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Storage-induced changes in functional properties of glycerol plasticized – Soybean protein concentrate films produced by casting

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ABSTRACT

Aging of soybean protein concentrate (SPC) films plasticized by glycerol obtained by casting and stored at 25 ± 2 °C and 65 ± 2% relative humidity was systematically investigated over a period of 90 days. Aging promoted the reorganization of the secondary structure of protein fraction in SPC into others with prevalence of extended β-sheet conformation. Stabilizing interactions also progressed to increasing contribution of disulfide cross-links and Maillard aggregates through the carbohydrate fraction of SPC. Such time-dependent structural changes were ineffective in retaining glycerol within the films. This caused increased strength and stiffness with time. Water vapor permeability values increased up to 90th day due to micro-cracks created by film contractions during storage, which create more permeable structures which hamper the determination of oxygen permeability on aged films. As a general trend, films functional properties remained stable for two weeks under specific storage conditions preserving enough mechanical and barrier properties to act as efficient protective food packaging materials.

1. Introduction

Motivated by petroleum shortage and market demand for sustainable and cost – effective sources, vegetable proteins are regarded as potentially viable feedstock options to depleting ones for the design of food packaging films. The biological nature of such materials provides abundant raw supplies with constant composition and favorable costs, along with the distinctive ability of being converted into biomass and harmless by-products through microbial activity, under appropriate waste management infrastructures. Soybean proteins are appealing raw materials for food packaging films because of their aptitude of protecting foodstuffs due to their good mechanical properties and oxygen barrier at medium and low relative humidity, and their processing ability by diverse methods (Ciannamea, Stefani, & Ruseckaite, 2014). From a technological point of view, the potential of soybean proteins films as packaging materials require controlled lifetime of the macroscopic properties to become competitive to the commonly used synthetic polymers (Orliac, Rouilly, Silvestre, & Rigal, 2003). However, biopolymers, such as soybean proteins suffer aging. To limit aging, it is important to identify and understand the mechanisms and reasons for the time-dependent physical and chemical changes (Olabarrieta, Cho, et al., 2006).

Physical aging or structural relaxation of a glassy matrix occurs at temperature below the glass transition temperature (T_g) and it is related with the evolution from a non-equilibrium state (i.e. glassy state) toward the equilibrium accompanied by molecular rearrangements. Chemical aging, on the other hand, is associated with chemical reactions undergoing with time such as protein aggregation and thiol oxidation (Morel, Bonicel, Micard, & Guilbert, 2000; Olabarrieta, Cho, et al., 2006; Olabarrieta, Gällstedt, Ispizua, Sarasua, & Hedenqvist, 2006; Ullsten et al., 2009). Accordingly the understanding and control of the causes driving such variations is crucial in predicting and ensuring the long-term stability of the functional properties of protein films.

Changes upon aging such as those due to the migration/diffusion of low mass components (i.e., plasticizers, additives), protein aggregation, denaturation and/or thiol oxidation have been reported for protein – based films. Aging experiments conducted on wheat gluten and corn zein films containing polyethylenglycol or glycerol (Gly) as plasticizers revealed increased tensile strength occurred after 20 days of storage associated with plasticizer migration (Park, Bunn, Weller, Vergano, & Testin, 1994). The greater stability of whey protein isolate films

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plasticized with sorbitol over those plasticized with Gly during storage at 23 °C, 50% relative humidity (RH) for 120 days was correlated with the out – diffusion of the latter over time (Anker, Stading, & Hermansson, 2001). On the contrary, whey protein isolate films with Gly were reported to be more stable than their counterpart containing sorbitol due to the migration and crystallization of sorbitol (Osés, Fernández-Pan, Mendoza, & Maté, 2009). Color variations upon aging linked with non enzymatic browning were also observed in whey protein concentrate (Trezza & Krochta, 2000) and fish myofibrillar protein –based films plasticized with saccharose (Cuq, Gontard, Cuq, & Guilbert, 1996). Examples of time-induced brittleness of wheat gluten films plasticized with diverse plasticizers are reported in the literature (Gueguen, Viroben, Barbot, & Subirade, 1998; Micard, Belamri, Morel, & Guilbert, 2000; Olabarrieta, Cho, et al., 2006). Particularly, the decrease in ductility of vital wheat gluten films obtained at pH 4 over time was associated to the loss of plasticizing molecules due to a lower degree of protein aggregation at acidic pH (Olabarrieta, Cho, et al., 2006). Similar findings were accounted for glycerol-plasticized vital wheat gluten films containing 4.5 wt % natural or quaternary ammonium salt modified montmorillonite clay (Olabarrieta, Gällstedt, et al., 2006), extruded glycerol-wheat gluten with salicylic acid and sodium hydroxide (Ullsten et al., 2009) exposed to identical aging conditions (23 °C, 50% RH, 120 days) and compression-molded glycerol-plasticized wheat gluten films exposed to 0% RH and 50% RH for 24 days (Gällstedt, Mattozzi, Johansson, & Hedenqvist, 2004). Hernández-Muñoz, López-Rubio, del-Valle, Almenar, and Gavara (2004) reported changes in mechanical and color properties of gluten films plasticized with glycerol while those incorporated with sorbitol and thriethanolamine remained stable after 1 year of storage at 25 °C and 65% RH. Similarly, sunflower protein films plasticized with glycerol and triethylenglycol were stable over a 3-month aging period while lighter plasticizers were lost over time (Orliac et al., 2003). In addition to variations in tensile, barrier and color properties, antioxidant activity also evolved with time as reported by Jongjareonrak, Benjakul, Visessanguan, and Tanaka (2008) for fish skin gelatin films incorporated with BHT (butylated-hydroxy-toluene) or a-tocopherol, stored at 28 °C and 50% RH for several weeks. Despite the industrial significance of the aging problem, a limited number of studies on soybean protein – based films have been reported in the literature. One study carried out on glycerol-plasticized soybean protein isolate (SPI) films made from intensively mixed materials stored at 25 °C and 50% RH over 14 weeks revealed that films evidenced a sticky surface due to the out – diffusion of the plasticizer. Thickness, tensile strength as well as percentage of elongation at break barely varied with aging, except for 40%Gly-added films which experienced a reduction in elongation of about 45% (Cunningham, Ogale, Dawson, & Acton, 2000). Compression-molded SPI plastics modified by different plasticizers and exposed at 25 °C, 50% RH for 180 days, showed an excess of enthalpy relaxation and all plastics tended to be stiff and brittle upon storage, being the plastics with Gly fairly stable after 60 days (Mo & Sun, 2003).

