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Rice bran protein-based films enriched by phenolic extract of fermented rice bran and montmorillonite clay

Películas a base de proteínas de salvado de arroz enriquecidas por el extracto fenólico de salvado de arroz fermentado y por la arcilla montmorillonítica

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Proteins extracted from rice bran were used to prepare bio-base films where a factorial experimental design was performed in order to evaluate the effect of protein and glycerol concentrations, and the addition of phenolic extract and montmorillonite (MMT) clay on their physicochemical properties. The phenolic extract was obtained from fermentation of rice bran in solid state with the fungus *Rhizopus oryzae*. Results showed that protein concentration affected ($p < 0.05$) the luminosity, opacity and solubility of the films. An increase of glycerol concentration strongly affects ($p < 0.05$) the values of mechanical properties and water vapor permeability of the films. The addition of phenolic extract to the films affected ($p < 0.05$) opacity, tensile strength, elongation, Young's modulus and water vapor permeability, while addition of MMT decreased ($p < 0.05$) the mechanical properties of the films. Films presented solubility values of less than 25%, luminosity above 80 (L^*), opacity above 14%, tensile strength of 8.6 MPa, elongation of 70%, elasticity above 600 MPa and water vapor permeability of 7.5 g.mm/d.m^2.kPa. These results show that rice bran protein can be used in the production of bio-based films to further use in food applications.

Keywords: rice bran; protein; phenolic extract; montmorillonite clay; bio-based films

Con el fin de evaluar el efecto que sobre las propiedades fisicoquímicas de las proteínas extraídas del salvado de arroz tienen distintas concentraciones de proteína y de glicerol, así como la adición de extracto fenólico y de arcilla montmorillonítica, las mismas se utilizaron para preparar películas de base biológica en el marco de un diseño experimental factorial. Mediante la fermentación de salvado de arroz en estado sólido con el hongo *Rhizopus oryzae,* se obtuvo el extracto fenólico. Los resultados demuestran que la concentración proteica afectó significativamente ($p < 0,05$) la luminosidad, la opacidad y la solubilidad de las películas. Asimismo, un aumento de la concentración de glicerina afectó fuertemente ($p < 0,05$) los valores de las propiedades mecánicas y de la permeabilidad al vapor de agua de las mismas. La adición de extracto fenólico a las películas afectó de manera significativa ($p < 0,05$) la opacidad, la fuerza de tensión, el alargamiento, el módulo de Young y la permeabilidad al vapor de agua, mientras que la adición de montmorillonita disminuyó ($p < 0,05$) sus propiedades mecánicas. Se constató que las películas mostraron valores de solubilidad inferiores a 25%, de luminosidad superiores a 80 (L*), de opacidad superiores a 14%, de fuerza de tensión de 8,6 MPa, de alargamiento de 70%, de elasticidad superiores a 600 MPa y de permeabilidad al vapor de agua de 7,5 g.mm/d.m^2.kPa. Estos resultados demuestran que, en el ámbito de los alimentos, la proteína de salvado de arroz puede utilizarse para la producción de películas de base biológica con el fin de adjudicarle usos adicionales.

Palabras claves: salvado de arroz; proteína; extracto fenólico; arcilla montmorillonítica; películas de base biológica

Introduction

Edible films can be used as semipermeable barriers in food with various purposes, such as: control respiration rate, retard moisture loss and color variation, improve texture and mechanical integrity, help retaining flavor and inhibit growth of micro-organisms (Olivas & Barbosahy-Canovas, 2009). Thus, development of edible or biodegradable films arises from the demand for high quality and safe food, as well as from environmental concerns with the disposal of non-renewable materials. It is also an opportunity to create a new market of raw materials for packaging (Fakhouri et al., 2007).

Proteins of plant origin are more often used than animal proteins for films' production due to availability and lower cost (Adebiyi, Adebiyi, Jin, Ogawa, & Muramoto, 2008; Rojas-Grau, Soliva-Fortuny, & Martín-Belloso, 2009). The byproducts from the cereal agro-industrial processing may be a source of protein that can be recovered for the production of protein-based films. An example is rice bran, resulting from the processing of the grain, which proteins were extracted to produce bio-based films (Adebiyi et al., 2008; Cipolatti et al., 2012; Oliveira et al., 2011).

The choice of film-forming materials will depend on the purpose, the nature of the product and the particular application sought. In the case of proteins, they require the use of plasticizers to improve processability, strength and elasticity of the films, being glycerol one of the most used plasticizers (Adebiyi et al., 2008; Cao, Fu, & He, 2007; Dangaran, Tomasula, & Qi, 2009).

Organic-inorganic hybrid systems, particularly those in which silicates are dispersed in a polymeric matrix, have been used in the formulation of films to improve their properties. MMT, characterized by its moderate negative surface charge (Tunc et al., 2007), has been included in the formulation of bio-based films and showed to improve mechanical strength, heat resistance and barrier properties of films (Chen & Zhang, 2006; Martins et al., 2012).

In addition, bio-based films have a great potential to carry active ingredients such as anti-browning agents, colorants,

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flavors, nutrients, spices and antimicrobial compounds that may extend shelf life reducing the risk of pathogens growth on food surfaces (Rojas-Grau et al., 2009). Phenolic compounds from rice bran fermentation have high antioxidant and antimicrobial activity (Oliveira, Dors, Souza-Soares, & Furlong, 2007; Souza, Oliveira, Rocha, & Furlong, 2010) with potential to be applied in the preparation of edible films. This study evaluated the effect of glycerol, phenolic extract and MMT clay on the physicochemical properties of films of protein obtained from rice bran.

