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ORIGINAL ARTICLE

Edible films from residual delipidated egg yolk proteins

Ismael Marcet[1] · Sara Sáez[1] · Manuel Rendueles[1] · Mario Díaz[1]

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Abstract Commercial extraction with organic solvents of valuable lipids from egg yolk produces a highly denatured protein waste that should be valorized. In this work, the delipidated protein waste remaining after ethanol extraction was used to prepare edible films. This material was also treated with transglutaminase, obtaining films that have also been characterized. When compared with gelatin and caseinate edible films, the films made with egg yolk delipidated protein showed poorer mechanical properties, but improved light barrier properties, low water solubility and a high degree of transparency. It is particularly interesting that the presence of phosvitin in the egg yolk gives the films important ferrous chelating properties. When the egg yolk delipidated protein was treated with transglutaminase, the strength of the film was improved in comparison with films made with untreated protein. Finally, addition of thymol and natamycin in the preparation of these films is shown to be an interesting alternative, providing them with antibacterial and antifungal capacities.

Keywords Egg yolk · Edible film · Protein · Mechanical properties · Solubility

✉ Manuel Rendueles
mrenduel@uniovi.es

[1] Department of Chemical and Environmental Engineering, University of Oviedo, C/ Julián Clavería 8, 33006 Oviedo, Spain

Introduction

Egg yolk is a source of highly valuable lipids, such as egg yolk lecithin which is composed mainly of phosphatidylcholine and phosphatidylethanolamine. These lipids have desirable functional properties widely utilized in the food industry and their emulsifying properties in particular have an important role but, on the other hand, the extraction of these compounds usually requires the use of organic solvents. Among the organic solvents which have been previously used for the extraction of these polar lipids is ethanol, a GRAS-grade organic solvent (Aro et al. 2009). However, the utilization of this type of lipid extraction method produces conformational changes in the protein structure and drastically reduces their solubility. Therefore, the delipidated protein obtained can be considered as a co-product of the lipid separation, with greatly decreased functional properties in comparison with its native state.

Egg yolk can be easily separated into two sub-fractions: egg yolk granules and plasma. The plasma fraction has a high lipid content (78%) and it has been described as an emulsifier (Le Denmat et al. 2000) and gelling agent (Kiosseoglou and Paraskevopoulou 2005). The granular fraction has a higher amount of protein (58%) than lipids (40%) (Laca et al. 2010) but this protein is only solubilized in high ionic strength mediums (>0.3 M NaCl), and therefore, its functional properties and the number of possible applications for this fraction are low. Since the revaluation of the egg yolk is associated with an increase in the number of applications developed for each of its fractions, the lipid and protein content of the egg yolk granules could be separated and used for different purposes in the food industry. In this sense, the highly denatured protein co-product obtained after egg yolk granules lipid extraction could be used to prepare edible films.

Research into edible films which can protect and increase the shelf life of food products has received increasing interest from the food industry and the scientific community in the last few years. Several sources of proteins have been used to produce protein-based edible films, such as casein (Rezvani et al. 2013) and whey (Galus and Kadzińska 2016) which are contained in milk, gelatin obtained from bovine hide (Sobral et al. 2001) or soy protein (Pan et al. 2014). These edible films, in addition to extending the shelf life of the food products, come from a renewable source, are highly biodegradable and could be an alternative to the use of environmentally harmful petroleum-derived plastics. Additionally, protein-based edible films can be easily combined with antimicrobial compounds, and their mechanical or water vapour capacities can be modulated by treating the proteins with crosslinkers (De Carvalho and Grosso 2004). Amongst the crosslinkers that can be used, one of the most intensively studied in food science is transglutaminase (EC 2.3.2.13), which is generally recognized as safe (GRAS) by the U.S Food and Drug Administration and by the U.S. Department of Agriculture, as well as being considered a processing aid in the European Union legislation (EU regulation 1332/2008), and therefore it is commonly used in the preparation of processed foods. Transglutaminase catalyses the formation of ε-(γ-glutamyl)-lysine crosslinks in proteins, so it is capable of introducing covalent bonds between chains of proteins, thus improving the mechanical properties of these films (Porta et al. 2015).

