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Properties and antioxidant activity of soy protein concentrate films incorporated with red grape extract processed by casting and compression molding

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ABSTRACT

Self-standing transparent soy protein concentrate (SPC) films plasticized by glycerol (30% w/w SPC dry basis) and supplemented with red grape extract (RGE) as natural antioxidant (0–10% w/w SPC dry basis) were prepared by two methods: casting and intensive mixing followed by compression molding. The influence of RGE on key film properties was analyzed at the light of the specific stabilizing interactions of SPC films. The addition of RGE had a favorable effect on moisture content, total soluble matter, water vapor permeability and percentage of elongation of casted films compare with compression molded counterparts. RGE induced a redistribution of hydrogen interactions of casted films replacing protein-protein hydrogen interactions by protein-polyphenol ones with no variations in disulfide bridges, while these last interactions were significantly reduced in compression molded films in favor of hydrophobic and hydrogen interactions, as disclosed by differential solubility assays and infrared spectroscopy. The antioxidant activity of the SPC films in terms of scavenging activity of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power, increased significantly irrespective of the manufacturing method, being the release of antioxidants from casted films lower than that from compression molded films in accordance with the strong interactions between SPC matrix and polyphenols.

1. Introduction

The main function of food packaging is to protect foodstuff by delaying or restraining the adverse effect of the environment on food, in order to extend its shelf-life (Marcos et al., 2014; Rooney, 2001; Woranuch, Yoksan, & Akashi, 2015). In this sense, active packaging is a concept that arose in response to consumer demands and market trends and is gaining increasing attention from researchers and food technology industry (Arrieta et al., 2014; Martucci, Gende, Neira, & Ruseckaite, 2015). These systems are based on the deliberate interaction of the packaging with the packed food or the environment and can provide certain functions lacking in conventional packaging systems, affording quality and hygiene benefits and shelf-life extension (Dainelli, Gontard, Spyropoulos, Zondervan-van den Beuken, & Tobback, 2008; Rooney, 2001). Active functions may include the removal of oxygen, moisture, antimicrobial activity, antioxidant activity, UV protection, etc. (Giménez, López de Lacey, Pérez-Santín, López-Caballero, & Montero, 2013; Robertson, 2009; Rooney, 2001).

The incorporation of antioxidants in the packaging material, that migrates from the packaging to the food, provides advantages compared to direct addition to food, since it allows one to extend the shelf-life of the product, while reducing the amount of chemical additives in direct contact with food (Li, Miao, Wu, Chen, & Zhang, 2014; Liang & Ludescher, 2011; Marcos et al., 2014). Currently, due to safety concerns associated with the use of synthetic active antioxidants in food packaging, many research works have focused on natural active substances (Giménez et al., 2013; Li et al., 2014; Marcos et al., 2014; Moreno, Atarés, & Chiralt, 2015), such as organic acids, sorbic (Ozdemir & Floros, 2001), ascorbic (Gemili, Yemenicioğlu, & Altınkaya, 2010; Le Tien, Vachon, Mateescu, & Lacroix, 2001), citric (Le Tien et al., 2001), tannic (Pyla, Kim, Silva, & Jung, 2010); α-tocopherol (Han & Krochta, 2007) and vegetal extracts and essential oils (Arancibia, López-Caballero, Gómez-

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Guillén, & Montero, 2014; Atarés, Bonilla, & Chiralt, 2010; Giménez et al., 2013; Gómez-Estaca, Bravo, Gómez-Guillén, Alemán, & Montero, 2009; Martucci et al., 2015; Moradi et al., 2012; Nie, Gong, Wang, & Meng, 2015; Pruneda et al., 2008; Tongnuanchan, Benjakul, & Prodpran, 2012). Among vegetal extracts, grape seed extracts are excellent alternatives to synthetic antioxidants, since they are recognized as GRAS (Generally Recognized as Safe) by the Food and Drug Administration US (FDA, US Food and Drug Administration) and are natural and renewable (Perumalla & Hettiarachchy, 2011). Grape extracts contains several polyphenolic compounds with antioxidant activity, including monomeric flavon-3-ols such as catechin, epicatechin and procyanidin dimmers and trimers (Chedea, Braicu, & Socaciu, 2010; Perumalla & Hettiarachchy, 2011).

One of the main challenges in the field of active packaging is the incorporation of natural active components into biogenic matrices to obtain completely bio-based active films. As a part of our continuous work on the development and characterization of soybean protein concentrate (SPC) films intended for food packaging (Ciannamea, Stefani, & Ruseckaite, 2014, 2015), our ongoing work was to evaluate the effect of the incorporation of red grape extract (RGE) as natural active agent. Besides the study on the potential activity induced by RGE, the work was focused on the possible alteration of the properties of SPC films due to the well reported interactions between proteins and polyphenols, which are the main components of RGE (Giménez et al., 2013; Sivarooban, Hettiarachchy, & Johnson, 2008; Tongnuanchan et al., 2012). Reportedly, the incorporation of phenolic compounds can alter protein film's properties, such increase the flexibility and lower water vapor permeability (Nie et al., 2015). Some phenolic compounds can react and establish covalent bonds with proteins (Hager, Vallons, & Arendt, 2012; Kroll, Rawel, & Rohn, 2003; Salgado, López-Caballero, Gómez-Guillén, Mauri, & Montero, 2012; Sivarooban et al., 2008; Tongnuanchan et al., 2012; Zhang et al., 2010). Furthermore, protein-polyphenol complexes may occur through various non-covalent interactions, such as hydrophobic interactions or hydrogen bonds (Salgado et al., 2012; Tongnuanchan et al., 2012; Zhang et al., 2010).

The influence of the addition of RGE (5 and 10% w/w SPC) on the formation of SPC based films and their physical, chemical, mechanical and barrier properties was studied. The effect of incorporating the RGE in films processed by the casting method and intensive mixing followed by compression molding was analyzed. Antioxidant capacity of the films through the ability to reduce iron (III) and radical scavenging using the stable radical DPPH was studied.

