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Influence of heating, protein and glycerol concentrations of film-forming solution on the film properties of Argentine anchovy (*Engraulis anchoita*) protein isolate

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ABSTRACT

This study was conducted to evaluate the influence of thermal treatment (74, 82, and 90 °C), glycerol (30%, 35%, and 40%, w/w) and protein concentrations (3.0%, 3.5%, and 4.0% w/w) of film-forming solution on the properties of Argentine anchovy (*Engraulis anchoita*) protein isolate (API) films produced by casting. The API presented 88.8% of proteins, 5.5% moisture, 1.3% lipids, 1.0% ash and 53.3% of polar amino acids. The DSC of protein isolate was observed at maximum temperature of 62.2 °C and ΔH 6.4 J/g. The thickness, water vapor permeability, color difference and opacity of the films were not affected by the experimental variables studied ($p > 0.05$). The lowest solubility, elongation, and highest tensile strength of the films occurred at low temperature, low protein and glycerol concentrations ($p < 0.05$). Micrographs obtained by scanning electron microscopy of the films showed homogeneous surfaces at low temperature.

ronmental impacts caused by the use of synthetic polymers, there is a great interest in developing biodegradable films that serve as packaging. Edible films can be used for versatile food products to reduce moisture loss, restrict oxygen absorption, lessen lipid migration, improve mechanical handling properties, provide physical protection, or offer an alternative to the commercial packaging materials (Falguera et al., 2011; Kowalczyk and Baraniak, 2011).

Though edible films can be prepared using protein, polysaccharide or lipid materials (Bourtoom et al., 2006; García and Sobral, 2005; Zavareze et al., 2012), recent developments put protein based edible film as the most interesting object of research. Proteins have been extensively used because of their relative abundance, their film-forming ability, and nutritional qualities (Bourtoom et al., 2006; Cuq et al., 1995; García and Sobral, 2005; Tongnuanchan et al., 2011). The mechanical properties of protein-based films are better than those of polysaccharides and lipids. Proteins have a unique structure (based on 20 different monomers) which confers a wider range of functional properties, especially a high intermolecular binding potential which can form bonds at different positions (Kokoszka et al., 2010).

proteins were capable of form- film-formation process (Bourtoom et al., 2006; García and Sobral, 2005; Limpan et al., 2010; Paschoalick et al., 2003). The myofibrillar proteins are insoluble in water, but can be made soluble by adjusting the pH of the solution (Iwata et al., 2000; Martins et al., 2011). Unexploited in Brazil, the Argentine anchovy (*Engraulis anchoita*), is a pelagic fish with coastal habits widely distributed from the Gulf of San Jorge in Argentina to Rio de Janeiro, in Brazil, more specifically between Cabo Frio (RJ) and Chui (RS) (Castello and Castello, 2003). This fact arouses the interest for obtaining protein for use in the preparation of films.

The formation of the films involves a complex series of chemical reactions; these are influenced by experimental conditions such as protein concentration, heating temperature and the addition of a plasticizer (García and Sobral, 2005; Sobral et al., 2005). The plasticizer is a small molecule of low volatility which, when added to polymeric materials, modifies the three-dimensional organization, decreases attractive intermolecular forces, and increases free volume and chain mobility. As a result of these changes in the molecular organization, plasticizers modify the functional properties of films by increasing extensibility, dispensability, and flexibility and by decreasing cohesion, elasticity, mechanical properties and rigidity (Cao et al., 2007; Vieira et al., 2011). The thermal treatment modifies the three-dimensional structure of the globular proteins, causing exposition of the SH groups and consequently producing

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intra- or intermolecular thiol/disulfide (SH/S–S) or thiol/thiol (SH/SH) interchange, while promoting the exposition of hydrophobic groups which proportion hydrophobic interactions during drying (García and Sobral, 2005; Perez-Gago and Krochta, 2001).

The use of edible films in food applications is related to the achievement of diverse characteristics such as cost, availability, functional attributes, mechanical properties (flexibility, tension), optical properties (brightness and opacity), the barrier effect against gas flow, structural resistance to water and microorganisms and sensory acceptability (Falguera et al., 2011). The objective of this research was to study the influence of heat treatment, plasticizer and protein concentrations of film-forming solution on the properties of films from the protein isolate of Argentine anchovy (*E. anchoita*).

2. Material and methods

2.1. Material

The fish used in this study was the Argentine anchovy (*E. anchoita*), provided by the Oceanographic Ship "Atlantico Sul" of the Federal University of Rio Grande, Brazil. Anchovywas washed in chlorinated water at a concentration of 5 ppm at 4 °C and then beheaded and gutted. After these operations, they were processed in a meat-bone separator (High Tech, model HT250, Chapecó, Brazil) that discarded skin and bones. The resulting mechanically separated meat was packaged in plastic bags and stored in a freezer at −18 °C. Glycerin, sodium hydroxide and hydrochloric acid were purchased from Synth (Diadema, São Paulo, Brazil).