In this study, always bearing in mind that the granular fraction is poorly valorised by the food industry, this fraction has been delipidated with ethanol and the protein obtained treated with transglutaminase and then used to prepare films in order to characterize the modifications induced by this crosslinking agent. To understand the strengths and weaknesses of these films, their properties have been compared with other highly-studied protein-based edible films created in a similar way but using gelatine from porcine skin and sodium caseinate.

Materials and methods

Obtaining delipidated egg yolk granules

Egg yolk granules were obtained and delipidated according to Marcet et al. (2014). Briefly, egg yolk was separated from the egg white and dried carefully using blotting paper. The vitelline membrane was broken using tweezers and the liquid yolk mixed with water in a proportion 1:1.5 (v/v). The pH of this diluted egg yolk solution was adjusted to 7.0 with 0.1 M NaOH, and the solution was centrifuged at 10,000×*g* for 45 min. The sediment (granules) was collected and lyophilized. The lyophilized egg yolk granules were delipidated using ethanol (96%) and gentle agitation for 2 h. Granules were recovered by filtration using Whatman no 1 paper and a vacuum pump. Delipidated granules were dried for 2 days at 40 °C in a heater and stored at −20 °C until use. The dry matter of the delipidated granules was 96% (w/w).

Film preparation

The composition of the film-forming solution was chosen according to the results of previous tests (data not shown). A 3% suspension of delipidated egg yolk granules in water was prepared by mixing 3 g of delipidated granules, 95.5 mL of water and 1.5 mL of 1 M NaOH. The granules were totally solubilised with sonication at 65 °C for 20 min using a MS 73 probe in a Sonopuls HD 2070 system (Bandelin, Germany). In this case the amplitude of sonication selected was 70% (100% amplitude equivalent to 212 μm). The amount of glycerine added was 50% of the total protein contained in the solution. Then, the film-forming solution was filtered using a vacuum pump and Whatman no 1 paper, degassed using a vacuum pump, and 20 mL cast in a Petri dish. This amount of film forming solution produces a dry film by evaporation of the water in an oven at 45 °C overnight (granules films). This temperature was selected to avoid the growth of microorganisms. With these parameters, the films can be completely removed from the mould, without their being sticky or brittle.

In the case of the granules treated with transglutaminase (GTT), the delipidated granules were solubilised as described above, and then the pH of the granules solution was adjusted to 8.6 with 1 M HCl. For each gram of protein contained in the sample, 10 U of transglutaminase (Probind TX, BDF ingredients, Spain) were added. In accordance with the manufacturer's instructions, this transglutaminase is mixed with maltodextrin and it has 100 U per gram of product, so, in 100 mL of film-forming solution with 3 g of delipidated egg yolk granules, 0.3 g of transglutaminase powder was added. According to the previous tests (data not shown), higher amounts of transglutaminase (0.4 g) did not produce any significant increase in the crosslinking of the granular proteins and further decreased the amount of protein per gram of film. The reaction was conducted in an oven at 45 °C for 90 min, after which the pH of the sample was adjusted to 10.0 with 1 M NaOH and the glycerin added in a similar proportion to that of the non-treated films. Finally, the film-forming solution was filtered, degassed and dried as described above.

The gelatine and sodium caseinate films were prepared in a similar way, mixing 3 g of powder of gelatine from

porcine skin (G2500, Sigma-Aldrich) or sodium caseinate (C8654, Sigma-Aldrich) with 97 mL of water. The protein solutions were heated in a water bath at 65 °C for 25 min. Once the protein was solubilised, the pH of the solutions was not adjusted. Then, an amount of glycerine equivalent to 50% of the protein contained in each solution was added, and the solutions were gently stirred for 5 min. The film-forming solutions showed a pH of 5.5 for the gelatin and of 6.7 for the preparation with caseinate. Finally, the film-forming solutions were filtered, degassed and dried as described above.