The functional properties of freshly prepared soybean protein concentrate (SPC) films plasticized with glycerol were studied after processing by casting and compression molding in our previous work (Ciannamea et al., 2014). In order to evaluate whether the functional properties remained stable during the foreseen shelf life of the material, films were stored for 90 days at 25 ± 2 °C and 65 ± 2% RH. Variations on tensile properties, color, moisture content, total soluble matter as well as water vapor permeability were assessed and related with the evolution of the stabilizing interactions in SPC films over time.

2. Experimental

2.1. Materials

Soy protein concentrate (SPC, Solcom S 110) with an average particle size of 100 mesh, and a proximate composition of 7% moisture, 69% protein, 1% fat, 3% fiber, 5% ash and about 15% non-starch polysaccharides (mainly cellulose, non cellulose polymers and pectin polysaccharides), was provided by Cordis S.A. (Villa Luzuriaga, Buenos Aires, Argentina). Glycerol analytical grade (Gly, 98%) was purchased from Anedra (Buenos Aires, Argentina) and used as a plasticizer. TRIZMA/hydrochloric acid, glycine and Na_2EDTA (Biopack, Buenos Aires, Argentina), Sodium dodecyl sulfate (SDS) and urea (Anedra, Buenos Aires, Argentina), and 2-mercaptoethanol (Aldrich, St. Louis, USA) were used in solubility tests. Buffer solution pH 10 (Anedra, Buenos Aires, Argentina) was used to adjust the pH level of the film-forming solutions. Sodium azide (Na_3N, Anedra, Buenos Aires, Argentina) was applied to prevent microbial growth during total soluble matter assays. Calcium chloride ($CaCl_2$; Aldrich, St. Louis, USA) was used as desiccant for water vapor transmission measurements. Copper sulfate ($CuSO_4$) and potassium and sodium double tartrate tetrahydrate (Anedra, Buenos Aires, Argentina) were used as received to prepare Biuret reagent. Trichloroacetic acid (TCA, Biopack, Buenos Aires, Argentina) was used as received.

2.2. Methods

2.2.1. Film formation

All films were produced by casting from their film – forming solutions according to the conditions previously reported elsewhere (Ciannamea et al., 2014). SPC powder was manually mixed with suitable amounts of glycerol (30%, 40% and 50% on dry SPC basis) for 15 min. Pre-mixes (5 g/100 ml solution) were subsequently dissolved in pH 10 buffer solution (pH > isoionic point, pI ~ 4.5) under constant stirring at 70 °C on a hot plate (Cole-Parmer, USA) for 30 min. After mixing, dispersions were sonicated in an ultrasonic bath (Testlab, 160 W, 40 KHz) for 15 min to remove bubbles and subsequently poured into leveled Teflon-coated Petri dishes, equipped with Teflon frames (thickness 0.5 cm) to control the amount of film – forming solutions per area. A uniform film thickness (target thickness ~ 150 μm) was maintained by casting the same solid content (i.e. approximately 35 ml/150 cm^2, for SPC-30% Gly). Water was evaporated in an air-circulating oven (Memmert, Germany) at 25 °C until reaching constant moisture content (about 24 h), and then peeled-off from the molds. Films were cut into the desired shapes for further testing and stored in a controlled humidity chamber at 25 ± 2 °C and 65 ± 2% RH for 48 h, prior testing. Films were labeled as **SPCX** where **X** corresponds to the percentage (on dry SPC weight) of glycerol.

2.2.2. Aging

After casting and conditioning, films were stored in a climate chamber at 25 ± 2 °C and 65 ± 2% RH. Preliminary experiments had shown that films reached the equilibrium moisture content within the first 48 h of storage. Twenty films were cast from each formulation and samples of adequate shape were subjected to FTIR analysis, differentiate solubility tests, tensile properties, opacity, color, total soluble matter and water vapor permeability as well as morphological changes with time. Experiment was performed during 90 days. Ün-aged refers to 0 day stored samples.

2.2.3. 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 test, opacity and moisture absorption experiments, four measurements were done on each specimen.

2.2.4. Tensile properties

Tensile tests were performed on an INSTRON 4467 Universal Test Machine (Buckinghamshire, England) equipped with a 0.5 KN load cell at a crosshead speed of 3 mm/min at room temperature, according to the procedure described in ASTM D1708-02a. Tensile strength (TS), Young modulus (E) and elongation at break values ($\varepsilon_b = \Delta L/L_0 \times 100$) were calculated as the average of ten replicates.

2.2.5. Light barrier and color properties

Visible light – barrier properties of films were determined by measuring their light absorption at wavelength ranging from 400 to 800 nm, using a UV–Visible spectrophotometer Agilent 8453 (China). Rectangular strips of films were placed directly in the spectrophotometer test cell and air was used as reference. Film opacity was expressed as the area under the recorded curve and was expressed as absorbance units (AU) × nm.

Color parameters were assessed using a portable colorimeter Lovi Bond Colorimeter RT 500 (Amesbury, United Kingdom) with a measuring area of 8 mm of diameter and recorded in the Hunter Lab color scale. The Hunter Lab scale consists of a luminance or lightness component (L^*, representing $L^* = 0$ for black and 100 for white) and two chromatic components: a^* value represents green ($a^* = -80$) to red ($a^* = +100$) while the b^* value represents blue ($b^* = -80$) to yellow ($b^* = +70$) colors. The colorimeter was calibrated using a standard white plate. The total color difference (ΔE), with respect to fresh SPC film, 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 values reported are the average of three replicates.

2.2.6. Equilibrium moisture content

Squared-shape \hskip strips of each sample (dimensions 4 cm^2) were weighed in an analytical balance (0.0001 g; Ohaus, USA) to determine the initial mass (m_i). Then the samples were dried in an air circulating oven (Memmert, Germany) at 105 °C for 24 h. Equilibrium moisture content (MC) was determined as the percentage of initial film weight lost during drying, calculated on wet basis.

$$\mathrm{MC}\ (\%) = 100 \cdot (m_i - m_d) \cdot m_i^{-1} \quad (2)$$

where m_i and m_d are the initial and final mass (dry) of the films, respectively. Reported values were the average of three replicates.

