Food Chemistry 146 (2014) 6–14

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Development of naturally activated edible films with antioxidant properties prepared from red seaweed *Porphyra columbina* biopolymers

Raúl E. Cian [a,b], Pablo R. Salgado [b,c], Silvina R. Drago [a,b], Rolando J. González [a], Adriana N. Mauri [b,c,*]

[a] Instituto de Tecnología de Alimentos, Facultad de Ingeniería Química, Universidad Nacional del Litoral, 1° de Mayo 3250, 3000 Santa Fe, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Rivadavia 1917, C1033AAJ Ciudad de Buenos Aires, Argentina
[c] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), CONICET CCT La Plata y Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 y 116 S/N°, B1900JJ La Plata, Argentina

ARTICLE INFO

Article history:
Received 14 March 2013
Received in revised form 18 June 2013
Accepted 29 August 2013
Available online 14 September 2013

Keywords:
Edible films
Red seaweeds
Phycobiliproteins
Phycocolloids
R-phycoerythrin
Antioxidant properties

ABSTRACT

The aim of this work was to study the physicochemical and antioxidant properties of phycobiliproteins–phycocolloids-based films, obtained from mixtures of two aqueous fractions extracted from *Porphyra columbina* red seaweed, one enriched in phycocolloids (PcF) and the other in phycobiliproteins (PF). Films with different ratios of PF:PcF (0, 25, 50, 75, 100% [w/w]) and without plasticizer addition were prepared by casting. PcF films had excellent mechanical properties (tensile strength ~50 MPa, elongation at break ~3% and an elastic modulus ~17.5 MPa). The addition of PF to formulations exerted a plasticizing effect on the PcF matrix, which was manifested in moisture content, water solubility and mechanical properties of the resulting films but not in its water vapour permeability. The antioxidant capacity (TEAC) of the PcF films was significantly increased by the addition of PF and a direct relationship between TEAC and the total phenolic compounds (r^2 = 0.9998) and R-phycoerythrin (r^2 = 0.9942) was observed.

1. Introduction

In recent years, marine resources have attracted attention in the search for bioactive compounds to develop new drugs and healthy foods. In particular, seaweeds are a very important and commercially valuable resource for food, fodder, soil conditioners and pharmaceuticals (Souza et al., 2012). Among the genus *Porphyra*, found on hard substratum in the Patagonia Argentina coasts, *Porphyra columbina* is a red seaweed with a high total dietary fibre and protein content (~45% and ~30% dry weight, respectively).

The most important phycocolloids of *P. columbina* are sulphated polysaccharides (carrageenans and agars). The main difference between the highly sulphated carrageenans, from the less sulphated agars, is the presence of D-galactose and anhydro-D-galactose in carrageenans and of D-galactose, L-galactose or anhydro-L-galactose in agars (Gómez-Ordóñez & Rupérez, 2011). These phycocolloids have been extensively used as gelling and thickening agents in food and industrial preparations. Several authors also developed phycocolloids-based films with good mechanical and barrier properties (Giménez, López de Lacey, Pérez-Santín, López-Caballero, & Montero, 2013; Rhim, 2012; Soliva-Fortuny, Rojas-Grau, & Martín-Belloso, 2012).

The traditional method to obtain these purified phycocolloids comprises successive extraction steps with cold and hot water. During this process, the first cold water extract – enriched in phycobiliproteins – was traditionally considered a waste (Cian, Martínez-Augustin, & Drago, 2012). Phycobiliproteins are oligomeric proteins, which are usually divided into three separate groups on the basis of their colour and absorption properties, namely, phycoerythrin, phycocyanin, and allophycocyanin (Niu, Chen, Wang, & Zhou, 2010); with R-phycoerthrin (R-PE) and phycocyanin being the most important pigments in red seaweeds. It was demonstrated that protein fractions from *P. columbina* have important bioactive properties, such as inmunomodulatory and antioxidant (Cian, López-Posadas, Drago, Sánchez de Medina, and Martínez-Augustin, 2012; Cian, Martínez-Augustin, et al., 2012). This antioxidant capacity was attributed to phenolic compounds found in red seaweed (Cian, Alaíz, Vioque, and Drago, 2012).

Regarding food packaging applications, one great advantage of edible films is that they can be used as vehicles for additives, such as antioxidant and antimicrobial agents, thus acting as a compound-releasing package that could help to improve the quality and preservation of food (Salgado, Molina Ortiz, Petruccelli, & Mauri, 2010). The current trend is to use natural products, whose activities take place, not only in packaged food, but also once the food has been ingested. Such products include peptides with

* Corresponding author at: Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), CONICET CCT La Plata y Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 y 116 S/N°, B1900JJ La Plata, Argentina. Tel./fax: +54 221 4890741.
E-mail address: anmauri@quimica.unlp.edu.ar (A.N. Mauri).

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http://dx.doi.org/10.1016/j.foodchem.2013.08.133

nutraceutical activities (Salgado, Fernández, Drago, & Mauri, 2011) and phenolic compounds (Salgado et al., 2010), among others.

Edible films could be prepared from the aqueous fractions extracted from red seaweed without ulterior purification steps, which implies a lower cost process than the use of purified phycocolloids fractions (e.g. carrageenans and agars). As far as we know, there is no literature related to the production of phycobiliproteins- or phycobiliproteins–phycocolloids-based films. Even less is known about the phycobiliproteins and phycocolloids present in the red seaweed *P. columbina*.

Taking into account the important antioxidant properties of the *P. columbina* protein fractions and previous observations that phycocolloid fractions can form flexible films without a plasticizer with a lower price than using purified carrageenan and agar, the aims of this work were: (i) to develop films composed by mixtures of phycobiliproteins and phycocolloids-enriched fractions from this seaweed and (ii) to assess the effect of different ratios of these fractions on microstructural, mechanical, water-barrier, light-barrier and antioxidant properties of the resulting films.

2. Materials and methods

2.1. Preparation of phycobiliproteins and phycocolloids-enriched fractions from P. columbina

One kilogram of different specimens of *P. columbina* were handpicked in Punta Maqueda (46°00′S, 67°34′W) in spring time. Punta Maqueda, a pristine beach far away from anthropogenic activities, is located within the San Jorge Gulf, 30 km to the south of Comodoro Rivadavia, Argentina. Samples were transported to the laboratory and stored at 4 °C inside plastic bags. To remove adherent seawater, sediment, organic debris, macro fauna and epibiota, the samples were scraped and rinsed with distilled water. *P. columbina* samples were dried to a constant weight (100 ± 4 °C), ground to obtain a powder, with a particle size lower than 1 mm, using a laboratory hammer mill (Retsch, Haan, Germany). Then the samples were passed through a 20-mesh sieve (0.85 mm) and stored at 4 °C in plastic bags until analysis.