The amount of protein in the delipidated egg yolk granules, the gelatine and the sodium caseinate powders was determined using an Elementar Vario EL analyzer (Elementar, Germany). Prior to testing, all films were conditioned for 2 days at 21 °C and 54 ± 2% RH in a closed chamber which contained a saturated $Mg(NO_3)_2$ solution.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

The transglutaminase activity on the delipidated granular proteins was evaluated using SDS-PAGE as described by Laemmli (1970). The stacking gel was formed by polymerising a 4% (w/v) acrylamide solution, whilst a 10% (w/v) acrylamide gel was used for the running phase. Proteins were stained with two different solutions. The first was normally used to specifically stain phosphoproteins (Coomassie blue 0.05%, acetic acid 10%, triton 1%, ethanol 25%, aluminium nitrate 0.1 M). The second was a standard Coomassie stain, which stains total protein (0.1% Coomassie blue, 50% methanol, 10% acetic acid and 40% water). SDS-PAGE Molecular Weight Standards, Broad Range (Bio-Rad) were used as protein standards.

Mechanical properties of the film

To test the strength and the elongation range of the films, a TA.XT.plus Texture Analyser (Stable Microsystems, UK) with a 5 kg load cell was used. For that purpose, the films were cut into squares with a geometry of 30 × 20 mm and placed on the test platform. The film was firmly attached by means of a plate which was screwed in position above the test platform, the film lying between them. The 5 mm diameter probe (P/5S) descends perpendicularly to the surface of the film, making contact with it through the orifice formed by two holes, one in each plate, thus stretching the film and measuring its puncture strength (PS) and puncture deformation (PD) values. These parameters have been used previously to characterize the mechanical properties of other protein-based films (Otero-Pazos et al. 2016). The PS and PD were calculated according to the following equations:

$$PS = Fm/Th \quad (1)$$

$$PD = \left(\sqrt{D^2 + R^2} - R\right)\Big/R \quad (2)$$

where Fm is the maximum force applied before film rupture and Th is the film thickness; D is the distance covered by the probe while it is in contact with the film until the film is broken; R is the radius of the orifice in the plates.

Scanning electron microscopy (SEM)

The microstructure of the film cross-section was analysed using a scanning electron microscope (SEM) (JSM-6610LV, JEOL, USA) according to Galus and Kadzińska (2016) with slight modifications. Briefly, film samples were freeze-dried and immediately cut into squares of 1 × 1 cm using a surgical blade. The film squares were mounted around stubs perpendicularly coated with gold.

Thermal properties

Thermo-gravimetric analyses (TGA) were carried out using an SDTA851e TGA analyser (Mettler-Toledo, Switzerland) from 25 to 650 °C under a nitrogen atmosphere. The heating rate was 10 °C/min. The first derivatives of the weight loss curve thermograms were calculated (DTG curves).

Film solubility and water vapour permeability (WVP)

The water solubility of the films was measured according to Blanco-Pascual et al. (2014). Briefly, the films were cut into circumferences of 40 mm in diameter and immersed in 50 mL of distilled water for 24 h under gentle stirring in an orbital shaker. To recover the insolubilized films, the water was filtered using a vacuum pump and Whatman no 1 paper. The dry mater of the recovered films was determined using a halogen moisture analyzer (HR80, Mettler-Toledo, Switzerland) at 105 °C for 12 h. The moisture content of each type of film before water immersion was previously determined using the same moisture analyser, and the values compared to calculate the amount of film solubilized during the experiment.