2.2. Preparation of Argentine anchovy protein isolate

Argentine anchovy protein isolate was prepared according to Freitas et al. (2011) and Nolsoe and Undeland (2009) with some modifications. The mechanically separated meat (MSM) of anchovy was homogenized with distilled water in a ratio of 1:9 (MSM: water, w/v). The reaction was conducted in a stainless steel reactor, under stirring and controlled temperature. The alkalinizing agent was (1 M) NaOH and the acidifying agent was (1 M) HCl. Alkaline solubilization was performed under pH 11.2 for 20 min at 4 °C. After solubilization, the sample was centrifuged at 9000*g* for 20 min. During centrifugation, the sample was separated in three phases. The upper phase (neutral lipids) was discarded, the middle phase (soluble proteins) was subjected to isoelectric protein precipitation carried out at pH 5.0 for 20 min at 4 °C, and the bottom phase (insoluble proteins) was discarded. The precipitated protein was dehydrated in an air circulation oven (Quimis, Q342, São Paulo, Brazil) for 16 h at 40 °C. It was then ground in a knife-mill, in order to standardize the particle size as 0.35 mm, and then used in the film elaboration.

2.3. Proximate analyses of protein isolate

Protein, ash, fat and moisture contents of API were determined according to AOAC (2000) method with the analytical N° 992.15, 923.03, 960.39 and 925.30 for protein, ash, fat and moisture analyses, respectively.

2.4. Determination of amino acid composition of protein isolate

The amino acid composition was determined according to a method proposed by Bidlingmeyer et al. (1984). The samples were previously hydrolysed with distilled 6 M HCl, at 22 h at 110 ± 1 °C.

2.5. Differential scanning calorimetry (DSC)

Thermal properties of API were determined using differential scanning calorimetry (DSC-Q20, TA Instruments, New Castle, USA) according to Thorarinsdottir et al. (2002) with some modifications. A sample, weighing 2–3 mg (accuracy of ±0.01 mg) was hermetically sealed in aluminum pans and scanned at a heating rate of 10 °C/min over the range 15–110 °C. The enthalpy of denaturation (ΔH) was computed from the endothermic peak observed in the thermogram.

2.6. Preparation of protein isolate films

The film from API was prepared according to the method of Limpan et al. (2010) with some modifications. The studied film-forming solution (FFS) compositions were as follows: protein, 3.0%, 3.5% and 4.0% (w/w, g of API/100 g of FFS); plasticizer, 30%, 35% and 40% (w/w, g of glycerin/100 g of API); pH kept 11.5 using (1 M) NaOH, and thermal treatment of 74, 82 and 90 °C/30 min with help of a bath with digital control of temperature. Distilled water (100 mL) was added to different concentrations of the API and homogenized at 500 rpm for 25 min at 25 °C. The pH was adjusted to 11.5 and different concentrations of glycerin were added, and the solution was homogenized at 10.000 rpm for 10 min using an Ultraturrax (IKA, T25 digital, Werke, Germany) homogenizer. The solution was heated at different temperatures for 30 min while stirring at 500 rpm to denature the API. The FFS obtained was filtered through a layer of cloth to remove undissolved debris. FFS (25 mL) were poured into an acrylic Petri dish (9 cm in diameter) and dried at 35 °C for 16 h in an air circulation oven (FANEM, 520, São Paulo, Brazil). The dried films were then peeled-off and conditioning in silica at 25 °C and 25 °C for 48 h prior to testing.

2.7. Determination of film properties

2.7.1. Film thickness

Thickness (mm) of the films was measured using a digital micrometer (Insize, IP54, São Paulo, Brazil) having a precision of 0.001 mm. Ten random locations around each film sample were used for average thickness determination.

2.7.2. Film solubility

Film solubility in water was determined according to the method by Gontard et al. (1994) with modification. A piece of film sized 2.0 cm in diameter was cut, dried in an oven (DeLeo A1 SED, Porto Alegre, Brazil) at 105 °C to constant weight to obtain the initial film dry weight. Films were individually placed into 50 mL of distilled water and the mixture was shaken at a speed of 100 rpm using a shaker (Cientec CT-712RNT, Piracicaba, Brazil) at 25 °C for 24 h. The amount of dry matter in final samples was determined by drying at 105 °C to constant weight. The weight of solubilized dry matter was calculated by subtracting the weight of insolubilized dry matter from the initial weight of dry matter and expressed as the percentage of total weight. Film solubility represented total soluble matter dissolved in water, mainly including water-soluble proteins and glycerol.

2.7.3. Water vapor permeability (WVP)

Water vapor permeability (WVP) was determined gravimetrically at 25 °C based on the method described by ASTM Standard Method E96-95 (ASTM, 1995) with some modifications. The film samples were sealed on a permeation cell (exposed area: 1.58×10^{-3} m^2) containing anhydrous calcium chloride (0% RH). The cell was placed in desiccators with a saturated sodium chloride solution (75% RH). The cell mass was observed in 24 h intervals for 48 h at 25 °C. WVP of the film was calculated as follows:

$$\text{WVP (g mm m}^{-2}\text{ d}^{-1}\text{ kPa}^{-1}) = wLA^{-1}t^{-1}(P_2 - P_1)^{-1}$$

where w is the weight gain of the cell (g); L is the film thickness (mm); A is the exposed area of film (m^2); t is the time of gain (days); $P_2 - P_1$ is the vapor pressure difference across the film (kPa).