Two fractions enriched with different colloids extracted from *P. columbina* were prepared as follows: *P. columbina* algae powder was dispersed at 50 g kg^{-1} in cold distilled water (at 4 °C) for 2 h and filtered through a 50-mesh sieve (0.297 mm). The residue of the filtration (particle size > 0.297 mm) was subjected to two washings with cold distilled water (at 4 °C), dispersed at 50 g kg^{-1} in hot distilled water (95 °C) for 2 h and then centrifuged at 3000*g* for 45 min at 45 °C. The supernatant obtained from the hot distilled water extraction was namely the phycocolloids-enriched fraction (PcF). On the other hand, the filtrated (particle size < 0.297 mm) obtained from the cold distilled water extraction was centrifuged at 3000*xg* for 30 min at 20 °C and the resulting supernatant was ultra-filtered through a 10 kDa cut-off Molecular/Por® Cellulose-Ester membrane and Molecular/Por® Stirred Cell S-43-70 system. The volume reduction factor was 2. The fraction with a molecular weight > 10 kDa was mainly the phycobiliprotein-enriched fraction (PF). Both fractions, PcF and PF, had 1 g 100 g^{-1} of dry solid.

2.2. Characterisation of phycobiliproteins and phycocolloids-enriched fractions from P. columbina

2.2.1. Chemical composition

The chemical composition was determined using the AOAC (1995) procedures. Proteins were determined by the Kjeldahl method ($N \times 6.25$). The total carbohydrates (expressed as galactose) and sulphate content were determined according to the method outlined by Dubois, Gillis, Hamilton, Rebers, and Smith (1956) and Dodgson and Price (1962), respectively. The R-phycoerythrin content (g 100 g^{-1} of protein) was estimated by measuring the absorbance at 566 nm and using a 3.9 mM R-phycoerythrin standard solution in 0.1 M phosphate buffer (pH 6.8) (Cian, López-Posadas, et al., 2012). The total phenolic compounds (TPC) were quantified according to Schanderl (1970) with modifications, using Folin–Ciocalteu reagent. A standard curve with gallic acid solutions was used for calibration. Results were expressed as g gallic acid 100 g^{-1} sample. All determinations were performed at least in triplicate.

2.2.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed according to Laemmli (1970), using a Mini-Protean II Electrophoresis cell (Bio-Rad) equipment with a Model 200/2.0 Bio-Rad source with stacking gel of 4% w/v polyacrylamide and separating gel of 12% w/v polyacrylamide in 25 mM Tris–HCl, pH 8.3, 0.18 M glycine and 0.1% w/v SDS. The separation was carried out at 180 V for 2 h. The polypeptides used as molecular weight markers were: phosphorylase b (94 kDa), albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa) (Bio-Rad cat. N°161–0304). Gel plates were fixed and stained with a solution containing 0.125% w/v Coomassie Blue R-250, 50% v/v methanol and 10% v/v acetic acid in water, and then destained with 25% v/v methanol and 10% v/v acetic acid.

2.2.3. FTIR analysis

Infrared spectra of **PF** and **PcF** were acquired using a Nicolet iS 10 Infrared Spectrometer (Thermo Scientific, Madison, WI, USA). Aqueous **PF** and **PcF** dispersions (1 g 100 g^{-1}) were dried at 60 °C and then placed on a sample holder. Measurements were performed at room temperature. Spectra were obtained in the 700–1500 cm^{-1} range by accumulation of 60 scans at 4 cm^{-1} resolution. FTIR spectra were recorded using the OMNIC software, version 7.3 (Thermo Electron Corporation, USA). Prior to sample collection, a background spectrum was recorded and subtracted from the sample spectra. The spectra were baseline corrected at 1812 cm^{-1} and normalised for comparison purposes. All experiments were performed in duplicate.

2.2.4. Intrinsic viscosity

Ten ml of aqueous PF and PcF dispersions (0.716–2.559 g solids/dl and 0.002 to 0.013 g solids/dl, respectively) were used to determine their flow time with a Cannon–Fenske capillary viscometer (Series 100, ASTM D2857-95, 2004) at 20 °C (±0.1 °C). Relative viscosity (η_{rel}), reduced viscosity (η_{red}) and inherent viscosity (η_{inh}) were determined as follows:

$$\eta_{rel} = \eta/\eta_0 = (t/t_0).(\rho/\rho_0) \quad (1)$$

$$\eta_{red} = (\eta_{rel} - 1)/C = [\eta] + [\eta]^2 \cdot k_H \cdot C \quad (2)$$

$$\eta_{inh} = \ln(\eta_{rel})/C = [\eta] + [\eta]^2 \cdot k_K \cdot C \quad (3)$$

where $[\eta]$ is the intrinsic viscosity; t and t_0 are the flow times of the solutions and water, respectively; ρ and ρ_0 are the corresponding densities; k_H and k_K are the Huggins' and Kramer's coefficients, respectively; and C is the concentration of PF or PcF solutions (g solids/dl).

Intrinsic viscosity, $[\eta]$, was obtained from Eqs. (2) and (3) (Huggin's and Kramer's, respectively), doing extrapolations to zero concentration. Measurements were performed in triplicate.

2.3. Film-forming dispersions

Film-forming dispersions (1 g 100 g^{-1}) with different PF:PcF ratios (0%, 25%, 50%, 75%, 100% [w/w]) were prepared at pH 7 without the addition of plasticizers. Five film-forming dispersions (named 0PF:100PcF, 25PF:75PcF, 50PF:50PcF, 75PF:25PcF and 100PF:0PcF) were prepared according to a two-component simplex-lattice mixture design and then evaluated as follows.

2.3.1. Rheology of film-forming dispersions

Rotational analyses of PF:PcF film-forming dispersions were performed at 25 °C in a Haake ReoStress 600 (Thermo Haake, Karlsruhe, Germany) with a 1 mm gap serrated plate–plate sensor system PP35. Dispersions were maintained at 25 °C by a circulating water bath (Circulator DC50 Thermo Haake, Germany) connected to the jacket surrounding the sensor system during testing. Shear stress (τ) was determined as a function of the shear rate (D). The shear rate increased from 0 to 500 s^{-1} in 2 min, maintained for 1 min and then decreased from 500 to 0 s^{-1} for 2 min. Apparent viscosities (η_{app}, in mPa s) were calculated in the upwards curves at 60, 300 and 500 s^{-1} for each film-forming dispersion. Flow (n) and consistency (K) indexes were determined by using the Ostwald de Waele model on experimental results (Eq. (4)). Determinations were carried out in triplicate.

$$\tau = K \cdot D^n \tag{4}$$

where τ is the shear stress (Pa), K is the consistency index (Pa s^n), D is the shear rate (s^{-1}) and n is the flow index (dimensionless).

2.3.2. Antioxidant capacity of film-forming dispersions

The antioxidant capacity of film-forming dispersions was measured using the $ABTS^{\cdot+}$ radical cation decolorisation assay according to Cian, Martínez-Augustin, et al. (2012). To estimate the Trolox Equivalent Antioxidant Capacity (TEAC) a concentration–response curve for the absorbance at 734 nm for $ABTS^{\cdot+}$ as a function of concentration of standard Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) solutions (0–2.5 mM) in 0.01 M PBS at pH 7.4 was performed. The absorbance reading was taken at 6 min following initial mixing. Determinations were carried out in triplicate.

