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Effect of whey protein purity and glycerol content upon physical properties of edible films manufactured therefrom

Óscar L. Ramos [a,b], Isabel Reinas [a], Sara I. Silva [a], João C. Fernandes [a], Miguel A. Cerqueira [c], Ricardo N. Pereira [c], António A. Vicente [c], M. Fátima Poças [a], Manuela E. Pintado [a], F. Xavier Malcata [b,d,*]

[a] CBQF/Escola Superior de Biotecnologia, Rua Dr. António Bernardino de Almeida, P-4200-072 Porto, Portugal
[b] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Avenida da República, P-2780-157 Oeiras, Portugal
[c] IBB – Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, P-4710-057 Braga, Portugal
[d] Department of Chemical Engineering, University of Porto, Rua Dr. Roberto Frias, P-4200-465 Porto, Portugal

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ABSTRACT

This manuscript describes the detailed characterization of edible films made from two different protein products – whey protein isolate (WPI) and whey protein concentrate (WPC), added with three levels of glycerol (Gly) – i.e. 40, 50 and 60%(w/w). The molecular structure, as well as barrier, tensile, thermal, surface and optical properties of said films were determined, in attempts to provide a better understanding of the effects of proteinaceous purity and Gly content of the feedstock. WPI films exhibited statistically lower ($p < 0.05$) moisture content (MC), film solubility (S), water activity, water vapor permeability (WVP), oxygen and carbon dioxide permeabilities (O_2P and CO_2P, respectively) and color change values, as well as statistically higher ($p < 0.05$) density, surface hydrophobicity, mechanical resistance, elasticity, extensibility and transparency values than their WPC counterparts, for the same content of Gly. These results are consistent with data from thermal and FTIR analyses. Furthermore, a significant increase ($p < 0.05$) was observed in MC, S, WVP, O_2P, CO_2P, weight loss and extensibility of both protein films when the Gly content increased; whereas a significant decrease ($p < 0.05$) was observed in thermal features, as well as in mechanical resistance and elasticity – thus leading to weaker films. Therefore, fundamental elucidation was provided on the features of WPI and WPC germane to food packaging – along with suggestions to improve the most critical ones, i.e. extensibility and WVP.

1. Introduction

Biopolymer-based films have been a subject of a rising interest in recent years, because of general concerns about limited natural resources as feedstock for, and environmental impacts caused by nonbiodegradable plastic-based packaging materials. A variety of polymers from renewable sources – e.g. polysaccharides, proteins, lipids and their composites, derived from plant and animal feedstocks, have thus been investigated toward development of edible/biodegradable, nontoxic packaging materials that might replace synthetic polymers (Kester & Fennema, 1986; Krochta & de Mulder-Johnston, 1997).

In particular, various whey protein products have been developed in recent decades – including whey protein concentrates (WPC) produced by ultrafiltration (UF), with protein contents ranging in 35–80%(w/w) on a dry basis, as well as whey protein isolates (WPI) produced by ion-exchange and subsequent UF, with protein contents above 90%(w/w) (Mulvihill & Ennis, 2003). Besides their distinct protein contents, WPI and WPC differ in the levels of such other constituents as lipids, minerals and lactose. These differences may influence markedly the intermolecular bonds in films manufactured therefrom – and consequently their barrier, mechanical and thermal properties, as a result of distinct molecular structures (Khwaldia, Perez, Banon, Desobry, & Hardy, 2004).

Use of whey protein to manufacture films has indeed received a great deal of attention – since they are edible and biodegradable, allow upgrade of a cheesemaking effluent, and possess interesting mechanical properties. Detailed reviews are already available on this subject (Gennadios, 2004; Khwaldia et al., 2004; Ramos, Fernandes, Silva, Pintado, & Malcata, 2012); the film-forming properties of whey proteins have accordingly been applied to manufacture transparent, flexible, colorless and odorless films

* Corresponding author. Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Avenida da República, P-2780-157 Oeiras, Portugal. Tel.: +351 96 8017 411; fax: +351 22 982 53 31.
E-mail address: fxmalcata@itqb.unl.pt (F.X. Malcata).

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(Fairley, Monahan, German, & Krochta, 1996a,b). With regard to manufacture itself, films based on whey proteins are usually obtained by casting and drying aqueous WPI and WPC; they have shown a moderate potential as moisture barriers (McHugh, Aujard, & Krochta, 1994), but a good potential as oxygen barriers (Maté & Krochta, 1996; McHugh & Krochta, 1994a).

On the other hand, formulation of protein-based films requires incorporation of plasticizer above a minimum threshold – to reduce their brittleness, allow easier removal from the forming support and confer plastic properties (Hernandez-Izquierdo & Krochta, 2008). The plasticizer molecules lead to decreases in intermolecular forces along the polymer chains, thus improving flexibility, extensibility, toughness and tear resistance of the film; however, they also decrease its mechanical resistance and barrier properties (Karbowiak et al., 2006). The most common plasticizers are polyols (e.g. glycerol, sorbitol and polyethylene glycol 400), mono-, di- or oligosaccharides, and lipids and derivatives thereof (Guilbert, 1986). Among these, glycerol (Gly) produces the best effects in whey protein films, thus leading to more stable, flexible and less brittle films under various relative humidities (RH) (Osés, Fernández-Pan, Mendoza, & Mate, 2009). Hence, Gly was chosen as plasticizer for this study.

There is large information available on the barrier and mechanical properties of whey protein-based films (Bodnár, Alting, & Verschueren, 2007; Fairley et al., 1996a,b; Krochta & de Mulder-Johnston, 1997; McHugh & Krochta, 1994a), but little information exists on the molecular structure of those films; hence, this work contributed to elucidate the relationships between barrier, tensile, surface and thermal features by providing data on said molecular structure, especially within wide ranges of Gly content. The aforementioned fundamental relationships are crucial to optimize film composition in a rational manner (in terms of protein feedstock and Gly level) toward pre-specified physical properties. Furthermore, one may therewith gain access to accurate prediction and successful manipulation of the physical properties of those films, using easily accessible formulation parameters.

In view of the above, one goal of this work was to provide an array of data to support comparative characterization of films obtained from WPI and WPC, at various levels of addition of Gly (i.e. 40, 50 and 60%, w/w). Moreover, moisture, solubility, density, water activity, molecular structure and surface hydrophobicity, as well as thermal, barrier, tensile and optical properties of these films were studied in attempts to shed light on the relationships holding among these properties and the nature of the proteinaceous feedstock and the plasticizer content utilized; this is a *sine qua non* for rational improvement of such films, toward their eventual application as edible packaging.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) was obtained from Armor Proteines (Saint Brice en Coglés, France), whereas whey protein concentrate (WPC) was obtained from Myprotein (Cheadle, UK); both had been characterized previously (Ramos, 2011), and their composition is depicted in Table 1. Ultrapure water (with a resistivity of 18.2 MΩ cm) was obtained with a Milli-Q Ultrapure water purification system (Millipore, Bedford MA, USA). Glycerol (Gly, 99% purity) was supplied by Panreac (Barcelona, Spain). All other chemicals were reagent-grade or better, and were used without further purification.

2.2. Film preparation

Film-forming solutions were prepared by slowly dissolving 10%(w/w) WPI and WPC powder in deionized water, following the procedure reported by Perez-Gago and Krochta (2002). Gly was added, at three different levels, to plasticize the films: 40, 50 and 60%(w/w), on a protein basis, and the resulting solutions were magnetically stirred for ca. 2 h. Subsequently, the solutions were heated in a water bath at 80 °C for 20 min, under stirring; this step is essential to formation of intermolecular bonds, which will in turn assist in establishment of a crosslinked polymeric network structure. Such a process is necessary to obtain a flexible film that is able to retain its structural integrity under high moisture environments (le Tien et al., 2000). The solutions were cooled for 1.5 h to room temperature, and then vacuum was applied for 30 min to remove any air incorporated during stirring (Seydim & Sarikus, 2006). The solution pH was adjusted to 7.0, using 0.1 M NaOH.

The solutions obtained were poured onto Teflon-coated plates (38 × 34 cm); to control film thickness, the amount of each film-forming solution poured was the same (300 mL). The spread solutions were allowed to dry at room conditions (ca. at 23 °C and 50% relative humidity, RH) for 24 h, following Gounga, Xu, and Wang (2007) and Osés et al. (2009). Once formed, the films were peeled off and conditioned at 23 ± 2 °C and 50 ± 2% RH, in a controlled temperature and humidity storage room, for at least 72 h prior to testing (ASTM, 2000).

Right before testing, the film thickness was measured using a micrometer Model m120 (from Adamel Lhomargy, Roissy en Brie, France), to the nearest 0.001 mm; the thickness was calculated as the average of five measurements, taken at different locations on each film sample.

2.3. Film characterization

2.3.1. Moisture content and solubility

The moisture content (MC) of the protein films was determined after drying in an oven at 105 °C, under forced air circulation for 24 h. Small specimens (0.200 g) of films were cut after adequate conditioning, and placed on Petri dishes – which were weighed before and after oven drying. MC values were determined as a fraction of initial film weight lost (ASTM, 1994) during drying, and reported on a wet basis.

The film solubility in water (S) was determined according to Gounga et al. (2007). The determinations of MC and S were performed in triplicate.

Table 1
Values (average ± standard deviation) of chemical composition of commercial whey protein isolate (WPI) and whey protein concentrate (WPC) powders.

Whey protein product	Composition							
	Protein (%, w/w)	Lactose (%, w/w)	Lipids (%, w/w)	Ash (%, w/w)	Moisture (%, w/w)	Calcium (mg 100 g^{-1})	Sodium (mg 100 g^{-1})	Potassium (mg 100 g^{-1})
WPI	92.0 ± 1.0	1.0 ± 0.1	1.0 ± 0.1	2.0 ± 0.1	3.0 ± 0.1	389.1 ± 12.2	100.1 ± 7.3	31.1 ± 1.3
WPC	82.0 ± 2.0	6.0 ± 0.4	1.6 ± 0.1	4.4 ± 0.1	3.3 ± 0.2	200.0 ± 11.0	400.1 ± 17.9	50.2 ± 2.4

2.3.2. Density

The film density (ρ^s) was calculated directly from the film weight and dimensions (Salgado, Ortiz, Petruccelli, & Mauri, 2010), according to:

$$\rho^s = m/A \times \delta \quad (1)$$

where A is the film area (12.6 cm^2 in our case), δ the thickness (cm), m the dry mass (g) and ρ^s the dry matter density (g cm^{-3}). The film density was expressed as the average of five independent determinations.

2.3.3. Water activity

The water activity (a_w) of preconditioned films was measured using a HygroLab 2 (from Rotronic, Bassersdrof, Germany). Pieces of films (ca. 0.5 g) were placed on the sample holder of the a_w device; a sealed system was formed by placing the a_w probe on top of the sample holder. The probe was equipped with a small fan to help circulate air inside the sample container, a thin film capacitance sensor able to measure RH from 0 to 100 ± 1.5%, and a platinum resistance temperature detector with a precision of ±0.3 °C. When a_w became constant (which usually took less than 1 h), its value was recorded. Calibration resorted to six saturated solutions of known a_w (viz. LiCl = 0.114, $MgCl_2$ = 0.329, K_2CO_3 = 0.443, $Mg(NO_3)_2$ = 0.536, NaBr = 0.653 and KCl = 0.821). These measurements were carried out in quadruplicate.