2.2.7. Total soluble matter

Total soluble matter (TSM) was expressed as the percentage of film dry matter solubilized after 24 h immersion in distilled water. The *wet* method was used for TSM determinations. Three samples were weighted (±0.0001 g) and subsequently were immersed in 30 ml of distilled water with traces of sodium azide (0.02%) to inhibit microbial growth, and stored at 25 ± 2 °C for 24 h. Insoluble dry matter was determined on the recovered samples after drying them in an air-circulating oven (Memmert, Germany) at 105 °C for 24 h. Dry matter was obtained by subtracting the moisture content calculated with Eq. (2) to the initial mass of the sample.

2.2.8. Protein solubility

Differentiate solubility of samples in specific buffer solutions which are known to disrupt specific protein interactions and bonds was used to elucidate the evolution of the stabilizing interaction with time (Guckian, Dwyer, O'Sullivan, O'Riordan, & Monahan, 2006; Hager, 1984; Jiang, Xiong, Newman, & Rentfrow, 2012; Mauri & Añón, 2006). The solutions were labeled as:

a) Solution 1 (S1): 0.086 M TRIZMA/HCl, 0.09 M glycine and 4 mM Na_2EDTA
b) Solution 2 (S2): S1 + 5 $g \cdot ml^{-1}$ SDS
c) Solution 3 (S3): S1 + 8 M urea
d) Solution 4 (S4): S1 + 5 $mg \cdot ml^{-1}$ SDS and 8 M urea
e) Solution 5 (S5): S1 + 5 $g \cdot ml^{-1}$ SDS, 8 M urea and 25 $mg \cdot ml^{-1}$ 2-mercaptoethanol (2-ME).

Film samples (approximately 150 mg) were carefully weighted and placed in test tubes with 3 ml of denaturing solution at 20 °C for 24 h. Afterward suspensions were centrifuged at 9000 rpm for 20 min (Labnet International, USA). Finally the protein content of 1 ml of supernatant was determined with Biuret reagent. In summary, 4 ml of Biuret reagent (1.5 g de $CuSO_4$, 6 g of potassium and sodium double tartrate tetrahydrate and 8 g of NaOH in 1000 ml of distilled water) and 1 ml of the test solution were mixed and incubated for 5 min at room temperature, and then the absorbance at 530 nm was measured in a UV–Visible spectrophotometer (Agilent 8453, China). Soluble protein content was expressed as the solubilized protein mass of the total protein mass in the film. For each solution (S1–S5) a calibration curve was performed using human albumin 20% (ZLB Behring), using solutions with protein contents varying from 0 to 10% (w/V). The procedure used to determine the protein content in solution S5 requires a previous stage of solvent separation, since the 2-ME interferes with the Biuret reagent. The protein dissolved in the supernatant was precipitated by adding 1 ml of TCA (600 $g \cdot L^{-1}$) and centrifuged at 9000 rpm for 20 min. The precipitate was separated and washed twice with 98% ethanol. Finally the precipitate was re-dissolved with 2 ml of 1 M NaOH prior to the Biuret assay.

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

ATR-FTIR spectra were carried out on a Thermo Scientific Nicolet 6700 spectrometer (Wisconsin, USA). The measurements were recorded between 400 and 4000 cm^{-1} using an attenuated total reflectance accessory (ATR) with a diamond ATR crystal. A total of 32 scans were performed at 4 cm^{-1} resolution. To resolve overlapping peaks and study the secondary structures of proteins the second derivative of the amide I band (1600–1700 cm^{-1}) was carried out.

2.2.10. Barrier properties

Water vapor permeability (WVP) was measured using the desiccant method, described in ASTM E96-00. Designed capsules were used according to the specifications of this standard. The desiccant ($CaCl_2$, PA, Aldrich, St. Louis, USA) was placed in the capsules, covering the whole surface and leaving a gap of 0.5 cm. The films were placed on the capsules, sealing with silicone grease, exposing a film area of 5 cm diameter (A). The system was placed in a controlled humidity chamber at 65% RH and 25 ± 2 °C. Mass increase was determined at specific intervals, until six measurements. The results were plotted as the mass change Δm (g) vs. t (h), from whose gradient was obtained the permeation rate of water vapor through the film G ($g \cdot h^{-1}$). Then, the permeability is obtained as:

$$WVP = G \cdot L \cdot [A \cdot S \cdot (HR_1 - HR_2)]^{-1} \quad (g/Pa \cdot h \cdot cm) \tag{3}$$

where L is the thickness of the film, S is the vapor pressure of pure water at the test temperature and ($HR_1 - HR_2$) is the relative humidity gradient used.

Oxygen permeability coefficient (OPC) determination was carried out using an oxygen permeability analyzer Model 8500 Systech Instruments (Oxon, UK), which operates according to ASTM 3985. Samples were cut circumferentially with a diameter of 14 cm and placed in the diffusion chamber of the equipment at 25°C. The computer monitors the amount of oxygen through the film per unit time and area to reach steady state. The results are reported as the oxygen transfer rate OTR ($cm^3/m^2 \cdot day$). OPC can be obtained by the following equation:

$$OPC = \left(OTR \cdot L\right) \cdot \Delta P^{-1} \quad \left(cm^3 \cdot \mu m \Big/ m^2 \cdot day\right) \tag{4}$$

where L is the thickness of the film (μm), ΔP is the partial pressure gradient of oxygen through the film (kPa).

2.2.11. Free amino groups

The extent of free amino groups in the obtained films was determined by UV–visible spectroscopy by using ninhydrin (2,2-dihydroxy-1,3-indanedione, NHN). Ninhydrin forms a purple complex (Ruhemann's purple) with the α-amino functionality of proteins. The absorbance of the solution measured at 570 nm (the wavelength of the blue–purple color) is proportional to the amount of free amino groups. Films were dried under vacuum at room temperature until constant weight. A precise amount of sample (100 ± 5 mg) was heated with ninhydrin solution (0.5 wt%) for 20 min. The absorbance of this solution was recorded on a UV–Visible spectrophotometer (Agilent 8453, China) at 570 nm.

2.2.12. Statistical analysis

Data values obtained in the experiments were statistically analyzed by one-way analysis of variance (ANOVA) employing OriginLab 8 software. Differences between pairs of means were assessed on the basis of confidence intervals using the Tukey test. The least significance difference was $P < 0.05$.

3. Results and discussion

3.1. Evolution of mass, moisture content and total soluble matter

Glycerol-plasticized SPC films lost mass upon aging (Fig. 1) mainly due to the diffusion of residual moisture from the film-forming procedure and/or to glycerol migration (Olabarrieta, Cho, et al., 2006). Aged SPC50 film showed the most significant weight difference ($P < 0.05$) compared with the un-aged counterpart. Since films were stored at constant relative humidity, this observation suggested that glycerol diffusion could be the main thing contributing to the mass variation due to its increased molecular mobility owing to its low molar mass (92 Da). This fact was also manifested by a stickier film surface and a reduction in film flexibility by the feeling of comparative hardness on handling as the storage time lengthened.