2.4. Films

2.4.1. Films preparation

Film-forming dispersions (1 g 100 g^{-1}) with different PF:PcF ratios (0%, 25%, 50%, 75%, 100% [w/w]) were prepared as mentioned before, were processed by casting. Films (0PF:100PcF, 25PF:75PcF, 50PF:50PcF, 75PF:25PcF and 100PF:0PcF) were obtained according to a two-component simplex-lattice mixture design. Film-forming dispersions were agitated in a magnetic stirrer for 1 h at room temperature. Twenty ml of each film-forming dispersion were poured on polystyrene Petri dishes (64 cm^2) and then dehydrated at 60 °C for 5 h in an oven, with air flow circulation (Yamato, DKN600, USA). Resulting films were conditioned for 48 h at 20 °C and 58% relative humidity (RH) in desiccators, with saturated solutions of NaBr, before being peeled from the casting surface for characterisation.

2.4.2. Films characterisation

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

2.4.2.2. Moisture content (MC). Small specimens of films collected after conditioning were cut and placed on Petri dishes, which were weighed before and after oven drying at 105 °C for 24 h (ASTM D644-99, 2004). MC values were determined in triplicate for each film, and calculated as the percentage of weight loss relative to the original weight.

2.4.2.3. Colour. The film colour was determined with a Konica Minolta Chroma metre CR-400 (Konica Minolta Chroma Co., Osaka, Japan) set to C illuminant/2° observer. A CIE-Lab colour scale was used to measure the degree of lightness (L^*), redness ($+a^*$) or greenness ($-a^*$), and yellowness ($+b^*$) or blueness ($-b^*$) of the films. The instrument was calibrated using a white standard plate with colour coordinates of $L^*_{standard} = 97.6$, $a^*_{standard} = -0.03$ and $b^*_{standard} = 1.73$, provided by Minolta. The films colour was measured on the surface of this standard plate and the total colour difference (ΔE^*) was calculated as follows:

$$\Delta E^* = [(L^*_{film} - L^*_{standard})^2 + (a^*_{film} - a^*_{standard})^2 + (b^*_{film} - b^*_{standard})^2]^{0.5} \tag{5}$$

colour can also expressed by the use of polar (or cylindrical) coordinates L^*, C^* and h^*, where L^* is the same as described previously, C^* is the chroma or saturation index and H^* is the hue. The following equations were used to convert $L^*a^*b^*$ coordinates to $L^*C^*h^*$ coordinates.

$$C^* = (a^{*2} + b^{*2})^{0.5} \tag{6}$$

$$h^* = \text{arc tan}(b^*/a^*) \tag{7}$$

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

2.4.2.4. Opacity. Each film specimen was cut into a rectangular piece and placed directly into a spectrophotometer test cell, and measurements were performed using air as the reference. A spectrum of each film was obtained in an UV–Vis spectrophotometer (Beckman DU650, Germany). The area under the absorption curve from 400 to 800 nm was recorded, and the opacity of the film (UA cm^{-1}) was calculated (Salgado et al., 2011). All determinations were performed in quadruplicate.

2.4.2.5. Water vapour permeability (WVP). Water vapour permeability tests were conducted according to the ASTM method E96-00 (ASTM., 2004), with some modifications. Each film sample was sealed over a circular opening of 0.00185 m^2 in a permeation cell, that was stored at 20 °C in desiccators. To maintain a 75% relative humidity (RH) gradient across the film, anhydrous silica (0% RH_c) was placed inside the cell and a saturated NaCl solution (75% RH_d) was used in the desiccators. The RH inside the cell was always lower than outside, and water vapour transport was determined from the weight gain of the permeation cell. When steady-state conditions were reached (about 1 h), eight weight measurements were made over 5 h. Changes in the weight of the cell were recorded and plotted as a function of time. The slope of each curve ($\Delta m/\Delta t$, g H_2O s^{-1}) was obtained by linear regression and the water vapour transmission rate was calculated from the slope divided by the permeation cell area (A, in m^2). The WVP (g H_2O Pa^{-1} s^{-1} m^{-1}) was calculated according to the following equation:

$$\text{WVP} = [\text{WVTR}/(P_V H_2O \cdot (RH_d - RH_c))] \cdot d \tag{8}$$

where WVTR = water vapour transmission rate (g H_2O s^{-1} m^{-2}), $P_V^{H_2O}$ = saturation water vapour pressure at test temperature (2339.27 Pa at 20 °C), $RH_d - RH_c$ = relative humidity gradient across the film – expressed as a fraction – (0.75), A = permeation (m^2) area, and d = film thickness (m). Each WVP value represents the mean value of at least three samples taken from different films.

2.4.2.6. Water solubility (WS). WS was determined following the method described by Gontard, Duchez, Cuq, and Guilbert (1994), with slight modifications. Film portions were weighed (diameter = 2 cm; $P_0 \sim 0.03$–0.05 g) and placed in an Erlenmeyer flask (250 ml) with 50 ml of distilled water (containing 0.02% w/v sodium azide) and then sealed and shaken at 100 rpm for 24 h at 20 °C (Ferca, TT400 model, Argentina). The solution was then filtered through Whatman n°1 filter paper (previously dried and weighed) to recover the remaining undissolved film, which was desiccated at 105 °C for 24 h (P_f). The WS was calculated as follows:

$$WS = [(P_0.(100 - MC)) - P_f] \cdot 100/[P_0 \cdot (100 - MC)] \quad (9)$$

where P_0 = initial film weight (g), P_f = final dry film weight (g), MC = moisture content (%). All tests were carried out in triplicate.

2.4.2.7. Mechanical properties. Tensile strength (TS), elastic modulus (EM) and elongation at break (EAB) of the films were determined using a texture analyzer TA.XT2i (Stable Micro Systems, Surrey, England) equipped with a tension grip system A/TG, according to the procedures outlined in the ASTM method D882-02 (ASTM, 2004). The measurements were made at 20 °C and 65% RH in a controlled room. Films probes of 70 mm length and 6 mm width were used, a minimum of six probes were prepared for each film. The initial grip separation was set at 50 mm and the crosshead speed at 0.5 mm s^{-1}. The curves of force (N) as a function of distance (mm) were recorded by the Texture Expert V.1.15 software (Stable Micro Systems, Surrey, England). Tensile properties were calculated from the plot of stress (tensile force/initial cross-sectional area) versus strain (extension as a percentile of the original length). TS and EAB were determined directly from the stresses-train curves, and EM was determined as the slope of the initial linear portion of this curve. Twelve replications were performed for each formulation.

