of Gram-positive bacteria and only films containing 15 wt.% TO showed significant antimicrobial activity against both types of bacteria and native microflora of two selected vegetables. Most of the thymol and carvacrol added remained in the formulations after processing, which resulted in a significant antioxidant activity, as indicated by the high percentage of inhibition obtained using the DPPH. Moreover, a continuous increase in antioxidant parameters was observed as the thyme oil concentration in the films increased. Thyme oil could protect the polymer WG matrix from oxidative degradation during processing and further the use of these biodegradable films.

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