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Biodegradable sunflower protein films naturally activated with antioxidant compounds

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ABSTRACT

The aim of this work was to study the formation of biodegradable films from sunflower protein isolates having different concentrations of phenolic compounds, which are present in sunflower seeds and are retained during the isolation of proteins from the residual pellet of the oil industry. Films were obtained by casting from aqueous dispersions of the isolates, using glycerol as plasticizer. Although proteins from different sunflower protein isolates presented different structural properties, such as surface hydrophobicity, the films obtained did not differ significantly regarding thickness (64–80 μm), density (~1.47 g/cm^{-3}), water content (~25%), water vapor permeability (~1.4 × 10^{-10} g H_2O/Pa.s.m), mechanical properties (traction), glass transition temperature, or type of interactions involved in the protein network, which in all cases were mainly hydrogen bonds, hydrophobic interactions, and disulfide bonds. Sunflower protein films contained phenolic compounds had antioxidant activity. As the concentration of these compounds increased the films also presented increasing opacity and greenish tones, with absorption maximums at wavelengths similar to those of chlorophyll (which suggests their potential use in mulching for impeding weed growth).

1. Introduction

In the last years there has been an increasing interest in the use of biodegradable polymers for the development of new materials as a potential solution to the problems of environmental contamination and solid residue handling posed by the accumulation of non-degradable synthetic containers, and also in response to the uncertain perspective of oil availability (Petersen et al., 1999). The use of biopolymers derived from agriculture products appears as an interesting alternative to synthetic polymers, especially for short term applications and/or when a rapid degradation constitutes an advantage (as in the case of food packaging and plastics used in agriculture).

With this aim, the ability of several agriculture-derived proteins as those from wheat gluten, corn zeins, soy, sunflower, rice, cotton, sorghum, and amaranth, to form edible and/or biodegradable films has been studied (Cuq, Gontard, & Guilbert, 1998; Gennadios, 2002). In general protein films present excellent barrier properties to oxygen, lipids and aromas, together with moderate mechanical properties, but have a high permeability to water vapor due to the hydrophilic character of these macromolecules (Gennadios, 2002). These properties are determined by the microstructure of the films, which in turn depend significantly on the initial protein structure and the preparation method (Mauri & Añón, 2006, 2008; Denavi et al., 2009).

One advantage of these materials is that they can serve as vehicles of several types of additives, including antioxidants and antimicrobial agents, vitamins, flavors and colorants. Thus, there are several reports on the inclusion of such additives in biopolymer-based formulations in order to prolong the life span of foods (Han & Krochta, 2007; Gómez Estaca, Giménez, Gómez-Guillén, & Montero, 2009).

Sunflower proteins have been shown to have the ability to form edible and/or biodegradable films (Ayhllon-Meixueiro, Vaca-Garcia, & Silvestre, 2000; Orliac, Rouilly, Silvestre, & Rigal, 2003; Rouilly, Mériaux, Geneau, Silvestre, & Rigal, 2006). While these proteins can be extracted from seeds, they can be also extracted from the residual cake produced in the oil industry, currently used for animal feeding only. Although the later alternative could reduce the production costs, it must be kept in mind that during oil extraction the proteins are subjected to treatment with organic solvents, and high pressures and temperatures, which modify their structure and, consequently, their functionality.

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Depending on the extraction procedure, proteinaceous products from sunflower retain different amounts of phenolic compounds, especially chlorogenic acid, which are virtually impossible to be totally removed due to their strong interactions with proteins (González-Pérez & Vereijken, 2008). The antioxidant activity of these compounds has been previously reported (Rice-Evans, Miller, & Paganga, 1995; Brand-Williams, Cuvelier, & Berset, 1995; Bastos, de Olivera, Teixeira, Carvalho, & Ribeiro, 2007), and there are also reports on their use during film production in order to endow films with such activity (León & Rojas, 2007; Flores, Conte, Campos, Gerschenson, & Del Nobile, 2007; Han & Krochta, 2007; Gómez Estaca et al., 2009).

The aim of the present work was to prepare biodegradable films from sunflower protein isolates having different levels of phenolic compounds, and to analyze the effect of the latter on the structural and functional properties of films, including their antioxidant activity.

2. Materials and methods

2.1. Sunflower protein preparation

Three sunflower protein isolates, which were obtained from the residual pellet of an oil industry (Santa Clara, Molinos Río de La Plata, Argentine) were used in film formation. The sunflower protein isolate (**SunI**) was obtained by solubilization of proteins at pH 9 (pellet:water 1:10 w/v), isoelectric precipitation at pH 4.5, dissolution at pH 9, and spray dry (in a Niro Atomizer Production Minor, operated at 170–190 °C and 80–90 °C air inlet/outlet temperatures). The $\mathbf{SunI}_W$ and $\mathbf{SunI}_R$ isolates were obtained following the same protocol, but with the addition of two washing steps of the pellet with water or 0.1% w/v Na_2SO_3 solution (pellet:water 1:15 w/v), respectively, before protein solubilization in order to eliminate phenolic compounds (Salgado, 2009). As a reference, films were also prepared from a commercial soy protein isolate (**SoyI**, Supro 500E, The Solae Company, Brazil).

Protein isolates were characterized regarding their protein content using Kjeldhal (AOAC 920.53, 1995), and their phenolic content using UV spectroscopy at 324 nm (Moores, McDermott, & Wood, 1948). The surface hydrophobicity of proteins was determined by fluorescence with the ANS probe (Kato & Nakai, 1980), and the denaturation degree was measured by differential scanning calorimetry (Molina, Petruccelli, & Añón, 2004).