2.7.4. Mechanical properties

Tensile strength (TS) and elongation at break (EAB) were determined as described by ASTM Standard Method D882-91 (ASTM, 1996) using a Texture Analyser ($TA.XT_{plus}$, Stable Micro Systems, England). The tensile tests were run using rectangular samples of 80 mm × 25 mm, initial grips separation of 50 mm and cross-head speed 0.8 mm s^{-1}. TS was calculated by dividing the maximum load to break the film by the cross-sectional area of the film and EAB was calculated by dividing film elongation at rupture by the initial gauge length of the specimen and multiplying by 100. Each type of film was prepared and evaluated in triplicate.

2.7.5. Color and opacity

Film color was determined using a colorimeter (Minolta, CR-400, Osaka, Japan) working with D_{65} (day light) using the CIELab color parameters and a measure cell with an opening of 30 mm, being expressed as the color difference (ΔE^*) was calculated as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* values are the difference between the color parameter of the samples and the color parameter of the white standard ($L = 97.39$, $a^* = -0.14$, $b^* = 1.94$) used as the film background.

The opacity was assessed according to the HunterLab method (1997) using the same equipment for color measure, also operating in the reflectance mode. The opacity (Y) of the samples was calculated as the relationship between the opacity of each sample on the black standard (Y_b) and the opacity of each sample on the white standard (Y_w). Measurements were made in triplicate and the results were presented as mean values:

$$Y(\%) = \frac{Y_b}{Y_w} \times 100$$

2.7.6. Scanning electron microscopy (SEM)

Studies of microstructure of films were performed by using scanning electron microscope (JEOL, JSM-6060, Japan). The film samples were mounted on an aluminum stub and sputtered with gold (Sputter coater SPI-Module, PA, USA) to make the sample conductive. The morphology of the surface of the films samples was visualized at an acceleration voltage of 10 kV.

2.8. Statistical analysis

The response surface methodology (contour surface) was used according to Monterrey and Sobral (1999) to analyse the effects of the following parameters: thermal treatment, protein isolate and glycerol concentrations on the mechanical properties (tensile strength and elongation), solubility, thickness, water vapor permeability, color and opacity. The values of the independent variables were calculated according to central composite design (CCD) 2^3 with three replicate at the central points. The statistical design considered and the values of the independent and coded variables are shown in Table 2. The interaction between the variables was evaluated using the software Statistica (Version 6.0, by StatSoft, Inc., Tulsa, USA) using a confidence interval of 95%.

3. Results and discussion

3.1. Proximate composition of protein isolate

Chemical analysis made in samples of API, presented 88.8% total proteins, 5.5% moisture, 1.3% lipids and 1.0% ash. The protein content obtained was higher than that obtained by Paschoalick et al. (2003) that obtained 80% for Nile tilapia protein isolate. García and Sobral (2005) obtained 86% of total protein in Thai Tilapia. The method used for the extraction of proteins, temperature, drying, centrifugal separation, and that used for a relative concentration of water-soluble sarcoplasmic proteins, among others, may have contributed to the different levels of protein isolates found (Nolsoe and Undeland, 2009).

3.2. Amino acid composition of protein isolate

Amino acid composition of API is presented in Table 1. The amino acids present in greater concentration in proteins were glutamic acid, lysine, isoleucine and aspartic acid. The polar amino acids correspond to about 53.3% of the total amino acids in the API. It can be observed that polar amino acids are in high concentration (aspartic acid, glutamic acid and lysine) such as in the muscle protein of Nile tilapia obtained by Paschoalick et al. (2003). The low concentration of cystine (0.3 g of amino acid/100 g of protein) implicated low potential for disulfide bonds (S–S). The low content of cystine results in a low density of covalent bonds, disulfide (S–S), which followed the intra- and intermolecular low-energy disulfide bonds of the protein becoming more soluble (Okamoto, 1978). Because of this, the films prepared on the basis of API have a hydrophilic characteristic, resulting in films with greater solubility and water vapor permeability.

3.3. Differential scanning calorimetry (DSC)

DSC has been widely used to characterize the thermal properties of food proteins, including heat-induced denaturation. The denaturation process is an intramolecular change involving the destruction of internal order, and in some cases, the complete unfolding of peptide chains with the formation of so-called "random coils" (Guerrero and Caba, 2010). The thermogram of the API is presented in Fig. 1. The endothermic peak was observed at 62.2 °C in the middle range between myosin and actin. Park and Lanier (1989) in their studies with tilapia (*Oreochromis aureus*), determined that myosin denatures at 58.3 °C and actin at 78.6 °C. However, Yongsawatdigul and Park (2003) found myosin

Table 1
Amino acid composition for Argentine anchovy protein isolate.