The WVP test was conducted according to Rezvani et al. (2013). Briefly, polyvinyl chloride cups were filled with water and the films were cut into circles matching the size of the cup mouth and sealed to it, leaving a gap of 1 cm between the water surface and the under surface of the films. The thickness of the film samples was measured at nine points and furthermore, they were previously

examined to avoid the use of film specimens with pinholes or any defects. The mounted cups were placed inside an environmental chamber which contained a saturated $Mg(NO_3)_2$ solution at 20 °C and 54 ± 2% RH. The weight loss was recorded hourly during the first 10 h and finally after 24 h. Three replicates of each film were evaluated. The weight loss was plotted against time and the water vapour transmission rate (WVTR: g m^{-2} h^{-1}) was estimated by dividing the slope in the linear region ($R^2 > 0.990$) by the film surface. The WVP was calculated using the following equation:

$$WVP = (WVTR \times Th)/\Delta P \quad (3)$$

where Th is the thickness of the film (mm) and ΔP is the water vapour partial pressure difference across the film (Pa).

Light transmission and transparency

The ultraviolet and visible light barrier properties of the films were tested at several wavelengths according to Hamaguchi et al. (2007), from 200 to 800 nm, using a spectrophotometer (Helios gamma, Thermo Fisher Scientific, USA). Briefly, a rectangular piece was cut from the film samples and placed directly into a spectrophotometer test cell. The measurements were performed using an empty test cell as the reference. The transparency of the films was calculated according to the following equation:

$$Transparency = A_{600}/x \quad (4)$$

where A_{600} is the absorbance of the film sample at 600 nm and x is film thickness (mm).

Film colour

The film colour properties were measured in the L*, a*, b* system using an UltraScan VIS spectrophotometer (HunterLab, USA). Films were measured on the surface of the white standard plate, which has L*, a*, b* values of 97.12, −0.14 and 0.13 respectively.

Ferrous ion chelating assay

The ferrous ion chelating ability of the films was measured according to the method of Decker and Welch (1990) with slight modifications. Briefly, pieces of film of several weights were placed in 4 mL of de-ionized water and immediately 0.1 mL of 2 mM $FeCl_2$ was added. The solution containing the film was stirred gently in an orbital shaker for 5 min, and then the pieces of film were removed and 0.2 mL of 5 mM ferrozine was added. After incubation at room temperature for 10 min, the absorbance was measured at 562 nm. The Fe^{2+}/ferrozine complex has a high absorbance at this wavelength, so high chelating ability is shown as a low absorbance. The chelating ability in percentage was calculated as follows:

$$Ferrous\ chelating\ ability\ (\%) = (A_{blank} - A_t/A_t) \times 100 \quad (5)$$

where A_t is the absorbance of the test sample.

Thymol and natamycin incorporation into the films and microbiological assays

Thymol (16254, Sigma-Aldrich) and natamycin (PHR1703, Sigma-Aldrich) were incorporated in the film-forming solution of the granules films and GTT films after the addition of glycerol. The thymol concentrations tested were 15 and 30% (w/w of protein), while the natamycin concentrations tested were 2.5 and 5% (w/w of protein). These film-forming solutions were processed in a similar way to those previously described in "Film preparation" section and then the antimicrobial performance of the films obtained was assayed.

Staphylococcus aureus CECT 240, *Pseudomonas taetrolens* LMG 2336 and *Penicillium roqueforti* for cheese cultures (Choozit, Dupont, USA) were selected and the inhibition assays were carried out using the agar diffusion test as follows: *Staphylococcus aureus* was cultured in a Tryptic Soy Broth medium for 16 h at 37 °C under agitation at 250 rpm in an orbital shaker. *Pseudomonas taetrolens* was cultured in a Nutrient Broth medium for 16 h at 30 °C and under agitation at 250 rpm. A solution which contained 300 × 10^6 spores/mL of *Penicilium roqueforti* was diluted in peptone water to 1 × 10^6 spores/mL. In each case, an inoculum of 200 µL was spread on the respective agar mediums and immediately the 10 mm diameter pieces of film were placed on the surface of the agar plates. In the case of *Penicilium roqueforti*, the agar medium selected was Plate Count Agar medium. Staphylococcus aureus plates were incubated for 10 h at 37 °C, *Pseudomonas taetrolens* for 16 h at 30 °C and *Penicillium roqueforti* for 4 days at 30 °C. At the end of the experiment, the plates were photographed.