2.2. Preparation of films

Films were prepared by dispersing **SunI**, $\mathbf{SunI}_W$, $\mathbf{SunI}_R$ or **SoyI** (5% w/v) and glycerol (1.5% w/v, Anedra, Argentine) in distilled water. Dispersions were agitated in a magnetic stirrer for 30 min at room temperature, their pH was adjusted to pH 11 with 2 N NaOH, and they were stirred again for 30 min. Ten milliliters of each film-forming dispersion were poured on polystyrene Petri dishes (64 cm^2) and then dehydrated at 60 °C for 5 h in an oven with air flow and circulation (Yamato, DKN600, USA). The dry films were conditioned at 20 °C and 58% relative humidity in desiccators with saturated solutions of NaBr for 48 h before being peeled from the casting surface for characterization.

2.3. Film thickness

Film thickness was measured by a digital coating thickness gauge (Check Line DCN-900, USA). Measurements were done at five positions along the rectangular strips for the tensile test, and at the center and at eight positions round the perimeter for the WVP determinations. The mechanical properties and WVP were calculated using the average thickness for each film replicate.

2.4. Moisture content (MC)

MC was determined after drying in an oven at 105 °C for 24 h. Small specimens of films collected after conditioning were cut and placed on Petri dishes that were weighed before and after oven drying. MC values were determined in triplicate for each film, and calculated as the percentage of weight loss relative to the original weight (ASTM D644-94, 1994).

2.5. Density

Film density (ρ^s) was determined directly from the film weight and dimensions (volume) and the values considered were the average of nine determinations.

2.6. Water vapor permeability (WVP)

Water vapor permeability tests were conducted using ASTM method E 96-80 (1989) with some modifications (Gennadios, McHugh, Weller, & Krochta, 1994). Each film sample was sealed over a circular opening of 0.00185 m^2 in a permeation cell that was stored at 20 °C in a desiccator. To maintain a 75% relative humidity (RH) gradient across the film, anhydrous silica (0% RH_c) was placed inside the cell and a saturated NaCl solution (75% RH_d) was used in the desiccator. The RH inside the cell was always lower than outside, and water vapor transport was determined from the weight gain of the permeation cell. When steady-state conditions were reached (about 1 h), eight weight measurements were made over 5 h. Changes in the weight of the cell were recorded and plotted as a function of time. The slope of each line was calculated by linear regression (Microsoft® Office Excel 2007) and the water vapor transmission rate (WVTR) was calculated from the slope (g H_2O/s) divided by the cell area (m^2). WVP (g H_2O/Pa.s.m) was calculated as:

$$\mathrm{WVP} = \frac{\mathrm{WVTR}}{P_v^{H_2O}\cdot(\mathrm{RH_d}-\mathrm{RH_c})\cdot A}\cdot d \qquad (1)$$

where $P_v^{H_2O}$ = vapor pressure of water at saturation (1753.35 Pa) at the test temperature (20 °C), HR_d = RH in the desiccator, HR_c = RH in the permeation cell, A = permeation area and d = film thickness (m). Each WVP value represents the mean value of at least three samples taken from different films.

2.7. Sorption isotherms

The water adsorption isotherms of the films were determined by the static method, using saturated saline solutions (NaOH, $KC_2H_3O_2$, $MgCl_2$, K_2CO_3, NaBr, $NaNO_2$, NaCl, KCl, and $BaCl_2$) to obtain different relative humidities (Labuza & Ball, 2000). Film samples were then conditioned in desiccators having the desired relative humidity (RH) (7, 2.49, 33.7, 43.1, 58.0, 65.5, 75.4, 85.3, 90.7% RH) and the samples were weighed until three measurements of constant weight were obtained. This implied that the films were equilibrated at the desired RH (21 days). The amount of water absorbed per gram of dry film (X_{eq}) was determined as the difference between the initial and the final weight. The Guggenheim–Anderson–de Boer (GAB) model (Eq. (2)) was used to fit film sorption isotherm data:

$$X_{eq} = \frac{X_o\cdot K\cdot C\cdot a_w}{(1-K\cdot a_w)\cdot(1-K\cdot a_w+C\cdot K\cdot a_w)} \qquad (2)$$

where X_{eq} is the humidity content of the sample (dry base) in the equilibrium (g H_2O/g dry film) at a given aqueous activity (a_w), X_o is the humidity content (dry base) of the monolayer (g H_2O/g dry film), C is the Guggenheim constant associated to the sorption heat of the monolayer, and K is the constant associated to the sorption heat of multilayers.

The parameters of the model (X_o, K, and C) were determined by quadratic regression of a_w/X_{eq} *versus* a_w using the Statgraphics version 5.1 software (Statgraphics, USA).

2.8. Solubility and diffusion coefficients of water in the film

The solubility coefficient of water in the films at 20 °C and 75% RH, β (g H_2O/Pa g of dry film), was determined using Eq. (3), according to Larotonda, Matsui, Sobral, and Laurindo (2005), based on the experimental water sorption isotherms, GAB model and Eq. (2).

$$\beta = \frac{C\cdot K\cdot X_o}{P_v^{H_2O}}\cdot\left[\frac{1}{(1-K\cdot a_w)\cdot(1-K\cdot a_w+C\cdot K\cdot a_w)} - \frac{a_w}{[(1-K\cdot a_w)\cdot(1-K\cdot a_w+C\cdot K\cdot a_w)]^2}[-K\cdot(1-K\cdot a_w+C\cdot K\cdot a_w)+(1-K\cdot a_w)\cdot(-K+C\cdot K)]\right] \quad (3)$$

The coefficients of water diffusion (D_{ef}) through the films at 20 °C and 75% RH were determined from data on water vapor permeability (WVP), solubility coefficient of water in the film (β) and film density (ρ^s), using Eq. (4).

$$D_{ef} = \frac{WVP}{\rho^s\cdot\beta} \quad (4)$$

2.9. Mechanical properties

The tensile strength, Young's modulus and elongation at break of the films were determined following the procedures outlined in the ASTM methods D882-91 (ASTM, 1991), taking an average of six measurements for each film and using at least two films per formulation. The films were cut into 6 mm wide and 80 mm long strips, and mounted between the grips of the texture analyzer TA.XT2i (Stable Micro Systems, Surrey, England). The initial grip separation was set at 50 mm and the crosshead speed at 0.5 mm/s. The tensile strength (σ = force/initial cross-sectional area) and elongation at break (ε) were determined directly from the stress–strain curves using Texture Expert V.1.15 software (Stable Micro Systems, Surrey, England), and the Young's modulus (E) was calculated as the slope of the initial linear portion of this curve.