2. Materials and methods

2.1. Materials

Soy protein concentrate (SPC) Solcom S110, with 69% of protein, 7% of moisture, 1% fat, 3% fiber, 5% ash and approximately 15% of non-starch polysaccharides, was provided by Cordis S.A. (Villa Luzuriaga, Buenos Aires, Argentina). Glycerol (Gly, 99% Anedra, Argentina) was used as a plasticizer without any prior purification. Food grade red grape extract (RGE) was obtained from a local pharmacy (Mar del Plata, Argentina) and was used as - received as antioxidant film additive. Phosphate buffer solution pH 10 (Anedra, Buenos Aires, Argentina) was used in film manufacturing processes. Calcium chloride ($CaCl_2$; Aldrich, St. Louis, USA) was used as desiccant for water vapor permeability tests. TRIZMA/hydrochloric acid, glycine and Na_2EDTA (Biopack, Buenos Aires, Argentina), sodium dodecyl sulfate (SDS) and urea (Anedra, Buenos Aires, Argentina), 2-mercaptoethanol (2-ME, Aldrich, St. Louis, USA) and trichloroacetic acid (TCA, Biopack, Buenos Aires, Argentina) were used for differential solubility test. Sodium azide (Na_3N, Anedra, Buenos Aires, Argentina) solution was used to prevent microbial growth during total soluble matter assays. Folin-Ciocalteu reagent (Sigma-Aldrich) was used to determine the total phenolic content of the RGE. Potassium hexacyanoferrate (III) ($K_3Fe(CN)_6$), Iron (III) chloride ($FeCl_3$) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were used for antioxidant activity assays.

2.2. Film processing

Films were obtained by two different processing methods, solution casting and intensive mixing followed by compression molding, according to the procedures reported in a previous work, with some modifications (Ciannamea et al., 2014). For thermoplastic processing SPC was manually mixed with Gly (30 and 40% w/w on dry SPC basis) and 50% of pH 10 buffer solution (pH > isoionic point, pI ~ 4.5) for 15 min. RGE was incorporated (0–10% w/w on dry SPC basis) to the premixes in a lab-scale roller mixer at 70 °C, 50 RPM for 30 min. Afterwards RGE-added mixtures were thermo-compressed in a three – step operation (150 °C for 5 min at 10 kg/cm^2, 150 °C for 2 min at 100 kg/cm^2 and finally cooling up to 30 °C at 100 kg/cm^2 for about 30 min) by using an hydraulic hot-press (EMS, Argentina). Solution casting films were prepared by mixing SPC with Gly (30–40% w/w on dry SPC basis) and RGE (0–10% w/w SPC) for 15 min. The premixes were then dispersed in pH 10 phosphate buffer solution at 70 °C under constant magnetic stirring for 30 min (Cole-Parmer, USA) to obtain 5% film-forming solutions (5 g SPC/100 cm^3 solution). After degassing under ultrasonic bath (Testlab, 160 W, 40 KHz) for 15 min, the film-forming solutions were poured onto leveled Teflon-coated Petri dishes (15 cm^2 area), left to dry in an air-circulating oven (Memmert, Germany) until reaching constant moisture content (about 24 h) and peeled-off from the plates. To control film thickness, the solid content casted on each plate was fixed.

Film samples were cut into the desired shapes and preconditioned in laboratory environmental chamber at 25 ± 2 °C and 65 ± 2% RH for 48 h prior testing. Casting and compression molding films were labeled as CSPC-XGly-YRGE and MSPC-XGly-YRGE, respectively, were X refers to glycerol and Y to RGE percentages, respectively (% w/w SPC).

2.3. Thickness

Film thickness was measured with a manual micrometer (0–25 ± 0.01 mm, Bta. China). Measurements were done at ten random points along the films. For tensile tests, opacity and moisture absorption experiments, four measurements were done on each specimen.

2.4. Attenuated total reflectance – Fourier transformed infrared (ATR-FTIR)

ATR-FTIR spectra were recorded on a Thermo Scientific Nicolet 6700 spectrometer (Wisconsin, USA). All runs were performed between 400 and 4000 cm^{-1} using an attenuated total reflectance accessory (ATR) with a diamond ATR crystal and 32 scans with resolution of 4 cm^{-1} resolution. Each assay was carried out by triplicate in random points along the preconditioned films.

2.5. Differential solubility tests

Samples were immersed in specific buffer solutions according to the procedure described in the literature (Ciannamea, Stefani, & Ruseckaite, 2015; Hager, 1984). Each solution is known to disrupt

specific protein interactions and bonds: Solution S1: 0.086 M TRIZMA/HCl, 0.09 M glycine y 4 mM Na_2EDTA; Solution S2: S1 + 5 g cm^{-3} SDS; Solution S3: S1 + 8 M urea; Solution S4: S1 + 5 mg cm^{-3} SDS y 8 M urea; Solution S5: S1 + 5 g cm^{-3} SDS, 8 M urea y 25 mg cm^{-3} 2-mercaptoethanol (2-ME). Film samples of approximately 150 mg were weight and set into tubes containing 3 cm^3 of solution at 20 °C. After 24 h the suspensions were centrifuged 20 min at 9000*g* (Labnet International, USA). Afterwards, 1 cm^3 of supernatant was mixed with 4 cm^3 of Biuret reagent (1.5 g de $CuSO_4$, 6 g de K and Na tartrate tetrahydrate and 8 g of NaOH in 1000 cm^3 of distilled water) to determine protein content, measuring the absorbance at 530 nm in an UV–Visible spectrophotometer (Agilent 8453, China) after 5 min of incubation at room temperature. The soluble protein content was expressed as the solubilized protein mass, referred to the total protein mass in the film. For each solution (S1–S5) a calibration curve was performed using human albumin 20% (ZLB Behring), from stock solutions with protein contents from 0 to 10% (w/v). The determination of the protein content in solution S5 requires a previous stage of solvent separation (Ciannamea et al., 2014). All samples were assayed in triplicate.

2.6. Equilibrium moisture content (MC) and total soluble matter (TSM)

MC of SPC films was analyzed by weighting samples of 2 cm × 2 cm in an analytical balance (m_i, ± 0.0001 g; Ohaus, USA) and drying them in an air-circulating oven at 105 ± 1 °C for 24 h (Memmert, Germany). MC was calculated as the percentage of mass lost during drying. TSM was measured according to Rhim, Gennadios, Weller, Carole, and Hanna (1998) and expressed as the percentage of film dry matter solubilized after 24 h of immersion in distilled water (Rhim et al., 1998). Reported values of MC and TSM were the average of three replicates.