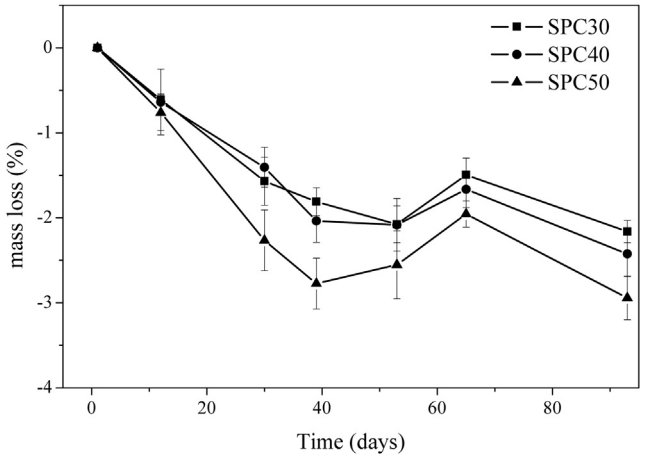

Fig. 1. Mass loss of SPC films during storage.

Un-aged samples exhibited slightly higher MC values with increasing glycerol content ($P < 0.05$). The reduction in moisture content experienced by all studied films (irrespective of glycerol content) with storage time (Table 1) may be related to the loss of the hydrophilic plasticizer as it has been previously discussed. Significant reduction in MC associated with plasticizer migration upon 16-weeks aging was described for gliadin films plasticized with glycerol (Hernández-Muñoz et al., 2004) and whey films plasticized with 50% (w/w) glycerol after 120 days of storage at 50% RH and 23 °C (Anker et al., 2001).

The total soluble matter (TSM) experienced a significant reduction after 90 days regardless of the glycerol content and in comparison with the un-aged counterparts ($P < 0.05$; Table 1). The time-induced variation in TSM is usually related with the leaching out of non-protein compounds remaining in the films after casting (i.e. plasticizer) and low molar mass polypeptides (Ullsten et al., 2009) and it can be taken as an indication of the intensity of protein–protein interactions in the film matrix. Since glycerol is fully soluble in water, the extractability of the plasticizer with time is highly probable. The lower TSM values quoted for aged SPC films (Table 1) suggested that structural changes during aging might restrict plasticizer migration (Olabarrieta, Cho, et al., 2006) and also gave a measure of the retained glycerol by the matrix at the end of the storage time (Hernández-Muñoz et al., 2004). According to this, one can hypothesize that glycerol molecules are distributed within SPC matrix in two phases; one of them which is weakly linked to the protein network (i.e. glycerol-rich domains) composed of those protein self-aggregates with relatively good compatibility to glycerol which are the most easily extracted. On the contrary, protein-rich domains consisted of glycerol molecules strongly retained by the protein matrix throughout associative interactions in different protein regions with distinct abilities to accept the glycerol

Table 1
Evolution of thickness, moisture content, total soluble matter and optical properties of SPC films with time.

	Time (days)	Gly (%)		
		30	40	50
Thickness (μm)	0	143 ± 22 a	153 ± 33 a	155 ± 19 a
	90	143 ± 24 a	154 ± 27 a	158 ± 27 a
MC (%)	0	17.7 ± 0.7 a	20.4 ± 0.7 a	23.6 ± 1.2 a
	90	17.9 ± 1.1 a	19.3 ± 0.9 b	22.8 ± 0.4 a
TSM (%)	0	52.8 ± 3.1 a	46.7 ± 3.2 a	45.3 ± 0.9 a
	90	32.1 ± 0.6 b	33.6 ± 2.3 b	40.0 ± 2.2 b
L^*	0	82.9 ± 1.4 a	82.5 ± 1.9 a	82.2 ± 1.6 a
	90	79.9 ± 1.9 b	79.9 ± 2.9 a	79.7 ± 1.0 a
a^*	0	0.8 ± 0.2 a	0.9 ± 0.3 a	0.9 ± 0.3 a
	90	0.9 ± 0.3 a	0.9 ± 0.5 a	0.8 ± 0.2 a
b^*	0	12.6 ± 2.6 a	12.7 ± 3.7 a	13.4 ± 2.8 a
	90	15.3 ± 2.8 a	14.4 ± 3.4 a	14.3 ± 1.9 a
ΔE	0	21.3 ± 2.9 a	21.5 ± 4.2 a	22.3 ± 3.2 a
	90	23.0 ± 3.3 a	22.2 ± 4.3 a	22.2 ± 2.1 a
Opacity (AU·nm)	0	955 ± 10 a	968 ± 24 a	1001 ± 16 a
	90	1049 ± 11 b	1032 ± 10 b	1075 ± 7 b

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

molecule (Chen & Zhang, 2005). Chen and Zhang (2005) demonstrated the presence of two T_g values at −28 °C to −64 °C and about 44 °C respectively, in SPI plasticized with glycerol in the range of plasticizer content between 25 and 50%, assigned to glycerol-rich and protein rich domains.

SPC protein fraction might undergo structural changes in the presence of glycerol (Subirade, Kelly, Guéguen, & Pézolet, 1998) therefore any variation in plasticizer content may affect functional properties of the resultant films. The loss of glycerol with storage time was experimentally evidenced by ATR-FTIR (Fig. 2) by comparing the spectra of SPC50 films before and after 90 days of aging. Both films exhibited peaks of relevance at 3500–3200, 1630, 1530, 1230 cm^{-1}, characteristic of free and bound OH and NH groups from SPC and Gly, amide I (C=O stretching), amide II (N–H bending), amide III (C–N and N–H stretching), respectively (Chen & Subirade, 2009). The peaks at 850 cm^{-1}, 900 cm^{-1}, 925 cm^{-1} (C–C vibrations), 1045 cm^{-1} (C–O stretching at C1 and C3), and 1117 cm^{-1} (C–O stretching at C2) were assigned to Gly. At the end of 90 days, ATR-FTIR spectra showed a quite similar general feature, differing mainly in the intensity of such bands (see inset in Fig. 2) confirming the loss of glycerol. Glycerol migration from aged protein – based films was observed in the past for gluten (Hernández-Muñoz et al., 2004), whey protein (Anker et al., 2001; Osés et al., 2009), wheat gluten films (Olabarrieta, Cho, et al., 2006) and also from sheets made of vital gluten (Ullsten et al., 2009), soybean plastics (Cunningham et al., 2000; Mo & Sun, 2003). From these findings it is anticipated that changes in glycerol concentration would play a key role in the evolution of films properties with storage time.