Materials and methods

Rice bran protein concentrate (RBPC) extraction

Rice bran was provided by companies in Rio Grande do Sul (Brazil). The protein concentrate used in films preparation was obtained from rice bran using the methodology adapted from Adebiyi et al. (2008). The rice bran samples (particle size 32 mesh) were degreased using petroleum ether (three times in the ratio 1:7 w/v) under orbital shaking at 100 rpm for 1 h at 25°C. After evaporation of excess petroleum ether (oven with air circulation at 40°C for 30 min), defatted rice bran was subjected to protein extraction in an alkaline medium (pH adjusted to 11.5 with 0.1 mol/L NaOH solution in the ratio 1:10 w/v for 30 min at 25°C) using an orbital shaking at 200 rpm. After centrifugation (15,200 *g*, 30 min, 10°C), the supernatant was collected and the residue submitted to a second extraction. The supernatants from both extractions were pooled and the extracted proteins were precipitated by adjusting the solution pH to 4.5 with a solution of 0.1 mol/L HCl. After standing overnight at 4°C, the proteins were separated by centrifugation (15,200 *g*, 30 min, 10°C), followed by washing the precipitate with distilled water. The protein concentrate was stored at −18°C until the physicochemical characterization and application. The quantification of protein (n° 955.04C; conversion factor of 5.7), lipid (n° 920.85), ash (n° 900.02A) and total fiber (n° 962.09) were determined according to the AOAC method (2000) and total carbohydrates estimated by difference.

Phenolic extract

The phenolic extract was obtained from solid-state fermentation of rice bran with the fungus *Rhizopus oryzae* (CCT 1217) according to the method proposed by Oliveira et al. (2011). Rice bran samples were incubated with a solution of 4×10^6 spores/g of bran in trays (12 cm × 8 cm × 4 cm) for 96 h at 30°C, whereas 45 mL of a nutrient solution (2 g/L of KH_2PO_4, 1 g/L of $MgSO_4$ and 5 g/L $(NH_4)_2SO_4$) was added for each 100 g of bran. The humidity of the medium was adjusted to 50% with sterile distilled water.

The phenolic compounds from fermented rice bran were extracted with methanol at the ratio 1:10 (w/v), according to the method described by M. Souza et al. (2010); 5 g aliquots were subjected to orbital shaking (150 rpm) for 3 h with methanol and filtered to obtain a crude extract that was subjected to a partition with 10 mL hexane (3 times). The organic solvent was evaporated on a rotary-evaporator at 50°C under reduced pressure and the phenolic compounds were resuspended with distilled (10 mL) water in an ultrasonic bath for 10 min. The resulting extract was clarified with 5 mL of 0.1 M $ZnSO_4$ and 5 mL $Ba(OH)_2$ 0.1 mol/L and allowed to rest for 20 min. After centrifugation (3200 *g*, 10 min, 25°C) the supernatant containing the phenolic compound was collected, lyophilized and quantified spectrophotometrically at 750 nm with Folin-Ciocalteau reagent using a standard curve of ferulic acid (2–20 mg).

Experimental design and effects analysis

The RBPC was used as the matrix for protein- films preparation being glycerol used as plasticizer. Phenolic compounds derived from rice bran fermentation and MMT were used as additives. A 2^4 factorial experimental design was performed to determine the effect of the concentration of each component in the films properties (Table 1). The results were evaluated by analysis of effects and response surface using *Statistica 7.0* software. Response surfaces that showed a correlation coefficient (R) greater than 0.9 and calculated F value for the mathematical model of at least two times higher than that of Fisher's F were generated, considering only the significant effects ($p < 0.05$) in each property.

Preparation of films

The RBPC obtained from rice bran and the phenolic extract (PE) obtained from rice bran fermentation were used in films' preparation, together with glycerol and MMT clay. The RBPC was resuspended in alkaline medium using a solution of 0.1 mol/L NaOH and adjusted to pH 10. The solution was heated to 85°C and the glycerol and MMT (pre-hydrated with distilled water for 16 h before being added) added keeping the mixture under stirring (150 rpm) for 30 min. The mixture was cooled to 40°C before addition of the phenolic extract and kept under magnetic stirring for 10 min and then was kept in an ultrasonic bath for 5 min to remove air bubbles. The films were casted by adding 20 mL of film solution in polystyrene Petri dishes (9 cm diameter) and dried in an oven with circulating air at 30°C for approximately 24 h. The films were kept in a desiccator containing a saturated solution of NaBr (58% RH) for 96 h before testing.

Characterization of the films

Solubility

Film samples with 2 cm diameter were dried at 105°C until constant weight, and then they were subsequently immersed in 50 mL distilled water and subjected to orbital shaking (70 rpm) at 25°C for 24 h. After this, samples were filtered on porous crucible with glass wool and dried to constant weight at 105°C. Solubility was determined by weight difference between the dry

Table 1. Levels of the independent variables used in experimental design.

Tabla 1. Niveles de las variables independientes usadas en el diseño experimental.

	Levels		
Factor	−1	0	+1
Protein (g/L)	20	30	40
Glycerol ($g/kg_{protein}$)	200	300	400
PE ($g/kg_{protein}$)	0	20	40
MMT ($g/kg_{protein}$)	0	50	100

Note: PE, phenolic extract; MMT, montmorillonite clay.

mass that was not solubilized in water and the initial mass. Three replicates were obtained for each film.

Optical properties

Luminosity (L*) and opacity (Op) parameters were determined using a Minolta colorimeter. Films' samples were overlapped to a white standard (Y = 93.5, x = 0.3114, y = 0.3190) for the measurement of luminosity. Opacity was calculated as the ratio of film opacity superimposed on a standard black and a standard white, as proposed by Carvalho and Grosso (2006), according to Equation (1):

$$\mathrm{Op}(\%) = \frac{P_{Black}}{P_{White}} \times 100 \quad (1)$$

where: Op = opacity; P_{Black} = Y coordinate value in black standard, P_{White} = Y coordinate value of the white standard. Tests were replicated at least six times for each film.

Mechanical properties

The tensile strength (TS) and percent elongation (%E) of films were obtained from the force deformation curves determined using a texturometer (TA.XT *plus*, Stable Micro Systems, Godalming, England) operating according to the standard method D882–02 (ASTM, 2001), with an initial separation of the jaws of 50 mm and a testing speed of 100 mm/min. The films were cut to form specimens of 80 mm long and 25 mm wide. The Young's modulus (YM) was calculated from the tangent of the initial linear portion of the stress–strain plot (Cao et al., 2007). TS, %E and YM tests were replicated at least five times for each type of film. Film thickness was measured using a digital micrometer (Insize, IP54, São Paulo, Brazil) with a sensitivity of 0.001 mm.