2.3.4. Surface hydrophobicity

The sessile drop method, based on the optical contact angle, was used to estimate the surface hydrophobicity of the films. The contact angle (θ) was determined with a face contact angle meter OCA 20 (from Dataphysics, Filderstadt, Germany), according to Kwok and Newman (1999): a 2 μL-droplet of ultrapure water was deposited on the film surface with a 500 μL precision syringe (Hamilton, Bonaduz, Switzerland), using a needle with a diameter of 0.75 mm. The image of the drop, taken by 5 s, was recorded with a video camera, and its profile was numerically solved and fitted to Laplace–Young equation. Ten replicated measurements of θ (upper and lower surfaces of the film) were obtained.

2.3.5. Differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements were performed with a Shimadzu DSC-50 calorimeter (from Shimadzu Corporation, Kyoto, Japan), equipped with STARe 6.1 Thermal Analysis System software. The instrument was calibrated with an indium standard, characterized by a T_m of 156.6 °C and a ΔH_m of 28.71 J g^{-1} (TA Instruments, New Castle DE, USA). Each sample was heated at a rate of 10 °C min^{-1}, from −150 °C (assured with liquid nitrogen) to 250 °C, under an inert atmosphere (100 ml min^{-1} of N_2).

The glass transition temperature (T_g) was recorded as the inflexion point of the baseline, caused by the discontinuity in the specific heat of the sample (Ghanbarzadeh & Oromiehi, 2008). The temperature of melting (T_m), observed as an endothermic peak, and the associated enthalpy (ΔH_m) were determined (and expressed as J g^{-1} protein) as reported by Ryan et al. (2008). These experiments were performed at least in duplicate, using punctured aluminum DSC pans (Al crimp Pan C.201-52090) containing 10 mg of dry sample. The samples were weighed with an automatic electrobalance AE 200 (from Mettler, Columbus OH, USA), with a precision of ±0.01 mg. An empty pan was used as reference.

2.3.6. Thermogravimetry

Thermogravimetric analyses (TGA) were performed with a TGA-50 apparatus (from Shimadzu Kyoto, Japan). Samples were placed in the balance system, and heated from 30 to 575 °C at 10 °C min^{-1}, under a nitrogen atmosphere. The samples were pre-weighed (10 mg) in aluminum pans (Al crimp Pan C.201-52943), using an empty pan as reference. The initial decomposition temperature (T_{di}), the derivate maximum decomposing rate temperature (DTG_{max}), and the corresponding weight losses – as well as the residual mass were all determined. The measurements were performed at least in duplicate.

2.3.7. FTIR–ATR analysis

The spectra of the films were determined using Fourier transform infrared spectrometry (FTIR) with a Perkin–Elmer 16 PC spectrometer (Boston MA, USA), under attenuated total reflectance (ATR) mode. The spectra were recorded in absorbance mode from 650 to 4000 cm^{-1}, using 16 scans at 4 cm^{-1} resolution. Three replicates were collected for each film surface sample. The spectra were input to a data analysis package (Barros, 1999) – and three spectral regions were preferentially selected (i.e. 800–1150, 1600–1700 and 3000–3600 cm^{-1}) owing to their relevance; for instance, to ascertain the protein secondary structure contents, spectra were curve-fitted in the 1600–1700 cm^{-1} region (amide I), using apporpriate Gaussian and Lorentzian functions.

For each region analyzed, a linear baseline was subtracted – and the absorbance was normalized to the peak maximum, so as to avoid undesirable intensity variations (Lefèvre, Subirade, & Pézolet, 2005); initial values of the peak positions were then determined by Fourier deconvolution. The parameters of Fourier deconvolution were chosen after several trials, so as to produce reasonable fits – and to obtain enough bands, thus narrowing the major components of the amide I band (Mangavel, Barbot, Popineau, & Gueguen, 2001). All data were treated with Peakfit software, v. 4.12 (from SYSTAT Software, Richmond CA, USA).

2.3.8. Water vapor permeability

The water vapor permeability (WVP) of films was gravimetrically assessed, according to the protocol B of ASTM (1995) – with the adaptations proposed by Debeaufort, Martin-Polo, and Voilley (1993) specifically for edible films. Circular aluminum cups, with a diameter of 8 cm and a depth of 5 cm, were accordingly used. Deionized water (30 mL) was placed in each test cup, to expose the lower film face to a high RH. The films samples were mounted with the upper surface facing the RH (50 ± 2%) of the environment-controlled room. The weight loss of the cups was monitored over a 72 h-period, with weights recorded at 4 h-intervals. WVP (expressed as g mm m^{-2} d^{-1} kPa^{-1}) of the film was calculated as follows:

$$WVP = (\Delta W \times FT)/(S \times \Delta p) \quad (2)$$

where ΔW is the weight loss of the cup per day (g d^{-1}) (i.e. the slope of the linear behavior), FT is the film thickness (mm), S is the area of exposed film (m^2) and Δp is the vapor pressure differential across the test film (kPa). At least 3 replicates were produced from each film type.

2.3.9. Oxygen and carbon dioxide permeability

Oxygen permeability (O_2P) and carbon dioxide permeability (CO_2P) were determined based on the reference method (ASTM, 2002a). A sample film was thus sealed between two chambers, each one with two channels – one for gas inlet and the other for gas outlet. In the lower chamber, O_2 (or CO_2) was supplied at a controlled flow rate, using an electronic flow meter ADM 2000 (from J & W Scientific, Folsom CA, USA) to keep the pressure constant inside that compartment. The other chamber was purged by a stream of nitrogen, also at a controlled flow rate; nitrogen acted as carrier for O_2 (or CO_2).

The values for O_2P and CO_2P (cm^3 mm m^{-2} d^{-1} kPa^{-1}) were determined by gas chromatography (Chrompack 9001, Middelburg, Netherlands), at 110 °C – with a molecular sieve 5 Å 80/100 mesh 1 m × 1/8″ × 2 mm column to separate O_2, and a Porapak Q 80/100 mesh 2 m × 1/8″ × 2 mm SS column to separate CO_2, using a thermal conductivity detector (TCD) at 110 °C. Helium at 23 mL min^{-1} was used as carrier gas. A standard mixture containing 10%(v/v) CO_2, 20%(v/v) O_2 and 70%(v/v) N_2 was used for calibration. Three replicates were obtained for each sample.

2.3.10. Tensile properties

The tensile properties of films – tensile strength (TS), elongation at break (EB) and Young's Modulus (YM), were measured according to the reference method (ASTM, 2002b), using a Universal Testing machine model 4501 (from Instron, Canton MA, USA), equipped with fixed grips (test method A). A 100 N-static load cell was used. The film samples were cut into strips (80 × 15 mm). The initial grip separation was set at 50 mm, and the crosshead speed at 4.8 mm min^{-1}. The TS, EB and YM values were determined using the Series IX Automated Materials Testing System software, v. 809.00 (Instron). At least ten strips of each film sample were analyzed.

2.3.11. Light transmission and film transparency

The ultraviolet (UV) and visible light barrier properties were measured on dried films at selected wavelengths (in the 200–800 nm range), using an UV–VIS Spectrophotometer (SPECORD S 600, from AnalytikJena, Jena, Germany). The film samples were cut into strips (4 × 1 cm) and attached to one side of a colorimetric cup – while the empty colorimetric cup was used as control. The relative transparency of films was measured at 600 nm, and calculated as (Han & Floros, 1997):

$$\text{Transparency} = A_{600}/X \tag{3}$$

where A_{600} is the absorbance at 600 nm and X the film thickness (mm). At least five strips of each film type were tested.

2.3.12. Color

The film color was evaluated using a portable Chroma meter CR-400 (from Minolta Chroma, Osaka, Japan). A CIELab color scale was employed to measure the degree of lightness (L), redness ($+a$) or greenness ($-a$), and yellowness ($+b$) or blueness ($-b$) of the films, under D_{65} (daylight). Film specimens were measured on the surface of the white standard plate, with color coordinates $L_{standard} = 97.6$, $a_{standard} = 0.01$ and $b_{standard} = 1.60$. The color of the films was expressed as the total difference in color (ΔE), calculated as

$$\Delta E = \left[\left(L_{film} - L_{standard}\right)^2 + \left(a_{film} - a_{standard}\right)^2 + \left(b_{film} - b_{standard}\right)^2\right]^{1/2} \tag{4}$$

For each condition, four samples were taken – and, on each film piece, four readings were made on each side.

2.4. Statistical analyses

Statistical analyses were performed using the Statistical Package for Social Sciences, v. 17.0 (SPSS, Chicago IL, USA), via one-way analysis of variance. The difference of means between pairs was resolved via confidence intervals, using Tukey's test. The significance level was set at $p < 0.05$.

3. Results and discussion

3.1. Film appearance

Both WPI- and WPC-based films were transparent, flexible and homogeneous. Their surfaces appeared smooth, without visible pores or cracks. These films did not undergo any change in appearance when different levels of plasticizer were used; however, WPC-based films exhibited a slightly yellowish color when compared with WPI ones.

Appearance of the two sides of the film was different for both WPI and WPC films. The film side facing the casting plate was indeed shiny, while the other was dull; this is likely an indication of some phase separation occurring in the solution during drying. Both types of film were easily separated from the casting plates, except for those containing 60%(w/w) Gly – which were rather sticky. Films manufactured from WPI with 10%(w/w) protein showed a thickness of 0.13 ± 0.04 mm, irrespective of Gly content – which is similar to those reported by Kokoszka, Debeaufort, Lenart, and Voilley (2010), Osés et al. (2009), and Simelane and Ustunol (2005) for the same protein concentration, i.e. 0.12 ± 0.08, 0.13 ± 0.01 and 0.14 ± 0.02 mm, respectively. WPC-based films exhibited a thickness of 0.17 ± 0.04 mm for the various Gly levels tested; this does not represent a significant increase ($p > 0.05$) relative to WPI ones. Furthermore, when the Gly level was increased in the film-forming solutions, the thickness values of both films (results not shown) did not exhibit any statistically significant differences either ($p > 0.05$).

3.2. Moisture content, solubility, density and water activity

The values obtained for the moisture content (MC), solubility (S), density (ρ^s) and water activity (a_w) for both whey protein products, as a function of the Gly level, are presented in Table 2.

WPI films exhibited significantly ($p < 0.05$) lower values of MC, S, and a_w, as well as significantly ($p < 0.05$) higher values of ρ^s than films manufactured from WPC. This observation may be rationalized by the differences in the film-forming product – especially the presence of higher contents of contaminants (i.e. lactose, lipids and minerals) in WPC (Table 1).

On the other hand, an increase in content of Gly from 40 to 60%(w/w) in WPI films produced a significant increase ($p < 0.05$) in MC – 3.60%, and in S – 6.41%; whereas no significant changes ($p > 0.05$) were observed for ρ^s and a_w. In the case of WPC films, a significant increase ($p < 0.05$) in MC – 3.80%, and in S – 5.90% was

Table 2
Values (average ± standard deviation) of moisture content (MC), solubility (S), density (ρ^s) and water activity (a_w) of whey protein isolate (WPI) and whey protein concentrate (WPC)-based edible films, with various glycerol (Gly) contents.