2.10. Differential solubility of proteins

Protein solubility of the films was determined according to the method described by Mauri and Añón (2006), with some modifications. Pieces of films (≅100 mg) were weighted and placed into a tube containing 1 ml of water or buffer. Five different buffer systems all at pH 7.5 were used: a) 0.1 M phosphate buffer containing 0.1 M NaCl (PB); b) PBD buffer: PB with 0.1% sodium dodecyl sulfate (SDS, Anedra, Argentine); c) PBU buffer: PB with 6 M urea (Riedel-deHaën, Germany); d) PBDU buffer: PB with 0.1% SDS and 6 M urea, and e) PBDUM buffer: PB with 0.1% SDS, 6 M urea and 2.5% mercaptoethanol (ME, Sigma–Aldrich, Germany). The tubes were shaken for 24 h at 20 °C. Suspensions were then centrifuged at 9000 × g for 20 min and the protein content in the supernatant was determined using a Bradford assay (Bradford, 1976). Standard curves using bovine serum albumin (BSA, Sigma–Aldrich Chemical Co., St. Louis, USA) were constructed for each buffer. For each type of film, at least two samples from four independent film preparations were solubilized. The soluble protein content was expressed as a percentage of the total amount of protein in the film, which was measured by the Kjeldahl method (AOAC 920.53, 1995).

2.11. SDS-PAGE electrophoresis

Film proteins solubilized in the different buffers were analyzed by SDS-PAGE according to Laemmli (1970), using a separating gel (12% w/v in polyacrylamide) and a stacking gel (4% w/v in polyacrylamide) in minislab arrangement (Bio-Rad Mini-Protean II Model). A continuous dissociating buffer system, containing 0.375 M Tris–HCl, pH 8.8, and 0.1% w/v SDS, was used for the separating gel, while the running buffer was 0.025 M Tris–HCl, 0.192 M glycine and 0.1% w/v SDS, pH 8.3. Electrophoresis was carried out at a constant voltage of 200 V. Samples were diluted with a buffer at pH 6.8 (0.125 M Tris–HCl, 0.1% w/v SDS, 40% v/v glycerol, 0.05% w/v bromophenol blue). Protein molecular weights were estimated using low MW markers (Pharmacia, Amersham, England) that included phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400). Gels were fixed, stained with R-250 Coomasie blue (0.1% w/v) in water/methanol/acetic acid (5:5:2) overnight, and destained with 25% (v/v) methanol and 10% (v/v) acetic acid. Images of the gels were analyzed with ImageJ software (Bethesda, MD: US National Institute of Health; http://resb.info.nih.gov/ij/).

2.12. Glass transition temperature (Tg)

Film glass transition temperature (Tg) was determined by differential scanning calorimetry, using a DSC TA 2010 calorimeter Q100 V9.8 Build 296 (TA Instrument, New Castle, Del., USA) controlled by a TA 5000 module with a quench cooling accessory. Temperature and heat flow calibration of the equipment were carried out according to ASTM Standards, using lauric and stearic acids and indium as standards. Hermetically sealed aluminum pans containing 10 mg of films were prepared, and the capsules were scanned at 10 °C/min over the range −100 to 220 °C. The glass transition temperature (Tg) was considered to be the inflexion point of the base line, caused by the discontinuity of the specific heat of the sample, and it was calculated with the help of the Universal Analysis V4.2E software (TA Instruments, New Castle, Del., USA) (Sobral, Menegalli, Hubinger, & Roques, 2001). All the assays were performed at least in duplicate.

2.13. Opacity

Each film specimen was cut into a rectangular piece and placed directly in a spectrophotometer test cell, and measurements were performed using air as the reference. A spectrum of each film was obtained in an UV–Vis spectrophotometer (Beckman DU650, Germany). The area under the absorption curve from 400 to

800 nm was recorded, and the opacity of the film (UA/mm) was calculated by dividing the absorbance at 500 nm by the film thickness (mm) (Cao, Fu, & He, 2007). All determinations were performed in quadruplicate.

2.14. Color

Film colors were determined using a Minolta Chroma meter (CR 300, Minolta Chroma Co., Osaka, Japan). A CIE Lab color scale was used to measure the degree of lightness (L), redness (+a) or greenness (−a), and yellowness (+b) or blueness (−b) of the films. The instrument was standardized using a set of three Minolta calibration plates. Films were measured on the surface of the white standard plate with color coordinates of $L_{standard} = 97.3$, $a_{standard} = 0.14$ and $b_{standard} = 1.71$. Total color difference (ΔE) was calculated from:

$$\Delta E = \sqrt{\left(L_{film} - L_{standard}\right)^2 + \left(a_{film} - a_{standard}\right)^2 + \left(b_{film} - b_{standard}\right)^2} \quad (5)$$

Values were expressed as the means of nine measurements on different areas of each film.