Water vapor permeability

The water vapor permeability (WVP) of the films was determined using an adaptation of the methodology proposed by Andreuccetti, Rosemary, Galicia-García, Martínez-Bustos, and Grosso (2011). Approximately 10 g of $CaCl_2$ (0% RH) were placed in polystyrene containers (area = 18.85 cm^2), sealed with the films and placed in a desiccator containing a saturated solution of NaCl (75% RH) at 25°C (films had initial moisture content of 149 ± 32 g/Kg). Every 24 h during 7 days the weight of the containers was taken. The water vapor transmission rate (WVTR) of the films was determined by the angular coefficient of the weight gain curve of water versus time. WVP was determined according to Equation (2):

$$\mathrm{WVP} = \mathrm{WVTR}\left(\frac{x}{\Delta P}\right) \quad (2)$$

where: WVTR = water vapor transmission rate, x = thickness; ΔP = partial vapor pressure difference. Three replicates were obtained for each film.

Scanning electron microscopy

The surface morphology of the films was examined using scanning electron microscopy (SEM) (Nova NanoSEM 200, Eindhoven, The Netherlands). Film samples, after drying and the stabilization process (described in *Preparation films* section), were mounted on aluminum base using carbon tape and coated by gold sputtering (approximately 10 nm in thickness).

Fourier-transform infrared (FTIR) spectroscopy

The infrared spectrum of the films was determined using the Infrared Fourier transform (Perkin-Elmer 16 PC spectrometer, Boston, USA) using the attenuated total reflectance technique and spectral analysis in the range between 4000 and 600 cm^{-1} using 16 scans. Signal averages were obtained at a resolution of 4 cm^{-1}.

Results and discussion

Characterization of the raw material

Rice bran, a by-product of rice processing, was extracted and used as main material for the production of bio-based films production. Table 2 shows rice bran and rice bran concentrate composition. Results show that rice bran has the protein content above 165 g/kg. RBPC was obtained from rice bran with protein content above 700 g/kg (dry basis).

The phenolic content increased about 80% during fermentation (Table 2). Phenolic compounds contained in cell walls of rice bran are linked to carbohydrates and lignin (Pourali, Asghari, & Yoshida, 2010) that during fermentation are released from structural rice bran by the action of microorganisms (Schmidt & Furlong, 2012). Phenols are prevalent in rice phenolic acids, mainly ferulic and *p*-coumaric acid, which have high antioxidant and antimicrobial activities (Schmidt, Gonçalves, Prietto, Hackbart, & Furlong, 2014; M. Souza et al., 2010), thus providing a potential ingredient for use in edible films.

Experimental design

Tables 3 and 4 showed that films properties are strongly affected by the materials used to prepare the film forming solution. Data obtained from experimental design were fitted as a function of the dependent variables that presented statistical significant effects ($p < 0.05$). To verification of mathematical models generated for each property, an analysis of variance (ANOVA) was performed (Table 5). The response surfaces were generated for WVP, opacity, YM and TS (Figure 1) that presented statistically significant models (Montgomery, 1991). Solubility showed a low variation explained by model, below 67%, while the luminosity although had an F ratio value high and a variance of 88%, being the protein concentration factor the only significant ($p < 0.05$) factor.

Table 2. Composition of rice bran and rice bran protein concentrated on dry basis.

Tabla 2. Composición del salvado de arroz y del concentrado de proteína de salvado de arroz con base seca.

Component (g/kg)	RB	RBPC
Protein	164.9 ± 6.9	716.7 ± 53.2
Lipids	188.6 ± 6.1	5.2 ± 3.1
Ash	94.1 ± 1.0	29.2 ± 3.3
Carbohydrate	552.6 ± 10.5	249.0 ± 39.8
Phenolic content	RB	RBF
g/kg	2.41 ± 0.01	4.33 ± 0.06

Note: Values are expressed as means ± SD. RB, rice bran; RBPC, rice bran protein concentrated; RBF, rice bran fermented.

Table 3. Values of solubility, lightness, opacity, tension strength, elongation, Young's modulus and water vapor permeability for each film formulation.

Tabla 3. Valores de solubilidad, de ligereza, de opacidad, de fuerza de tensión, de alargamiento, de los módulos de Young y de permeabilidad al vapor de agua para cada fórmula de película.