Protein film	Gly (%)	MC (%)	S (%)	ρ^s (g cm^{-3})	a_w
WPI	40	15.10 ± 0.14^a	63.91 ± 0.32^a	1.32 ± 0.00^a	0.46 ± 0.01^a
	50	16.82 ± 0.25^b	67.60 ± 0.44^b	1.35 ± 0.03^a	0.47 ± 0.01^a
	60	18.70 ± 0.49^c	70.32 ± 0.51^c	1.38 ± 0.08^a	0.48 ± 0.01^a
WPC	40	17.91 ± 0.32^d	78.32 ± 0.13^d	1.26 ± 0.05^b	0.51 ± 0.01^b
	50	19.62 ± 0.17^e	81.83 ± 0.22^e	1.29 ± 0.02^b	0.53 ± 0.02^b
	60	21.71 ± 0.31^f	84.22 ± 0.30^f	1.31 ± 0.00^b	0.53 ± 0.01^b

Note: $^{a, b, c, d, e, f}$ Means within the same column, labeled with the same letter, do not statistically differ from each other ($p > 0.05$).

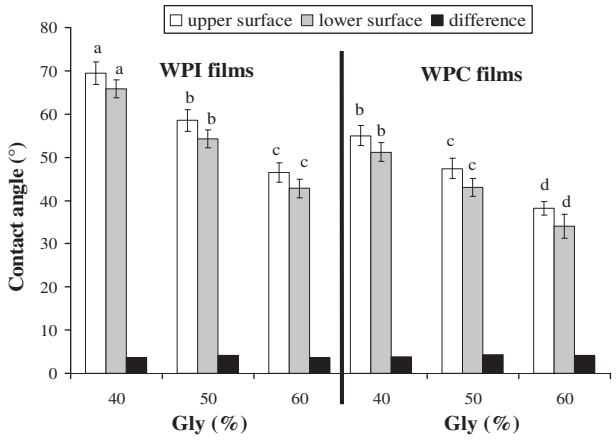

Fig. 1. Water contact angle values (average ± standard deviation, $n = 10$) for upper and lower surface of 10%(w/w) WPI- and WPC-edible films, with various glycerol (Gly) contents. Means labeled with the same letter do not statistically differ from each other ($p > 0.05$).

also observed, when Gly was increased from 40 to 60%(w/w), yet no significant changes ($p > 0.05$) were observed for ρ^s and a_w. The observed increase may be attributed to the hygroscopic nature of Gly – which attracts and holds water molecules, thus favoring wetting of the film surface and moisture absorption thereby (Kokoszka et al., 2010).

In the case of S, our results proved that WPI films kept their integrity after 24 h of immersion in water. The partial insolubility of these films may be attributed to establishment of stronger intermolecular bonds (e.g. disulfide bonds, as a result of the heat treatment) between protein molecules in the matrix of WPI films (McHugh, Avena-Bustillos, & Krochta, 1993; McHugh & Krochta, 1994b). This will likely account for the proteinaceous polymeric network of such films being highly stable, since only small molecules – e.g. small peptides, monomers and nonprotein material, are soluble (Yoshida & Antunes, 2004).

3.3. Surface hydrophobicity

Surface hydrophobicity of protein films was evaluated via measuring the contact angle of water (θ) upon the film surface by the sessile drop method. In general, films with higher θ values exhibit a higher surface hydrophobicity (Tang & Jiang, 2007); quantitative differentiation between "hydrophobic" and "hydrophilic" surfaces is indeed based on whether $\theta > 65°$ or $\theta < 65°$, respectively (Vogler, 1998).

From inspection of Fig. 1, films from WPI containing 40%(w/w) Gly can be considered to have hydrophobic surfaces, since θ took values of 69.5 ± 2.6° and 65.8 ± 2.1° for the upper and lower surfaces, respectively. Conversely, WPI-based films with higher Gly content (i.e. 50 and 60%, w/w) and WPC-based films (for all Gly contents) could be considered to have hydrophilic surfaces, since their values for θ were below 65°. Furthermore, WPI films exhibited higher θ values on both (upper and lower) surfaces when compared with WPC films; statistically significant differences ($p < 0.05$) were recorded between the two film products for a given content of glycerol – see Fig. 1.

The results for the contact angle suggest that the surface hydrophobicity of WPI and WPC films does not depend on which surface (upper or lower) is tested – since statistically significant differences were not obtained ($p > 0.05$). It is also apparent in Fig. 1 that θ (for the upper and lower surfaces) of WPI and WPC films decreased proportionally to the increase in Gly; once again, such a behavior was expected due to the hygroscopic nature of Gly (Sobral, dos Santos, & García, 2005).

In order to complement the data produced, the behavior of a water droplet on the upper surface of WPI and WPC-based films is depicted in Fig. 2, as a function of Gly content. It is apparent that both the whey product and the Gly content had a strong influence on the shape of said drop. A higher hydrophobicity could be attributed to WPI films, because of the lower enlargement of the water droplet as compared with their WPC counterparts. On the other hand, as the content of Gly increased, the enlargement of the water droplet also became more notorious – see Fig. 2. This result is consistent with the claim by Sobral et al. (2005), who reported that increasing concentrations of Gly facilitate water absorption and transport within the films.

3.4. Thermal properties

The properties of WPI and WPC films, at various levels of Gly, were also analyzed in terms of thermal performance via differential scanning calorimetry (DSC) and thermogravimetry (TGA).

DSC thermograms (Fig. A.1) showed two thermal transitions for WPI and WPC films, irrespective of their content of Gly; a glass transition for the amorphous fraction, and a melting transition for the crystalline one. The glass transition temperature (T_g), the melting temperature (T_m) and the melting enthalpy (ΔH_m) values are summarized in Table 3.

WPI films exhibited T_g values significantly higher ($p < 0.05$) than those obtained for WPC films, at a given content of Gly (see Table 3) – thus suggesting stronger films. In addition, WPI films exhibited values for T_m and ΔH_m significantly higher ($p < 0.05$) in the case of films with 40 and 50%(w/w) Gly, thus unfolding more heat-stable films; however, they showed lower T_m and ΔH_m values ($p > 0.05$) in the case of films with 60%(w/w) Gly, when compared with WPC films – see Table 3. The aforementioned differences between WPI and WPC films may arise from the higher hydrophilic nature of the

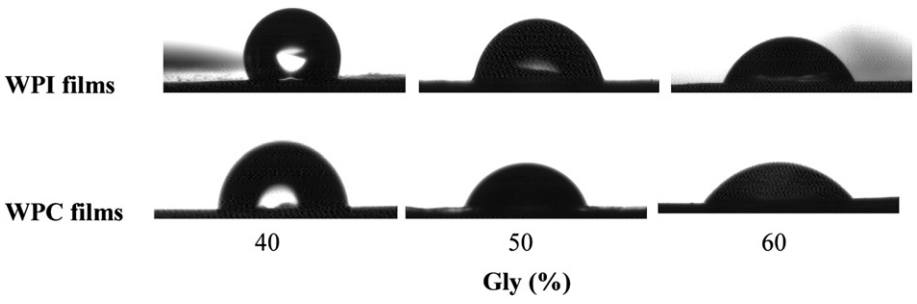

Fig. 2. Shape and behavior of water droplets on the upper surface of 10%(w/w) WPI- and WPC-edible films, with various glycerol (Gly) contents, by 5 s of exposure.

Table 3
Values (average ± standard deviation) of thermal properties, obtained from viz. differential scanning calorimetry (DSC) and thermogravimetry (TGA) analyses of whey protein isolate (WPI) and whey protein concentrate (WPC)-based edible films, with various glycerol (Gly) contents, in terms of glass transition temperature (T_g), melting temperature (T_m), enthalpy of melting (ΔH_m), initial decomposition temperature (T_{di}), derivate maximum decomposing rate temperature (DTG_{max}), weight loss and residual mass.

Protein film	Gly (%)	DSC			TGA				
		T_g (°C)	T_m (°C)	ΔH_m (J g^{-1})	T_{di} (°C)	Weight loss (%, w/w)	DTG_{max} (°C)	Weight loss (%, w/w)	Residual mass (%, w/w)
WPI	40	50.2 ± 0.7^a	184.5 ± 1.3^a	209.9 ± 2.4^a	298.4 ± 1.4^a	45.0 ± 0.2^a	369.3 ± 2.2^a	64.0 ± 0.6^a	3.2 ± 0.3^a
	50	46.9 ± 0.5^b	168.0 ± 1.2^b	186.9 ± 1.4^b	292.1 ± 0.8^b	46.0 ± 0.4^b	362.7 ± 1.5^b	65.4 ± 0.4^b	2.1 ± 0.1^b
	60	42.9 ± 0.4^c	152.0 ± 1.3^c	180.0 ± 1.1^c	281.9 ± 1.6^c	47.5 ± 0.5^c	350.3 ± 1.3^c	66.4 ± 0.3^c	1.6 ± 0.2^c
WPC	40	43.6 ± 0.6^d	172.8 ± 1.0^d	193.8 ± 2.1^d	291.0 ± 1.9^d	46.0 ± 0.3^b	366.8 ± 1.6^d	65.1 ± 0.5^b	2.9 ± 0.2^a
	50	41.3 ± 0.2^e	161.7 ± 1.0^e	183.9 ± 1.5^e	286.1 ± 1.2^e	47.7 ± 0.5^c	353.6 ± 1.2^e	66.8 ± 0.3^c	0.9 ± 0.1^d
	60	36.5 ± 0.2^f	156.8 ± 2.9^c	181.0 ± 1.4^c	280.1 ± 2.1^f	48.7 ± 0.3^d	340.1 ± 1.5^f	67.5 ± 0.2^d	0.6 ± 0.1^e

Note: $^{a, b, c, d, e, f}$ Means within the same column, labeled with the same letter, do not statistically differ from each other ($p > 0.05$).

latter (associated also with higher MC, S and a_w, as well as lower θ as reported above).

From inspection of Fig. A.1 and Table 3, it is possible to conclude that T_g and T_m decreased as Gly content increased from 40 to 60%(w/w). This trend is a consequence of the plasticizing effect of Gly molecules – which typically increase the free volume of the polymer network and the segmental mobility of the polymer chains, thus decreasing both T_g and T_m (Sobral, Menegalli, Hubinger, & Roques, 2001; Sobral, Monterrey-Quintero, & Habitante, 2002). For WPI and WPC films (see Fig. A.1a and b, respectively), T_g decreased when the Gly content was raised from 40 to 60%(w/w) – and such a decrease was statistically significantly ($p < 0.05$) for both protein films (see Table 3).

The DSC thermograms showed that T_m and ΔH_m decreased when the Gly content increased, for both WPI and WPC films (see Table 3); in both cases, this decrease was statistically significant ($p < 0.05$). Such a decrease in thermal stability was affected by the presence of Gly, which reduced the interaction between proteins, and thus stabilized the network structure (Barreto, Pires, & Soldi, 2003); in other words, higher Gly content required a lower enthalpy to disrupt inter-chain interactions.