Statistical analysis

Analysis of variance (ANOVA) was applied. Least significant differences (LSD) were calculated by Fisher's test to determine significant differences between the tested samples. These analyses were performed using the statistical software Statgraphics® V.15.2.06.

Results and discussion

To get some idea about the physical parameters obtained for the new granules and GTT films in relation with other protein-based edible films, these values were compared with those obtained for gelatin and caseinate films. Since the relative humidity and the type and amount of plasticizer have relevance for the film properties, these films were prepared in a similar way to those made using granules protein. The amount of glycerine selected (50% w/w of protein) was the optimal quantity for the egg yolk protein films. In the case of gelatin and caseinate films, similar levels of glycerol were successfully used by other authors (Audic and Chaufer 2005; De Carvalho and Grosso 2004).

SDS-PAGE

In the SDS-PAGE shown in Fig. 1a, at each time tested, it is possible to detect a decrease in the intensity of the apo-HDL bands of 110 and 100 kDa, and at the same time, an appreciable increase in the amount of protein which cannot enter or can hardly enter the separation gel. In fact, after 30 min of reaction, these high molecular weight proteins almost disappear from the reaction medium, not being detectable after 90 min of reaction. This experiment suggests a high capacity of the transglutaminase to catalyse the crosslinking reaction for these proteins. However, the action of the transglutaminase on the other proteins of lower size was limited. It should be taken into account that the proteins involved in the crosslinking reaction must have a glutamine and a lysine residue, and they must be accessible to the enzyme. Furthermore, the amino acid sequence and the secondary structure in which the glutamine and lysine are included appear to be important for recognition by the enzyme (Rachel and Pelletier 2013).

Microstructure

The surface microstructure of all tested films was completely smooth and with no difference between them (data not shown). However, the micrographs of the film cross-section showed particularities in the way the proteins form the film in each case (Fig. 1b). In the granules films the cross-sectional area was observed to be slightly heterogeneous, with protein agglomerations appearing throughout the film matrix. The GTT film cross-sectional area showed a flaky, fibrous appearance, which produced stronger egg yolk protein packaging. The gelatin film showed a smooth and structured cross-sectional area, with a high degree of homogeneity, and this resulted in the film with the best mechanical properties, as reported below. Finally, in the caseinate film, as in the case of the gelatin film, the cross-sectional area looked uniform except for the presence of some protein aggregates in the upper half.