	[P]	[G]	[PE]	[MMT]	Solubility	Lightness	Opacity	TS	Elongation	YM	WVP
Sample	g/L	$g/kg_{protein}$	$g/kg_{protein}$	$g/kg_{protein}$	%	L*	%	MPa	%	MPa	$g.mm/d.m^2.kPa$
1	−1 (20)	−1 (200)	−1 (0)	−1 (0)	37.2 ± 3.1	76.9 ± 0.8	18.1 ± 1.5	5.2 ± 0.2	26.1 ± 3.7	216.0 ± 81.4	7.7 ± 0.2
2	1 (40)	−1 (200)	−1 (0)	−1 (0)	24.7 ± 0.8	61.6 ± 4.7	26.5 ± 2.0	6.5 ± 1.0	7.8 ± 0.9	218.6 ± 31.3	10.2 ± 1.2
3	−1 (20)	1 (400)	−1 (0)	−1 (0)	29.4 ± 2.5	81.0 ± 2.4	15.5 ± 1.0	3.3 ± 0.5	29.0 ± 0.2	84.5 ± 4.1	11.7 ± 1.2
4	1 (40)	1 (400)	−1 (0)	−1 (0)	30.1 ± 0.4	63.9 ± 3.8	23.3 ± 1.7	4.3 ± 0.2	52.7 ± 4.2	98.7 ± 2.3	17.1 ± 0.4
5	−1 (20)	−1 (200)	1 (40)	−1 (0)	36.0 ± 1.7	77.7 ± 4.3	15.4 ± 1.5	8.6 ± 1.9	1.6 ± 0.9	617.5 ± 97.4	10.6 ± 0.3
6	1 (40)	−1 (200)	1 (40)	−1 (0)	31.6 ± 2.1	66.3 ± 3.6	19.6 ± 1.7	6.9 ± 0.7	0.8 ± 0.2	551.9 ± 94.3	10.4 ± 0.2
7	−1 (20)	1 (400)	1 (40)	−1 (0)	36.4 ± 1.3	80.1 ± 2.5	14.1 ± 1.4	3.3 ± 0.6	33.4 ± 4.2	51.3 ± 2.4	11.8 ± 1.1
8	1 (40)	1 (400)	1 (40)	−1 (0)	34.5 ± 1.5	65.3 ± 4.3	20.1 ± 1.7	2.3 ± 0.2	56.8 ± 6.9	41.1 ± 4.1	21.3 ± 1.1
9	−1 (20)	−1 (200)	−1 (0)	1 (100)	33.7 ± 1.0	80.5 ± 2.6	16.3 ± 1.4	3.1 ± 0.1	17.9 ± 1.1	57.7 ± 11.6	7.5 ± 0.5
10	1 (40)	−1 (200)	−1 (0)	1 (100)	28.1 ± 0.7	60.2 ± 5.6	26.9 ± 3.5	5.8 ± 0.7	4.9 ± 2.3	147.4 ± 3.2	9.3 ± 0.9
11	−1 (20)	1 (400)	−1 (0)	1 (100)	40.2 ± 5.1	80.2 ± 1.7	15.4 ± 1.1	1.5 ± 0.1	31.2 ±4.1	16.7 ± 2.9	13.1 ± 1.3
12	1 (40)	1 (400)	−1 (0)	1 (100)	30.2 ± 1.6	65.0 ± 3.7	23.5 ± 2.2	2.4 ± 0.1	31.2 ± 6.8	46.0 ± 5.9	19.5 ± 0.6
13	−1 (20)	−1 (200)	1 (40)	1 (100)	31.7 ± 1.5	73.4 ± 8.7	16.5 ± 1.8	6.1 ± 0.7	1.6 ± 0.4	261.4 ± 69.6	7.5 ± 0.6
14	1 (40)	−1 (200)	1 (40)	1 (100)	26.1 ± 0.6	64.0 ± 3.7	21.7 ± 1.8	7.6 ± 0.3	1.6 ± 0.3	318.9 ± 55.5	10.8 ± 1.3
15	−1 (20)	1 (400)	1 (40)	1 (100)	37.8 ± 0.9	79.3 ± 2.4	16.4 ± 0.8	2.3 ± 0.1	22.2 ± 3.7	30.9 ± 2.4	12.2 ± 1.9
16	1 (40)	1 (400)	1 (40)	1 (100)	32.2 ± 0.4	64.2 ± 1.6	21.8 ± 0.5	2.4 ± 0.1	30.9 ± 3.7	35.8 ± 2.3	24.8 ± 2.1
17	0 (30)	0 (300)	0 (20)	0 (50)	36.3 ± 0.9	76.4 ± 1.9	16.4 ± 1.5	2.5 ± 0.2	69.6 ± 1.2	33.8 ± 4.8	12.8 ± 0.5
18	0 (30)	0 (300)	0 (20)	0 (50)	34.2 ± 0.5	74.9 ± 2.2	17.7 ± 1.3	2.3 ± 0.3	68.5 ± 9.1	24.3 ± 3.7	13.7 ± 1.5
19	0 (30)	0 (300)	0 (20)	0 (50)	35.8 ± 0.2	74.1 ± 1.6	16.7 ± 1.2	2.2 ± 0.2	70.1 ± 16.6	23.7 ± 1.0	12.8 ± 0.9

Note: [P], protein concentration; [G], glycerol concentration; [PE], phenolic extract concentration; [MMT], montmorillonite concentration; TS, tensile strength; YM, Young's modulus; WVP, water vapor permeability.

Table 4. Effect of protein, glycerol, phenolic extract and montmorilonite clay concentration on films properties.

Tabla 4. Efecto de las concentraciones de proteína, de glicerol, de extracto fenólico y de arcilla montmorillonítica en las propiedades de las películas.

	Solubility (%)		Lightness (L*)		Opacity (%)		TS (MPa)		Elongation (%)		YM (MPa)		WVP ($g.mm/d.m^2.kPa$)	
Factor	Effect	*p*-value	Effect	*p*-value	Effect	*p*-value	Effect	*p*-value	Effect	*p*-value	Effect	*p*-value	Effect	*p*-value
Mean	32.96	0.0001	71.83	<0.0001	19.04	0.0001	4.14	0.0001	29.36	<0.0001	151.37	0.0001	12.87	0.0001
[P]	−5.61	0.0093	−14.82	0.0015	6.98	0.0026	0.56	0.0212	2.97	0.0180	15.29	0.0329	5.16	0.0025
[G]	2.70	0.0386	2.29	0.0580	−1.35	0.0629	−3.51	0.0006	28.15	0.0002	−248.06	0.0001	7.18	0.0013
[PE]	1.58	0.1014	0.11	0.8625	−2.51	0.0196	0.93	0.0079	−6.48	0.0039	127.92	0.0005	1.68	0.0230
[MMT]	0.02	0.9778	−0.73	0.3311	0.74	0.1721	−1.12	0.0055	−8.32	0.0024	−120.62	0.0006	0.49	0.1984
[P] × [G]	1.42	0.1215	−0.73	0.3328	−0.14	0.7307	−0.34	0.0545	11.00	0.0013	−5.77	0.1800	3.29	0.0062
[P] × [PE]	1.24	0.1515	2.16	0.0648	−1.75	0.0391	−0.87	0.0090	4.86	0.0068	−18.64	0.0225	1.16	0.0469
[P] × [MMT]	−1.09	0.1842	−0.16	0.8086	0.36	0.4187	0.71	0.0135	−4.05	0.0098	30.06	0.0088	0.86	0.0797
[G] × [PE]	1.17	0.1659	−0.41	0.5501	1.15	0.0836	−1.20	0.0048	6.27	0.0041	−149.58	0.0004	0.51	0.1892
[G] × [MMT]	2.51	0.0442	0.35	0.6044	0.30	0.4902	−0.01	0.9109	−5.75	0.0049	84.05	0.0011	1.44	0.0308
[PE] × [MMT]	−2.72	0.0379	−1.36	0.1420	1.07	0.0954	0.48	0.0290	−0.76	0.2005	−33.12	0.0073	−0.19	0.5399

Note: [P], protein concentration; [G], glycerol concentration; [PE], phenolic extract concentration; [MMT], montmorillonite concentration; TS, tensile strength; YM, Young's modulus; WVP, water vapor permeability.