In addition, the DSC thermograms in Fig. A.1 suggest that Gly was compatible with whey protein, and confirmed the effectiveness of plasticization – since only one T_g followed by an endothermic peak (T_m) was observed (Sobral et al., 2001; 2002). If a polymer and the plasticizer, or two different polymers were immiscible, the mixture would in fact exhibit two T_g values, corresponding to the two pure phases (Arvanitoyannis, Psomiadou, Nakayama, Aiba, & Yamamoto, 1997; Carvalho & Grosso, 2004; Vanin, Sobral, Menegalli, Carvalho, & Habitante, 2005).

TGA thermograms of WPI and WPC films are depicted in Fig. A.2, as a function of Gly content; the initial decomposition temperature (T_{di}), the derivate maximum decomposing rate temperature (DTG_{max}), the corresponding weight losses and the residual mass are shown in Table 3. WPI and WPC films displayed an initial weight loss of ca. 10%(w/w), irrespective of Gly content, which is observed up to ca. 130 °C; this can be related to the loss of free water adsorbed on the films (Nuthong, Benjakul, & Prodpran, 2009; Su et al., 2010). WPI and WPC started decomposing at 180–230 °C – thus leading to a sharp weight loss between 280 and 500 °C; this is chiefly associated with degradation of the major protein component, as well as with the plasticizer incorporated in the film matrix. Said degradation pattern was similar to that undergone by other protein films, e.g. sodium caseinate and gelatin (Barreto et al., 2003).

From the data tabulated in Table 3, one noticed that T_{di} and DTG_{max} of WPI films were significantly higher ($p < 0.05$) than their WPC counterparts. Furthermore, WPI films exhibited a lower mass loss during the heating scan than WPC films; statistically significant differences from each other ($p < 0.05$) were found at the same Gly content. On the other hand, the WPI films exhibited the highest residue mass for 40%(w/w) Gly; but the difference to WPC films was only statistically significant when the Gly content was 50 or 60%(w/w). Higher contents of Gly led to significant decreases ($p < 0.05$) of T_{di} and DTG_{max}, as well as significant increases in weight loss ($p < 0.05$) of the protein films tested. The higher weight loss of WPI and WPC films became significant above 180 °C (Fig. A.2); this can be explained by the relatively high vapor pressure of glycerol (Guerrero & de la Caba, 2010).

TGA results corroborated the conclusions drawn from DSC data: TGA indicated that whey protein films decomposed at temperatures of ca. 180 °C, whereas DSC unfolded T_m values ranging from 152.0 ± 1.3 to 184.5 ± 1.3 °C. In addition, the T_{di} and DTG_{max} values decreased when the Gly content increased – similarly to what happened with T_g and T_m. Finally, TGA thermograms showed that all films exhibited a single T_{di}, which is an indication of a good compatibility between protein and Gly.

3.5. FTIR–ATR analysis

The FTIR spectra of WPI and WPC films, with various contents of Gly, are shown in Fig. 3. The main absorption peaks were located in the spectral range: (i) 800–1150 cm^{-1}, thus being attributed to absorption bands of glycerol; (ii) 1200–1350 cm^{-1}, related to combination of N–H in-plane bending with C–N stretching vibrations (amide III); (iii) 1400–1550 cm^{-1}, associated to N–H bending (amide II); (iv) 1600–1700 cm^{-1}, governed by stretching vibration of C=O and C–N groups (amide I); (v) 2850–2980 cm^{-1}, assigned to C–H stretching; and (vi) 3000–3600 cm^{-1}, attributed to free and bound O–H and N–H groups (Karnnet, Potiyaraj, & Pimpan, 2005; Lodha & Netravali, 2005; Schmidt, Giacomelli, & Soldi, 2005). However, only three spectral regions were selected for further discussion – owing to their particular interest toward a better understanding of the interactions among proteins and Gly, and the underlying molecular mechanisms responsible for the specific functional properties displayed.

The first spectral region (from 800 cm^{-1} to 1150 cm^{-1}), attributed to absorption bands of Gly, produced five peaks for either protein film corresponding to vibrations of C–C and C–O bonds (Guerrero, Retegi, Gabilondo, & de la Caba, 2010). Comparing these spectra, it can be concluded that changes occurred in the characteristic peaks of WPI- and WPC-based films when the Gly content increased. In particular, the bands associated with the backbone C–C bond and stretching of the C–O linkage increased progressively their frequency toward that recorded for pure Gly: i.e. 850 cm^{-1}, 925 cm^{-1} and 995 cm^{-1}, for the former; 1045 cm^{-1}, for stretching of the C–O bond in C1 and C3; and 1117 cm^{-1}, for stretching of C–O in C2 (Guerrero et al., 2010) – when Gly increased from 40 to 60%(w/w). Hence, it can be realized that a gradual increase in Gly content led to an increase in intensity of

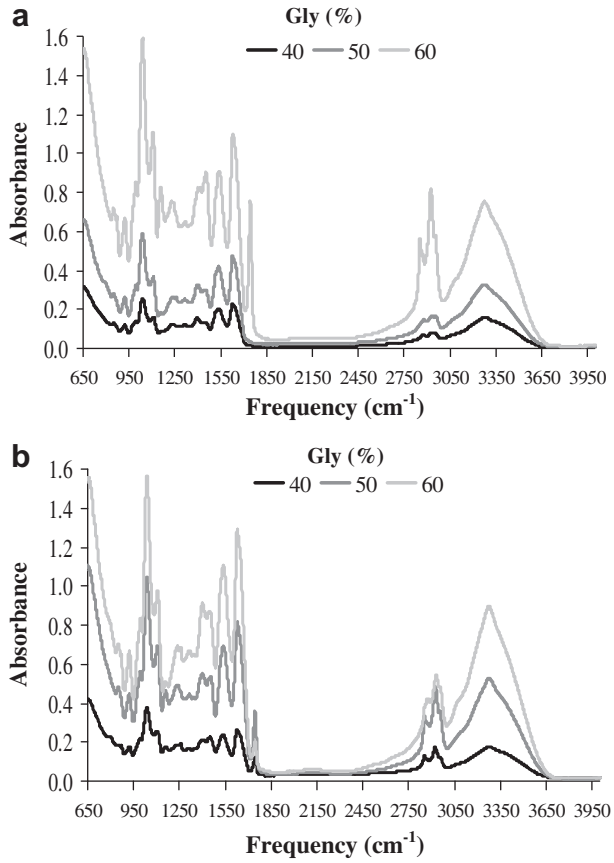

Fig. 3. FTIR absorbance spectra of 10%(w/w) WPI- (a) and WPC- (b) edible films, with various glycerol contents.

the bands in that spectral region, for both protein films – meaning that the number of free hydroxyl groups of Gly increased, thus becoming available to bind water molecules that may contribute to increase the MC of films formulated with more concentrated Gly. This observation is in agreement with results presented below – which showed significantly higher values of MC in the case of films with higher Gly content. Furthermore, a statistically lower ($p < 0.05$) intensity was observed in the case of WPI than WPC films, at both 40 and 50%(w/w) Gly; however, no significant differences ($p > 0.05$) were observed at 60%(w/w) Gly (see Fig. 3).

The second spectral region (from 3000 cm^{-1} to 3600 cm^{-1}) was characterized by a broad absorption band at 3263 cm^{-1}, for both protein films at each Gly level; it was attributed to free and bound O–H and N–H groups (le Tien et al., 2000). Several studies on proteins in this spectral region indicated that the band corresponding to N–H appears generally at 3254 cm^{-1} (Bandekar, 1992). Hence, this band shift could be due to presence of other components in the film formulation, especially Gly – owing to a large amount of hydroxyl groups brought thereby (le Tien et al., 2000). Within this region of the spectrum, WPI films showed significantly lower ($p < 0.05$) band intensity than that observed with WPC films, for all values of Gly content – but with a particular emphasis at 50 and 60%(w/w); and that an increase of Gly from 40 to 60%(w/w) led to an increase in said band intensity (see Fig. 3).

The aforementioned observations can be rationalized on the basis of protein crosslinking. In fact, the lower width of the band observed for WPI films was probably derived from a higher degree of crosslinking of the protein network – with chains closer to each other, as promoted by more frequent hydrogen bonding; hence, fewer free –OH groups were available, and a lower susceptibility to hydration was attained (Fairley et al., 1996a,b; McHugh et al., 1993; McHugh & Krochta, 1994b). It is also consistent with the data discussed above pertaining to thermal stability – which showed that WPI films were more thermostable, and thus entertained lower weight losses probably because of a highly crosslinked network. It could also explain the insolubility of WPI films (mentioned before as well), since the protein polymer network of such films was highly stable (Yoshida & Antunes, 2004).

On the other hand, the observed increase in intensity of the band when the Gly content increased could be explained by Gly reacting with protein through covalent bonds (Jiang, Li, Chai, & Leng, 2010), which may interfere with the hydrogen bonds established between the protein molecules that released –OH groups. It is expected that amino or hydroxyl groups of non-crosslinked proteins can form hydrogen bonds with –OH groups of water molecules, thus turning to be more susceptible to hydration. However, these groups become more involved in protein hydrogen bonding upon crosslinking, so they are accordingly less susceptible to hydration.

The third spectral region corresponds to the absorption of Amide I (from 1600 cm^{-1} to 1700 cm^{-1}) that is sensitive to the secondary structure of the protein, and is mainly governed by stretching vibration of C=O (70–85%) and C–N groups (10–20%) (Pereira, Souza, Cerqueira, Teixeira, & Vicente, 2010). By deconvolution of this region, eight bands were observed in the range 1616–1682 cm^{-1} and 1618–1683 cm^{-1} within the spectra of WPI and WPC films, respectively, for all Gly concentrations – see Fig. 4.

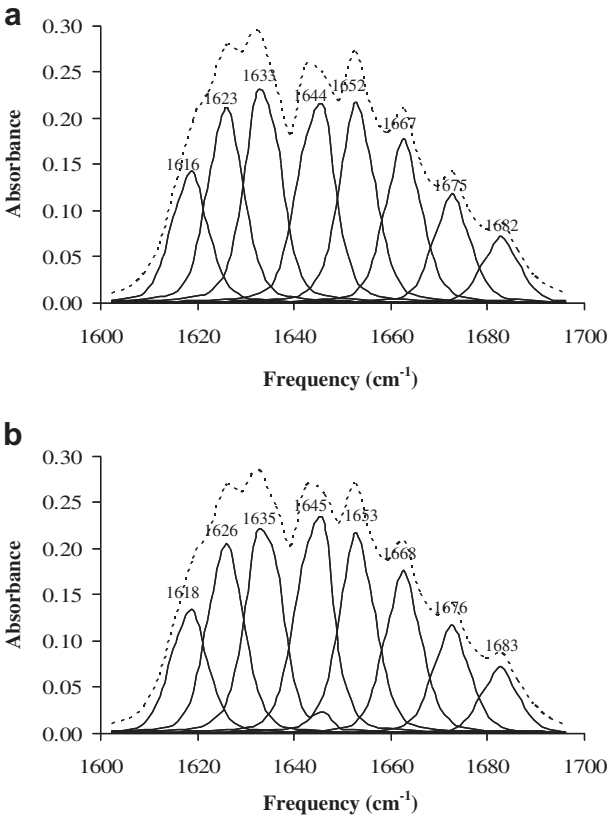

Fig. 4. Fourier self-deconvolution and curve-fitting of FTIR absorbance spectra, under attenuated total reflectance (ATR) mode, in the 1600–1700 cm^{-1} region (amide I), of 10%(w/w) WPI- (a) and WPC- (b) edible films, with 50%(w/w) glycerol.