2.7. Color and light barrier properties

Color parameters were measured with a portable colorimeter (Lovi Bond RT 500, United Kingdom) with a measuring diameter of 8 mm and recorded in the Hunter Lab scale: $L^* = 0$ (black) to $L^* = 100$ (white); $a^* = -80$ (green) to $a^* = 100$ (red); and $b^* = -80$ (blue) to $b^* = 70$ (yellow). The colorimeter was calibrated using a standard white plate. The total color difference (ΔE), was calculated using the following equation:

$$\Delta E = \left[\left(L^*_{standar} - L^*_{sample}\right)^2 + \left(a^*_{standar} - a^*_{sample}\right)^2 + \left(b^*_{standar} - b^*_{sample}\right)^2\right]^{0.5} \quad (1)$$

All reported values are the average of five replicates. The opacity of films was calculated from at least four repetitions by dividing the area under the absorption curve obtained in the range of 400–800 nm by film thickness based on the method described by Gontard, Guilbert, & Cuq, 1992 (Gontard et al., 1992). Spectra were recorded using a UV–Visible Agilent 8453 (United States). The results are the average of four readings.

2.8. Tensile properties

Tensile tests were carried out at room temperature on an INSTRON 4467 Universal Testing Machine (Buckinghamshire, England) equipped with a 0.5 kN cell, with a crosshead speed of 3 mm/min and, following the procedure described in ASTM D1708-02a. The tensile strength (TS), elongation at break (ε_b) and elastic modulus (E) were calculated as the average of ten replicates.

2.9. Barrier properties

Water vapor permeability (WVP) was determined according the desiccant method following ASTM E96-00. Films were placed on poly (methyl methacrylate) cups containing dry $CaCl_2$, with an exposed area of 5 cm diameter (A). The system was placed in a controlled humidity chamber at 65% RH and 25 °C. Cups were weighed at 30 min intervals over a 10 h period. Results were plotted as the mass change Δm (g) vs. t (h), and from the gradient the permeation rate of water vapor through the film G (g/h) was obtained. Then, the permeability was calculated as:

$$WVP = G.L.[A.S.(HR_1 - HR_2)]^{-1} \quad \left(Kg.m/m^2.s.Pa\right) \quad (2)$$

where L is the thickness of the film, S is the vapor pressure of pure water at the test temperature, and (HR1, HR2) is the relative humidity gradient used in the assay.

Oxygen permeability coefficient (OPC) determination was assessed using an oxygen permeability analyzer Model 8500 Systech Instruments (Oxon, UK), according to ASTM D3985-02. Film samples with a diameter of 14 cm were placed in the analyzer at 25 °C. The results were reported as the oxygen transfer rate OTR (cm^3/m^2 day) and the oxygen permeation coefficient (OPC) was calculated as:

$$OPC = (OTR \cdot L).\Delta P^{-1} \quad \left(cm^3.\mu m/(m^2.day.kPa)\right) \quad (3)$$

where L is the thickness of the film (μm), ΔP is the partial pressure gradient of oxygen through the film (kPa). Three measurements of WVP and OPC were recorded for each film.

2.10. Total phenolic content (TPC)

The total phenolic content in the red grape extract was determined by the Folin-Ciocalteu assay as described in the literature (Singleton & Rossi, 1965; Singleton, Orthofer, & Lamuela-Raventós, 1999; Siripatrawan & Harte, 2010). Briefly, 0.1 cm^3 of extract was mixed with 7 cm^3 of distilled water and 0.5 cm^3 of Folin-Ciocalteu reagent (Sigma-Aldrich). Mixture was incubated for 8 min at room temperature before adding 1.5 cm^3 of a Na_2CO_3 solution (200 mg/cm^3) and 0.9 cm^3 of distilled water. The mixture was kept in dark at room temperature for 2 h. Absorbance was measured at 765 nm using a UV–visible spectrophotometer (Agilent 8453, United States). A calibration standard curve of gallic acid was prepared and the results were expressed as μg gallic acid equivalents/cm^3. Each assay was carried out by triplicate.

2.11. Antioxidant activity

2.11.1. Ferric reducing antioxidant power assay (FRAP)

Frap assay was carried out according to the method described by Oyaizu (1986), with some modifications (Oyaizu, 1986). Films samples (0.4 g) were placed in tubes containing 3 cm^3 of methanol and then incubated at room temperature for 24 h. Then an aliquot of 1 cm^3 was taken and mixed with 2.5 cm^3 of phosphate buffer (pH 7) and 2.5 cm^3 of potassium hexacyanoferrate (III) ($K_3Fe(CN)_6$, 1%). The mixture was incubated at 50 °C for 20 min and then 2.5 cm^3 of TCA was added, and the mixture was centrifuge at 5000 rpm for 10 min (Sartorius type 4–15, Germany). A 2.5 cm^3 aliquot of the supernatant was mixed with 2.5 cm^3 of distilled water 0.5 cm^3 of 0.1% $FeCl_3$ solution, and the absorbance at 700 nm was measured in a UV–visible spectrophotometer (Agilent 8453, China). Increase

absorbance of the reaction mixture indicates greater reducing power. The reducing capacity of RGE was measured likewise. Ascorbic acid (AA) was used as reference material and the final results were expressed as ppm of ascorbic acid equivalents (AAE). All tests were performed in triplicate.

2.11.2. Determination of DPPH• radical scavenging activity

The DPPH• method (Brand-Williams, Cuvelier, & Berset, 1995) measures the antioxidant activity in terms of the ability to capture radicals using the stable radical DPPH• (2,2-diphenyl-1-picrilhidrazilo). To evaluate their antioxidant activity, films were weighed (0.4 g) and placed in 3 cm^3 of methanol at room temperature for 24 h. An aliquot of 2 cm^3 of solution was taken and 1 cm^3 of fresh solution of DPPH• (74 $\mu g/cm^3$) in methanol was added. The mixture was reacted at room temperature in dark for 30 min and the absorbance measured at 517 nm using an UV–visible spectrophotometer (Agilent 8453, China). The antioxidant activity of red grape extract was evaluated in the same methanol solution (0–10% v/v), following the same procedure. All determinations were performed in duplicate. Radical scavenging activity (RSA) was expressed as:

$$RSA\% = \left([DPPH\cdot]_{bnk} - [DPPH\cdot]_t / [DPPH\cdot]_{bnk} \right) \times 100 \quad (4)$$

where $[DPPH\bullet]_t$ is the concentration in the sample and $[DPPH\bullet]_{bnk}$ is the concentration in the blank (no sample).

2.12. Statistic analysis

Results were statistically analyzed by one-way analysis of variance (ANOVA) employing Origin Lab 8 software. Tukey's test for comparison of means at a 5% of significance level was used ($\alpha = 0.05$).