3.2. Evolution of protein secondary structure and stabilizing interactions in SPC films upon aging

The evolution of protein secondary structures upon aging was assessed by analyzing in depth the ATR-FTIR spectral region of amide I in Fig. 3. To improve the spectral resolution and identify the main components that build the amide I band in SPC films, the second derivative (Lacroix et al., 2002) was applied to the 1700–1600 cm^{-1} region of the spectra and an overlaid of the second derivative spectra is shown in Fig. 3. Amide I band is composed by the superposition of several absorption bands related to different secondary structures. Amide I band of un-aged films is composed by at least six visible constituents at 1693, 1683, 1651, 1643, 1626 and a shoulder at 1619 cm^{-1}. According to data reported for glycinin (Subirade et al., 1998) and beta conglycinin (Wang & Damodaran, 1991), the assignment of such bands can be summarized as follows: the two bands at 1626 and 1683 cm^{-1} are characteristics of amide groups involved in extended beta sheets, while that at 1651 is related to alpha-helix and 1643 cm^{-1} to random structures. The small peak at 1693 cm^{-1} can be assigned to beta–turns while that at 1619 cm^{-1} could be interpreted as intermolecular beta sheet associated with protein aggregation. These observations suggest that beta sheet structures are essential for network formation in soybean films (Subirade et al., 1998). It is important to note that this is an SPC sample therefore comprising of many proteins, although the predominant proteins are β-conglycinin (7S) and glycinin (11S); the FTIR spectrum recorded is therefore the average absorbance of the protein concentrate.

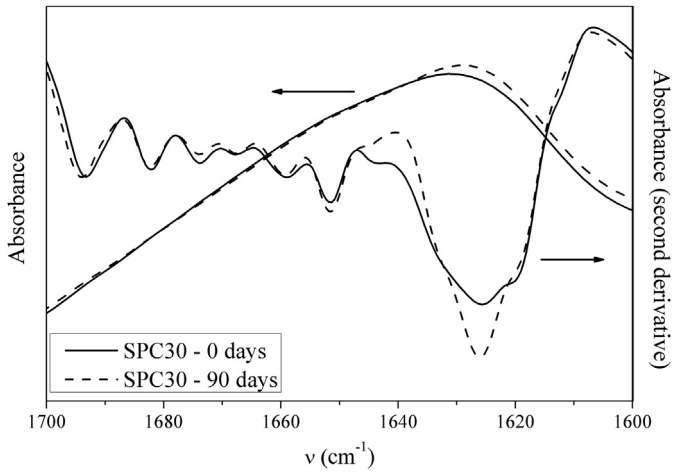

Fig. 3. ATR-FTIR spectra: Amide I band (1700–1600 cm^{-1}) of SPC-30Gly films and its second derivative at 0 and 90 days of storage.

Aging seemed to cause conformational rearrangements in the protein fraction of SPC. The main spectral difference observed was the significant increment in the intensity and reduction in amplitude of the peak at 1626 cm^{-1}, at expenses of other structures such as alpha-helix and random structures. This result suggests that aging promoted the reorganization of the secondary structure of protein fraction in SPC into others with prevalence of extended beta-sheet conformation. This result suggests that glycerol migration leads to an increase in beta structures. Glycerol as most of the alcohols used as protein plasticizers are reported as helicogenic agents even in the case of proteins known to have non-helical native structures (Subirade et al., 1998). Therefore the loss of glycerol could be the main factor responsible for the conversion of other secondary structures to beta-sheets. The lower amplitude of such band could be ascribed to a weakening in H-bonds between peptide chains.

Reshuffle of intra-molecular disulfide bridges into intermolecular ones through thiol-disulfide exchange, aggregation and evolution of hydrogen, hydrophobic and electrostatic interactions during storage were verified for other globular protein-based films (Hernández-Muñoz et al., 2004; Olabarrieta, Cho, et al., 2006; Ullsten et al., 2009) but not data were found for soybean protein films. Reportedly all these processes might take place even under temperature and relative humidity conditions where the molecular mobility is reduced due to protein aggregation (Morel et al., 2000; Olabarrieta, Cho, et al., 2006; Ullsten et al., 2009). The development of a three dimensional network during film formation depend on the establishment, distribution and extent of inter- and intra-

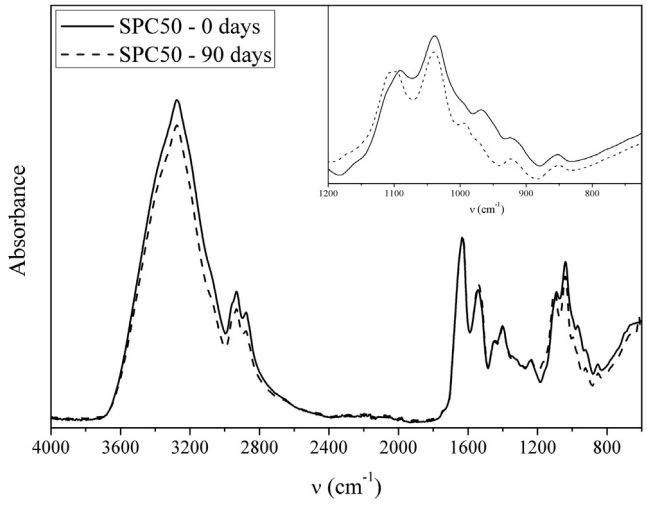

Fig. 2. ATR-FTIR spectra of the SPC-50Gly films at 0 and 90 days of storage. Inset: Region 1200–800 cm^{-1}, peaks assignable to Gly.