The bands observed at 1623 and 1633 cm^{-1}, and at 1626 and 1635 cm^{-1}, for WPI and WPC films, respectively, are characteristic of amide groups involved in the extended β-sheet structure (Allain, Paquin, & Subirade, 1999), whereas bands at 1616 and 1682 cm^{-1}, in the case of WPI films, and at 1618 and 1683 cm^{-1}, in the case of WPC ones, were associated with formation of intermolecular antiparallel β-sheets (Lefèvre et al., 2005). In addition, the bands observed at 1644 and 1652 cm^{-1} in the case of WPI films, and at 1645 and 1653 cm^{-1} in the case of WPC ones were attributed to unordered and α-helix structures, respectively (Allain et al., 1999; le Tien et al., 2000); whereas the bands at 1667 and 1675 cm^{-1}, and at 1668 and 1676 cm^{-1}, for WPI and WPC films, respectively, correspond to turns (Goormaghtigh, Cabiaux, & Ruysschaert, 1990). A shift to a low wavenumber suggested stronger crosslinking via hydrogen bonds (Gilbert et al., 2005; Lefèvre & Subirade, 2000).

In the case of WPI films, the most intense band corresponds to β-sheet structures (i.e. 1633 cm^{-1}) – see Fig. 4a; this accounts for an area of 18%. Conversely, the strong band for WPC films resulted from unordered structure (i.e. 1645 cm^{-1}) – Fig. 4b, attributed to 19% thereof. In fact, the area associated with peaks at 1616, 1623, 1633 and 1682 cm^{-1} indicates that 46.1% of the amide I region of WPI films is due to intermolecular and intramolecular β-sheets; therefore, ca. 46.1% of the amino acids are likely engaged in β-sheet aggregation (Jiang et al., 2010), whereas 16.5% correspond to α-helix – and 37.4% to other structures, e.g. random coil segments and turns. On the other hand, WPC films presented 43.1% of β-sheets and 56.9% of the remaining structures – with 15% being attributed to α-helix. Significant differences ($p < 0.05$) were found between the protein films with regard to the percent area of β-sheets, whereas no significant differences ($p > 0.05$) were observed between the contents of α-helix. A high content of β-sheet structures is commonly found in aggregated proteins, especially those for which thermal denaturation was extensive; moreover, aggregation is followed by frequent formation of intermolecular antiparallel β-sheets (Fabian et al., 1999; Lefèvre et al., 2005). Therefore, the higher content of β-sheet structures observed in WPI than in WPC films likely derives from the high purity of the former, associated with the higher content of calcium (see Table 1). It has been suggested (Nicolai, Britten, & Schmitt, 2011; Vardhanabhuti, Foegeding, McGuffey, Daubert, & Swaisgood, 2001) that higher contents of calcium in whey protein products led systematically to higher aggregation rates, thus contributing to the increasing gel strength and to the development of intermolecular antiparallel β-sheets. This finding implies that stronger crosslinking occurred between WPI than WPC aggregates. Furthermore, it is possible that intermolecular disulfide bonds were established during aggregation in WPI gelation (Nicolai et al., 2011; Sothornvit, Olsen, McHugh, & Krochta, 2007). However, physical interactions including hydrophobic effects and hydrogen bonds are necessarily involved (McHugh et al., 1994).

No significant differences ($p > 0.05$) were found in the position of each aforementioned band (in this spectral region) for either whey protein, irrespective of the Gly content of the film. This is the reason why the FTIR spectrum of WPI and WPC films with 50%(w/w) Gly was used below to compare the deconvolution of the Amine I region – see Fig. 4a and b, respectively.

3.6. Barrier properties

Results pertaining to water vapor, oxygen and carbon dioxide permeabilities (WVP, O_2P and CO_2P, respectively) of WPI and WPC films, at different levels of Gly, are shown in Table 4. It is apparent that films made from WPI exhibited significantly lower ($p < 0.05$) values of WVP, O_2P and CO_2P than their WPC counterparts, for a given content of Gly. These results are consistent with the higher ρ^S values observed for WPI films, as well as with the results from the thermal and FTIR studies – showing that WPI films were more stable than WPC films, as a likely consequence of a network strongly crosslinked via non-covalent and covalent bonds. This piece of evidence contributes markedly to reduction of the interstitial spacing between molecules, thus leading to a more compact matrix in WPI films; as a consequence, lower diffusion rates for water and gas molecules resulted, arising from obstruction to transport through the more closely packed protein network. Similar results were reported by Anker, Stading, and Hermansson (2000), when studying the relationship between microstructure and barrier properties of whey protein films. The presence of a higher content of lactose in WPC powder (as apparent in Table 1) may have contributed to this finding, since this compound has a relatively low molecular weight and exerts a plasticizing effect on the protein polymer (Ghanbarzadeh & Oromiehi, 2008) – with consequent increases in permeability to water and gases (Hong & Krochta, 2006).

For both films, an increasing Gly content led to a higher permeability to water vapor – see Table 4; there were indeed significant differences ($p < 0.05$) between the WVP values of those films manufactured with a different Gly content. This could be explained by the fact that Gly reduces internal hydrogen bonding of protein molecules, and thus increases intermolecular spacing – so the permeability of protein films is promoted (Cuq, Gontard, Aymard, & Guilbert, 1997).

A significant increase ($p < 0.05$) in O_2P and CO_2P was observed for WPI and WPC films, when the Gly level increased from 40 to 60%(w/w) – see Table 4. This confirms data reported for other edible polymer films (Alves, Costa, & Coelhoso, 2010; Dole, Joly, Espuche, Alric, & Gontard, 2004). Gly may compete with water for the active sites on the polymer, thus enhancing water clustering and increasing the free volume between molecules in the film matrix – which contributes to a higher diffusivity and an increased permeability (Lieberman & Gilbert, 1973). The O_2P values obtained for WPI films were lower than those observed by Gounga et al. (2007) for 9%(w/w) WPI films, with 28, 33 and 50%(w/w)

Table 4
Values (average ± standard deviation) of barrier properties, viz. water vapor permeability (WVP), oxygen and carbon dioxide permeability (O_2P and CO_2P, respectively) of whey protein isolate (WPI) and whey protein concentrate (WPC)-based edible films, with various glycerol (Gly) contents.

Protein film	Gly (%)	WVP (g mm m^{-2} d^{-1} kPa^{-1})	O_2P (cm^3 mm m^{-2} d^{-1} kPa^{-1})	CO_2P (cm^3 mm m^{-2} d^{-1} kPa^{-1})
WPI	40	8.25 ± 0.31^a	0.20 ± 0.00^a	1.02 ± 0.01^a
	50	10.11 ± 0.20^b	0.29 ± 0.00^b	1.21 ± 0.03^b
	60	11.92 ± 0.10^c	0.37 ± 0.01^c	1.41 ± 0.01^c
WPC	40	10.81 ± 0.23^d	0.41 ± 0.01^d	1.58 ± 0.02^d
	50	12.72 ± 0.27^e	0.53 ± 0.01^e	1.75 ± 0.04^e
	60	14.04 ± 0.34^f	0.62 ± 0.01^f	1.98 ± 0.04^f

Note: $^{a, b, c, d, e, f}$ Means within the same column, labeled with the same letter, do not statistically differ from each other ($p > 0.05$).

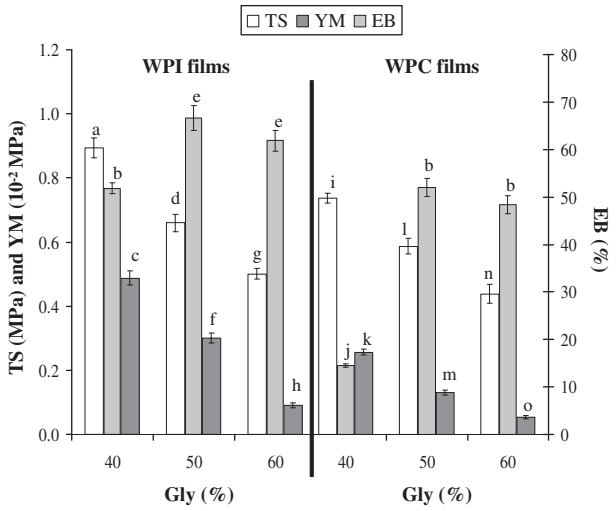

Fig. 5. Values (average ± standard deviation, $n = 10$) of TS, EB and YM of 10%(w/w) WPI- and WPC-edible films, with various glycerol (Gly) contents. Means labeled with the same letter do not statistically differ from each other ($p > 0.05$).

Gly – i.e. ca. 0.23, 0.38 and 0.50 g mm m^{-2} d^{-1} kPa^{-1}, respectively. These differences were probably due to the different protein concentration used. According to those authors, a high concentration of protein increases the density of the film solution, thus reducing the interstitial spacing within the matrix of polymeric films – and, consequently, producing lower O_2P values.

The O_2P values observed were low when compared with alternative protein films – e.g. collagen, wheat gluten and soy protein (McHugh & Krochta, 1994a); as well as with a few synthetic films – e.g. low-density and high-density polyethylenes (Miller & Krochta, 1997), under similar RH and temperature. This could be related to the more polar nature and more linear structure of the whey protein matrix film that leads to a higher cohesive energy density and a lower free volume (Miller & Krochta, 1997). Such a relatively low O_2P of whey protein films can be taken advantage of in attempts to enhance chemical quality – including oxidative damage of lipid ingredients and deterioration brought about by aerobic microflora, as happens in nuts, confectionary, fried products, and fresh fruits and vegetables, as well as colored produce (Baldwin, Nispero-Carriedo, Hagenmaier, & Baker, 1997).

On the other hand, WPI and WPC films displayed significantly higher ($p < 0.05$) values of CO_2P than those recorded for O_2P – which were 5.10-, 4.17- and 3.81-fold, for 40, 50 and 60%(w/w) Gly, respectively, in the case of WPI films; and 3.85-, 3.30- and 3.19-fold, for 40, 50 and 60%(w/w) Gly, respectively, in the case of WPC counterparts (see Table 4). This result was somehow expected; it can be attributed to the solubility of CO_2 in water, which can go up to 35-fold that of O_2. According to Mujica-Paz and Gontard (1997), this is the major reason why CO_2 diffuses much faster, and thus leads to much higher readings of permeability. Since CO_2 is essential for respiration of living tissues, films bearing higher CO_2P would be more appropriate for fresh fruits and vegetables (Ayranci & Tunc, 2003). However, the scarce information available on CO_2P of edible polymers – and whey protein films in particular, hampers more extensive conclusions.