Amino acid	(g amino acid/ 100 g of API[a])
Alanine	5.6
Arginine	4.1
Aspartic acid	9.2
Cystine	0.3
Glutamic acid	14.4
Glycine	4.3
Histidine	3.0
Isoleucine	10.2
Leucine	6.4
Lysine	11.4
Methionine	3.9
Phenylalanine	5.1
Proline	4.2
Serine	3.0
Threonine	4.7
Tyrosine	4.1

[a] API: Argentine anchovy protein isolate.

Table 2
Color difference, opacity, mechanical properties, thickness, solubility and water vapor permeability of Argentine anchovy protein isolate film obtained as a function of temperature, protein isolate and glycerol concentrations.

Experiment	API (X_1)[a] (w/w)	Glycerol (X_2)[a] (w/w)	T (X_3)[a] (°C)	ΔE[a]	Opacity (%)	TS (MPa)	EAB (%)	Thickness (mm)	S (%)	WPV (g mm /m^{2}d kPa)
1	3.0(−1)	30(−1)	74(−1)	64.7 ± 0.3	15.7 ± 0.3	3.9 ± 0.0	9.1 ± 1.1	0.172 ± 0.010	27.3 ± 0.8	8.2 ± 0.3
2	4.0(+1)	30(−1)	74(−1)	66.2 ± 0.0	15.1 ± 0.2	2.9 ± 0.1	15.3 ± 0.2	0.176 ± 0.030	29.8 ± 0.4	8.3 ± 0.1
3	3.0(−1)	40(+1)	74(−1)	60.6 ± 0.3	15.5 ± 0.3	1.8 ± 0.1	17.0 ± 1.3	0.136 ± 0.020	35.3 ± 0.9	10.5 ± 0.8
4	4.0(+1)	40(+1)	74(−1)	67.6 ± 0.2	15.6 ± 0.1	1.1 ± 0.0	29.5 ± 0.8	0.166 ± 0.000	41.3 ± 1.0	12.2 ± 0.7
5	3.0(−1)	30(−1)	90(+1)	69.5 ± 0.3	17.3 ± 0.8	1.6 ± 0.4	14.5 ± 0.1	0.142 ± 0.030	37.5 ± 0.2	8.5 ± 0.2
6	4.0(+1)	30(−1)	90(+1)	70.4 ± 0.2	18.1 ± 0.6	1.4 ± 0.0	20.0 ± 0.5	0.139 ± 0.010	40.4 ± 0.5	8.6 ± 0.1
7	3.0(−1)	40(+1)	90(+1)	62.4 ± 0.1	17.5 ± 0.4	0.6 ± 0.5	27.8 ± 1.1	0.113 ± 0.010	45.2 ± 0.2	11.6 ± 0.6
8	4.0(+1)	40(+1)	90(+1)	71.4 ± 0.2	16.6 ± 0.2	0.6 ± 0.2	35.2 ± 1.2	0.140 ± 0.010	50.1 ± 0.8	11.8 ± 0.7
9	3.5(0)	35(0)	82(0)	53.6 ± 0.4	13.0 ± 0.4	1.5 ± 0.7	23.8 ± 0.8	0.130 ± 0.000	34.0 ± 0.9	9.4 ± 0.9
10	3.5(0)	35(0)	82(0)	53.2 ± 0.1	13.3 ± 0.0	1.5 ± 0.1	24.0 ± 0.5	0.149 ± 0.010	35.7 ± 0.1	9.6 ± 0.3
11	3.5(0)	35(0)	82(0)	53.2 ± 0.2	13.5 ± 0.6	1.5 ± 0.2	24.7 ± 0.3	0.138 ± 0.010	33.4 ± 1.2	10.5 ± 0.2

[a] Independent variables values (the values between brackets are the coded variables); Values are given as mean ± SD (n = 3); API: Argentine anchovy protein isolate (w/w, g/100 g FFS); Glycerol (w/w, g/100 g API); T: temperature; ΔE^*: color difference; TS: tensile strength; EAB: elongation at break; S: solubility; WVP: water vapor permeability.

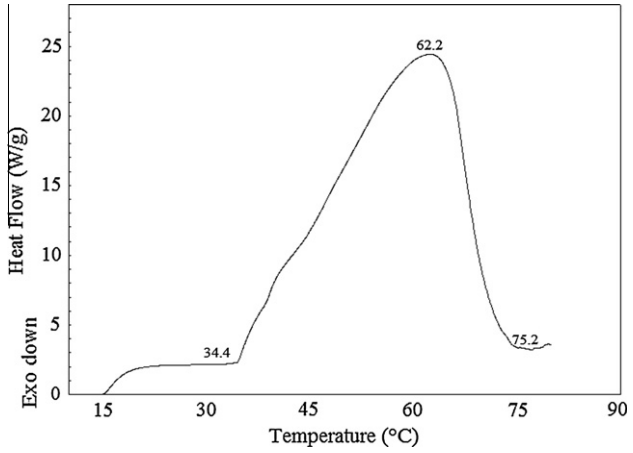

Fig. 1. DSC thermogram of protein isolate prepared from Argentine anchovy (*Engraulis anchoita*).

denaturation at 35 °C. The enthalpy of denaturation was 6.4 J/g for API, extracted from a solubilizing alkaline of pH 11.2. (Tahergorabi et al. 2012) evaluated denaturation of isolate protein from chicken breast and found a enthalpy of denaturation was 2.0 J/g extracted from a solubilizing alkaline of pH 11.5. The slight differences in endothermic transitions between our results and theirs might be partially due to the different sample conditions. Temperatures for these endothermic peaks tend to vary depending on the muscle type, pH and heating conditions.