molecular stabilizing interactions, such as ionic interaction, hydrogen bond, hydrophobic interaction and disulfide bonds (Jiang et al., 2012). The major associative forces involved in SPC film formation and their evolution with time was analyzed by solubility assays in 5 denaturing solutions and representative results for SPC30 are shown in Fig. 4. For un-aged SPC30 films the lowest protein solubility experienced in S1 solution (around 340 $\mu g \cdot g^{-1}$) indicated that non-electrostatic interactions most likely stabilized the film network (Ciannamea et al., 2014; Guckian et al., 2006). When SPC30 film was exposed to S2 solution, the solubility increased in around 13% with respect to that in S1, evidencing a significant contribution of hydrophobic interactions in the network stabilization (Mauri & Añón, 2006; Utsumi, Damodaran, & Kinsella, 1984). When S3 was used as solubilizing agent, the increase in solubility was noticeable (i.e. 602 μg/g), giving experimental proof of the involvement of hydrogen bonds as the main associative forces for the formation of SPC films by casting. The addition of 2-mercaptoethanol (S5) induced not statistically significant variations in solubility ($p > 0.05$), signifying a negligible contribution of disulfide (covalent) stabilizing bonds (Ciannamea et al., 2014). Utsumi et al. (1984) have proposed that SH/S–S exchange participate in soybean gelation depending on the treatment temperature. The 11S fraction has higher SH concentration and denaturation temperature (i.e. $T > 80$ °C) than 7S fraction, therefore at the temperature used in this work (c.a.70 °C) the major contribution to S–S become from 7S fraction, therefore a scarce contribution to the gel is anticipated. Utsami et al. (1984) suggested that SH/S–S exchange was involved in the elasticity of the films but not necessary in gel formation. From these results, it can be inferred that hydrogen bonds and hydrophobic interactions are the main forces involved in the formation of SPC films obtained by casting in accordance to results (Ciannamea et al., 2014; Guckian et al., 2006; Jiang et al., 2012; Mauri & Añón, 2006). The low contribution of intermolecular covalent bonds between the polypeptide chains in un-aged SPC films was responsible for the films' slightly lower water resistance in terms of MC and TSM. Differences with reported data for other proteins further demonstrated the differences in behavior of proteins according to their nature and probably the length and hydrophobicity of their chains, compatibility with different plasticizers and the amount of plasticizer tolerated by the protein matrix and the processing method (Orliac et al., 2003).

Solubility results revealed a smaller contribution of non-covalent interactions accompanied by the concomitant increment of covalent S–S bonds when comparing with un-aged counterpart after 90 days (Fig. 4). The solubility in S4 was meaningfully reduced ($p < 0.05$) being 481 μg/g at the 90th day of aging therefore it became more difficult to dissolve SPC films by attempting to break secondary bonds. On the contrary, the solubility in S5 experienced an increment of about 13% evidencing the development of intermolecular disulfide bonds during aging. Solubility results agree well with those reported for aged wheat gluten films (Olabarrieta, Cho, et al., 2006; Ullsten et al., 2009).

Protein aggregation through Maillard reaction could also be possible in SPC films (Leerahawong, Tanaka, Okazaki, & Osako, 2012). Such reaction begins with a carbonylamine formation when amino side-chain groups from protein chain condensed reversibly with carbonyl groups from non-reducing sugars followed by water elimination giving rise to Schiff's base (Singh, Gamlath, & Wakeling, 2007). Casting of SPC films was performed in alkaline film – forming solutions (pH ~ 10, higher than pI of lysine residues) guaranteeing the nucleophilic character of amino-side chain groups on soybean chains. The likely contribution of Maillard aggregates was explored by determining the free amino groups before and after 90 days of storage (Sun, Lin, Weng, & Chen, 2006). The reduction in the absorption band at 570 nm associated with the formation of the Ruhemann's complex (Table 2) could be an indication of the involvement of amino groups in Maillard complexes. In addition, and as it will be discussed later, aged films exhibited increased yellowness, which can be more likely attributable to the existence of Schiff's bases from Maillard reactions. Maillard aggregates were previously observed for myofibrilar proteins plasticized with sucrose (Cuq et al., 1996), whey protein films (Trezza & Krochta, 2000), and fish gelatin films modified with glycerol (Leerahawong et al., 2012).

From these findings is worth noting aging induces conformational changes in protein fraction of SPC mostly to extended β-sheets. Covalent structures including disulfide bridges (Ullsten et al., 2009) and Maillard complexes also contribute suggesting that carbohydrate fraction in SPC also evolved with time. In line with our results are those reported by others confirming the reorganization of globular protein conformations with time (Lefèvre, Subirade, & Pézolet, 2005; Olabarrieta, Cho, et al., 2006; Ullsten et al., 2009).

3.3. Evolution of the visual appearance, color parameters and light barrier properties

Changes in the film thickness may be indicative of variations in network structure and is hence presented at an early stage. However, no substantial variation ($P > 0.05$) in such parameter was

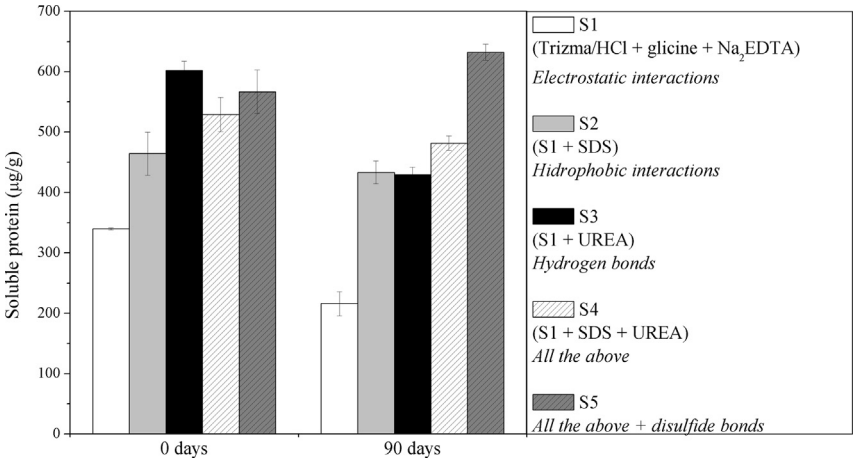

Fig. 4. SPC-30 film solubility in different denaturing solutions during aging: S1: 0.086 M TRIZMA/HCl, 0.09 M glycine and 4 mM Na_2EDTA; S2: S1 + 5 g ml^{-1} SDS; S3: S1 + 8 M urea; S4: S1 + 5 mg ml^{-1} SDS and 8 M urea; S5: S1 + 5 g ml^{-1} SDS, 8 M urea and 25 mg ml^{-1} 2-ME.

Table 2
Evolution of free amino groups with time determined by UV–visible spectroscopy by using ninhydrin.