2.15. Antioxidant capacity

The $ABTS^{\bullet+}$ radical (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) scavenging capacity of the samples was determined according to a modified version of the method of Re et al. (1999). The stock solution of $ABTS^{\bullet+}$ radical consisted of 7 mM ABTS (Fluka, Sigma–Aldrich, Germany) in 2.45 mM potassium persulfate (Anedra, Argentine), kept in the dark at room temperature for 12–16 h. An aliquot of the stock solution was diluted with distilled water in order to prepare the working solution of $ABTS^{\bullet+}$ radical with absorbance of 0.70 ± 0.03 at 734 nm. A sample of the protein film (5 mg) was added with 50 μl of sodium phosphate buffer (0.01 M, pH 7.4) and 950 μl of the solution containing the $ABTS^{\bullet+}$ radical. The mixture was vortexed for 2 min and then centrifuged for 3 min at 9000 × g (A15, B. Braun Biotech International, USA). The supernatant was collected and its absorbance at 734 nm (Abs_s) was measured 6 min after the addition of the $ABTS^{\bullet+}$ solution. To obtain a reaction blank (Abs_{rb}) the same procedure was carried out but the protein film was replaced with 25 μl of sodium phosphate buffer. The antioxidant capacity (AC), as measured by the ability to scavenge the $ABTS^{\bullet+}$ radical, was calculated with the equation (Eq. (6)). All determinations were performed at least in triplicate.

$$AC = \frac{Abs_{rb} - Abs_s}{Abs_{rb}} \cdot 100 \quad (6)$$

2.16. Statistical analysis

Results were expressed as mean ± standard deviation and were analyzed by analysis of variance (ANOVA). Means were tested with the Fisher's least significant difference test for paired comparison, with a significance level $\alpha = 0.05$, using the Statgraphics Plus version 5.1 software (Statgraphics, USA).

3. Results and discussion

3.1. Characterization of protein isolates

The characterization of sunflower protein isolates and a soy protein isolate used as control is shown in Table 1. It can be observed that sunflower isolates presented similar protein concentrations, around 70%, represented mostly by 11S globulins (Molina et al., 2004) which were partially denatured but conserved about 40% of their native conformation (as estimated from the denaturation enthalpy −14.5 J/g-reported by González-Pérez et al. (2004) for sunflower native proteins extracted from seeds not subjected to thermal treatment).

Table 1
Content of proteins and phenolic compounds, denaturation degree, and surface hydrophobicity of sunflower protein isolates (**SunI**, **SunI$_W$** and **SunI$_R$**) and soy protein isolates (**SoyI**) used for preparing biodegradable films.

Protein isolates	Protein content (%)	Phenolic compounds (%)	Denaturation degree (%)	Surface hydrophobicity (UA mL/mg)
SunI	70.35 ± 0.75^b	2.51 ± 0.14^b	62.5 ± 2.9^a	50.4 ± 0.3^a
SunI$_W$	70.07 ± 1.39^b	2.15 ± 0.12^a	59.8 ± 2.4^a	74.6 ± 4.0^b
SunI$_R$	66.69 ± 0.80^a	1.82 ± 0.04^a	62.8 ± 2.3^a	91.1 ± 8.9^c
SoyI	85.02 ± 1.20^c	ND	100^b	75.4 ± 2.6^b

Reported values for each protein isolate are means ± standard deviation. Values means followed by the same letter are not significantly ($p \leq 0.05$) different according to Fisher's test. ND: no detected.

These isolates differed in their content of phenolic compounds, that were both free (mainly chlorogenic acid) and interacting with proteins, forming stable dispersions with particle size smaller than 45 μm (data not shown). While the phenolic content did not affect the denaturation degree detected by DSC, it significantly ($p > 0.05$) influenced the surface hydrophobicity of the proteins, since the later increased as the phenolic content of isolates decreased. The soy protein isolate presented a higher protein concentration (85%), and its DSC thermogram did not exhibit the typical denaturation endotherms at 78.2 °C and 92.4 °C that correspond to its major protein fractions (7S and 11S globulins, respectively) (Mauri & Añón, 2006). Phenolic compounds were not detected in the soy protein isolate, and its proteins had surface hydrophobicities intermediate to those of sunflower protein isolates.

3.2. Characterization of biodegradable films

The thickness and water content of films obtained by casting from **SunI**, **SunI$_W$**, **SunI$_R$** or **SoyI** using glycerol as plasticizer are shown in Table 2. All the films had thickness ranging from 71 to 80 μm, densities ranging from 1.46 to 1.51 g/cm^3, and moisture content close to 25%, with no significant differences ($p \leq 0.05$) according to the protein isolate used. Therefore, the differences or similarities in the properties considered would be attributed to the proteins and to the way they interact within the film matrix.

3.2.1. Film's water susceptibility

The water vapor barrier properties of the protein films were studied. The values obtained for water vapor permeability (WVP) are shown in Table 3. While the sunflower isolates with lower phenolic content (**SunI$_W$** and **SunI$_R$**) had proteins with higher surface hydrophobicity than the **SunI** isolate (Table 1), no

Table 2
Thickness, moisture content, and density of films obtained from sunflower protein isolates (**SunI**, **SunI$_W$** and **SunI$_R$**) and soy protein isolates (**SoyI**).

Protein films	Thickness (μm)	Moisture content (%)	Density (g cm^{-1})
SunI	71 ± 11^a	25.88 ± 2.38^a	1.46 ± 0.07^a
SunI$_W$	74 ± 13^a	26.34 ± 1.13^a	1.47 ± 0.03^a
SunI$_R$	80 ± 10^a	24.93 ± 1.35^a	1.51 ± 0.06^a
SoyI	75 ± 17^a	23.97 ± 1.45^a	1.46 ± 0.06^a

Reported values for each protein isolate are means ± standard deviation. Values means followed by the same letter are not significantly ($p \leq 0.05$) different according to Fisher's test.

Table 3
Water vapor permeability (WVP), water solubility coefficient in the film (β), effective diffusivity (D_{ef}), and parameters of the GAB model for sorption isotherms of sunflower protein films (**SunI**, **SunI$_W$** and **SunI$_R$**) and soy protein films (**SoyI**).