3.4. Determination of film properties

Solubility, TS and EAB of API obtained according to the full experimental factorial design are shown in Table 2. Data were fitted as functions of the dependent variables. Eqs. (1)–(3) represent the coded models for solubility, TS and EAB are shown in Table 3. According to the variance analysis (ANOVA), the models represented by these equations were significant at a 95% level of significance ($p < 0.05$). The contour surfaces were generated for the statistically significant models (Montgomery, 1991). The contour surfaces generated using Eqs. (1)–(3) are shown in Fig. 2. The thickness, WVP, color difference and opacity of the API film were not significantly correlated ($p > 0.05$).

3.4.1. Film solubility

API films retained their integrity after immersion in water under constant stirring. The solubility of the films increased from 27.3% to 50.1%, Table 2, for films prepared with 3.0% of API, 30% of glycerol at 74 °C and 4.0% of API, 40% of glycerol at 90 °C, respectively. Similar results were found for films based on protein isolate from red tilapia muscle by Tongnuanchan et al. (2011), these authors observed solubilities ranging from 21.4% to 56.0%.

The water solubility of the films increased with glycerol concentration, Fig. 2a. The results revealed a great influence of the plasticizer on this property. The increase in glycerol content caused an increase in film solubility in water, because of the hydrophilicity of glycerol (Vieira et al., 2011). The solubility of the film increased from 35.3% to 41.3% by adding 3.0% to 4.0% of API for the same glycerol content and heat treatment (40% glycerol and 74 °C), Fig. 2b, respectively. According to Paschoalick et al. (2003) fish protein films have high water solubility because of the hydrophilicity of the amino acids of the protein molecules (Paschoalick et al., 2003). In this study the polar amino acids correspond to about 53.3% of the total of amino acids in the API, showed in Table 1.

The temperature favoured an increase in solubility of the films, as showed in Fig. 2b. The increase in heat treatment may have caused increased exposure of monomers and peptides of low molecular weight and non-protein material, leading to an increased solubility of these films (Stuchell and Krochta, 1994). Bourtoom et al. (2006) reported that fish protein based films showed that an increase in heating temperature of film solution from 60 to 70 °C resulted in a decrease in film solubility. However, when the heating temperature of the film solution was raised higher than 70 °C, it yielded an increase in film solubility.

3.4.2. Mechanical properties

Mechanical properties of films from API are shown in Table 2. TS and EAB of the films range from 0.6 to 3.9 MPa and 9.1% to 35.2%, respectively. TS values determined in this work were lower than those found by Limpan et al. (2010) in films from fish myofibrillar protein, which had a tensile strength of 5.6 MPa. They observed that fish myofibrillar protein isolate film had an elongation of 25%. The elongation determined in this study was higher than that determined by those authors.

Tests 1 and 2 had higher values of TS, when 3.0% and 4.0% of API, 30% of glycerol and 74 °C were used, respectively. The influence of glycerol content on TS of film is showed in Fig. 2c. It shows that TS decreased significantly with an increase in glycerol content. The glycerol reduces the density of protein–protein interactions, increases the mobility of polymer chains and consequently, makes the films less resistant. The addition of a plasticizing agent is necessary in order to overcome the brittleness of the film, to improve flexibility (Cao et al., 2007). Sobral et al. (2005) observed that the TS of films based on protein isolates from muscle of Thai Tilapia decreased with increasing glycerol.

Table 3
Contour surface regression models for the parameters solubility, tensile strength and elongation at break.

Parameters	Equations	R^2	F	$F_{(95\%)}$
Solubility	$y = 37.3 + 2.0\,X_1 + 4.6\,X_2 + 4.9\,X_3$ (1)	0.90	23.6	4.1
Tensile strength	$y = 1.7 - 0.2\,X_1 - 0.3\,X_2 - 0.7\,X_3 + 0.2\,X_1.\,X_3 + 0.2\,X_2.\,X_3$ (2)	0.98	93.0	5.1
Elongation at break	$y = 21.9 + 3.9\,X_1 + 6.3\,X_2 + 3.3\,X_3 + 1.02\,X_1X_2 - 0.7\,X_1\,X_3 + 0.8\,X_2\,X_3$ (3)	0.96	15.6	6.2

y: Function of response; X_1: protein isolate concentration (g/100 g of FFS); X_2: glycerol concentration (g/100 g of API); X_3, thermal treatment (°C); R^2: coefficient of determination ($p < 0.05$); F: test F ($p < 0.05$).