	Gly (%)	Reduction in the absorption band at 570 nm after 90 days
Casting	30	−12.2
	40	−28.7
	50	−28.3

evidenced after 90 days of storage (Table 2). According to Anker et al. (2001) who reported comparable behavior for whey protein films plasticized with glycerol and sorbitol, the loss of plasticizer appeared to be too small to affect thickness (Anker et al., 2001). Olabarrieta, Cho, et al. (2006) and Olabarrieta, Gällstedt, et al. (2006) observed the opposite effect with films based on glycerol-plasticized wheat gluten (Olabarrieta, Cho, et al., 2006). Over 120 days films suffered substantial variations in thickness accompanied by increased roughness and heterogeneity. This further demonstrated the variations in behavior of films based on protein of different nature (Orliac et al., 2003).

A visual inspection of aged films revealed minor changes in color but opacity increased progressively over 90 days. To better understand the evolution of the optical properties of SPC films, Hunter Lab color values (L^*, a^*, b^*), total color difference (ΔE), and opacity values were analyzed and values are summarized in Table 2. As a general trend, opacity increased with time, i.e. for SPC30 values varied from 955 ± 10 AU·nm up to 1049 ± 11 AU·nm. Plasticizers are reported to enhance transparency in the visible region by interfering with protein–protein interactions (Sothornvit & Krochta, 2001). Therefore the reduction in transparency was correlated with the loss of glycerol and moisture during storage. Jongjareonrak et al. (2008) found a slight degree of opalescence with time in fish gelatin films.

Un-aged films exhibited initial values for lightness ($L^* = 82–83$), yellowness ($b^* = 12.5–13.5$) and greenness ($a^* = 0.8–0.9$) characterized of low color packaging films. Storage provoked a slight reduction in lightness in accordance with opacity measurements as well as a small increment in b^* value, although it was not statistically significant ($P > 0.05$). The slight raise in yellowness could be interpreted as a concentration effect, due to the inherent light yellow color of raw SPC intensified by the plasticizer loss during storage. Yellow–brown coloration is also characteristic of Maillard products (Martucci & Ruseckaite, 2009) therefore increased b^* values could be an indirect experimental evidence of the presence of Maillard (non-enzymatic browning) aggregates upon aging. Browning was observed in myofibillar protein films plasticized with sucrose and highly intense color was ascribed to non-enzymatic browning (Cuq et al., 1996). Increments in b^* parameter with time was reported for whey concentrate (Trezza & Krochta, 2000) and fish skin gelatin films (Leerahawong et al., 2012).

3.4. Evolution of tensile properties with time

Mechanical strength and extensibility are generally required for a packaging film to maintain its integrity and withstand external stress. Tensile properties of un-aged and aged films are reported in Table 3. The addition of glycerol in newly prepared SPC films reduced significantly TS and E values while increased ε_B (Table 3) because plasticizer disrupt protein chain-to-chain interactions that keep the soybean protein molecules together to maintain film integrity (Ciannamea et al., 2014). Fig. 5 displays representative stress–strain curves for SPC30 films after selected aging times.

Table 3
Evolution of mechanical and barrier properties of SPC films with time.

	Time (days)	Gly (%)		
		30	40	50
TS (MPa)	0	2.3 ± 0.2 a	2.1 ± 0.4 a	1.7 ± 0.3 a
	90	2.8 ± 0.6 a	2.7 ± 0.3 b	2.3 ± 0.2 b
ε (%)	0	15 ± 2 a	16 ± 3 a	18 ± 2 a
	90	18 ± 4 b	23 ± 4 b	19 ± 7 a
E (MPa)	0	48 ± 7 a	39 ± 7 a	33 ± 9 a
	90	55 ± 14 a	45 ± 8 a	31 ± 7 a
WVP·10^{13} (kg·m/m^2·s·Pa)	0	3.1 ± 0.3 a	4.8 ± 0.8 a	6.3 ± 1.0 a
	90	3.6 ± 0.5 a	5.4 ± 0.8 a	5.5 ± 0.4 a
OPC (cm^3·μm/(m^2·day·kPa))	0	8.5 ± 0.8	18.5 ± 2.4	–
	90	–	–	–

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

Stiffness and strength experienced a monotonically time-induced increment (Anker et al., 2001; Cunningham et al., 2000; Gällstedt et al., 2004; Leerahawong et al., 2012; Mo & Sun, 2003; Orliac et al., 2003; Ullsten et al., 2009) accompanied by a slight increase in extensibility. TS and E values did not vary significantly ($P > 0.05$) during the first two months of storage but values changed considerably at 90th day (Table 3). Young modulus and TS values clearly increased ($P < 0.05$) over three months of storage, i.e. for SPC30 22% and 15%, respectively. These results correlated well with the reduction in MC and the loss of glycerol previously discussed, since plasticizers are usually applied to overcome the brittleness of films resulting from high intermolecular forces, by increasing the mobility of polymer chains, making them stretchable and flexible (Sothornvit & Krochta, 2001). Hernandez-Muñoz et al. (2004) reported a 1.7-fold increase in strength in glutenin films prepared at pH 5 and aged for 112 day. Olabarrieta, Cho, et al. (2006) and Olabarrieta, Gällstedt, et al. (2006) observed 32-fold of increment in TS values of wheat gluten film obtained at pH 4 whereas those produced at pH 11 increased 2-fold accompanied by reductions in elongation. Anker et al. (2001) reported an increment in TS values of whey protein films plasticized with 50% Gly, from 2.8 to 8.4 MPa, while elongation at break reduced from 33% to 8%, over 45 days of aging (Anker et al., 2001). The evolution in tensile properties upon aging can be related to several processes taking place during storage. Apart from glycerol migration and structural reorganization, chemical aging might take place, as verified by solubility assays. The increased chemical cross-linking through intermolecular

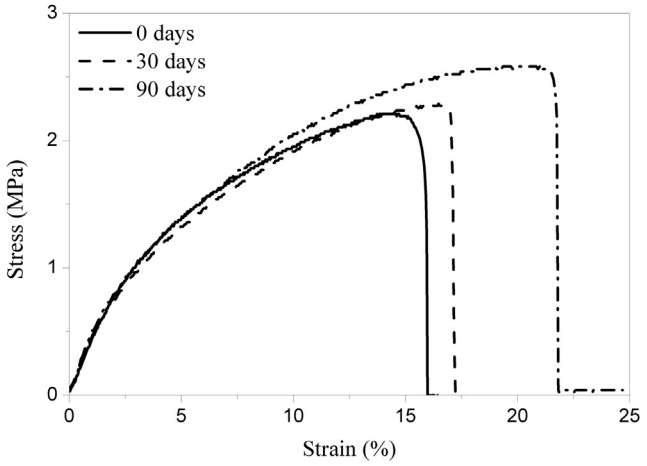

Fig. 5. Evolution of stress–strain curves of SPC-30Gly films with time.

disulfide bridges and the formation of Maillard conjugates could contribute to the increased TS and *E* values. Leerahawong et al. (2012) assigned the increment in TS in gelatin films plasticized with glycerol upon aging with cross-linking and aggregation through Maillard reactions.