Protein films	WVP × 10^{10}*	$\beta \times 10^{4}$*	$D_{ef} \times 10^{7}$*	Sorption isotherms – GAB model			
				X_o*	C	K	r^2
SunI	1.45 ± 0.01[a]	7.7	1.28	0.2416	50.40	0.8724	0.9840
SunI$_W$	1.49 ± 0.07[a]	8.5	1.17	0.1756	86.15	0.9459	0.9761
SunI$_R$	1.46 ± 0.01[a]	7.9	1.23	0.1793	41.52	0.9265	0.9761
SoyI	1.49 ± 0.07[a]	8.5	1.21	0.1647	9.98	0.9508	0.9879

*Units: WVP: g H_2O Pa^{-1} s^{-1} m^{-1}; X_o: g H_2O/g of dry film; β: g H_2O/Pa g of dry film; D_{ef}: m^2 s^{-1}. Reported values for each protein isolate are means ± standard deviation. Values means followed by the same letter are not significantly ($p \leq 0.05$) different according to Fisher's test.

statistically significant differences ($p \leq 0.05$) were observed between the films regarding WVP values. Since this property depends both on solubility (β) and on the effective water diffusivity (D_{ef}) in the protein film (Larotonda et al., 2005), the later measures were also determined, with no differences ($p \leq 0.05$) being detected between the films (Table 3). Similarly, no differences were found between the WVP, β and D_{ef} parameters of sunflower and soy protein films in spite of the different amino acidic composition of these proteins (Molina et al., 2004). The WVP values found were within the range reported for other protein films (Gennadios, Brandenburg, Weller, & Testin, 1993; Cuq et al., 1998; Rhim, Gennadios, Handa, Weller, & Hanna, 2000).

The sorption isotherms of the protein films analyzed are shown in Fig. 1. All the isotherms had a sigmoideal shape ($C > 2$), becoming asymptotic when a_w tended to 1, which is typical of products rich in proteins or starch. Films obtained from **SunI** were the ones with higher water absorption for aqueous activities lower than 0.85, while for higher a_w values all the films had the same behavior. As shown in the figure, the GAB model was adequate for describing mathematically the sorption isotherms ($r^2 > 0.97$), but the model diverged from experimental data at high relative humidity values ($a_w > 0.9$) because it assumes physical adsorption in multilayers, and in this zone the sorbate presents properties of pure water. The parameters obtained by adjusting experimental data with the GAB model are shown in Table 3. It can be observed that the humidity value in monolayer (X_o) is higher for **SunI** films (that contained the highest carbohydrate content and had proteins with lowest surface hydrophobicity). Water adsorption values found in this study were similar to those reported by other authors (Gennadios et al., 1994; Cuq, Gontard, & Guilbert, 1997).

3.2.2. Mechanical properties

Stress–strain curves obtained in traction experiments are shown in Fig. 2. All the sunflower protein films had the same mechanical behavior, with no statistically significant differences ($p \leq 0.05$) in values of tensile strength (≈4 MPa), elongation at break (≈24%), or Young's module (≈0.58 MPa). Ayhllon-Meixueiro et al. (2000) obtained by casting sunflower proteins films that had similar tensile strength values (3.9 MPa) but higher values of deformation at break (250%). In that work, however, the starting protein dispersion had a higher concentration (10% w/w) than the one used in the present work and the glycerol proportion was also higher (50% w/w respect to isolate mass, twice the percentage used in the present work). Most probably, the greater thickness of those films (170–200 μm) and the higher content of plasticizer would have a negative influence on the barrier properties of such films to water vapor (McHugh, Avena-Bustillos, & Krochta, 1993; Ghorpade, Li, Gennadios, & Hanna, 1995; Gennadios, Weller, Hanna, & Froning, 1996). On the other hand, the **SoyI** films evaluated in the present work had similar resistance but higher values of deformation at break and elastic module (about 4-fold and 1.5-fold, respectively) than those obtained with sunflower protein isolates. The ability to deform before reaching the rupture point (ε_{max}) and the Young's module (E) are the first results to suggest the existence of differential characteristics between the protein matrices of sunflower and soy. Since all the films had the same concentration of proteins and plasticizer (water and glycerol), the differences noted are probably associated to the form in which proteins interacted in the matrix forming the film.

3.2.3. Protein solubility

To further characterize the phenomenon mentioned above, the differential solubility of film proteins in buffer systems with the capacity to disrupt different types of interactions was studied. Such systems were: water (W), which can dissolve free polypeptides not strongly linked to the protein matrix; phosphate buffer (PB), which affects protein electrostatic interactions; PBD, which contains SDS and disrupt mainly hydrophobic interactions and also interacts with proteins increasing their charge/mass ratio; PBU, which contains urea and disrupts the water structure affecting hydrogen bonds and also hydrophobic interactions; PBDU, which disrupts all the interactions mentioned above and also modifies protein charge; and PBDUM, which also disrupts disulfide bonds because it contains β-mercaptoethanol. The results obtained are shown in Fig. 3. Films obtained with **SunI** had moderate values of protein solubility in water (≈40%), indicating that during film formation there is an important protein cross-linking. The protein solubility of the film decreased with PB (0.1 M sodium phosphate pH 7.5),

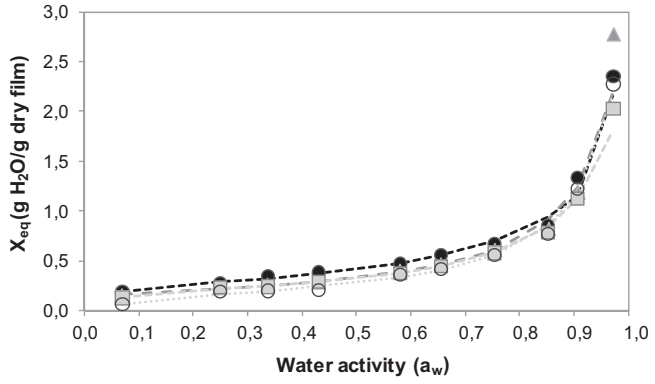

Fig. 1. Sorption isotherms of films prepared from sunflower proteins: **SunI** (●), **SunI$_W$** (▲) and **SunI$_R$** (■), or from soy proteins: **SoyI** (○). Filled symbols correspond to experimental data, and dotted lines indicate data adjusted with the GAB model.