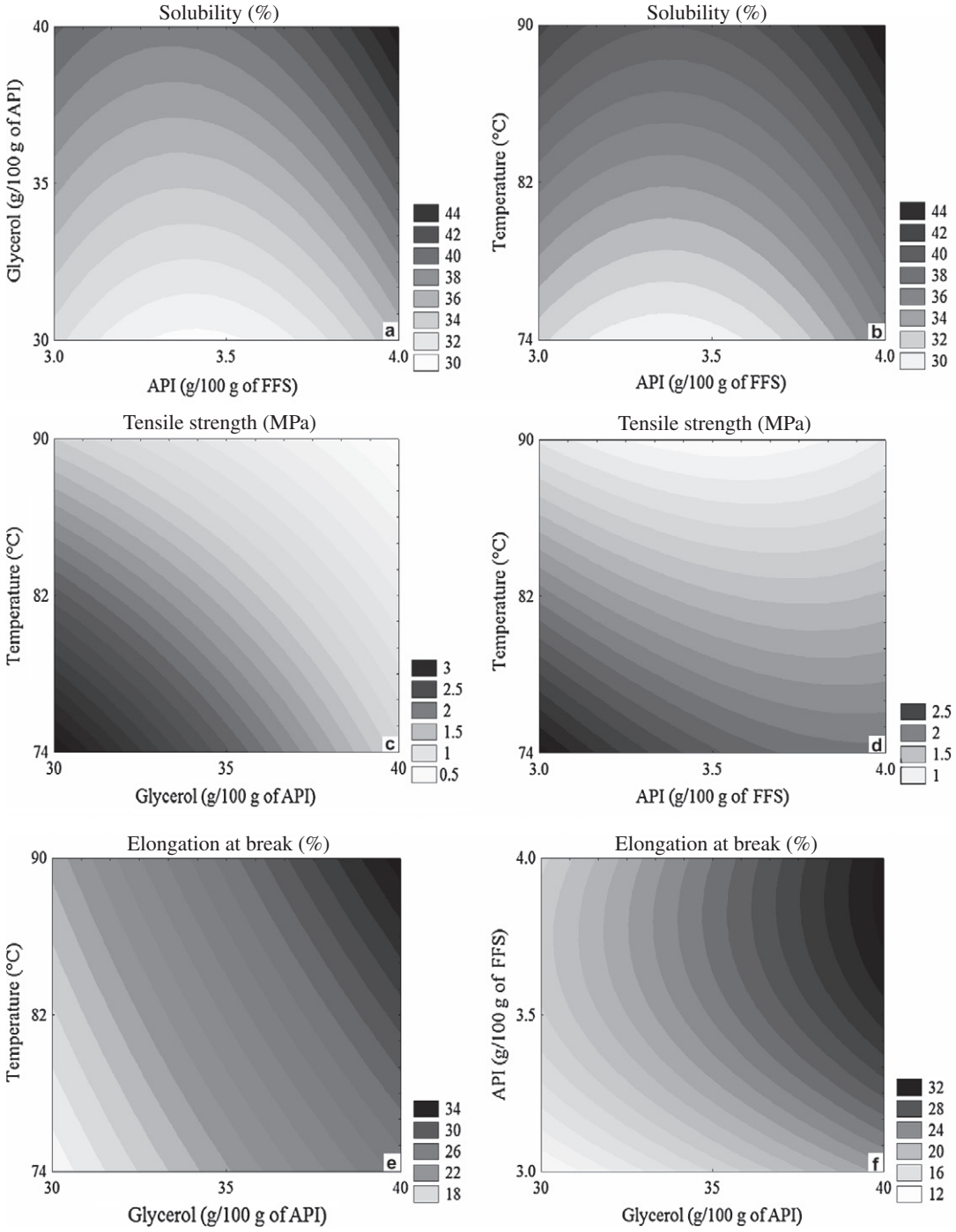

Fig. 2. Contour curves for solubility as a function of glycerol and API (a) and as a function of temperature and API (b); tensile strength as a function of temperature and glycerol concentration (c) temperature and API (d); the elongation as a function of temperature and glycerol concentration (e) API and glycerol concentrations (f).

The TS decreased significantly with increased protein concentration, Fig. 2d. The same behavior was verified by Iwata et al. (2000) where TS decreases with the increase of sarcoplasmic proteins (from 2% to 4%) in film-forming solution. However, Sobral

et al. (2005) reported that films prepared with 2 g of muscle protein of a Thai Tilapia/100 g of FFS were more resistant than those prepared with 1 g of muscle protein of a Thai Tilapia/100 g of FFS. This can be explained by the possible effect of protein concentration on the reaction kinetics of sulfur cysteine residues, present in proteins, thereby increasing the concentration of protein may have favoured the formation of the sulfide bridges between the protein chains due to heat treatment (Perez-Gago and Krochta, 2001). In this study a low cysteine content was found (amino acid 0.3 g/100 g of API), Table 1, which may have influenced the decrease in TS and an increase of EAB when API increased.