2.4.2.8. Differential scanning calorimetry (DSC). The glass transition temperature (Tg) of the films was determined by differential scanning calorimetry, using a DSC TA 2010 calorimeter Q100 V9.8 Build 296 (TA Instrument, New Castle, Del., USA), controlled by a TA 5000 module with a quench cooling accessory. Temperature and heat flow calibration of the equipment were carried out according to the ASTM methods, using lauric and stearic acids and indium as standards. Hermetically sealed aluminium pans containing 5 mg of films were prepared, and the capsules were scanned as follows: (a) 1 min at 20 °C (to equilibrate samples), cooling until −80 °C at 10 °C min^{-1}, isotherm for 5 min and heating to 150 °C at 10 °C min^{-1}. The Tg was considered to be the inflexion point of the base line, caused by the discontinuity of the specific heat of the sample (ASTM D3418-03, 2004), and it was calculated using the Universal Analysis V4.2E software (TA Instruments, New Castle, Del., USA). All the assays were performed at least in duplicate.

2.4.2.9. Antioxidant capacity of film. The antioxidant capacity of the films was evaluated as described above (Section 2.3.2). Films were dispersed in water at 1% w/w and dispersions were used for the $ABTS^{\cdot+}$ assays. The antioxidant capacity was expressed as trolox equivalent antioxidant capacity (TEAC).

Table 1
Content of protein, R-phycoerythrin, total carbohydrates, sulfate, total phenolic compounds and ash of *phycobiliproteins and phycocolloids*-enriched fractions (PF and PcF respectively) from *P. columbina*.

	PF	PcF
Protein (g 100 g^{-1} dw)	28.3 ± 0.6[b]	14.3 ± 0.2[a]
R-phycoerythrin (g 100 g^{-1} of proteins)	3.5 ± 0.0[b]	0.2 ± 0.0[a]
Total carbohydrates (g 100 g^{-1} dw)*	50.1 ± 3.9[a]	72.0 ± 3.3[b]
Sulfate content (g 100 g^{-1} of total carbohydrates)	6.1 ± 0.2[a]	12.6 ± 0.1[b]
Total phenolic compounds (g 100 g^{-1} dw)**	0.7 ± 0.0[b]	0.1 ± 0.0[a]
Ash (g 100 g^{-1} dw)	14.4 ± 0.2[b]	6.4 ± 1.1[a]

* Total carbohydrate content is expressed as galactose.
** Total phenolic compounds content is expressed as gallic acid. Results are expressed as mean value ± standard deviation, dw (dry weight). Different letters in the same row mean significant differences between samples ($p < 0.05$), according to Fisher's least significant difference test.

2.5. Statistical analysis

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

3. Results and discussion

3.1. Characterisation of phycobiliproteins and phycocolloids-enriched fractions extracted from P. columbina

The chemical composition of phycobiliproteins and phycocolloids-enriched fractions (PF and PcF, respectively) from red edible seaweeds *P. columbina* is shown in Table 1. The crude protein content of PF was 2.0-fold higher than that obtained for PcF ($p < 0.05$). R-phycoerythrin (R-PE) and phycocyanin – to a lesser extent – are the most important phycobiliprotein pigments in red seaweeds (Cian, López-Posadas, et al., 2012). The R-PE content of PF was ~17 times higher than that of PcF ($p < 0.05$), in agreement with that reported by Denis, Massé, Fleurence, and Jaouen (2009) for the ultrafiltered fraction obtained from red macroalga *Grateloupia turuturu*.

The presence of R-PE in both fractions was confirmed by SDS–PAGE (Fig. 1.A). R-PE (lane 2) featured 3 components: one near 30 kDa and two between 14.4 and 21.5 kDa. The first one could be attributed to γ subunit (molecular weight ~30–33 kDa), while the others probably corresponded to the α and β subunits (molecular weight 17 and 19 kD, respectively) (Niu et al., 2010). PF showed three components, in agreement with R-PE electrophoresis profile (lane 3). Additional components were also detected, with an apparent molecular weight between 45–66.2 kDa and 14.4 kDa, representing other protein components in PF different from the phycobiliproteins. On the other hand, PcF only showed components with apparent molecular weights lower than 21.5 kDa (lane 4). Therefore, the components with apparent molecular weight higher than 30 kDa were completely removed during cold water extraction. The bands between 14.4 and 21.5 kDa might belong to α and β subunits from R-PE. It is appropriate to note that phycocyanin also had two bands (α and β) with similar apparent molecular weight (Minkova et al., 2003). Also, the components with apparent molecular weight near 14.4 kDa present in both fractions (PF and PcF), were found in different red seaweeds SDS–PAGE patterns such as *Chondrus crispus*, *Gracilaria verrucosa*, *Palmaria palmata* and *Porphyra umbicalis* (Rouxel, Daniel, Jerome, Etienne, & Fleurence, 2001).

The carbohydrate content of PF was not negligible (Table 1) and was similar to that reported by Jiménez-Escrig, Gómez-Ordóñez,