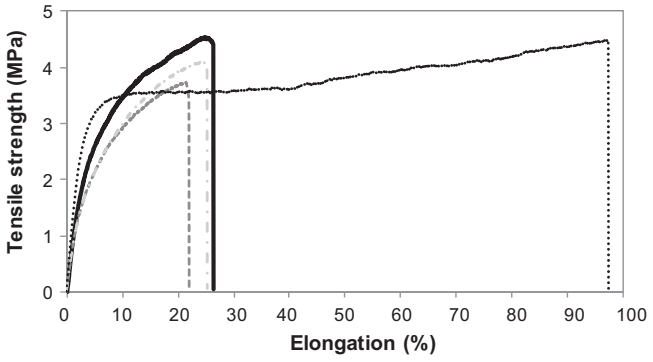

Fig. 2. Mechanical properties as from measured by traction of films prepared from sunflower proteins –**SunI** (——), **SunI$_W$** (– – –) and **SunI$_R$** (– · –)– and soy proteins –**SoyI** (▪▪▪▪▪▪)–.

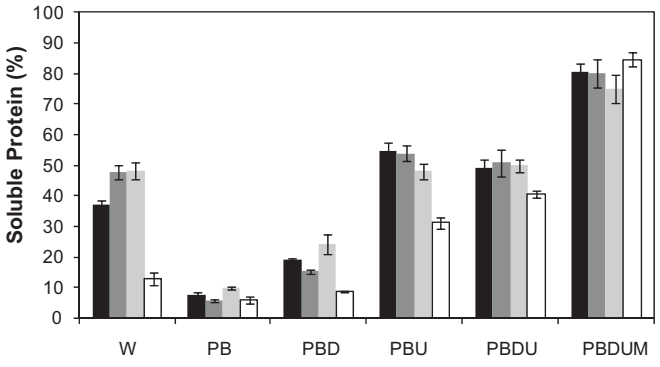

Fig. 3. Differential protein solubility of films prepared with **SunI** (■), **SunI$_W$** (■), **SunI$_R$** (■) and **SoyI** (□) in media with different chemical activity: Water (W), 0.1 M sodium phosphate buffer (PB), PB containing 0.1% w/v SDS (PBD), PB containing 6 M urea (PBU), PB containing both 0.1% SDS and 6 M urea (PBDU), and PBDU with 2.5% v/v β-mercaptoethanol (PBDUM), all at pH 7.5. Reported values for each protein isolate are means ± standard deviation.

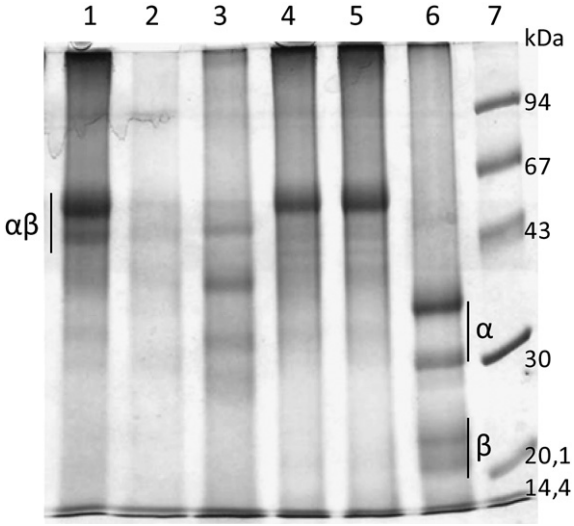

Fig. 4. SDS-PAGE analysis of **SunI** film soluble proteins in 1) Water (W), 2) 0.1 M sodium phosphate buffer (PB), 3) PB containing 0.1% w/v SDS (PBD), 4) PB containing 6 M urea (PBU), 5) PB containing both 0.1% SDS and 6 M urea (PBDU), and 6) PBDU with 2.5% v/v β-mercaptoethanol (PBDUM). Low molecular weight markers are shown in lane 7.

possibly because this buffer may favor ionic interactions between polypeptide chains, producing a *salting out* effect (Mauri & Añón, 2006). The solubility increase observed when films were treated with buffers containing SDS and urea (PBD and PBU, respectively) would indicate that hydrophobic interactions and hydrogen bonds stabilize the film structure, the later interactions being more important. No significant differences ($p \leq 0.05$) in solubility were observed between the buffer containing urea (PBU) and the one that also contained SDS (PBDU), which can be attributed to the fact that the effect of urea on hydrogen bond formation modified the structure of water favoring protein unfolding, also associated to hydrophobic interactions in the film, thus masking the effect of SDS. The increased solubility obtained with the addition of β-mercaptoethanol to the buffer (PBDUM) evidences the important role of disulfide bonds in the formation of the protein network, since the disruption of this type of bonds makes possible to reach solubility values between 80% and 90%. As shown in Fig. 3, sunflower protein films obtained from **SunI$_W$** and **SunI$_R$** were more soluble than those of **SunI** only in water, but the behavior of the three films was similar for the remaining buffers. A marked difference ($p > 0.05$) in water solubility can be observed between films obtained from sunflower proteins and those from soy proteins, evidencing the structural differences between these proteins which in turn determine differences in the predominance of each type of interactions within the film structure. Notwithstanding, similar results were obtained with the strongest buffers that affect various types of interactions simultaneously. For both sunflower and soy films solubility values reached 90% only in the presence of β-mercaptoethanol, indicating that interactions through disulfide bonds are important in the formation of the film matrix.