3. Results and discussion

3.1. Characterization of red grape extract, formulation and processing conditions

The amount and distribution of polyphenols in grapes depends on several factors, including grape varieties, climate and cultivation places, as well as harvesting season among others (Brewer, 2011; Sagdic et al., 2011). The prevailing polyphenol compounds present in red grape extract (RGE) include monomeric flavon-3-ols such as catechin, epicatechin and procyanidin dimmers and trimers (Chedea et al., 2010; Perumalla & Hettiarachchy, 2011). According to the literature RGE consist of 5–30% monomers, 17–63% oligomers, 11–39% polymers and 2–50% large polymers (>24 monomeric units) (Nakamura, Tsuji, & Tonogai, 2003; Waterhouse, Ignelzi, & Shirley, 2000). The antioxidant activity of the commercial RGE employed in this work was measured as the reducing power (FRAP) and the radical scavenging activity (RSA%). In Fig. 1, RSA exhibited a dose-dependent increase reaching 95% for 5% w/w RGE. Higher RGE concentrations did not induce significant changes in RSA% indicating a saturation level around 5% w/w RGE. FRAP also revealed a sustained dose-dependent increment up to 6% w/w, followed by a minor increase up to 10% w/w RGE with no saturation level at least for the concentration analyzed. These results are in agreement with the high content of phenolic compounds as determined by Folin-Ciocalteu method which was 1055.5 ppm GAE. It is well reported that phenolic compounds are free radical acceptors that delay or inhibit the autoxidation initiation step (Sivarooban et al., 2008). The amount of total phenolic compounds of the extract used in this study is comparable with that of the red seed grape extract variety “Negro Amaro”, being 1111 ppm of GAE (Negro, Tommasi, & Miceli, 2003).

Taking into account the antioxidant capacity of the extract and the potential effect of its addition on the sensory properties of SPC films (as determined through exploratory studies), films were processed by solution casting and compression molding techniques (Ciannamea et al., 2014) with 0, 5 and 10% w/w RGE as a natural antioxidant additive. Properties of SPC active films were compared with control ones (without RGE) to assess the influence of the active agent on the final properties.

3.2. Effect of RGE on protein stabilizing interactions

There are several studies in the literature that demonstrate the existence of interactions between proteins and polyphenols containing active compounds, which can alter the physicochemical properties of the films, such as solubility and mechanical and barrier properties. In order to get a better understanding of the modifications induced by the RGE in protein conformation and stabilizing interactions of SPC films, ATR-FTIR and differential solubility studies were conducted.

SPC based films were analyzed by ATR-FTIR and the second derivative of amide I band (1700–1600 cm^{-1}; Fig. 2a) was evaluated to analyze possible modifications of the secondary structures of proteins (Le Tien et al., 2000). Differences in the spectra associated with processing were formerly explained elsewhere (Ciannamea et al., 2014). The main difference was detected around 1620 cm^{-1} associated with the presence of aggregates (hydrogen bonded β-sheet) which was more important in thermo compressed films than in casted ones, as reported for other globular proteins processed through thermoplastic procedures (Kuktaite et al., 2011; Ullsten, Gällstedt, Johansson, Gräslund, & Hedenqvist, 2006). The incorporation of RGE in casted films induced a slight increase in α-helix structures (1651 cm^{-1}), in agreement with Kanakis et al. (2011). Authors reported a slight increase in α-helix structures of β-lactoglobulin after the incorporation of green tea polyphenols, associating the results with the interaction and formation of polyphenol-protein complexes (Kanakis et al., 2011). By contrast, minor change in this region was observed for films processed by thermo-compression, so it can be assumed that the extract barely influences protein secondary structures in these films.

The amide II band, which is related to the bending of NH bond and mainly responds to changes in hydrogen bonding because of the medium was also analyzed in detail (Fig. 2b) (Almutawah, Barker, & Belton, 2007; Vasconcelos, Freddi, & Cavaco-Paulo, 2008). No changes were observed in the amide II band of films processed by thermo-compression due to the incorporation of RGE. Contrary, the extract induced a slight reduction in the intensity of this band when films where obtained by casting. The same trend was previously observed due to the incorporation of Gly (Ciannamea et al., 2014). Therefore, this result suggests that some compounds of the extract could be acting as plasticizers in the films obtained by casting, in agreement with the increase in α-helix structures. It has been reported that the incorporation of plasticizers such as polyols have helicogenic activity (Gilbert et al., 2005; Lefèvre, Subirade, & Pézolet, 2005). Hydroxyl groups in RGE are able to interact with proteins, thus modifying the structure and related function (Nie et al., 2015). According to Sivarooban et al. (2008), soy protein molecules associate with grape polyphenols mainly through hydrogen bonding (Sivarooban et al., 2008). For example, polar groups of polyphenols, such as OH groups, can form hydrogen bonds with C=O amide groups of the protein, replacing hydrogen bonds interactions between NH or SH and C=O groups of proteins. By reducing protein-protein interactions flexible domains are created and chain mobility within the film matrix is enhanced. Therefore, when such interactions dominate, for example if

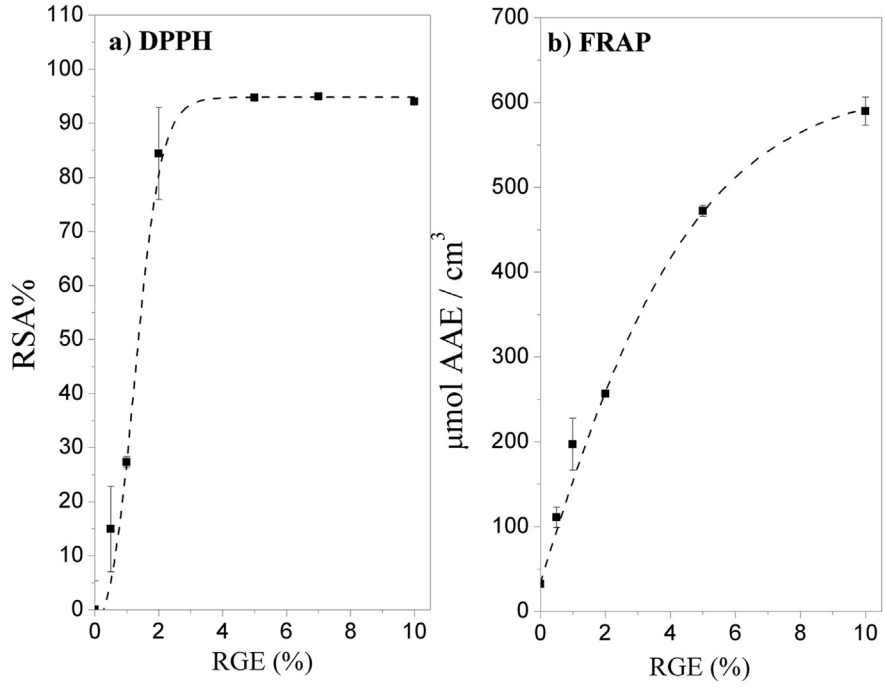

Fig. 1. Antioxidant activity of commercial RGE: a) RSA; b) FRAP.