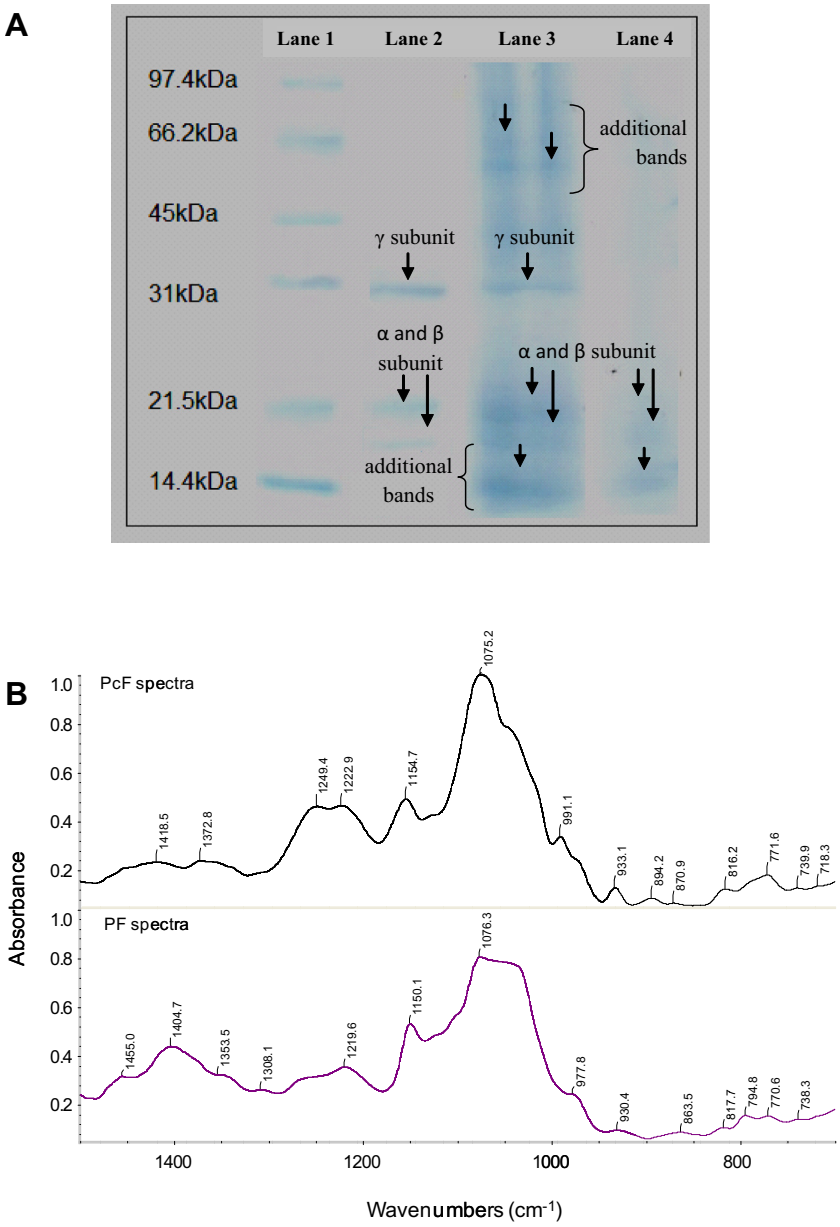

Fig. 1. (A) SDS–PAGE patterns of fractions from red seaweed *P. columbina*. Lane 1: low molecular weight markers, Lane 2: R-phycoerythrin (Sigma 52412-F), Lane 3: Phycobiliproteins-enriched fraction (PF), and Lane 4: Phycocolloids-enriched fraction (PcF). (B) FTIR spectrum (1500 to 700 cm^{-1}) of phycocolloids-enriched fraction (PcF) and phycobiliproteins-enriched fraction (PF).

and Rupérez (2012) for the water extract of red seaweed *Gigartina pistillata* (~47 g 100 g^{-1} dw). The carbohydrate content of PF was lower than that of the PcF fraction ($p < 0.05$), which is probably due to the water temperature (4 °C) used during extraction process. Maciel et al. (2008) studied the extraction of sulfated polysaccharides from *Gracilaria birdiae* using cold water and showed that the low yield obtained was due to the low extraction temperature. Regarding PcF, the carbohydrate content was similar to that obtained for a water extract of red seaweeds *Porphyra capensis* (72 g 100 g^{-1} dw) (Zhang et al., 2005).

Also, the sulfate content of PF was twofold lower than that of PcF ($p < 0.05$) due to the greater solubilisation of sulfated polysaccharides in hot water. The sulfate content of PF was similar to that reported by Maciel et al. (2008) for the cold water extract of *G. birdiae* (6.4 g 100 g^{-1}). The sulfate content of PcF was similar to that reported by Zhang et al. (2005) for *P. capensis* (12.1 g 100 g^{-1}) and higher than that found by Maciel et al. (2008) for red seaweeds, *G. birdiae* (8.4 g 100 g^{-1}).

The total phenolic compound content (TPC) of PF was seven times higher than that of PcF ($p < 0.05$), indicating that cold water extraction removed most of the water soluble phenolic compounds. The TPC of PF was similar to that reported by Kuda, Tsunekawa, Goto, and Araki (2005) for the *Porphyra* spp. water extract (0.94 g 100 g^{-1} dw). Epicatechin and gallic acid were the most abundant phenolic compounds in PF, whereas cathechin, coumaric acid, quercetin, and kaempferol were also present in this fraction, but in a lesser amount (Cian, López-Posadas, et al., 2012).

FTIR spectrum (1500–700 cm^{-1}) of both the PcF and PF fractions from red edible seaweeds *P. columbina* are shown in Fig. 1.B. This region was used to obtain information on the structure of phycocolloids (e.g. agars and carrageenans). The PcF spectra exhibits the characteristic bands of agarocolloids, such as *porphyran*

(1372, 1249, 1154, 1075, 933, 893, 816 and 771 cm^{-1}). These results are in concordance with those reported by Zhang et al. (2005) for *P. capensis*. The bands at 1372 and 1249 cm^{-1} can be attributed to the S=O vibration of the sulphate groups. The important band at 1075 cm^{-1} corresponds to the skeleton of galactans, while the band at 893 cm^{-1} could be assigned to agar specific bands. The bands at 933 cm^{-1} could be attributed to the C–O–C group of 3,6-anhydro-α-L-galactose (Souza et al., 2012). Additionally, the bands at 845, 830 and 820 cm^{-1} are used to infer the position of the sulphate group in agarocolloids and are assigned to the 4-sulphate, 2-sulphate and 6-sulphate of the D-galactose units, respectively (Gómez-Ordóñez & Rupérez, 2011; Maciel et al., 2008). The FTIR spectrum of PcF shows a low intensity band at 845 cm^{-1}, attributed to the sulphate substitution at the C-4 of the galactose. The presence of a shoulder close to 820 cm^{-1} could also indicate a small degree of substitution at the C-6 position. The absence of bands at 830 cm^{-1} indicates that 2-sulphate galactose and sulphate on the C-2 of 3,6-anhydro-α-L-galactose were not present. These results are in agreement with those reported by Melo, Feitosa, Freitasa, and de Paula (2002) and Maciel et al. (2008) who worked with two different red seaweeds. Finally, the bands at 1154 and 771 cm^{-1} cannot be assigned, as in the case of those reported by Maciel et al. (2008) and Melo et al. (2002) for FTIR spectrum of red seaweed *G. birdiae* and *Gracilaria cornea*, respectively. On the other hand, the PF spectrum (Fig. 1.B) exhibits bands at 1307, 1219, 1149, 1075, 929, 862, 794 and 770 cm^{-1}, and was very different from that obtained for PcF. Thus, the region around 1075 cm^{-1} – assigned to skeleton of the galactans – became more important, but the band around 890 cm^{-1} – attributed to the agar specific bands – decreased in intensity. Also, the bands around 1370 and 1250 cm^{-1} attributed to the S=O vibration of the sulphate groups are very weak. These results suggest that the proportion of carrageenan:agar in PF is greater than in PcF, but with a lower degree of sulphatation.

3.2. Characterisation of film-forming dispersions

Table 2 shows the calculated protein and polysaccharide contents of the film-forming dispersions as well as its measured total phenolic compounds content (TPC). As the PF proportion increased in the film-forming dispersions (from 0PF:100PcF to 100PF:0PcF), the protein content and TPC increased ($p < 0.05$), the polysaccharide content decreased ($p < 0.05$) and the sum of the biopolymers (proteins and polysaccharides) slightly decreased. These differences were manifested in their antioxidant and rheological properties (see Table 2). With regard to rheological properties of the PF:PcF film-forming dispersions, the power-law (or the Ostwald de Waele model) fitted satisfactorily with the experimental data ($r^2 > 0.98$ in all cases). The corresponding fitting parameters, n and K are shown in Table 2. The PcF dispersion (0PF:100PcF) exhibited a shear-thinning behaviour ($n < 1$), while that corresponding to PF (100PF:0PcF) showed a Newtonian behaviour ($n = 1$). The incorporation of PF to PcF dispersions decreased its consistency (K decreased) ($p < 0.05$) and its shear-thinning behaviour increased (n increased) ($p < 0.05$). The dispersion behaviour changed to Newtonian for formulations with PF contents higher than 50%. A shear-thinning behaviour of carrageenan aqueous dispersions, at similar concentrations and temperature, was previously reported by Marcotte, Hoshahili, and Ramaswamy (2001) and Lizarraga, De Piante Vicin, González, Rubiolo, and Santiago (2006), but there is not information about the flow behaviour of phycobiliproteins aqueous dispersions in the bibliography.