Effect of protein concentration on films properties

The inherent properties of proteins make them excellent materials for films productions. The distribution of charged, polar and non-polar amino acids along the protein chain creates a potential chemical interaction between materials, resulting in a cohesive protein film matrix. Films are formed and stabilized through electrostatic interactions, hydrogen bonding, van der Waals forces, covalent bonding and disulfide bridges (Dangaran et al., 2009). Table 4 shows that protein concentration was the most important factor affecting solubility of the films, resulting in a decrease of 10% in solubility values, being obtained some films with solubility below 25%. This behavior is explained by the increasing protein–protein interactions caused by the presence of intermolecular disulfide bonds (Gounga, Xu, & Wang, 2007). However, films' solubility values were lower than those obtained from other protein sources such as whey (Ozdemir & Floros, 2008), soy protein isolate and cod gelatin (Denavi et al., 2009), which presented minimal solubility values of 37%, 84% and 87%, respectively.

Opacity is indicative of the light passing through a material (Lima et al., 2010) and can be used to control the incidence of light on a food protecting it against oxidative processes. Protein concentration was the factor that most affected the optical properties of the films, being the only factor that affected luminosity ($p < 0.05$). Luminosity values of the films were similar to those reported by Guerrero, Stefani, Ruseckaite, and Caba (2011) for

Table 5. ANOVA of the films properties using a confidence interval of 95%.

Tabla 5. Análisis de varianza ANOVA de las propiedades de las películas utilizando un intervalo de confianza de 95%.

Properties	Sources of variations	SS	DF	MS	*F* ratio	R^2
Solubility (%)	Regression	209.55	4	52.39	2.33	0.67
	Residual	101.31	14	7.24		
	Total	310.86				
Lightness (L*)	Regression	877.94	1	877.94	29.08	0.88
	Residual	115.31	17	6.78		
	Total	993.25				
Opacity (%)	Regression	232.19	3	77.40	8.91	0.85
	Residual	39.64	15	2.64		
	Total	271.83				
Tensile strength (MPa)	Regression	70.77	8	8.85	2.08	0.84
	Residual	13.84	10	1.38		
	Total	84.61				
Elongation (%)	Regression	4582.66	9	509.18	0.24	0.44
	Residual	5937.78	9	659.75		
	Total	10520.44				
Young's modulus (MPa)	Regression	497870.18	9	55318.91	2.23	0.88
	Residual	70322.45	9	7813.61		
	Total	568192.63				
WVP (g.mm/d.m^2.kPa)	Regression	380.92	6	63.49	14.70	0.96
	Residual	17.30	12	1.44		
	Total	398.22				

Note: Where: SS, sum of squares; DF, degrees of freedom; MS, mean square; *F* ratio, relation between test *F* value and *F* of Fisher's value at 95% confidence interval; WVP, water vapor permeability.

soy protein isolate films, with a yellowish tonality (chromaticity coordinate b*+, data not included).

The increase in protein concentration in the film forming solutions had positive effects ($p < 0.05$) on the mechanical properties and water vapor permeability of the resulting films. Proteins are very different depending on the origin, structure and amino acid composition. Protein–protein interactions involved in film formation determine the degree of crosslinking and their hydrophilic/hydrophobic properties and are also related to the physicochemical, mechanical and barrier properties of the films (Denavi et al., 2009).

Effect of glycerol concentration

Production of protein-based films generally needs the incorporation of a minimal content of plasticizer. Plasticizers act by weakening intermolecular forces between adjacent polymer chains of protein matrix reducing its brittleness. This incorporation results in an increase of film extensibility and flexibility while decreases the elasticity, mechanical resistance and barrier property of the films (Gounga et al., 2007). These modifications are shown in this study (Table 4), where the increase of glycerol concentration changes the solubility, WVP and mechanical properties of the films. The effect of glycerol on the solubility of rice bran protein films was also verified by Adebiyi et al. (2008), who found that increasing the concentration of glycerol can open the polymer structure and enhance the permeability of moisture at higher concentrations.

Addition of glycerol produced a negative effect on TS, leading to a decrease of 3.5 MPa, while its interaction with proteins promoted a positive effect on %E. With respect to elasticity (YM), the increase of glycerol concentration resulted in weakening of the connections between molecules, reducing films elasticity. The effect of increasing concentrations of glycerol on mechanical properties of biodegradable films was also reported by Cao et al. (2007), who observed that the tension and elasticity decreased while elongation increased.

With respect to WVP, the glycerol content should be the lowest possible to avoid moisture transfer between the food and the external atmosphere (Dias, Muller, Larotonda, & Laurindo, 2010). The WVP of films was mainly affected by glycerol and protein concentrations (Table 4). Increasing the concentration of these components, an increase of WVP values of the films were promoted; moreover, the interaction between these two variables also caused an increase of the permeation rate. Similar results were observed by Kokoszka, Debeaufort, Hambleton, Lenart, and Voilley (2010) for soy protein-based films. The lower values of WVP (below 7.5 g.mm/d.m^2.kPa) were obtained at low protein and glycerol concentrations (Figure 1a). An increase in the concentration of plasticizer generally causes an increase in films' WVP due to reorganization of the protein network and consequent increase in free volume (Dias et al., 2010; Gounga et al., 2007).

Effect of phenolic extract concentration

Phenolic compounds derived from rice bran fermentation have antioxidant and antimicrobial activity (Oliveira et al., 2007; M. Souza et al., 2010). The extract phenolic content added to the films, on the level of the highest concentration, was estimated upon the maximum that protein in solution could support without precipitating these proteins. This corresponded to about 25 times greater than the EC_{50} value of the extract phenolic (Schmidt et al., 2014). Therefore, antioxidant and antimicrobial effects should be expected. However, the inclusion of an additive can modify the properties of edible films due to the physical changes induced in the film's structure, promoting changes in polymer–polymer interactions, reacting at more than one site on the protein structure and inducing cross-links between proteins (Dangaran et al., 2009). Table 4 shows the effect of the addition of phenolic extract in films properties.

Optical, mechanical and WVP properties were affected by increasing phenolic extract, that in interaction with the protein concentration also affects the films' opacity ($p < 0.05$). The highest opacity value (27%) was obtained at lower phenolic extract concentrations and higher protein concentrations (Figure 1b).

Phenolic extract addition leads to a positive effect on TS and a negative effect on %E, being also the factor that most contributed to increase of YM values of films (Table 4). Figure 1c shows that the elasticity of films was greater for higher phenolic extract concentrations and lower glycerol concentrations, with values above 600 MPa, much higher than those found for films with soy protein isolate and gelatin, whose maxima were around 160 MPa (Guerrero et al., 2011).