polyphenols are in excess, the material behavior is similar to a plasticized protein matrix and polyphenols act similarly to a plasticizer (Gómez-Estaca, Giménez, Montero, & Gómez-Guillén, 2009; Hager et al., 2012; Tongnuanchan et al., 2012; Zhang et al., 2010).

ATR-FTIR studies were complemented with differential solubility analysis. Adding up to 10% of RGE did not modify significantly the solubility of proteins of casting films in any of the five solutions tested ($p > 0.05$; Fig. 3). This result suggests that interactions involved in the stabilization of casting films were not affected by the addition of the extract (Jiang, Xiong, Newman, & Rentfrow, 2012; Nuthong, Benjakul, & Prodpran, 2009). While RGE polyphenols can interfere with protein-protein hydrogen interactions, these interactions are replaced by protein-polyphenol hydrogen bonds. Therefore, assessing the solubility of the films, i.e. in the presence of urea (capable of breaking hydrogen bridges) the same amount of protein could be extracted.

In contrast, RGE induced significant changes in the stabilizing interactions of films processed by compression molding (Fig. 3). MSPC control films, as discussed in a previous work (Ciannamea et al., 2014), shown a significant difference in the protein solubility between S4 and S5 solutions (350 ± 33 μg/g to 675 ± 4 μg/g, $p < 0.05$), indicating that disulfide interactions have an relevant role in the formation and stabilization of thermo-molded SPC films (Hager, 1984; Liu & Hsieh, 2008; Zárate-Ramírez, Martínez, Romero, Partal, & Guerrero, 2011). In this case, the incorporation of RGE in the formulation of thermo-compressed films slightly reduced the solubility of proteins in solution S1, indicating a lower contribution of electrostatic interactions, while causing a increase in the solubility in solutions S2 (S1 + SDS), S3 (S1 + urea) and S4 (S1 + SDS + urea) statistically no significant ($p > 0.05$), revealing a greater contribution of non-covalent interactions, both hydrophobic and hydrogen bonding. However, protein solubility in the presence of 2-ME, did not induce significant hanges compared to control films. Therefore, the major contribution of non-covalent interactions and the constancy in the solubility S5 solution after the addition of the RGE, suggests that disulfide bonds contribution is lower in active films, indicating a redistribution of interactions. Polyphenols in RGE, such as catechin, epicatechin, proanthocyanidins, phenolic acids, etc., may associate with free polar groups of proteins through hydrogen bonds, interfering with the formation of covalent disulfide bonds. These results agree with those reported by Kroll et al. (2003) who showed that the interaction between phenolic compounds and proteins cause the blocking of hydrophilic groups such as amino and thiol. Moreover, phenolic compounds have been intentionally used for disrupting the formation of disulfide bonds to enhance the upper temperature limit of the processing window of wheat gluten (Ullsten et al., 2006).

Differential solubility tests and ATR-FTIR studies led to the conclusion that RGE affected the secondary structures of soy proteins and interactions present in the stabilization of the films, and that this effect was clearly different according the processing method used. As a consequence, it can be foresee that the incorporation of RGE might alter protein film's properties, such as mechanical and barrier properties.

3.3. *Effect of RGE on film properties*

3.3.1. *Visual aspect, color and light barrier properties*

Film thickness range between 135 and 155 μm. The result indicates the incorporation of RGE in SPC films did not induced significant differences in the thickness values ($p > 0.05$, Table 1). Hence properties like opacity, mechanical and barrier properties can be considered independent of this parameter.

Active films with 5 and 10% w/w of RGE were visually similar to control films, either processed by solution casting or compression molding (Ciannamea et al., 2014). In fact, the addition of the RGE did not significantly change color parameters, confirming visual observations ($p > 0.05$, Table 1). A slight increase in the total color difference (ΔE), was detected in the films obtained by casting due to the incorporation of RGE, possibly associated with the inherent color of the extract, although in general differences were not statistically significant ($p > 0.05$). The addition of 5% of RGE slightly reduced the opacity of the films, regardless the processing method (Table 1). This result could be related to the plasticizing action of

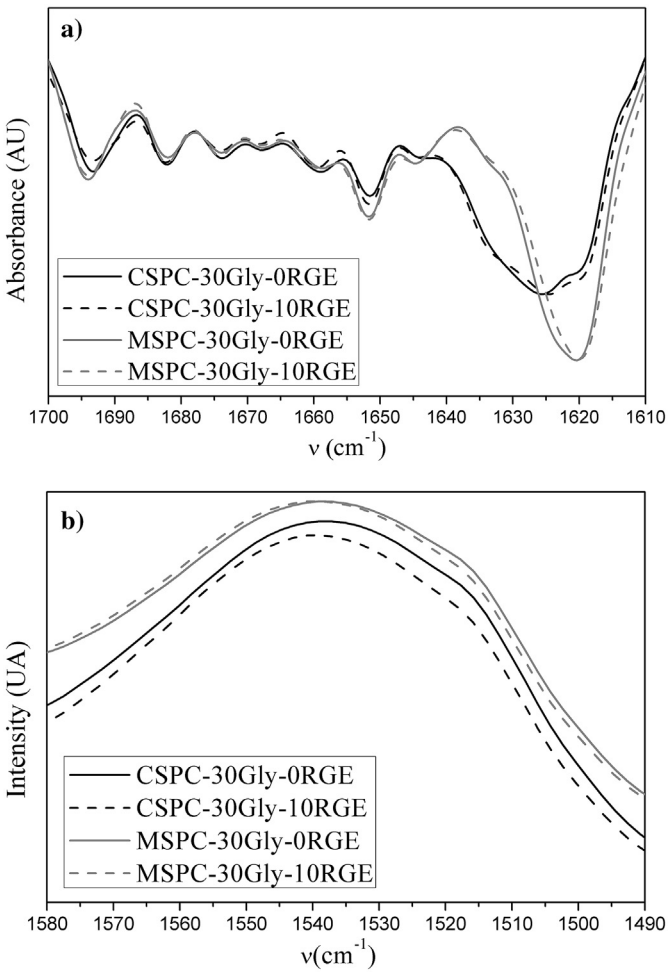

Fig. 2. (a) Amide I band: Second derivative of ATR-FTIR spectra of active and control SPC-films. (b) Amide band II of control and active films.