Apparent viscosity of PcF film-forming dispersions (calculated at 60, 300 and 500 s^{-1}) exhibited higher values compared with viscosity of PF ($p < 0.05$) (Table 2). These results could be attributed, at least in part, to differences in the intrinsic viscosity of polymers, 80.9 ± 2.40 dl g^{-1} *vs.* 0.38 ± 0.02 dl g^{-1} for PcF and PF, respectively. This suggests that the polymer–polymer interactions are more important for PcF and a greater number of water molecules are immobilised in PcF than in PF film-forming dispersions. As the PF fraction increases in the formulations, the apparent viscosity of dispersions significantly decreased ($p < 0.05$). This behaviour could be explained by the higher content of lower molecular weight water soluble compounds (such as phycobiliproteins and phenolic compounds) of PF, which would reduce the resistance to flow. The rheological properties of the dispersions are mainly responsible for the presence of defects in the film matrix. For example, the homogenisation of high viscosity dispersions is difficult, and as a consequence, films with heterogeneities in composition are obtained. Besides, high viscosity dispersions prevent air bubble escaping from the film and pores (holes) which are consequently formed. On the contrary, low viscosity dispersions obtained with dilute solutions lead to very thin films (Han & Gennadios, 2005).

The antioxidant capacity (TEAC) of film-forming dispersions (pre-casting) is shown in Table 2. All film-forming dispersions exhibited $ABTS^{\cdot+}$ scavenging capacity. In PcF dispersions, the TEAC can be attributed mainly to the presence of sulphated polysaccharides. Rocha de Souza et al. (2007) reported the *in vitro* antioxidant activities of iota, kappa and lambda carrageenans. On the other hand, the PF film-forming dispersions (100PF:0PcF) presented the highest TEAC ($p < 0.05$). As previously mentioned, phycobiliproteins have antioxidant capacity (Cian, Martínez-Augustin, et al., 2012), and may contribute to an increase in the TEAC of film-forming dispersions. However, many researchers have attributed the TEAC mainly to the phenolic compounds present in the seaweed (Matanjun, Mohamed, Mustapha, Muhammad, & Ming, 2008; Wang, Jónsdóttir, & Ólafsdóttir, 2009). As shown in Table 2, the incorporation of PF to the PcF film-forming dispersions significantly increases the TPC ($p < 0.05$). Therefore, the higher TEAC of the PF based film-forming dispersion may be due to the higher phycobiliproteins and TPC contents. This result is in agreement with that reported by Jiménez-Escrig et al. (2012) and Wang et al. (2009), who attribute the antioxidant capacity of seaweed

Table 2
Content of protein, total carbohydrate and total phenolic compounds (TPC), antioxidant capacity (TEAC) and rheological properties of film-forming dispersions obtained from mixtures of *phycobiliproteins* (PF) and *phycocolloids*-enriched fractions (PcF) from *P. columbina*.

Mixtures	Proteins (g 100 g^{-1} dispersion)	Carbohydrate (g 100 g^{-1} dispersion)	TPC (mg gallic acid 100 g^{-1} dispersion)	TEAC (μg Trolox g^{-1} dry solids)	Ostwald de Waele parameters		Apparent viscosity (mPa s)		
					K (Pa s^n)	n	D = 60 s^{-1}	D = 300 s^{-1}	D = 500 s^{-1}
0PF:100PcF	0.14	0.72	1.03 ± 0.01^a	75.7 ± 1.2^a	4.072 ± 0.434^e	0.39 ± 0.01^a	337.3 ± 23.5^d	124.0 ± 6.6^e	89.2 ± 4.2^e
25PF:75PcF	0.18	0.67	5.06 ± 0.13^b	131.7 ± 1.2^b	0.331 ± 0.015^d	0.66 ± 0.01^b	79.7 ± 1.2^c	48.7 ± 1.2^d	40.3 ± 1.1^d
50PF:50PcF	0.21	0.61	6.72 ± 0.12^c	215.7 ± 1.2^c	0.033 ± 0.005^c	0.87 ± 0.01^c	18.0 ± 3.7^b	15.6 ± 1.3^c	14.6 ± 1.4^c
75PF:25PcF	0.25	0.56	6.99 ± 0.13^d	255.3 ± 4.0^d	0.004 ± 0.001^b	1.00 ± 0.01^d	4.3 ± 0.3^b		
100PF:0PcF	0.28	0.50	7.40 ± 0.10^e	288.7 ± 0.4^e	0.003 ± 0.001^a	1.00 ± 0.01^d	2.9 ± 0.5^a		

nd: not determined. Results are expressed as mean value ± standard deviation. Different letters in the same column mean significant differences between samples ($p < 0.05$), according to Fisher's least significant difference test.

Table 3
Thickness, moisture content (MC), water solubility (WS), water vapour permeability (WVP) and glass transition temperature (Tg) of films obtained from mixtures of *phycobiliproteins* (PF) and *phycocolloids*-enriched fractions (PcF) from *P. columbina*.

Mixtures	Thickness (μm)	MC (g 100 g^{-1})	WS (%)	WVP × 10^{11} (g H_2O Pa^{-1} s^{-1} m^{-1})	Tg (°C)
0PF:100PcF	28.25 ± 1.23^{d}	11.48 ± 0.84^{a}	81.77 ± 5.38^{a}	8.90 ± 0.38^{c}	−32.7 ± 1.2^{a}
25PF:75PcF	27.23 ± 0.60^{d}	11.19 ± 0.49^{a}	80.68 ± 3.34^{a}	6.02 ± 0.53^{b}	−33.9 ± 1.1^{a}
50PF:50PcF	22.43 ± 1.00^{c}	12.08 ± 0.42^{a}	82.97 ± 2.24^{a}	3.47 ± 0.03^{a}	−34.0 ± 1.8^{a}
75PF:25PcF	17.70 ± 0.44^{b}	14.70 ± 0.38^{b}	97.65 ± 1.09^{b}	3.23 ± 0.58^{a}	−33.3 ± 1.2^{a}
100PF:0PcF	12.90 ± 0.42^{a}	20.72 ± 0.59^{c}	97.85 ± 0.80^{b}	nd	−32.0 ± 1.5^{a}

nd: not determined. Results are expressed as mean value ± standard deviation. Different letters in the same column mean significant differences between samples ($p < 0.05$), according to Fisher's least significant difference test.

extracts to different bioactive compounds, such as sulphated polysaccharides, polyphenolic compounds, proteins and peptides.