The addition of phenolic extract to films caused a significant increase ($p < 0.05$) of WVP values (Table 4) explained by the increase of hydrophilicity of the films with the addition of phenolic extracts. Similar behavior was observed by Ou, Wang, Tang, Huang, and Jackson (2005).

Effect of MMT concentration

MMT can form stable suspensions in water due to its hydrophilic character. This property of MMT facilitates its dispersion in water soluble polymers (Tunç & Duman, 2010). Various authors have used MMT in films preparation, aiming the improvement of their physical properties. Tunç and Duman (2010) developed

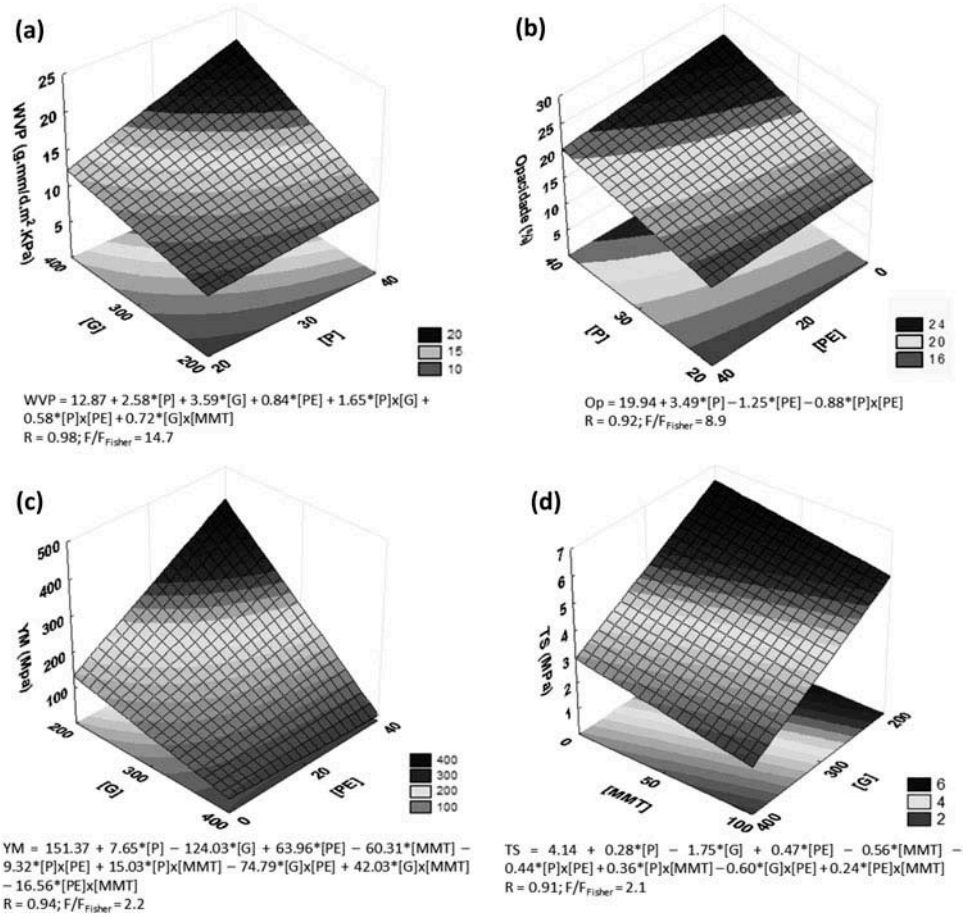

Figure 1. Response surfaces that relate: (a) water vapor permeability with protein and glycerol concentrations; (b) opacity with protein and phenolic extract concentrations; (c) Young's modulus with glycerol and phenolic extract concentrations; (d) tensile strength with glycerol and MMT clay, where: [P], protein concentration (g/L); [G], glycerol concentration ($g/kg_{protein}$); [PE], phenolic extract concentration ($g/kg_{protein}$); [MMT], montmorillonite concentration ($g/kg_{protein}$); TS, tensile strength; YM, Young modulus; WVP, water vapor permeability. R, correlation coefficient; *F,* value *F* of mathematic model; $F_{Fisher,}$ value *F* of Fisher.

Figura 1. Superficies de respuesta que relacionan: (a) la permeabilidad al vapor de agua con concentraciones de proteína y de glicerol; (b) la opacidad con concentraciones de extractos proteicos y fenólicos; (c) los módulos de Young con concentraciones de extracto fenólico y de glicerol; (d) la fuerza de tensión con el glicerol y la arcilla montmorillonítica. Donde: [P], concentración proteica (g/L); [G], concentración de glicerol ($g/kg_{protein}$); [PE], concentración de extracto fenólico ($g/kg_{protein}$); [MMT], concentración montmorillonítica ($g/kg_{protein}$); TS, fuerza de tensión; YM, módulos de Young; WVP, permeabilidad al vapor de agua. R, coeficiente de correlación; *F,* valor *F* del modelo matemático; $F_{Fisher,}$ valor *F* de Fischer.

gluten-based films with the addition of MMT and highlighted that the presence of MMT induced reduction of the water sensitivity of the films. Lee and Kim (2010) developed composite films of soy protein isolate and MMT and obtained better mechanical and permeability properties when compared with films without MMT.

The addition of MMT to the films negatively affected ($p < 0.05$) mechanical properties (Table 4), once higher values of TS (8.6 MPa) were obtained without the addition of MMT and for lower glycerol concentrations (Figure 1d). MMT addition at the levels reported here did not affect ($p < 0.05$) the other properties, only its interactions with glycerol increased solubility and WVP of films, while the interaction with the phenolic extract decreased solubility of the films.

MMT is a type of clay that has been used in the preparation of biodegradable films in order to improve their mechanical properties (Chen & Zhang, 2006). In this study the use of clay MMT showed no significant improvements in the films' properties for the reported concentrations. This can be explained by the incomplete exfoliation of the clay in the film forming solution, difficult because of the wide lateral dimension of the layers, due to high intrinsic viscosity of the polymer and the strong tendency of clay tactoids to agglomerate (Ray & Bousmina, 2005; Zhu & Wool, 2006).