polyphenols, given that plasticizers interfere with protein-protein interactions, reducing the absorbance in the visible region (Shaw, Monahan, O'Riordan, & O'Sullivan, 2002; Sothornvit & Krochta, 2001). However, films processed with 10% w/w of RGE were more opaque than those containing 5%. Possibly, at higher levels of RGE, the effect described above is being compensated by an increased amount of polyphenols, capable of absorbing light in the visible region, such as anthocyanins, which absorb between 270–280 nm and 465–550 nm (González García, Iglesias, Laguna, Martínez, & González Lavaut, 2011). It has been reported that the addition of grape seed extract in SPI films significantly modifies color parameters, resulting in darker and more yellow-reddish films (Sivarooban et al., 2008). The difference between results reported by other authors as Sivarooban et al. (2008) and those shown in this work probably lies in the differences between the nature and concentration of the extract used.

3.3.2. Equilibrium moisture content and total soluble matter

The total soluble matter of control and RGE incorporated SPC films obtained by casting was invariable ($p > 0.05$, Table 1), in agreement with differential solubility results and with results reported by others (Bodini, Sobral, Favaro-Trindade, & Carvalho, 2013; Gómez-Estaca, Giménez et al., 2009). By contrast, the addition of increasing amounts of RGE in SPC films obtained by compression molding caused a significant increment ($p < 0.05$; Table 1) in TSM (i.e. from 26.3 ± 0.5 to 36.8 ± 5.7% by adding 10% w/w RGE in MSPC-30Gly films). Confirming differential solubility results, the increased solubility in distilled water of active films could be associated with a lower cross-linking degree through disulfide bridges relative to control films, due to interferences of polyphenols with protein-protein interactions, preventing aggregation as previously reported by Ullsten et al. (2006) for wheat gluten-salicylic acid films processed by extrusion.

Moisture content also varied with the incorporation of RGE in film's formulation (MC, Table 1). The incorporation of 5% w/w RGE in SPC casting films resulted in a small contraction of MC values ($p < 0.05$), while further adding RGE did not significantly affect this parameter ($p > 0.05$). Similar behavior was observe for green tea-chitosan films obtained by casting and was ascribed to the interactions between polyphenols and chitosan, which reduce the availability of hydroxyl and amino groups, restricting chitosan-water interactions (Wang, Dong, Men, Tong, & Zhou, 2013). Whereas the redistribution of hydrogen protein-protein interactions to protein-polyphenol could not be confirmed by differential solubility test, a reduction in the intensity of the amide II band was evidenced, indicating protein-polyphenol hydrogen interactions. Thus, the lower MC of active films can be explained

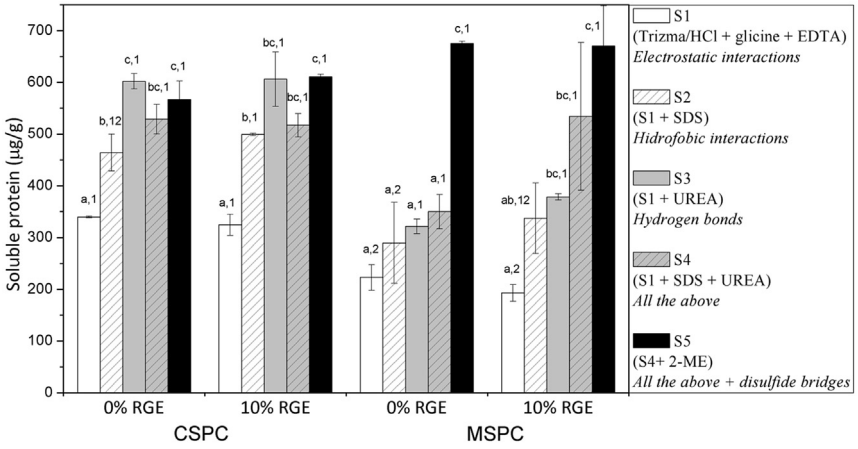

Fig. 3. Protein solubility of active SPC-30Gly films in different denaturing solutions. Mean values ± standard deviations. Different letters (a, b, c) indicate significant differences between denaturing solutions for the same formulation ($p < 0.05$). Different numbers (1, 2) indicate significant differences among the different formulations for the same denaturing solution ($p < 0.05$).

Table 1
Thickness, optical properties, moisture content and total soluble matter of SPC films.

	Gly (%)	RGE (%)	Thickness (μm)	Opacity (UA.nm)	ΔE	MC (%)	TSM (%)
CSPC	30	0	143 ± 22 ab	955 ± 10 a	21.3 ± 2.9 a	17.7 ± 0.7 a	34.7 ± 2.7 a
		5	157 ± 3 ab	918 ± 3 b	24.9 ± 0.2 ab	15.9 ± 0.1 b	39.0 ± 4.4 a
		10	144 ± 15 ab	939 ± 12 ab	25.4 ± 1.9 ab	16.0 ± 0.6 b	38.3 ± 1.3 a
	40	0	153 ± 33 ab	968 ± 24 a	21.5 ± 4.2 a	20.4 ± 0.7 c	36.7 ± 2.8 a
		5	167 ± 36 b	953 ± 10 ac	25.4 ± 1.9 b	17.4 ± 1.1 a	41.0 ± 3.5 a
		10	155 ± 10 ab	931 ± 2 bc	25.9 ± 0.6 ab	16.6 ± 0.7 ab	40.5 ± 1.0 a
MSPC	30	0	139 ± 14 ab	668 ± 4 de	25.4 ± 2.1 ab	21.3 ± 0.8 c	26.3 ± 0.5 b
		5	126 ± 22 a	609 ± 26 f	25.6 ± 2.2 ab	23.1 ± 0.8 d	35.0 ± 4.7 a
		10	131 ± 25 a	659 ± 13 d	23.7 ± 0.8 ab	22.4 ± 0.5 d	36.8 ± 5.7 a
	40	0	135 ± 14 ab	618 ± 7 f	24.9 ± 1.5 ab	28.1 ± 0.4 e	26.7 ± 0.1 b
		5	131 ± 25 a	589 ± 17 f	21.4 ± 0.9 a	28.8 ± 0.6 ef	35.2 ± 7.3 a
		10	137 ± 4 ab	694 ± 3 e	25.5 ± 1.1 ab	29.8 ± 0.7 f	35.4 ± 2.3 a

Mean values ± standard deviations. Mean values within the same column followed by the same letter are not significantly different ($p > 0.05$, Tukey test).

taking into account the interactions between proteins and polyphenols from the extract. If hydrophilic groups of the protein chains are associated with polyphenols via hydrogen bonds, these groups are not available to be associated with water molecules, reducing the MC of the films (Kroll et al., 2003).