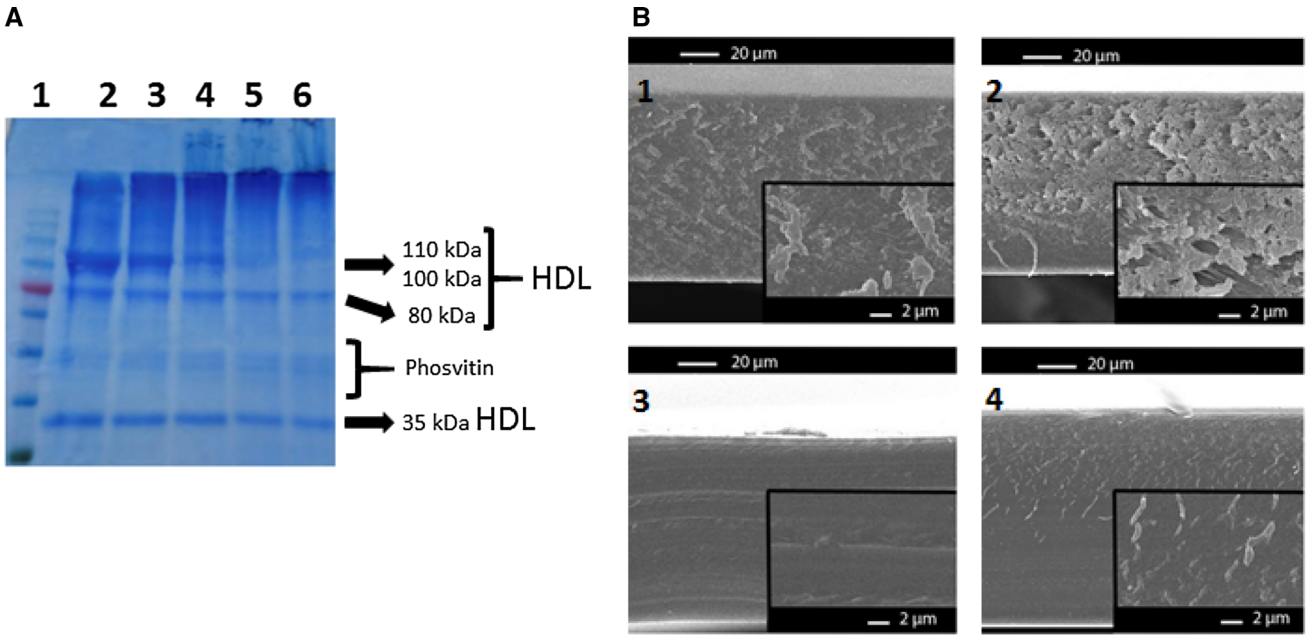

Fig. 1 Effect of transglutaminase on the delipidated granules proteins and on the microstructure of the films obtained. **a** Electrophoresis. 1: Molecular weight markers. 2: Transglutaminase-treated granules. 3: 15 min of reaction. 4: 30 min of reaction. 5: 60 min of reaction. 6: 90 min of reaction. **b** Micrographs of the film cross-Section. 1: Untreated granules. 2: Transglutaminase-treated granules. 3: Gelatin. 4: Caseinate

Mechanical properties

To study the mechanical properties of the granules-based films with and without the transglutaminase treatment, the PS and PD values were obtained (Table 1). These values were compared with those obtained for the gelatin and caseinate films.

The gelatin films obtained the highest PS and PD values. This is possibly because the mechanical properties of the protein-based films are modulated by the chain-to-chain interactions produced between the proteins (hydrogen, electrostatic and hydrophobic bonds). So, the primary structure of the proteins as well as the degree of extension of the protein chains are factors that have an influence on the strength and flexibility of the film formed (Bourtoom 2008). In this case, the high PS and PD values of the gelatin film are due to the gelatin being obtained from collagen, which is a fibrous protein with structural functions that can form intermolecular interactions easily.

The PS value of the caseinate films was similar to that of the gelatin films. The casein structure has a high degree of flexibility and confers on this protein a good chance of establishing interactions with other proteins (Kinsella and Morr 1984). It could explain the formation of a highly interconnected net of proteins, forming strong films.

The PS and PD values of the granules films were found to be the lowest of those obtained by the evaluated films, this being in accordance with the micrographs shown previously. The granular fraction is composed mainly of high density lipoproteins, which are globular-type proteins, and since the amount of protein and glycerol was similar to that used for the gelatin and caseinate films, it is possible that the number and/or quality of protein interactions produced in the granules film matrix is lower than those in the other films tested.

Furthermore, when this egg yolk protein was treated with transglutaminase, the PS value of the films obtained was increased by 54%. Increments in the strength of the protein-based films after treatment with transglutaminase were also reported by other authors. Weng and Zheng (2015) made films using gelatin and transglutaminase (10 U/g of protein) and obtained an increase in the tensile strength of 29%. However, the effect of the transglutaminase on the flexibility of the films was slight. This marginal effect corresponds with other reported films in which the transglutaminase had no effect or had a negative effect on this film property (Jiang et al. 2007; Porta et al. 2015).