In this study it is observed that an increase of temperature from 74 to 90 °C results in a decrease in TS, as shown in Fig. 2d. Perez-Gago and Krochta (2001), studied the effect of temperature on the mechanical properties of films based on whey protein isolate, and observed that the increase of thermal treatment from 80 to 90 °C for 20 min caused a reduction from 14 to 9 MPa of TS, respectively. Hoque et al. (2011) found that increasing the heat treatment from 70 to 90 °C contributes to the reduction of TS from 8.9 to 4.9 MPa of cuttlefish skin gelatin film. These behaviors may have occurred because the more intensive thermal treatment (>70 °C) caused protein denaturation and consequently bury free –SH groups in the hydrophobic pockets such that they may remain unavailable for disulfide bond formation (García and Sobral, 2005). The thermogram of API is presented in Fig. 1. It can be seen that 75.2 °C is the end of denaturation. Nevertheless, the impact of heating on the film properties might be varied with the protein sources, particularly the bonding involved (Hoque et al., 2010).

Perez-Gago and Krochta (2001) observed that EAB of whey protein isolate film increased as the temperature of FFS increased. The same behavior was observed in this study, there was an increase in EAB from 9.1% to 35.2% (from 74 to 90 °C), which is shown in Fig. 2e. According to Hoque et al. (2010), the increase in elongation of the film when heated at temperatures higher than 70 °C indicated that higher temperatures resulted in the formation of a film network with lower rigidity.

High concentration of the protein and plasticizers in FFS contributes to the increase in EAB of the films, as shown in Fig. 2f. Iwata et al. (2000) observed that the increase of sarcoplasmic protein concentration from 2% to 4% increased the elongation at break of films. García and Sobral (2005) developed muscle protein based films from Tilapia found that when glycerol was increased from 15% to 45% in the formulation of the films, elongation increased. Thus, addition of glycerin to the film increased extensibility of the film, whilst it reduced the mechanical strength. Without glycerine, the composite films tended to be brittle and difficult to handle, which mainly resulted from the brittleness of film (Cao et al., 2007).

3.4.3. Film thickness

The thickness of API films is shown in Table 2. In this work, according to the analysis of the effects of the factorial design variables (data not shown), there was no influence of film formulation on thickness. The thickness of the films ranges from 0.113 to 0.176 mm. These results were higher than those found for films based on protein isolate from red tilapia muscle, which showed thicknesses ranging from 0.034 to 0.036 mm (Tongnuanchan et al., 2011). According García and Sobral (2005), higher protein isolate and glycerol concentrations used in the formulation of the films, induces an increase in solids in the polymer matrix formed after drying the film-forming solutions and therefore increases the thickness of the films. Films produced with constant concentration of API (3.0% of API) and glycerol (30% of glycerol), but different temperature (74 and 90 °C) had a thickness ranging from 0.172 to 0.142 mm.

3.4.4. Color and opacity

The color difference (ΔE^*) and opacity of films from API are shown in Table 2. Among the important optical properties of the films for use in packaging, color and opacity are highlighted. Color is an important parameter in the characterization of the films; it is related to the raw material used to make the products.

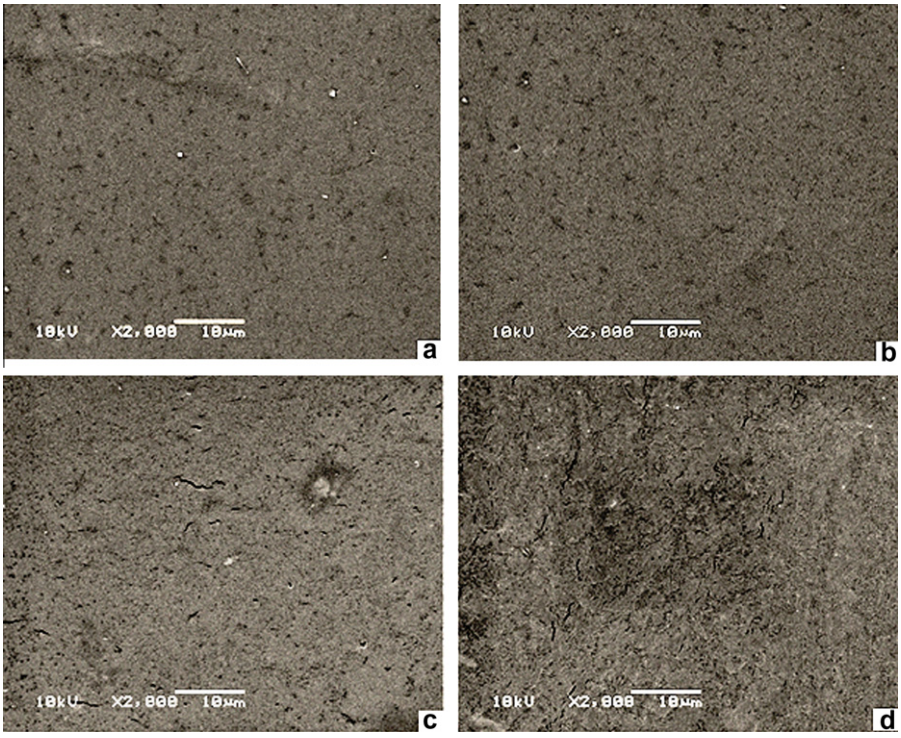

Fig. 3. Micrographs of the surface of films from Argentine anchovy protein isolate in magnification of 2000× (a) 3.0% of API and 30% of glycerol (b) 3.0% of API and 40% of glycerol at 74 °C (c) 3.0% of IPA and 30% of glycerol and (d) 3.0% of IPA and 40% of glycerol at 90 °C.