3.7. Tensile properties

Data pertaining to the tensile properties of WPI and WPC films, with different levels of Gly, are shown in Fig. 5. WPI films showed significantly higher values ($p < 0.05$) of tensile strength (TS), elongation at break (EB), and Young's Modulus (YM) than those for WPC films with the same Gly content. Hence, films made from WPI are stronger and more flexible than those made from WPC, owing to their higher mechanical resistance (i.e. higher TS), higher stiffness (i.e. lower YM) and higher extensibility (i.e. higher EB). The aforementioned data are in agreement with our thermal and FTIR observations, which showed that WPI films are stronger and more stable than WPC films.

For both types of film, when the content of Gly increased, TS and YM decreased – thus leading to weaker films. Significantly lower values ($p < 0.05$) of both parameters were attained for those films – see Fig. 5. Moreover, when the Gly content was increased from 40 to 50%(w/w), EB increased significantly ($p < 0.05$) for both protein films (i.e. 15 and 38%, for WPI and WPC, respectively). On the other hand, when the Gly content increased from 50 to 60%(w/w), EB decreased (i.e. 5 and 4% for WPI and WPC, respectively); however, such a decrease was not statistically significant ($p > 0.05$) (Fig. 5). According to Barreto et al. (2003), a rising Gly content increases film elasticity and elongation because it constrains establishment of hydrogen bonds between the protein chains – thus increasing intermolecular spacing, and therefore chain mobility (as explained before). However, such an increase only took place up to some level of Gly – in our case, the threshold was 50%(w/w); above this value, the increase in Gly content did not produce any increase in elongation, probably as a result of film matrix saturation with Gly. This observation is in agreement with our FTIR analysis of the spectral regions, from 800 to 1150 cm^{-1} and from 3000 to 3600 cm^{-1} – which unfolded a significantly higher intensity ($p < 0.05$) at a Gly content of 60%(w/w), thus meaning that, at concentrations above ca. 50%(w/w), Gly did not react with the protein molecules to establish covalent bonds; consequently, the number of free hydroxyl groups of Gly increased, but was unable to enhance EB.

Table 5
Values (average ± standard deviation, $n = 5$) of optical properties, viz. light transmission (%) and transparency (A_{600}/mm) of whey protein isolate (WPI) and whey protein concentrate (WPC)-based edible films, with various glycerol (Gly) contents.

Film		Gly (%)	Wavelength (nm)								Transparency
			200	280	350	400	500	600	700	800	
Protein	WPI	40	0.0 ± 0.0^a	1.8 ± 0.0^a	14.8 ± 0.7^a	28.6 ± 0.1^a	35.5 ± 1.1^a	38.2 ± 0.9^a	42.6 ± 1.2^a	44.3 ± 1.4^a	3.21 ± 0.22^a
		50	0.0 ± 0.0^a	1.3 ± 0.0^a	10.9 ± 0.2^a	24.4 ± 0.4^a	31.4 ± 0.5^a	35.5 ± 0.6^a	37.3 ± 0.9^a	38.9 ± 1.1^a	3.43 ± 0.38^a
		60	0.0 ± 0.0^a	1.5 ± 0.0^a	12.5 ± 0.4^a	26.1 ± 0.3^a	33.5 ± 1.0^a	37.0 ± 1.4^a	39.6 ± 0.9^a	41.3 ± 1.3^a	3.01 ± 0.17^a
	WPC	40	0.0 ± 0.0^a	2.0 ± 0.1^a	36.5 ± 0.8^b	43.2 ± 1.3^b	46.2 ± 0.1^b	55.3 ± 1.5^b	59.3 ± 0.9^b	64.6 ± 1.6^b	1.29 ± 0.15^b
		50	0.0 ± 0.0^a	1.5 ± 0.0^a	30.7 ± 1.0^b	35.3 ± 1.1^b	40.7 ± 1.1^b	43.7 ± 0.7^b	53.5 ± 1.3^b	58.4 ± 1.3^b	1.47 ± 0.21^b
		60	0.0 ± 0.0^a	1.7 ± 0.0^a	34.3 ± 1.1^b	39.2 ± 0.9^b	43.1 ± 1.4^b	46.5 ± 1.3^b	55.7 ± 1.4^b	60.5 ± 1.1^b	1.11 ± 0.24^b
Synthetic[a]	LDPE	–	13.1	67.5	79.9	83.4	85.6	86.9	87.8	83.6	3.05
	OPP	–	4.6	80.0	86.2	87.9	88.8	89.1	89.3	89.6	1.67
	PE	–	0.3	0.3	68.3	73.6	82.1	83.5	84.2	84.9	1.51

Note: [a, b] Means within the same column, labeled with the same letter, do not statistically differ from each other ($p > 0.05$).
[a] From Shiku et al. (2003): LDPE: low-density polyethylene; OPP: oriented polypropylene; PE: polyester.

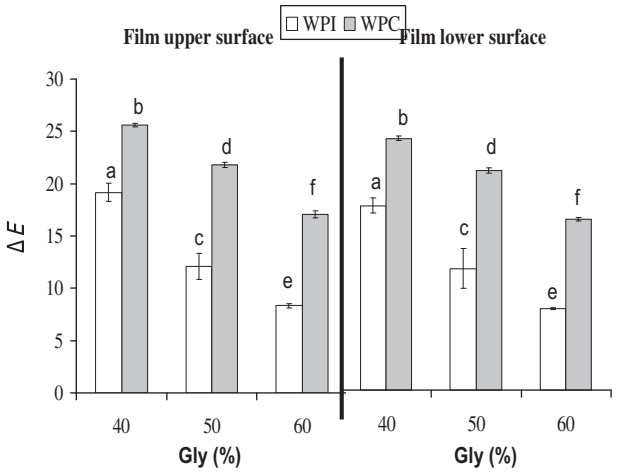

Fig. 6. Values (average ± standard deviation, $n = 4$) of ΔE for the upper and lower surface of 10%(w/w) WPI- and WPC-edible films, with various glycerol (Gly) contents. Means labeled with the same letter do not statistically differ from each other ($p > 0.05$).

3.8. Light transmission and film transparency

Light transmission (T) in the UV–Vis range, as well as transparency values pertaining to WPI- and WPC-based films, at different contents of Gly, are displayed in Table 5.

Negligible transmission was noted at 200 nm for both WPI and WPC films, while at 280 nm transmission values ranged from 1.3 ± 0.0% to 1.8 ± 0.0%, and from 1.5 ± 0.0% to 2.0 ± 0.1%, in the case of WPI and WPC films, respectively. Hence, the films made from both whey products held excellent barrier properties in the UV region – probably owing to the high content of aromatic amino acids in the protein-based structure that are able to absorb radiation (Limpan, Prodpran, Benjakul, & Prasarpran, 2010). On the other hand, synthetic polymer films cannot in general prevent passage of UV light above 280 nm, except for polyester (see Table 5). These results suggest that whey protein films might be able to retard lipid oxidation induced by UV light in food systems.

In the visible range (350–800 nm), T ranged from 10.9 ± 0.2 to 44.3 ± 1.4%, and from 30.7 ± 1.0 to 64.6 ± 1.6%, for WPI and WPC films, respectively (see Table 5); significantly lower ($p < 0.05$) values of T were thus obtained for WPI films when compared with WPC ones. Such an observation may have arisen from differences in the film-forming product – as explained above. The T values obtained for WPI and WPC films were significantly lower than those obtained by Gounga et al. (2007) in the case of 7%(w/w) WPI with 20%(w/w) Gly, and by Fang, Tung, Britt, Yada, and Dalgleish (2002) using 12%(w/w) WPI with 40%(w/w) Gly and 10 mM of Ca^{2+}; hence, our WPI and WPC films blocked the passage of visible light more effectively. In addition, those films exhibited better barrier properties (in the visible range) than those by synthetic polymer films (see Table 5).

In the UV–Vis range, the Gly content did not significantly ($p > 0.05$) affect the T values of WPI and WPC films (see Table 5).

On the other hand, the transparency obtained for WPI films was significantly higher ($p < 0.05$) than that of WPC counterparts. As happened with the T values of WPI and WPC films, the Gly content did not significantly ($p > 0.05$) affect their transparency values (Table 5).

Our WPI films exhibited a transparency similar to that obtained for low-density polyethylene films – i.e. 3.05%; however, they exhibited a higher transparency than films made from marlin myofibrillar protein (Shiku, Hamaguchi, & Tanaka, 2003), from skin gelatin with 50%(w/w) Gly – i.e. 1.82% (Jongjareonrak, Benjakul, Visessanguan, Prodpran, & Tanaka, 2006), and from synthetic polymer films, e.g. oriented polypropylene and polyethylene – i.e. 1.67 and 1.51%, respectively.

3.9. Color

Color attributes are of prime importance because they directly influence product appeal and consumer acceptability. The total color difference (ΔE) observed between WPI- and WPC-based films, containing various levels of Gly, is shown in Fig. 6. ΔE provides a good measure of the color difference, since it takes into account all three color parameters: lightness (L), red-green (a) and yellow-blue (b) components. WPI films showed lower ΔE values than WPC ones, and these differences were significant ($p < 0.05$). In addition, WPC films exhibited higher values of b than WPI ones (data not shown); this fact was consistent with the slightly yellowish color observed in WPC films, which may be attributed to presence of contaminants – especially fat and phospholipids (Lorenzen & Schrader, 2006). For practical uses, however, such a minor defect of WPC films can be overcome via addition of coloring agents, as frequently done in food packaging films, or else by lamination with opaque outer layers (Hong & Krochta, 2006).

Inspection of Fig. 6 indicates that statistically significant differences ($p > 0.05$) were not obtained for ΔE values between the upper and lower surfaces of WPI and WPC films – so such a distinction was not pursued hereafter, as it will likely be irrelevant for industrial level processing. On the other hand, ΔE values of WPI and WPC films decreased significantly ($p < 0.05$) when the content of Gly increased from 40 to 60%(w/w). Since Gly is a colorless component, the effect of such a plasticizer was probably related to dilution of the proteins and other components, due to its increasing concentration in the film-forming solution (Sobral et al., 2005). In addition, the increase in Gly level enhanced the reflection of light on the film surface, thus producing increased L values (data not shown).

4. Conclusions

This work provided a better understanding of the relationships between several physical parameters and the molecular structure of WPI- and WPC-edible films, for several distinct Gly contents. The whey protein films studied exhibited good mechanical and excellent oxygen barrier properties, much better than competitive protein- (e.g. corn zein, wheat gluten and soy protein isolate) or polysaccharide-based (e.g. starch, cellulose, carrageenan and pectin) films; they were even comparable to the best synthetic polymer films available in the market. Furthermore, they held excellent barrier properties in the UV–Vis range, clearly better than their synthetic counterparts. WPI films possessed statistically lower ($p < 0.05$) moisture content, solubility, water activity, water vapor, oxygen and carbon dioxide permeabilities, and color change values, as well as statistically higher ($p < 0.05$) density, surface hydrophobicity, mechanical resistance, elasticity, and transparency than their WPC counterparts, for a given content of Gly. Film MC, S, ρ^s and a_w, as well as surface, thermal, molecular, barrier, tensile and optical properties were also influenced by the Gly content. Hence, the film appearance, stability, consistence and barrier properties can be manipulated to some extent by choosing the base material and the level of addition of Gly.