Fig. 4 depicts the electrophoretic patterns of protein fractions soluble in each buffer system for **SunI** films, used as an example. It can be observed that the main species soluble in water (lane 1) were some αβ subunits of helianthin (45–62 kDa), α polypeptides, and soluble aggregates of high molecular weight that could not enter the gel. The lower solubility in phosphate buffer (PB) was evidenced in the electrophoretic pattern of lane 2, where only tiny bands associated to αβ subunits, both associated and free, were observed. The SDS-containing buffer (lane 3) lead mainly to the dissolution of α polypeptides, while those containing urea (lanes 4 and 5) dissolved mainly αβ subunits and high molecular weight aggregates. The addition of a reducing agent (β-mercaptoethanol) led to the dissociation of the αβ subunits in their constituent α and β polypeptides (lane 6), and to a lesser extent to the dissociation of high molecular weight aggregates that could not be resolved in the gel. These results indicate that the 11S globulin has a preponderant role in the formation of the film structure, and that it is present in the film in two forms: one weakly associated (water soluble) and other strongly associated (extractable with PBDUM).

It has been reported that materials with higher capacity to establish covalent interactions through disulfide bonds form more resistant and elongable matrices (Pérez-Gago & Krochta, 2001). In the present study no differences ($p \leq 0.05$) in resistance were found between soy films and those made from sunflower proteins, but the former were four times more deformable. This behavior might be due to a different distribution of disulfide bonds in the protein matrices that constitute soy and sunflower films. Bases on amino acid composition analysis and also primary sequence of storage proteins, sunflower protein isolates have higher of methionine and cysteine content than soybean protein isolates, but soybean proteins are more charged and less hydrophobic than sunflower proteins (Molina et al., 2004). These differences in amino acid composition might be responsible of the differences observed in the deformability of proteins films; soybean proteins sulfhydryl groups are more exposed than sunflower's ones, because the electrostatic repulsion in soybean favor a major exposition. This phenomena favors the formation of disulfide bonds inter and intra chain (Darby & Creighton, 1995) therefore soybean protein film are more elongable than sunflowers ones. In contrast, in the case of sunflower proteins, which partially maintain their native conformation, part of the potential disulfide bonds would be stabilizing the soluble aggregates observed in Fig. 4.

3.2.4. Glass transition temperatures (Tg)

The DSC thermograms of all the protein films studied presented two vitreous transition temperatures, one close to −69 °C and a second close to −28 °C, with no significant differences ($p \leq 0.05$) for the different isolates, even when comparing sunflower and soy films. Since all the films had the same content of plasticizer (glycerol and water), eventual differences in transition temperatures would had been attributed to differences in the degree of cross-linking. This partially corroborates that the observed differences in the formation of disulfide bonds are more related to the strategic location of the bonds in the network, which contribute to elasticity,

Table 4
Hunter color values (L, a and b), total color difference (ΔE) and opacity of sunflower (**SunI**, **SunI$_W$** and **SunI$_R$**) and soy protein films (**SoyI**).

Protein films	Hunter-lab color parameters				Opacity (UA mm^{-1})
	L	a	b	ΔE	
SunI	29.49 ± 0.86^a	−1.49 ± 0.55^b	2.72 ± 1.13^a	67.85 ± 0.83^a	22.34 ± 1.63^c
SunI$_W$	37.27 ± 2.10^b	6.59 ± 0.10^c	15.06 ± 2.94^b	61.92 ± 1.38^b	17.75 ± 1.00^b
SunI$_R$	43.10 ± 1.52^c	15.21 ± 0.64^d	24.62 ± 2.11^c	60.79 ± 0.57^b	16.81 ± 0.47^b
SoyI	93.23 ± 0.69^d	−3.83 ± 0.32^a	16.91 ± 1.53^b	16.24 ± 1.65^c	1.50 ± 0.48^a

Reported parameters for each film are means ± standard deviation. Means followed by the same letter are not significantly different ($p \leq 0.05$) according to Fisher's test.

than to the number of bonds formed, such that the cross-linking is not greatly affected. This would also explain the lack of differences regarding WVP.

The presence of two vitreous transition temperatures is typical of systems having phase separation (Sobral et al., 2001). In the case of the films studied here, such temperatures may be attributed to the existence of zones enriched in the different components, Tg_1 (−69 °C) corresponding to a glycerol-rich phase and Tg_2 (−28 °C) corresponding to a protein-rich phase. The same behavior was reported by Cherian, Gennadios, Weller, and Chinachoti (1995) for wheat gluten protein films plasticized with sucrose, and by Sobral et al. (2001) for films made from fish sarcoplasmic and myofibrilar proteins using glycerol as plasticizer. In contrast, only one Tg has been reported for glycerol-plasticized films prepared from milk whey (Anker, Stading, & Hermansson, 1999), wheat gluten (Gontard & Ring, 1996) or soy (Shaw, Monahan, ÓRioran, & ÓSullivan, 2002; Denavi et al., 2009).

3.2.5. Optical properties

Although films prepared from sunflower isolates with different content of phenolic compounds did not differ in terms of thermal, mechanical and barrier properties, they presented a very distinct aspect due to differences in color. The color and opacity parameters of the different films are shown in Table 4. Films obtained from **SunI**, which presented a dark green tone, had the lowest L, b and a values (the later being negative). In contrast, films prepared from sunflower isolates with a lower content of phenolic compounds (**SunI$_W$** and **SunI$_R$**) had a more brownish and light tone, characterized by higher values of a, b and L, that resulted in a lower general color (lower ΔE). These differences in film color are directly related to the color of the sunflower protein isolates used to prepare them. This property develops during the alkaline extraction of proteins, and is due to oxidation of the phenolic compounds to o-quinones, which can condense or react with the cationic groups of proteins (Sosulski, 1979). Soy protein films, which presented a light yellow color, had the highest L values, moderate a and b values, and the lowest ΔE.