As already mentioned, the elongation at break values increased upon storage, being the differences with un-aged films significant ($P < 0.05$), i.e. for SPC30 ε_B increased 20% (Table 3). The overall final result is more fragile but more extensible materials. This effect appears contrary to that is normally observed for other protein-based films exposed to aging (Cunningham et al., 2000; Leerahawong et al., 2012; Olabarrieta, Cho, et al., 2006; Ullsten et al., 2009) and cannot be assigned to moisture content since such parameter reduced with time (Table 2). A plausible explanation for this behavior can be proposed at the light of ATR-FITR results (Fig. 3). Aged films evidenced an increment in the intensity of the absorption band associated with β-sheets structures due to glycerol migration, which are reported to contribute to film flexibility (Baimark, Srihanam, & Srisuwan, 2009; Bergo & Sobral, 2007).

3.5. Evolution of barrier properties with time

Packaging films should provide extended protection against water vapor and oxygen in order to ensure the freshness, hygiene and safety of the packed food. WVP values of un-aged films increased with glycerol content ($P < 0.05$, Fig. 6) (Ciannamea et al., 2014). Hydrophilic plasticizers such glycerol and sorbitol are known to enhance the water vapor permeability of hydrocolloid based films. In addition high plasticizer concentrations significantly modify the molecular organization of the protein network and increase the free volume resulting in less dense network that results in films that are more permeable to water. It is recognized that an increase in plasticizer is directly proportional to an increase in water vapor permeability. The higher WVP of SPC films, compared to those based on SPI, could be explained by the high carbohydrate content in the SPC (about 15%), which confers higher hygroscopic character to the resultant films (McHugh, Avena-Bustillos, & Krochta, 1993). Plasticizers modify the molecular organization of the protein network and increase the free volume resulting in less dense network that results in films more permeable to water. For the same reason the OPC values of films containing high Gly level were significantly higher ($P < 0.05$; Table 3) (Ciannamea et al., 2014).

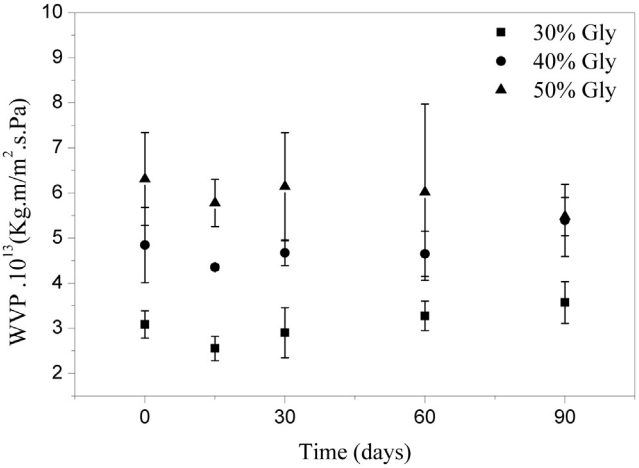

Fig. 6. Evolution of water vapor permeability of SPC-Gly films during storage.

A reduction in WVP was observed within the first 2 weeks of storage although differences were not statistically significant ($P > 0.05$, Fig. 6). Such reduction could be a function of the loss of glycerol and moisture with time (Olabarrieta, Cho, et al., 2006). Afterwards, WVP values increased up to attain higher values than those of 30% and 40% glycerol-plasticized un-aged SPC films at the 90th day. As an example, WVP values in SPC30 films ranged between 3.1 ± 0.3 and 3.6 ± 0.5 $.10^{13}$ kg·m/m^2·s·Pa considering both initial and final time. The increased WVP suffered by SPC50 could be a result of higher porous and small crack concentration created due to glycerol migration as revealed by SEM in a previous work (Ciannamea et al., 2014). Hoque, Benjakul, and Prodpran (2011) suggested that reduction in MC and plasticizer, together with an increased cross-linked degree, possibly induced a non-homogeneous contraction of film structure, producing micro-cracks which could participate in creating more permeable structures (Hoque et al., 2011). Our results are in contrast to those obtained by Anker et al. (2001) who reported no variations in WVP of whey protein films and Leerahawong et al. (2012) who showed almost constant WVP values of gelatin-glycerol films with time. Accordingly authors suggested that WVP is governed by film porosity which remained invariable during storage since no alteration in thickness or micro-structures was evidenced (Leerahawong et al., 2012).

It is expected that if films loss plasticizing molecules during aging the oxygen permeability would decrease (Olabarrieta, Cho, et al., 2006). Oxygen causes undesirable sensorial and nutritional changes which reduce quality and shelf life of the packed food (Janjarasskul & Krochta, 2010). OPC was determined and results for fresh SPC films are reported in Table 3. OPC values of fresh SPC films increased with glycerol content ($P < 0.05$). Glycerol might easily insert between protein chains increasing free volume facilitating oxygen transport through the films, and consequently incrementing their permeability. In addition, the higher the glycerol content the higher the MC of films (Table 1). It is well reported in the literature that increased MC increased molecular mobility favoring oxygen diffusion (Sothornvit & Krochta, 2000). OPC values of SPC films were comparable to the previously reported for SPI and other alternative protein films (Gällstedt et al., 2004; Park & Chinnan, 1995). Contrary to WVP determinations, OPC could not be measured after 90 days of storage since aged SPC films resulted too fragile and easily broken upon oxygen pressure application. The creation of cracks and pits (Ciannamea et al., 2014) gave rise to unusually high OPC values with large dispersions only attributable to structural defects.

4. Conclusions

The continuous assessment of the evolution of functional properties induced by aging provides valuable practical information about the integrity and potential protective efficiency of SPC-based films containing glycerol and obtained by casting as packaging materials. Results revealed that glycerol-plasticized SPC films were stable at least during 2 weeks of storage at 65% RH and 25°± 2 °C, preserving enough resistance to act as mechanical barrier, chain mobility to be flexible and adequate moisture barrier for the intended application without significant plasticizer migration. Nonetheless the continuous changes in the molecular structure and film properties suggested that aging of SPC-Gly films did not reach the equilibrium even 90-days storage period under specific conditions. Further work is currently in progress to carry out a global migration study of the plasticizer to food simulants to facilitate a rational design and safe application of such new packaging materials.

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