3.3. Characterisation of films

3.3.1. Thickness and water susceptibility of films

All phycobiliproteins–phycocolloids-based films were found to be homogeneous and flexible even without the addition of a plasticizer. Table 3 shows the thickness, moisture content (MC), water solubility (WS) and water vapour permeability (WVP) of the obtained films. The PcF films (0PF:100PcF) had a 28.3 μm thickness. This property significantly decreased ($p < 0.05$) as the PF proportion in films increased (from 0PF:100PcF to 100PF:0PcF), reaching 12.9 μm for the PF films (100PF:0PcF). This decrease in film thickness directly correlates with the total polysaccharides content ($r^2 = 0.972$) and the sum of proteins and polysaccharides ($r^2 = 0.974$) present in the film-forming dispersions. In contrast, the PcF films (0PF:100PcF) exhibited the lowest moisture content (~11%) and it only significantly increased in the films with a higher content of PF (75PF:25PcF and 100PF:0PcF) ($p < 0.05$). It is noteworthy that, in spite of the use of the same total solid mass and drying conditions for preparing all the films, those containing PcF were thicker even with their lower moisture content. These results indicate a higher degree of compaction of the film matrix when PF was present.

On the other hand, the films' water solubility showed a similar behaviour to MC. PcF (0PF:100PcF) films had the lowest WS (~82%) (Table 3). This property only significantly increased in the PF based film (75PF:25PcF and 100PF:0PcF) ($p < 0.05$), which became almost completely soluble in water (~98%). These findings agree with the fact that PF is composed mainly of highly water soluble compounds, such as phycobiliproteins and phenolic compounds, and suggest that these compounds would contribute to plasticize the PcF network. It is noteworthy that the high water solubility values reported in the presented work would indicate a poor water resistance, although for some applications this property could be advantageous, for example, as a carrier of bioactive compounds.

Table 3 also shows the WVP of the films, with the exception of PF films (100PF:0PcF). This film could not be handled properly to evaluate its permeability and mechanical properties due to its high hygroscopicity. On the other hand, the PcF films (0PF:100PcF) exhibited the highest WVP value (8.9 × 10^{-11}g H_2O Pa^{-1} s^{-1} m^{-1}). This parameter significantly decreased ($p < 0.05$) as the PF proportion increased in the films. The reduction achieved in WVP values was ~64% in the 75PF:25PcF films. This effect could be related to the lesser thickness of films with a high PF proportion. As reported previously for other hydrophilic films (pectin, amylose, cellulose ethers, sodium caseinate, and soybean proteins), the WVP increases with film thickness (Ghorpade, Li, Gennadios, & Hanna, 1995).

3.3.2. Mechanical properties of films

Tensile strength (TS), elongation at break (EAB) and elastic modulus (EM) of the films are shown in Fig. 2A, B and C, respectively. Mechanical properties of PcF films (0PF:100PcF) were very interesting. Even without the requirement of a plasticizer, films were flexible and had a very high TS (~50 MPa) and EM (~17.5 MPa) but a low EAB (~3%). Similar values of TS and EM were reported by Rhim (2012) for an agar/κ-carrageenan blend film.

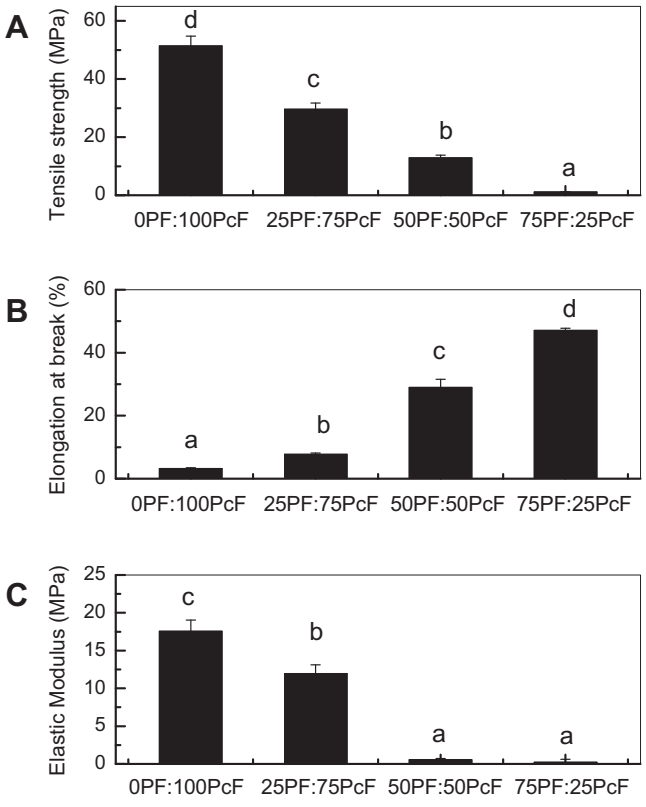

Fig. 2. Mechanical properties measured in tensile tests: (A) tensile strength (TS), (B) elongation at break (EAB), and (C) elastic modulus (EM) of films obtained from mixtures of phycobiliproteins and phycocolloids-enriched fractions (PF and PcF respectively) from *P. columbina*.

As the PF content increased in the formulation (from 0PF:100PcF to 75PF:25PcF), the films progressively decreased in their TS and EM ($p < 0.05$), and gradually increased in their EAB ($p < 0.05$). These results also suggest that PF would be acting as a plasticizer of the PcF network.

According to Sothornvit and Krochta (2000) there are two main types of plasticizers: (1) molecules capable of forming hydrogen bonds, thus interacting with polymers by interrupting polymer–polymer bonding and maintaining the furthers distance between polymers chains, (2) molecules capable of interacting with large amounts of water to retain more water molecules, thus resulting in a higher moisture content and larger hydrodynamic radius. In our case, since there was an increase in the MC and WS of films

Table 4
CIE-Lab colour parameters (L^*, a^* and b^*), total colour difference (ΔE^*), chroma (C^*), hue (h), opacity and antioxidant capacity (TEAC) of films obtained from mixtures of *phycobiliproteins* and *phycocolloids*-enriched fractions (PF and PcF respectively) from *P. columbina*.