Scanning electron microscopy

The microscopic observation of the surface of the films provides information on the integrity, continuity and also about the structural organization of the filmogenic polymeric matrix (Souza, Cerqueira, Teixeira, & Vicente, 2010). The surface of the films produced only with rice bran protein at low glycerol concentration showed no visibly different structural characteristics when compared to the films with phenolic extract (Figure 2a and b), where pinholes were observed on the surface, possibly due to air bubbles. Some irregularities were also observed on their surfaces, possibly due to the presence of more than one macromolecule in the polymer matrix. The RBPC had about 25% of polysaccharides in its composition (Table 2), which could

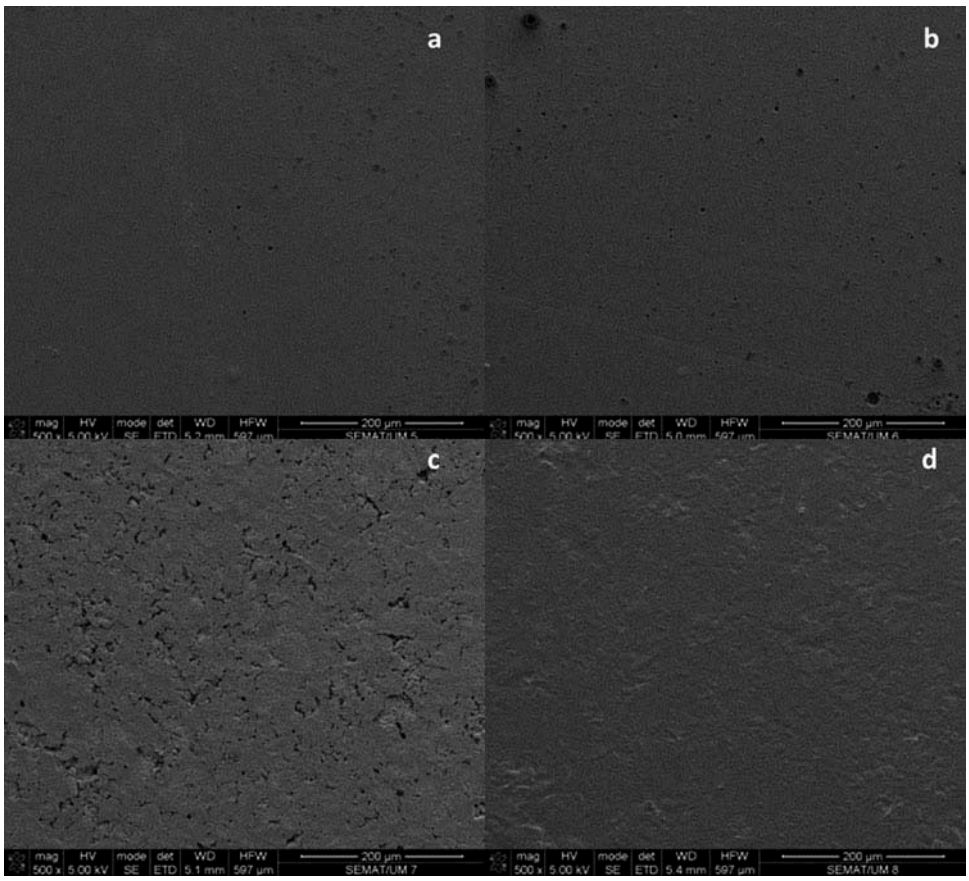

Figure 2. SEM images of surface of rice bran protein films developed from formulations containing: 20 g/L of protein and 200 g/kg$_{protein}$ of glycerol (a); 20 g/L of protein and 200 g/kg$_{protein}$ of glycerol and 40 g/kg$_{protein}$ of phenolic extract (b); 20 g/L of protein and 200 g/kg$_{protein}$ of glycerol and 100 g/kg$_{protein}$ of MMT (c); 30 g/L of protein and 300 g/kg$_{protein}$ of glycerol and 20 g/kg$_{protein}$ of phenolic extract and 50 g/kg$_{protein}$ of MMT (d).

Figura 2. Imágenes SEM de la superficie de las películas de proteína de salvado de arroz desarrolladas de fórmulas que contienen: 20 g/L de proteína y 200 g/kg$_{protein}$ de glicerol (a); 20 g/L de proteína y 200 g/kg$_{protein}$ of glicerol y 40 g/kg$_{protein}$ de extracto fenólico (b); 20 g/L de proteína y 200 g/kg$_{protein}$ de glicerol y 100 g/kg$_{protein}$ de MMT (c); 30 g/L de proteína y 300 g/kg$_{protein}$ de glicerol y 20 g/kg$_{protein}$ de extracto fenólico y 50 g/kg$_{protein}$ de MMT (d).

influence the films' homogeneity. A similar effect was reported by Dias et al. (2010) in rice flour films.

MMT addition to the films in concentrations of 5% w/v appear to correct some imperfections (Figure 2d), decreasing the porosity and forming a denser surface; however, concentrations of 10% w/v induced excessive cracks on the surfaces of the films (Figure 2c) which confirms the negative effects produced in TS, %E and YM (Table 4). When the clay is found in high concentrations, some of it may not establish interactions with the protein, being subject to loss of water during drying, thus causing cracks in the surface of the films.

FTIR spectroscopy

Infrared spectroscopy is a rapid and a non-destructive technique that has been widely used to characterize different biomaterials. Moreover, FTIR spectroscopy is a powerful technique to evaluate polymer blend miscibility. When chemical groups interact at the molecular level, changes are seen in FTIR spectra such as the shifting of absorption bands. These changes can be an indication of good miscibility of polymers as well of other chemical interactions. In this work, FTIR showed that the absorption spectra of films from different formulations had similar absorption regions, differing only in the bands' absorption intensity. Films with phenolic extract (Figure 3c) showed higher absorptions across the spectrum when compared to films without phenolic extract (Figure 3b), indicating a greater interaction of compounds explained by the presence of hydroxyls that increase the number of hydrogen bonds in the protein matrix. Absorption bands at 1600 and 1520 cm^{-1} can be attributed to C = C bond vibrations typical of aromatic systems. A strong contribution of the –OH deformation can be found in the region 1410–1260 cm^{-1}. Strong valence vibrations between 1150 and 1040 cm^{-1} overlap aromatic impressions of bands at 1225–950 cm^{-1}. CH_3 symmetric deformation vibrations occur in the region 1190–1370 cm^{-1} (Edelmann, Diewok, Schuster, & Lendl, 2001). These bands coincided with the bands presented by the RBPC (Figure 3a).