Thermal properties

The TGA curves of the films are shown in Fig. 2a. The tested films showed behaviour typical of this type of material, with a loss of weight with increasing temperature

Table 1 Puncture strength (PS), puncture deformation (PD), water solubility (WS), water vapour permeability (WVP), thickness, moisture content (MC), colour, light transmission and transparency of the tested films

	PS (N/mm)	PD (%)	WS (%)	WVP (g mm/m^2 h kPa)	Thickness (μm)	MC (%)	Colour	Light transmission (nm)							Transparency
							L*	a*	b*	200	280	350	400	500	600	
Granules	79.0 ± 8.4^c	50.0 ± 2.1^c	22.1 ± 2.0^c	3.38 ± 0.10^a	122 ± 5^a	29.5 ± 1.2^a	95.10 ± 0.30^c	−0.53 ± 0.07^c	12.80 ± 1.00^a	0.1	0.1	52	78.4	96	96	0.18
GTT	122.0 ± 9.1^b	58.0 ± 3.0^b	24.5 ± 2.0^c	3.05 ± 0.05^b	119 ± 4^a	30.5 ± 0.5^a	94.50 ± 0.40^c	−0.58 ± 0.01^c	13.70 ± 0.19^a	0.1	0.1	53.5	78.3	95.0	96.2	0.18
Gelatin	190.6 ± 21.5^a	75.7 ± 5.6^a	48.9 ± 2.5^b	2.90 ± 0.05^c	100 ± 10^b	23.2 ± 1.0^b	98.48 ± 0.20^a	−0.40 ± 0.12^b	3.50 ± 0.70^b	0.1	25.6	83.5	92.4	95.1	97.3	0.21
Caseinate	188.6 ± 13.8^a	30.5 ± 5.2^d	99.0 ± 1.0^a	2.93 ± 0.30^c	115 ± 3^a	21.0 ± 0.3^c	97.60 ± 0.25^b	−0.10 ± 0.01^a	4.40 ± 0.50^b	0.1	0.1	42.1	66.9	84.0	84.5	0.65

Different letters in the same column indicate significant differences ($P < 0.05$)