The influence of RGE on the MC was less significant in films processed by thermo-compression and, in fact, showed an inverse tendency respect active films obtained by casting (Table 1), the incorporation of different percentages of RGE resulted in an increase in MC ($p < 0.05$). While, the interaction between phenolic compounds and proteins cause blocking of hydrophilic groups such as hydroxyl, amino and thiol, an increase in RGE concentration will cause an increase in the content of both non-polar groups (ie. aromatic rings) and polar groups (ie. hydroxyl and carboxyl groups) corresponding to the polyphenols (Kroll et al., 2003). A greater amount of not bonded polar groups will contribute to moisture absorption by the film. Nie et al. (2015) reported an increased in MC due to the incorporation grape seed procyanidins and green tea polyphenol in miofibrillar protein-based films, associated to the hydrophilic property of added phenolic compound.

3.3.3. Mechanical properties

The addition of RGE in CSPC-30Gly films did not induce significant changes in TS and E ($p > 0.05$; Table 2). In contrast, RGE caused a considerable increment in the elongation at break of CSPC films ($p < 0.05$), i.e. from 14.52 ± 2.18 to 19.82 ± 2.97% for CSPC-30Gly films with 0 and 10% RGE, respectively. In the case of the CSPC films containing 40% of Gly, the addition of increasing amounts of RGE caused a significant contraction in TS and E values ($p < 0.05$), accompanied by a significant increase in ε_b. These results reveal a plasticizing action of polyphenols in SPC films obtained by casting, in line with results reported by other authors for protein matrices modified with polyphenols. Bodini et al. (2013) reported that tensile strength and elastic modulus of gelatin films were reduced by incorporating propolis extract and Tongnuanchan et al. (2012) observed that the addition of citrus essential oils decrease the interactions between gelatin molecules, causing a decrease in rigidity and a consequent increase in the extensibility of the films. Similar arguments were raised by Gomez-Estaca et al. (2009) and Gómez-Guillén, Ihl, Bifani, Silva, and Montero (2007) for gelatin films and borage and murta extract, respectively.

In contrast, the incorporation of RGE in thermo-compressed films resulted in lower elongation at break percentages ($p < 0.05$) and higher elastic modulus ($p < 0.05$), in agreement with results reported by Min, Song, and Zheng (2008) for wheat gluten films processed by thermo-compression with different reducing agents. The redistribution of stabilizing interactions produced by the incorporation of RGE in these films could be responsible for the observed changes in the mechanical properties of active MSPC films (Min et al., 2008).

3.3.4. Barrier properties

In order to assess the potential application of these films in food packaging the effect of the addition of RGE on the water vapor and oxygen barrier properties was analyzed. The incorporation of increasing amounts of RGE in films obtained by casting significantly reduced WVP values, regardless of the percentage of Gly ($p < 0.05$, Table 2). In example, the addition of 5% of RGE in CSPC-40Gly formulation induced a reduction in WVP of approximately 45%, comparing with control film. This result suggests that RGE has a favorable effect on WVP of SPC films obtained by casting, probably associated with the lower MC observed in active films. If polyphenols associate with polar groups of protein chains, these groups will not be available to associate with water molecules, reducing not only the MC of the films, but also the transfer rate of water molecules through the matrix (Bodini et al., 2013). The reduction in WVP has been previously reported in other protein systems caused by the addition of polyphenolic compounds, such as gelatin films with thyme extract (Pires et al., 2011), propolis (Bodini et al., 2013), murta (Gómez-Guillén et al., 2007), citrics (Tongnuanchan et al., 2012) and green tea (Giménez et al., 2013) and miofibrillar protein-based films with grape seed procyanidins and green tea polyphenols (Nie et al., 2015).

Contrary, the incorporation of RGE in films processed by compression molding had a negative influence on the WVP in a concentration-dependant manner. A slight increase was observed in this property after adding the RGE, although the differences were not significant ($p > 0.05$). These results are consistent with the higher percentage of MC observed for these films as a result of the reduction in disulfide covalent bonds. Water vapor permeability of SPC-Gly-RGE films was comparable to reported for other active protein films, such as gelatin films with BHT and α-tocopherol (WVP: 1.20–2.0 10^{13} kg m/m^2 s Pa) (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2008), agar-gelatin films with green tea extract (WVP: 1.63–2.04 10^{13} kg m/m^2 s Pa) (Giménez et al., 2013), and chitosan with tea extract (WVP 1.2 10^{13} kg m/m^2 s Pa) (Wang et al., 2013).

The addition of 10% of RGE in thermo-compressed films resulted in a lower oxygen permeability (Table 2), which could be related to the redistribution of interactions toward a greater contribution of hydrophobic and hydrogen bonds interactions, and to a more hydrophilic matrix, cause by the incorporation of polar groups corresponding to polyphenols (ie. hydroxyl and carboxyl groups) (Kroll et al., 2003). On the other hand, OPC of casting films could not be evaluated since the films were drilled during the test due to the flow of oxygen. This was previously observed in CSPC films with

Table 2
Mechanical properties, water vapor and oxygen permeability of SPC-Gly-RGE films.