Both whey protein films, and particularly whey protein isolate plasticized with 40 or 50%(w/w) glycerol, displayed good mechanical properties susceptible to minimize the decay

permitted by minimal processing of fresh fruits and vegetables that are still metabolically active – while providing partial barriers to moisture and gas exchange helpful in constraining moisture loss and/or reducing oxygen uptake from the environment (as slow respiration rates hamper spoilage). Moreover, they could improve the visual appeal of fruits and vegetables that directly influence consumers' acceptability. However, existing whey protein films are still characterized by lower percent elongation and higher water vapor permeability than synthetic polymer films. Hence, further research is warranted in attempts to improve the current whey protein films, besides ascertaining the impact of using such films for packaging a wider variety of food products.

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Appendix A

Fig. A.1. DSC thermograms, showing the glass transition temperature (T_g), the melting temperature (T_m) and the enthalpy of melting (ΔH_m), of 10%(w/w) WPI- (a) and WPC- (b) edible films, with various glycerol contents.

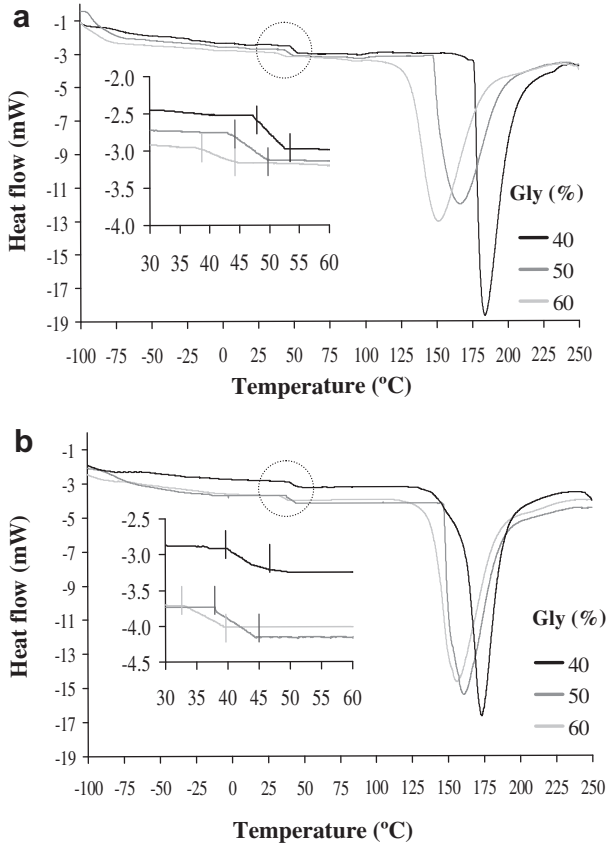
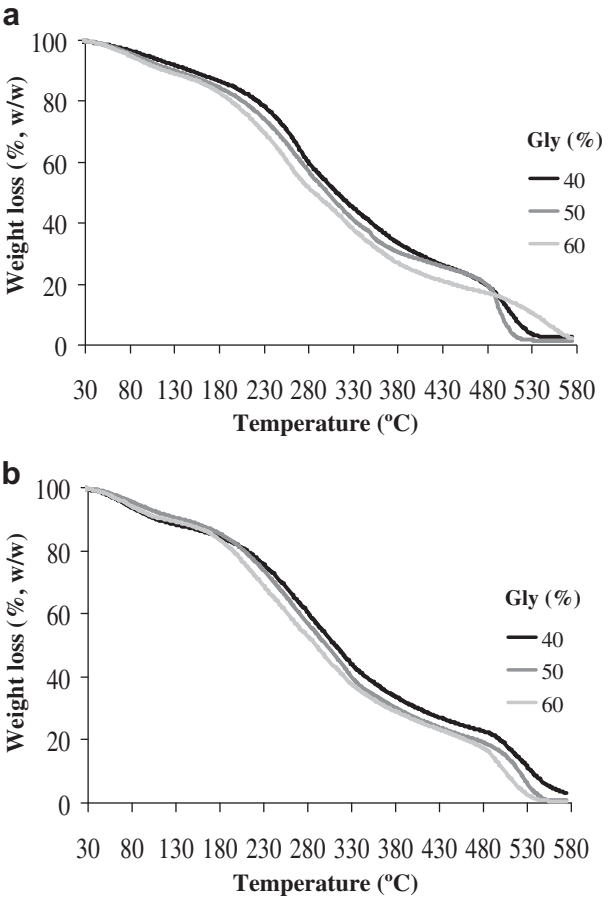


Fig. A.2. TGA thermograms, showing weight loss as a function of temperature of 10%(w/w) WPI- (a) and WPC- (b) edible films, with various glycerol contents.



References

Allain, A. F., Paquin, P., & Subirade, M. (1999). Relationships between conformation of β-lactoglobulin in solution and gel states as revealed by attenuated total reflection Fourier transform infrared spectroscopy. *International Journal of Biological Macromolecules, 26*, 337–344.

Alves, V. D., Costa, N., & Coelhoso, I. M. (2010). Barrier properties of biodegradable composite films based on *k*-carrageenan/pectin blends and mica flakes. *Carbohydrate Polymers, 79*, 269–276.

Anker, M., Stading, M., & Hermansson, A. (2000). Relationship between the microstructure and the mechanical and barrier properties of whey protein films. *Journal of Agricultural and Food Chemistry, 48*, 3806–3816.

Arvanitoyannis, I., Psomiadou, E., Nakayama, A., Aiba, S., & Yamamoto, N. (1997). Edible films made from gelatin, soluble starch and polyols, Part 3. *Food Chemistry, 60*, 593–604.

ASTM. (1994). Standard test method for moisture content of paper and paperboard by oven drying. In *Annual book of ASTM standards* (pp. 1–2). Philadelphia, PA: ASTM D 644-94.

ASTM. (1995). Standard test methods for water vapor transmission of materials. In *Annual book of ASTM standards* (pp. 697–704). Philadelphia, PA: ASTM E96-95.

ASTM. (2000). Standard practice for conditioning plastics for testing. In *Annual book of ASTM standards*. West Conshohocken, PA: ASTM D 618-00.

ASTM. (2002a). Standard test method for oxygen gas transmission rate through plastic film and sheeting using a coulometric sensor. In *Annual book of ASTM standards 15.09*. Philadelphia, PA: ASTM D 3985-02.

ASTM. (2002b). Standard test method for tensile properties of thin plastic sheeting. In *Annual book of ASTM standards* (pp. 1–9). Philadelphia, PA: ASTM D 882-02.

Ayranci, E., & Tunc, S. (2003). A method for the measurement of the oxygen permeability and the development of edible films to reduce the rate of oxidative reactions in fresh foods. *Food Chemistry, 80*, 423–431.

Baldwin, E. A., Nispero-Carriedo, M. O., Hagenmaier, R. D., & Baker, R. A. (1997). Use of lipids in coatings for food products. *Food Technology, 51*, 56–64.

Bandekar, J. (1992). Amide modes and protein conformation. *Biochimica et Biophysica Acta, 1120*, 123–143.

Barreto, P. L. M., Pires, A. T. N., & Soldi, V. (2003). Thermal degradation of edible films based on milk proteins and gelatin in inert atmosphere. *Polymer Degradation and Stability, 79*, 147–152.

Barros, A. (1999). *Contribution à la sèlection et la comparaison de variables caractéristiques*. Ph.D. thesis. Paris, France: Institut National Agronomique Paris-Grignon.

Bodnár, I., Alting, A. C., & Verschueren, M. (2007). Structural effects on the permeability of whey protein films in an aqueous environment. *Food Hydrocolloids, 21*, 889–895.

Carvalho, R. A., & Grosso, C. R. F. (2004). Characterization of gelatin based films modified with transglutaminase, glyoxal and formaldehyde. *Food Hydrocolloids, 18*, 717–726.

Cuq, B., Gontard, N., Aymard, C., & Guilbert, S. (1997). Relative humidity and temperature effects on mechanical and water vapor barrier properties of myofibrillar protein-based films. *Polymer Gels and Networks, 5*, 1–15.

Debeaufort, F., Martin-Polo, M., & Voilley, A. (1993). Polarity and structure affect water vapour permeability of model edible films. *Journal of Food Science, 58*, 428–434.

Dole, P., Joly, C., Espuche, E., Alric, I., & Gontard, N. (2004). Gas transport properties of starch-based films. *Carbohydrate Polymers, 58*, 335–343.

Fabian, H., Fälber, K., Gast, K., Reinstädler, D., Rogov, V. V., Naumann, D., et al. (1999). Secondary structure and oligomerization behavior of equilibrium unfolding intermediates of the λ Cro repressor. *Biochemistry, 38*, 5633–5642.

Fairley, P., Monahan, F. J., German, J. B., & Krochta, J. M. (1996a). Mechanical properties and water vapor permeability of edible films from whey protein isolate and sodium dodecyl sulfate. *Journal of Agricultural and Food Chemistry, 44*, 438–443.

Fairley, P., Monahan, F. J., German, J. B., & Krochta, J. M. (1996b). Mechanical properties and water vapor permeability of edible films from whey protein isolate and N-ethylmaleimide or cysteine. *Journal of Agricultural and Food Chemistry, 44*, 3789–3792.

Fang, Y., Tung, M. A., Britt, I. J., Yada, S., & Dalgleish, D. G. (2002). Tensile and barrier properties of edible films made from whey proteins. *Journal of Food Science, 67*, 188–193.

Gennadios, A. (2004). Edible films and coatings from proteins. In R. Yada (Ed.), *Proteins in food processing*. Cambridge, UK: Woodhead Publishing.

Ghanbarzadeh, B., & Oromiehi, A. R. (2008). Studies on glass transition temperature of mono and bilayer protein films plasticized by glycerol and olive oil. *Journal of Applied Polymer Science, 109*, 2848–2854.

Gilbert, V., Rouabhia, M., Wang, H., Arnould, A.-L., Remondetto, G., & Subirade, M. (2005). Characterization and evaluation of whey protein based biofilms as substrates for *in vitro* cell cultures. *Biomaterials, 26*, 7471–7480.

Goormaghtigh, E., Cabiaux, V., & Ruysschaert, J. M. (1990). Secondary structure of the membrane-bound form of the pore-forming domain of colicin A – an attenuated total-reflection polarized Fourier-transform infrared spectroscopy study. *European Journal of Biochemistry, 193*, 409–420.

Gounga, M. E., Xu, S.-Y., & Wang, Z. (2007). Whey protein isolate-based edible films as affected by protein concentration, glycerol ratio and pullulan addition in film formation. *Journal of Food Engineering, 83*, 521–530.

Guerrero, P., & de la Caba, K. (2010). Thermal and mechanical properties of soy protein films processed at different pH by compression. *Journal of Food Engineering, 100*, 261–269.

Guerrero, P., Retegi, A., Gabilondo, N., & de la Caba, K. (2010). Mechanical and thermal properties of soy protein films processed by casting and compression. *Journal of Food Engineering, 100*, 145–151.

Guilbert, S. (1986). Technology and application of edible protective films. In M. Mathlouthi (Ed.), *Food packaging and preservation: Theory and practice* (pp. 371–394). New York: Elsevier Applied Science.

Han, J. H., & Floros, J. D. (1997). Casting antimicrobial packaging films and measuring their physical properties and antimicrobial activity. *Journal of Plastic Film and Sheet, 13*, 287–298.