The color of films was more affected by the protein concentration than by the thermal treatments. The ΔE^* of the films ranged from 67.6 to 71.4, this can be observed when increasing the temperature from 74 to 90 °C for API and glycerol content constants. Some authors (Chinabhark et al., 2007; Hoque et al., 2010) reported that color changes due to Maillard reaction are always associated with the heat-induced process.

There was an increase in ΔE^* for films with higher addition of the API for the same glycerol content and temperature. The raw material used for the preparation of the films in this study was protein isolate obtained from Argentine anchovy. This fish has dark muscle, resulting in an increase of color in the film. Dark muscle was reported to contain a higher amount of myoglobin than ordinary muscle (Chaijan et al., 2004). Those pigments most likely contributed to the darker color of films compared with films from other muscles.

The opacity of the films also presented an increasing tendency when the temperature increased. The opacity of the films from API ranged from 15.1% to 18.1%, this can be observed when the API content is 4.0%, glycerol concentration of 30% and the thermal treatment increased from 74 to 90 °C. Paschoalick et al. (2003) in their studies of films prepared with 1 g of muscle proteins of Nile tilapia/100 g FFS treated at 40 and 65 °C for 30 min observed opacity ranging from 2% to 7%. According to Monterrey-Quintero and Sobral (2000) the sarcoplasmic proteins of Nile Tilapia denature at around 41 °C. Therefore, the denaturation of sarcoplasmic proteins, present in the muscle proteins, occurred during the thermal treatments and may have caused the increase of opacity of film.

3.4.5. Water vapor permeability (WVP)

WVP of films from API is shown in Table 2. The WVP of the films ranges from 8.2 to 12.2 g mm/m^2 d kPa, it was more permeable than the films based on myofibrillar proteins of argentine croaker (*Micropogonias furnieri*) which ranged from 2.25 to 4.10 g mm/m^2 d kPa (Zavareze et al., 2012). With the increase of glycerol content from 30% to 40% for the same content of API (3.0% of API) and heat treatment (74 °C), an increase from 8.2 to 10.5 g mm/m^2 d kPa in the WVP occurred. An increase in glycerol content may cause an increase of WVP, due to its high hydrophilic character (Vieira et al., 2011). Glycerol as a low molecular weight substance can probably penetrate into protein network easily thereby increasing WVP. Glycerol could result in more effective disruption of intermolecular interaction among polypeptide chains (Vieira et al., 2011).

Fish muscle protein is known for its hydrophilic characteristics; therefore, WVP barrier properties of protein films are expected to be poor, compared to those from other non-hydrophilic material (García and Sobral, 2005). The hydrophilic character of fish muscle is associated with polar amino acids present in the same (Tongnuanchan et al., 2011). However, in this study the differences in API content and temperature of FFS did not apparently influence WVP.

3.5. Microstructure of film

Micrographs of the surface of API films are shown in Fig. 3. The tests selected to represent the morphology of the surface were 1 and 3 (3.0% of API, plasticizers, 30% or 40% of glycerol and temperature of 74 °C) and are shown in Fig. 3a and b; 5 and 7 (3.0% of API, plasticizers 30% or 40% of glycerol and a temperature of 90 °C) are shown in Fig. 3c and d. The micrographs of the surfaces of the films with different plasticizers concentrations and thermal treatment were presented because they showed the greatest effect on EAB, TS and solubility, in shown Table 2.

The microstructure showed in Fig. 3a and b revealed a homogeneous and continuous structure. The homogeneity may be related to a better compatibility between the substances present in FFS, glycerol and API concentrations. This resulted in improved mechanical properties of films. However, Fig. 3d showed the highest roughness compared to the other experiments. The cracks on the film surface, Fig. 3c and d decrease the TS of the film. According to Monterrey-Quintero and Sobral (2000) a rough or rougher surface suggests a characteristic of brittle film.

4. Conclusion

The results of this study showed that it is possible to prepare homogeneous and flexible films from Argentine anchovy protein isolate. Nevertheless the effect on the thickness, color difference, opacity and water vapor permeability, were not significantly influenced by the variables studied. The films with higher protein and glycerol content and with higher thermal treatment were more water soluble. With the excessive heating, 90 °C for 30 min, API degradation occurred and the corresponding film showed increased elongation, but lower TS. Furthermore, the increase in glycerol concentration resulted in a film with greater elongation. The films made from the more protein concentrated FFS were less resistant than the films with less API in the FFS. The increase in heat treatment of FFS resulted in a film with a rough surface and less homogeneous. In conclusion, protein isolates of fish with low commercial value can be used as a component of new polymeric films for packaging and other applications.

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