	Gly (%)	RGE (%)	TS (MPa)	ε_b (%)	E (MPa)	WVP 10^{13} (Kg m/m^2 s Pa)	OPC (cm^3 μm/(m^2 día KPa))
CSPC	30	0	2.27 ± 0.20 ad	14.52 ± 2.18 a	48.1 ± 7.3 ae	3.09 ± 0.30 a	8.5 ± 0.8 a
		5	2.17 ± 0.67 a	16.83 ± 2.65 ab	50.1 ± 24.5 ae	1.46 ± 0.06 b	–
		10	2.35 ± 0.32 ad	19.82 ± 2.97 b	48.2 ± 11.5 ae	1.79 ± 0.10 bd	–
	40	0	2.12 ± 0.47 a	15.82 ± 2.89 a	38.8 ± 8.3 ab	4.85 ± 0.83 c	18.5 ± 2.4 c
		5	1.59 ± 0.32 b	19.98 ± 1.17 be	26.9 ± 9.1 bc	2.70 ± 0.19 ad	–
		10	1.32 ± 0.22 b	17.89 ± 2.38 abe	23.5 ± 4.6 cd	2.42 ± 0.29 ad	–
MSPC	30	0	3.54 ± 0.18 c	27.96 ± 1.73 c	56.6 ± 7.5 ef	1.60 ± 0.04 bd	15.2 ± 2.0 bc
		5	3.47 ± 0.22 c	24.01 ± 4.62 df	76.1 ± 8.8 g	1.90 ± 0.11 bd	13.2 ± 5.3 abc
		10	3.43 ± 0.19 c	21.56 ± 2.24 de	65.1 ± 8.5 fg	2.17 ± 0.14 bde	9.7 ± 2.7 ab
	40	0	2.63 ± 0.30 d	29.82 ± 3.56 c	26.5 ± 2.2 bd	2.99 ± 0.07 ae	27.1 ± 0.0 d
		5	2.15 ± 0.22 a	26.17 ± 4.01 cf	33.7 ± 4.1 bf	3.06 ± 0.23 a	23.2 ± 1.0 de
		10	2.23 ± 0.11 ad	24.03 ± 3.11 df	37.2 ± 3.2 abd	3.07 ± 0.10 a	18.8 ± 2.7 ce

Mean values ± standard deviations. Mean values within the same column followed by the same letter are not significantly different ($p > 0.05$, Tukey test).

50% Gly (Ciannamea et al., 2014). As was discussed above, the plasticizing effect of polyphenols resulted in films with lower mechanical resistance.

3.3.5. Antioxidant activity

The antioxidant activity (Table 3) of films processed by compression molding and casting was determined in terms of DPPH radical scavenging activity (RSA) and the reducing power of $FeCl_3$ (FRAP). Interestingly, control films (without RGE), showed antioxidant activity, not negligible in both experimental methods. This result agrees with those informed by other authors for protein films derived from different sources, i.e. different gelatin types and has been assigned to the antioxidant capacity of amino acids such as glycine and proline (Gómez-Estaca, Giménez et al., 2009; Gómez-Guillén et al., 2007; Haddar et al., 2012; Kang, Kim, You, Lacroix, & Han, 2013). Moreover, Kang et al. (2013) have shown that some amino acids of soy proteins, such as cysteine, tyrosine, tryptophan and histidine, are strong free radicals scavengers, thus acting as antioxidants (Kang et al., 2013). Also, it has been reported that soy contains a high concentration of isoflavones that in their natural state are attached to proteins. The concentration of these polyphenols in soy flour is around 150–200 mg/100 g (Singh, Kumar, Sabapathy, & Bawa, 2008). Therefore, the antioxidant activity of control films could also be related to polyphenols present in SPC.

The influence of the RGE on the antioxidant activity of the films was different according to the processing method used. In the films obtained by casting, the incorporation of RGE increased the antioxidant activity, from 24.5 to 43.2% and from 0.70 to 0.87 mmol AAE/g, according to the methods of RSA and FRAP, respectively, when RGE concentration was varied from 0 to 10%. On the other hand, incorporation of RGE in compression molding films had a greater influence on the antioxidant activity, resulting in films with increased activity. In this case, the addition of 5% of RGE produced a significant increase in antioxidant activity, determined by both methods ($p < 0.05$), from 58.1 to 81.4% (RSA) and from 0.52 to 0.83 AAE/g (FRAP). Higher concentrations of RGE (10% w/w) did not affect significantly the antioxidant activity ($p > 0.05$).

Table 3
Antioxidant activity of SPC-30Gly films.

	RGE (%)	RSA (%)	FRAP (mmol AAE/g films)
CSPC	0	24.5 ± 7.0 a	0.70 ± 0.05 ab
	5	30.3 ± 3.4 a	0.79 ± 0.04 a
	10	43.2 ± 0.8 b	0.87 ± 0.14 ac
MSPC	0	58.1 ± 4.2 b	0.52 ± 0.04 b
	5	81.4 ± 5.5 c	0.83 ± 0.11 ac
	10	88.0 ± 3.0 c	0.99 ± 0.04 c

Mean values ± standard deviations. Mean values within the same column followed by the same letter are not significantly different ($p > 0.05$, Tukey test).

These results indicated that RGE is less retained into SPC matrix in compression molding films than in casted counterparts, more likely due to differences in the interactions between proteins and polyphenols, as disclosed by differential solubility assays. Furthermore, the formation of protein-polyphenol complexes can mask the antioxidant activity, if active antioxidant polyphenol groups are involved in interactions with proteins (Giménez et al., 2013; Salgado et al., 2012). The effectiveness of these films in protecting foodstuff against oxidation will be analyzed in future works through *in vitro* and *in vivo* migrations assays.

4. Conclusions

The incorporation of RGE produced changes in stabilizing interactions of films, affecting their mechanical and barrier properties and moisture resistance. In the films obtained by casting the RGE acted mainly as a plasticizer and reduced WVP. In the films processed by compression molding the studies indicated that RGE produced a redistribution of the interactions, interfering with the formation of disulfide bonds and favoring hydrophobic and hydrogen interactions, which was reflected mainly in an increase in TSM of films. The antioxidant activity of the films processed by thermo-compression was higher than the AA of casting films.

In the light of the obtained results, MSPC-30Gly-10RGE films showed the best set of properties for packaging applications, having superior oxygen barrier properties (9.7 cm^3 μm/(m^2 day KPa)) and greater *in vitro* antioxidant activity in term of RSA (88%) and FRAP (0.99 mmol AAE/g film), while similar resistance (TS: 3.43 ± 0.19 MPa) and water vapor permeability (2.17 ± 0.14 10^{-13} kg m/m^2 s Pa), than casted films. In a future work the effectiveness of SPC based films incorporated with RGE in extending the shelf-life of foodstuff will be elucidated in the light of in-vitro and in-vivo migrations assays.

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