Hernandez-Izquierdo, V. M., & Krochta, J. M. (2008). Thermoplastic processing of proteins for film formation – a review. *Journal of Food Science, 73*, 30–39.

Hong, S.-I., & Krochta, J. M. (2006). Oxygen barrier performance of whey-protein-coated plastic films as affected by temperature, relative humidity, base film and protein type. *Journal of Food Engineering, 77*, 739–745.

Jiang, Y., Li, Y., Chai, Z., & Leng, X. (2010). Study of the physical properties of whey protein isolate and gelatin composite films. *Journal of Agricultural and Food Chemistry, 58*, 5100–5108.

Jongjareonrak, A., Benjakul, S., Visessanguan, W., Prodpran, T., & Tanaka, M. (2006). Effects of plasticizers on the properties of edible films from skin gelatin of bigeye snapper and brownstripe red snapper. *European Food Research and Technology, 222*, 229–235.

Karbowiak, T., Hervet, H., Léger, L., Champion, D., Debeaufort, F., & Voilley, A. (2006). Effect of plasticizers (water and glycerol) on the diffusion of a small molecule in ι-carragennan biopolymer films for edible coating application. *Biomacromolecules, 7*, 2011–2019.

Karnnet, S., Potiyaraj, P., & Pimpan, V. (2005). Preparation and properties of biodegradable stearic acid-modified gelatin films. *Polymer Degradation and Stability, 90*, 106–110.

Kester, J. J., & Fennema, O. R. (1986). Edible films and coatings. A review. *Food Technology, 40*, 47–59.

Khwaldia, K., Perez, C., Banon, S., Desobry, S., & Hardy, J. (2004). Milk proteins for edible films and coatings. *Critical Reviews in Food Science and Nutrition, 44*, 239–251.

Kokoszka, S., Debeaufort, F., Lenart, A., & Voilley, A. (2010). Water vapour permeability, thermal and wetting properties of whey protein isolate based edible films. *International Dairy Journal, 20*, 53–60.

Krochta, J. M., & de Mulder-Johnston, C. D. (1997). Edible and biodegradable polymer films: challenges and opportunities. *Food Technology, 51*, 61–74.

Kwok, D. Y., & Neumann, A. W. (1999). Contact angle measurement and contact angle interpretation. *Advances in Colloid and Interface Science, 81*, 167–249.

Lefèvre, T., & Subirade, M. (2000). Molecular differences in the formation and structure of fine-stranded and particulate β-lactoglobulin gels. *Biopolymers, 54*, 578–586.

Lefèvre, T., Subirade, M., & Pézolet, M. (2005). A molecular description of the formation and structure of plasticized globular protein films. *Biomacromolecules, 6*, 3209–3219.

Lieberman, E. R., & Gilbert, S. G. (1973). Gas permeation of collagen films as affected by cross-linkage, moisture, and plasticizer content. *Journal of Polymer Science, 41*, 33–43.

Limpan, N., Prodpran, T., Benjakul, S., & Prasarpran, S. (2010). Properties of biodegradable blend films based on fish myofibrilar protein and polyvinyl alcohol as influenced by blend composition and pH level. *Journal of Food Engineering, 100*, 85–92.

Lodha, P., & Netravali, A. N. (2005). Thermal and mechanical properties of environment-friendly 'green' plastics from stearic acid modified-soy protein isolate. *Industrial Crops and Products, 21*, 49–64.

Lorenzen, P. C., & Schrader, K. (2006). A comparative study of the gelation properties of whey protein concentrate and whey protein isolate. *Le Lait, 86*, 259–271.

Mangavel, C., Barbot, J., Popineau, Y., & Gueguen, J. (2001). Evolution of wheat gliadins conformation during film formation: a Fourier transform infrared study. *Journal of Agricultural and Food Chemistry, 49*, 867–872.

Maté, J. I., & Krochta, J. M. (1996). Comparison of oxygen and water vapor permeabilities of whey protein isolate and β-lactoglobulin edible films. *Journal of Agricultural and Food Chemistry, 44*, 3001–3004.

McHugh, T. H., Aujard, J. F., & Krochta, J. M. (1994). Plasticized whey protein edible films: water vapor permeability properties. *Journal of Food Science, 59*, 416–419.

McHugh, T. H., Avena-Bustillos, R. D., & Krochta, J. M. (1993). Hydrophilic edible films: modified procedure for water vapor permeability and explanation of thickness effects. *Journal of Food Science, 58*, 899–903.

McHugh, T. H., & Krochta, J. M. (1994a). Sorbitol vs. glycerol-plasticized whey protein edible films: integrated oxygen permeability and tensile property evaluation. *Journal of Agricultural and Food Chemistry, 42*, 41–45.

McHugh, T. H., & Krochta, J. M. (1994b). Plasticited whey protein edible films: water vapor permeability properties. *Journal of Food Science, 59*, 416–423.

Miller, K. S., & Krochta, J. M. (1997). Oxygen and aroma barrier properties of edible films: a review. *Trends in Food Science and Technology, 8*, 228–237.

Mujica-Paz, H., & Gontard, N. (1997). Oxygen and carbon dioxide permeability of wheat gluten film: effect of relative humidity and temperature. *Journal of Agricultural and Food Chemistry, 45*, 4101–4105.

Mulvihill, D. M., & Ennis, M. P. (2003). Functional milk proteins: production and utilisation. InFox, P. F., & McSweeney, P. L. H. (Eds.). (2003). *Advanced dairy chemistry, vol. 1, Part B* (pp. 1176–1228). New York: Kluwer.

Nicolai, T., Britten, M., & Schmitt, C. (2011). β-Lactoglobulin and WPI aggregates: formation, structure and applications. *Food Hydrocolloids, 25*, 1945–1962.

Nuthong, P., Benjakul, S., & Prodpran, T. (2009). Characterization of porcine plasma protein-based films as affected by pretreatment and cross-linking agents. *International Journal of Biological Macromolecules, 44*, 143–148.

Osés, J., Fernández-Pan, I., Mendoza, M., & Mate, J. I. (2009). Stability of the mechanical properties of edible films based on whey protein isolate during storage at different relative humidity. *Food Hydrocolloids, 23*, 125–131.

Pereira, R. N., Souza, B. W. S., Cerqueira, M. A., Teixeira, J. A., & Vicente, A. A. (2010). Effects of electric fields on protein unfolding and aggregation: influence on edible films formation. *Biomacromolecules, 11*, 2912–2918.

Perez-Gago, M. B., & Krochta, J. M. (2002). Formation and properties of whey protein films and coatings. In A. Gennadios (Ed.), *Protein-based films and coatings* (pp. 159–180). Boca Raton FL, USA: CRC Press.

Ramos, O. L. (2011). *Development and characterization of bioactive, edible whey protein films and coatings to improve quality and safety of food products*. Ph.D. thesis in Technological and Engineering Sciences – Institute of Chemical and Biological Technology (ITQB) of the New University of Lisbon.

Ramos, O. L., Fernandes, J. C., Silva, S. I., Pintado, M. E., & Malcata, F. X. (2012). Edible films and coatings from whey proteins: a review on formulation, and on mechanical and bioactive properties. *Critical Reviews in Food Science and Nutrition, 52*, 533–552.

Ryan, M., McEvoy, E., Duignan, S., Crowley, C., Fenelon, M., O'Callaghan, D. M., et al. (2008). Thermal stability of soy protein isolate and hydrolysate ingredients. *Food Chemistry, 108*, 503–510.

Salgado, P. R., Ortiz, S. E. M., Petruccelli, S., & Mauri, A. N. (2010). Biodegradable sunflower protein films naturally activated with antioxidant compounds. *Food Hydrocolloids, 25*, 525–533.

Schmidt, V., Giacomelli, C., & Soldi, V. (2005). Thermal stability of films formed by soy protein isolate-sodium dodecyl sulfate. *Polymer Degradation and Stability, 87*, 25–31.

Seydim, A. C., & Sarikus, G. (2006). Antimicrobial activity of whey protein based edible films incorporated with oregano, rosemary and garlic essential oils. *Food Research International, 39*, 639–644.

Shiku, Y., Hamaguchi, P. Y., & Tanaka, M. (2003). Effect of pH on the preparation of edible films based on fish myofibrillar proteins. *Fisheries Science, 69*, 1026–1032.

Simelane, S., & Ustunol, Z. (2005). Mechanical properties of heat-cured whey protein-based edible films compared with collagen casings under sausage manufacturing conditions. *Journal of Food Science, 70*, 131–134.

Sobral, P. J. A., Menegalli, F. C., Hubinger, M. D., & Roques, M. A. (2001). Mechanical, water vapor barrier and thermal properties of gelatin based edible films. *Food Hydrocolloids, 15*, 423–432.

Sobral, P. J. A., Monterrey-Quintero, E. S., & Habitante, A. M. Q. B. (2002). Glass transition of Nile Tilapia myofibrillar protein films plasticized by glycerin and water. *Journal of Thermal Analysis and Calorimetry, 67*, 499–504.

Sobral, P. J. A., dos Santos, J. S., & García, F. T. (2005). Effect of protein and plasticizer concentrations in film forming solutions on physical properties of edible films based on muscle protein of Thai Tilapia. *Journal of Food Engineering, 70*, 93–100.

Sothornvit, R., Olsen, C. W., McHugh, T. H., & Krochta, J. M. (2007). Tensile properties of compression-molded whey protein sheets: determination of molding condition and glycerol-content effects and comparison with solution-cast films. *Journal of Food Engineering, 78*, 855–860.

Su, J.-F., Huang, Z., Zhao, Y.-H., Yuan, X.-Y., Wang, X.-Y., & Li, M. (2010). Structure and properties of carboxymethyl cellulose/soy protein isolate blend edible films crosslinked by Maillard reactions. *Carbohydrate Polymers, 79*, 145–153.

Tang, C.-H., & Jiang, Y. (2007). Modulation of mechanical and surface hydrophobic properties of food protein films by transglutaminase treatment. *Food Research International, 40*, 504–509.

le Tien, C., Letendre, M., Ispas-Szabo, P., Mateescu, M. A., Delmas-Patterson, G., Yu, H. L., et al. (2000). Development of biodegradable films from whey proteins by cross-linking and entrapment in cellulose. *Journal of Agricultural and Food Chemistry, 48*, 5566–5575.

Vanin, F. M., Sobral, P. J. A., Menegalli, F. C., Carvalho, R. A., & Habitante, A. M. Q. B. (2005). Effects of plasticizers and their concentrations on thermal and functional properties of gelatin-based films. *Food Hydrocolloids, 19*, 899–907.

Vardhanabhuti, B., Foegeding, E. A., McGuffey, M. K., Daubert, C. R., & Swaisgood, H. E. (2001). Gelation properties of dispersions containing polymerized and native whey protein isolate. *Food Hydrocolloids, 15*, 165–175.

Vogler, E. A. (1998). Structure and reactivity of water at biomaterial surfaces. *Advances in Colloid and Interface Science, 74*, 69–117.

Yoshida, C. M. P., & Antunes, A. J. (2004). Characterization of whey protein emulsion films. *Brazilian Journal of Chemical Engineering, 21*, 247–252.