On the contrary, addition of MMT to the films decreased absorption peaks over the whole range of the spectrum (Figure 3d), especially in the region around 3400 cm^{-1} and between 1700 and 1500 cm^{-1}, regions which are characteristic of –NH absorption groups of the amino acids and amide I and II bands of proteins, indicating the interaction of MMT with these protein groups which results in a reduced vibration of these functional groups of proteins.

The spectra in the infrared region are commonly influenced by the contribution of various vibrational modes between 3000 and 3700 cm^{-1} and below 1700 cm^{-1}. Due to this, glycerol and MMT

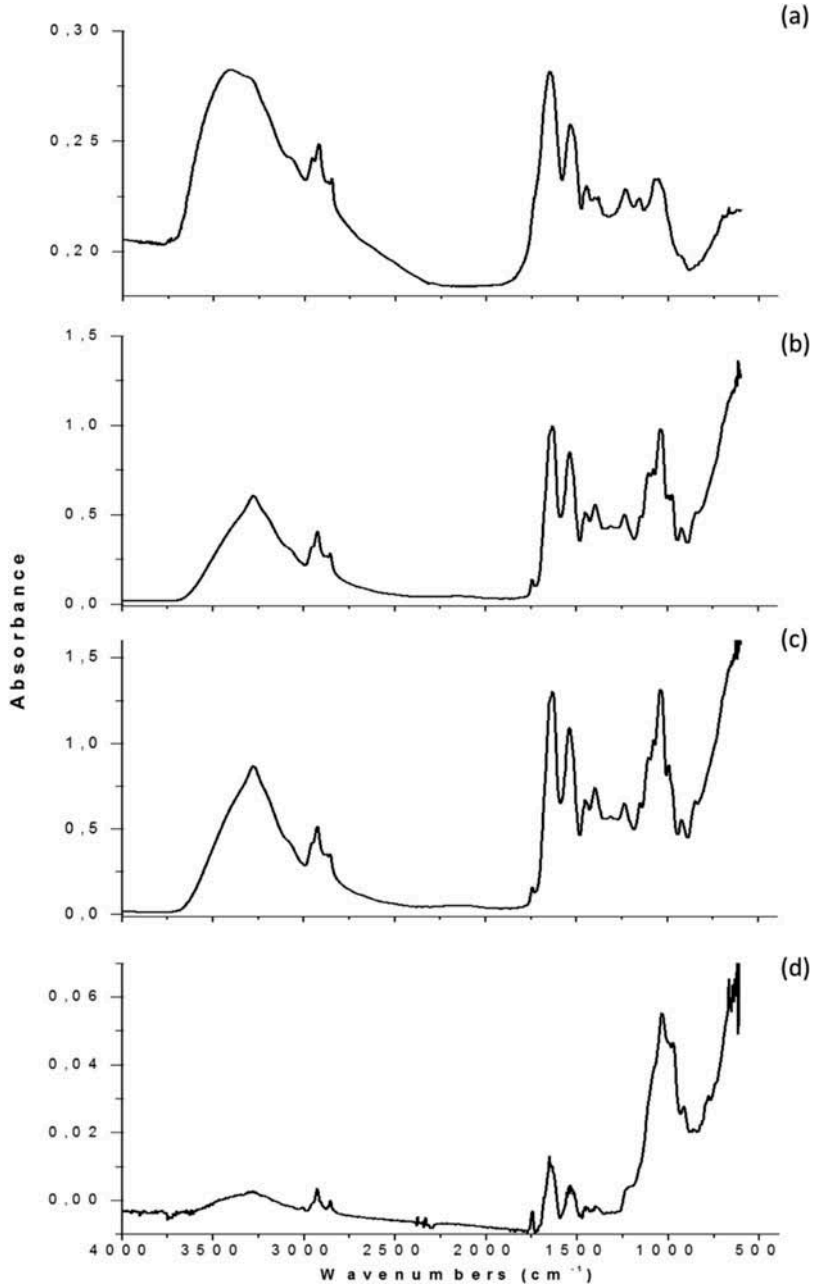

Figure 3. FTIR spectra of rice bran protein concentrated (a) and rice bran protein films developed from formulations containing 20 g/L of protein and 200 g/kg$_{protein}$ of glycerol (b); 20 g/L of protein and 200 g/kg$_{protein}$ of glycerol and 40 g/kg$_{protein}$ of phenolic extract (c); 20 g/L of protein and 200 g/kg$_{protein}$ of glycerol and 100 g/kg$_{protein}$ of MMT (d).

Figura 3. Espectro FTIR de concentrado de proteína de salvado de arroz (a) y películas de proteína de salvado de arroz desarrolladas a partir de fórmulas que contienen: 20 g/L de proteína y 200 g/kg$_{protein}$ de glicerol (b); 20 g/L de proteína y 200 g/kg$_{protein}$ de glicerol y 40 g/kg$_{protein}$ de extracto fenólico (c); 20 g/L de proteína y 200 g/kg$_{protein}$ de glicerol y 100 g/kg$_{protein}$ de MMT (d).

could not be identified because their vibrational modes are overlapped by several bands from protein and phenolic compounds.

Rice bran protein films showed distinct characteristics, which may find application in the production of biodegradable packaging and protective films. Their use as edible coatings has been evaluated as a good alternative for preservation of whole or minimally processed fruits and vegetables, since these coatings can create a semi-permeable barrier to gases and water vapor, maintaining product quality, as well as being potential carriers for additives to help preserve or even improve the quality of the product (Dangaran et al., 2009; Olivas & Barbosa-Canovas, 2009).

Conclusions

The best properties were obtained for rice bran protein films with lower protein and glycerol concentrations and with addition of phenolic extract without the presence of MMT clay. Protein concentration was the factor that most affected solubility, luminosity and opacity these films. The mechanical properties of the films were affected by glycerol concentration, while the addition of phenolic extract to the films affected opacity, tensile strength, elongation, Young's modulus and water vapor permeability. Due to not fully exfoliated, the addition of MMT not favored the mechanical properties of the films. The obtained films could be

applied as packaging material for fruits and vegetables, whole or minimally processed.

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