Film opacity tended to vary in a way similar to that described for ΔE. Sunflower protein films had higher opacity than those of **SoyI**, with **SunI** films presenting the highest opacity value. Film-forming dispersions were translucent. The concentration process that occurred during drying the dispersions, which concluded in film formation, could favor the formation of protein aggregates. This aggregation was more important in films contained higher concentration of phenolic compounds than in film contained small amount of phenols, therefore the presence of phenols promoted protein aggregation and film's opacity.

Undoubtedly, the intense color limits some potential applications of these materials in food packaging. For example, they could not be used for products that should be easily visible through the package (such as minimally processed vegetables) because the impaired visualization may reduce the acceptability of potential consumers. In contrast, such films could be used, if their properties are adequate, for applications in which color is irrelevant or in those in which color may have an additional usefulness, as in the case of plastics used for agriculture. In addition, the use of biodegradable materials in intensive agriculture is very important given the difficulty for disposing plastic residues in agriculture settings.

The absorbance of sunflower and soy protein films in the visible spectrum is shown in Fig. 5. The soy protein film absorbed in the region of the spectrum evaluated (400–800 nm). The absorbance of sunflower films in such region increased for increasing concentrations of phenolic compounds. Films prepared with **SunI** proteins presented two absorption maxima close to 420 nm and 670 nm, which partly coincide with the absorbance of chlorophyll, whose spectrum is also shown in Fig. 5. This would be advantageous for the use of these films in mulching, since these materials would block visible light for impeding weed growth (Ali et al., 2004).

3.2.6. Antioxidant properties

Since it has been reported that phenolic compounds, such as the chlorogenic acid present in sunflower isolates, might have antioxidant activity, this property was studied in the protein films

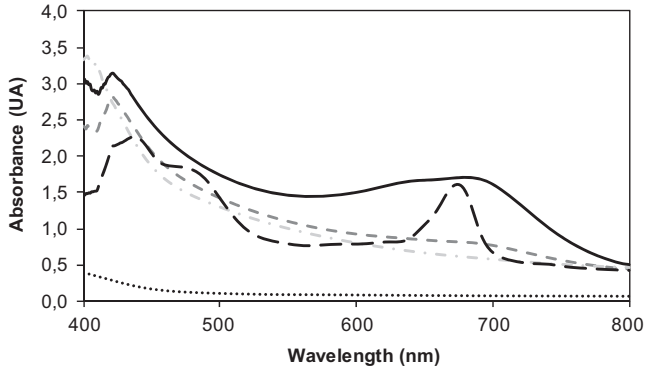

Fig. 5. Absorbance spectrum in the visible range of films prepared from sunflower proteins: **SunI** (——), **SunI$_W$** (– – –) and **SunI$_R$** (– · –) or from soy proteins: **SoyI** (▪▪▪▪▪▪), compared to that of a chlorophyll solution extracted from spinach (*Spinaca oleracea*).

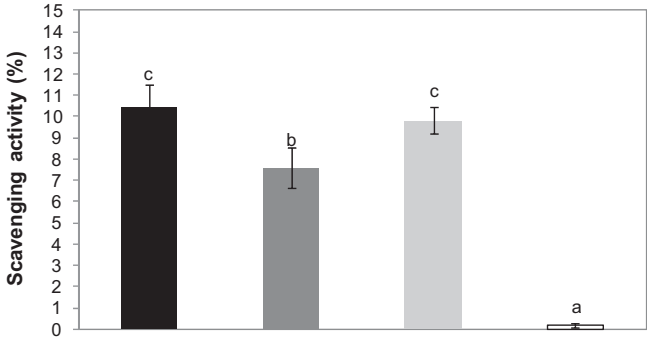

Fig. 6. Antioxidant activity of films prepared from sunflower proteins –**SunI** (■), **SunI$_W$** (■) and **SunI$_R$** (■)– or from soy proteins –**SoyI** (□)–, measured as the $ABTS^{\cdot+}$ radical scavenging capacity. Reported values for each protein isolate are means ± standard deviation. Values means followed by the same letters are not significantly ($p \leq 0.05$) different according to Fisher's test.

obtained in this work. As measured by the $ABTS^{\bullet+}$ radical scavenging capacity (Fig. 6), all the sunflower protein films had antioxidant properties, which is absent in soy films. This result clearly demonstrated the antioxidant capacity of sunflower films although the number of sunflower samples included in this work is not sufficient to show a correlation between $ABTS^{\bullet+}$ radical scavenging capacity and phenolic compounds content (Table 1). **$SunI_R$** films showed an antioxidant activity that did not have the same correlationship with its phenol content than the one obtained for **SunI** and **$SunI_W$**, the differences in the relationship can be attributed to a residual amount of sulfite. Nevertheless the comparison of the antioxidant values between sunflower and soybean films whose major different in chemical composition is caused by phenols compounds demonstrated the importance of these compounds in conferring antioxidant activity. This is an interesting characteristic of sunflower films, since they would be naturally acting as carriers of antioxidant compounds.

4. Conclusions

The sunflower protein isolates obtained from the residual pellet of oil industry were adequate for preparing flexible biodegradable films by casting, with mechanical and barrier properties in the same range of other protein films. The films were mainly stabilized by hydrogen and disulfide bonds, and to a lesser extent by hydrophobic and ionic interactions. The content of residual phenolic compounds in the isolates modified the color and opacity of the films, but conferred them antioxidant properties of potential usefulness for preserving oxidation-sensitive products. While color may limit some potential applications of the films in packaging, these materials could be used, if their properties are adequate, for mulching with herbicide effect in intensive agriculture.

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