Mixtures	Color parameters						Opacity (UA/cm)	TEAC (μg Trolox g^{-1} dry solids)
	L^*	a^*	b^*	ΔE^*	C^*	h		
0PF:100PcF	67.1 ± 0.2[a]	−4.9 ± 0.0[a]	31.8 ± 0.1[a]	43.1 ± 0.2[c]	32.2 ± 0.2[a]	98.7 ± 0.1[e]	4.9 ± 0.1[d]	74.5 ± 0.5[a]
25PF:75PcF	70.8 ± 1.0[b]	−2.8 ± 0.1[b]	32.0 ± 0.7[a]	40.5 ± 1.0[b]	32.1 ± 0.9[a]	94.9 ± 0.1[d]	4.1 ± 0.3[c]	133.5 ± 2.9[b]
50PF:50PcF	72.4 ± 0.7[c]	−0.1 ± 0.0[c]	33.2 ± 0.1[b]	40.3 ± 0.6[b]	33.2 ± 0.1[a]	90.2 ± 0.1[c]	4.1 ± 0.0[c]	216.3 ± 0.3[c]
75PF:25PcF	74.3 ± 0.4[d]	2.9 ± 0.1[d]	33.9 ± 1.5[b]	39.8 ± 0.3[b]	34.0 ± 2.1[a]	85.2 ± 0.3[b]	3.1 ± 0.1[b]	256.0 ± 0.0[d]
100PF:0PcF	78.4 ± 1.1[e]	4.7 ± 0.1[e]	33.5 ± 0.4[b]	37.4 ± 0.8[a]	33.8 ± 0.6[a]	81.9 ± 0.1[a]	1.8 ± 0.0[a]	289.0 ± 2.7[e]

Results are expressed as mean value ± standard deviation. Different letters in the same column mean significant differences between samples ($p < 0.05$), according to Fisher's least significant difference test.

and a decrease in thickness with increasing the proportion of PF (see Table 3), the plasticizing effect of PF could not be attributed to only one of the described mechanisms.

Moreover, molecules often used as plasticizers in materials based on biopolymers are small, such as glycerol, sorbitol, and even water, but larger molecules may also act as a plasticizer, if their presence interferes with the formation of a more cross-linked matrix or increase its water content (because water acts as a plasticizer in these materials). In this way, the addition of a bovine plasma hydrolysate (65% protein content and 19.4% hydrolysis degree) to formulations based on soybean or sunflower protein, besides activating the respective films with interesting antioxidant properties, caused a decrease in tensile strength, elastic modulus and glass transition temperature of the films, as well as an increase in their elongation at break and water vapour permeability. Salgado et al. (2011) suggested that this hydrolysate had a plasticizing effect on film properties despite its wide distribution of molecular weights (14 kDa < MW < 43 kDa).

However, plasticizing effect of PF in PF:PcF resulting films was not observed by DSC analysis (Table 3). All PF:PcF films had no significant differences in glass transition temperatures (Tg ~ −33 °C) ($p > 0.05$). Other assays, such as dynamic mechanical analysis (DMA) should be performed in order to confirm the PF-plasticization hypothesis.

3.3.3. Optical properties of films

CIE-Lab colour parameters (L^*, a^* and b^*), total colour difference (ΔE^*), chroma (C^*), hue (h) and opacity of films are shown in Table 4. These results give an idea of the visual aspect of the developed materials. PcF films (0PF:100PcF) had the lowest L^* and a^* values and the highest ΔE^* and h values ($p < 0.05$), indicating that their colour tends to be green (a^* negative). On the other hand, PF films (100PF:0PcF) tend to be a light red colour (a^* positive). As the PF proportion increased in film formulations, L^* and a^* parameters gradually increased ($p < 0.05$) while ΔE^* and h values progressively decreased ($p < 0.05$), indicating the film changes from a dark green to a light red. Two relationships between a^* or h and PF content in developed films were found. The Pearson correlation coefficients were $r = 0.998$ and $r = -0.998$ for a^* and h, respectively. These results could be due to the addition of R-PE given by PF fraction to the PF:PcF films. This phycobiliprotein is a red pigment involved in light harvesting in red algae (Niu et al., 2010). As mentioned above, with increasing PF ratio, the protein content increases, therefore the R-PE content in the PF films (100PF:0PcF) is higher than that of PcF films (0PF:100PcF) (see Tables 1 and 2).

Also, the addition of the PF fraction decreased the opacity of the resulting films ($p < 0.05$) (Table 4). In this sense, an inverse relationship between the L^* value and opacity was obtained ($r = -0.986$). Therefore, PF films (100PF:0PcF) were lighter and less opaque than the PcF ones (0PF:100PcF).

Undoubtedly, intense colour limits some potential applications of these materials in food packaging. For example, they could not be used for products that should be easily visible through the package (such as minimally processed vegetables) because the impaired visualisation may reduce the acceptability to potential consumers. In contrast, such films could be used, if their properties are adequate, for applications in which colour is irrelevant or in those in which colour may have an additional usefulness, as in the case of packaging products that are sensitive to visible light radiations (i.e. that can be altered or degraded by 400–800 nm light radiations), such as dairy products, baby food, soy based sauces, nutritional or medicinal products (Krikor, Tarrago, & Jansen, 2008; Salgado et al., 2010).

3.3.4. Antioxidant capacity of film

The antioxidant capacity (TEAC) of phycobiliprotein-phycocolloids-based films is shown in Table 4. The PcF films (0PF:100PcF) exhibited the lowest TEAC, while the PF films (100PF:0PcF) presented the highest activity ($p < 0.05$). The TEAC of the PF:PcF films was PF-concentration dependent ($p < 0.05$). Also, there were no significant differences between the TEAC from films and film-forming dispersions ($p > 0.05$) (see Tables 4 and 2). This means that the drying process did not affect the antioxidant capacity present in original film-forming dispersions. This result is very important because it indicates that the bioactive compounds do not undergo degradation by heat at 60 °C over 5 h. In this sense, a direct relationship between TEAC and TPC ($r^2 = 0.9998$) or TEAC and R-PE ($r^2 = 0.9942$) supplied by PF was obtained. These results are in agreement with those reported by Matanjun et al. (2008) for eight species of seaweeds from north Borneo. As mentioned above, the sulphated polysaccharides also have antioxidant capacity; however, an inverse relationship between total carbohydrate and TEAC from films was obtained ($r^2 = 0.9874$). This result would confirm that the TEAC of films is due mainly to phenolic compounds and phycobiliproteins supplied by PF.

4. Conclusions

Usually, PF, enriched in phycobiliproteins, is discarded during phycocolloids extraction. However, the excellent antioxidant properties of this fraction made it a potential byproduct. Moreover, PcF, mainly composed by phycocolloids, extracted from red seaweed without an ulterior purification steps gives a low cost potential source of hydrocolloids for producing an edible film. In this sense, it was possible to prepare edible films naturally activated with antioxidant properties from mixtures of both fractions. In particular, PcF films had excellent mechanical properties and PF films showed important antioxidant capacity. Films prepared by mixing different proportions of PF:PcF fractions showed intermediate properties which correlated with its formulation.

Taking into account that the antioxidant activity of phycobiliproteins and phenolic compounds was preserved during film forming processing (drying step), these natural polymeric matrices may

be evaluated for protecting other bioactive compounds added to any specific purpose.

Acknowledgements

The authors are thankful to the National Agency of Scientific and Technological Support (SECyT, PICT-2010-1837), the Universidad Nacional de La Plata (11-X494) and the Universidad Nacional del Litoral (CAI + D 2009-PI-54-258) for their financial support.

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