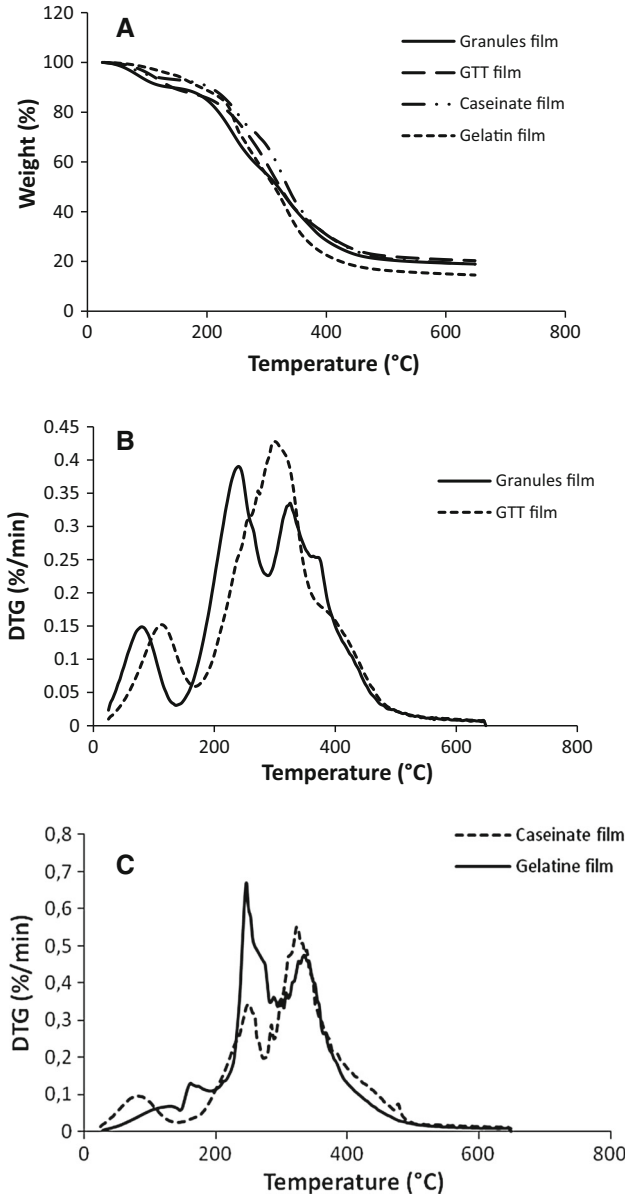

Fig. 2 Films TGA (**a**) and DTG (**b**, **c**) curves

that can be divided into three different stages. In the first stage, from 40 to 150–160 °C, the adsorbed water is evaporated from the film (Guo et al. 2014). In the second phase, from around 150–160 °C to 270–280 °C, the glycerol is volatilized from the sample (Hoque et al. 2011). Finally, the third stage starts with the thermal degradation of the protein, and it begins at 270–280 °C (Ge et al. 2015). For a better understanding of the thermal degradation of the films, the derivatives of the TGA thermograms were calculated and they are shown in the form of DTG curves (Fig. 2b, c). Furthermore, some parameters were calculated from TGA and DTG curves and they are shown in Table 2. In the DTG curve of the granules film the three stages described above can be clearly observed in the form of three peaks. However, when the DTG curve of the GTT films was analysed, the peak corresponding to the glycerol evaporation could not be observed, and appeared to be combined with the protein degradation stage. This could be due to an improvement in the compatibility between the glycerol and the egg yolk protein (Ramos et al. 2013), probably produced by a better interaction between the glycerol molecules and the protein chains in the film matrix. Furthermore, the GTT film showed an increase in the amount of residue at 650 °C and a higher T_{50} value with respect to the granules film, evidencing an improvement in the thermal properties of the transglutaminase-treated films. In agreement with this, several authors have reported similar effects on the thermal properties of this type of packaging material when using different crosslinking agents (Ge et al. 2015). In comparison with the other films tested, the caseinate film showed the highest T_{50} value and a low level of thermal degradation in the glycerol evaporation stage. This suggests a good compatibility between the glycerol and the caseinate and the best thermal resistance among the films tested. Finally, the gelatin film had the lowest values of T_{50} and amount of residue at 650 °C and it also showed higher thermal degradation in the glycerol and in the protein degradation stages than did the granules and GTT films.

Film solubility, water vapour permeability (WVP)

Films made using delipidated egg yolk granules showed low water solubility in comparison with the gelatin and caseinate films (Table 1). The egg yolk granules proteins are soluble in water at high ionic strength (>0.3 M), so the films obtained using these proteins are also expected to be insoluble in distilled water. In fact, the conditions for solubilizing the egg yolk granules cannot be easily found in the food industry, and therefore, these films could be suitable for covering food. The caseinate films dissolved almost immediately in distilled water, and the gelatin films showed a water solubility value that was intermediate between that of egg yolk and caseinate films. The WVP value of the films tested varied within narrower limits, although the differences were statistically significant. This could be explained by the fact that this film property is strongly modulated by the type of biopolymer used and by the amount and type of plasticizer (Wihodo and Moraru 2013). In this sense, all films have a similar protein-based composition, with a similar amount of protein and glycerol in the film forming solution. Among them, the gelatin and caseinate films showed a slightly lower WVP value than that of the granules. This could be due to better packaging of the proteins in the film matrix, leaving less free space to facilitate the migration of water through the film. This granules film property was improved by the

Table 2 Thermal property values obtained from Fig. 2

Films	Decomposition stage	Temperature range (°C)	DTG peak	Weight loss (%)	Residue at 650 °C	T_{50}^{a} (°C)
Granules	1	25–140	85	11	18.9	313
	2	140–291	243	32.6		
	3	291–500	335	35.8		
GTT	1	25–170	120	12.5	20.3	319
	2 and 3	170–500	303	65.4		
Caseinate	1	25–167	85	7.5	18.9	332
	2	167–260	259	15.7		
	3	260–500	325	55.6		
Gelatin	1	25–145	139	5	14.5	313
	2	145–278	249	32.5		
	3	278–500	335	46.2		

[a] Temperature at which 50% of the film weight was lost

transglutaminase treatment, which increased the degree of packaging of the proteins and decreased the WVP of the film. This effect caused by the transglutaminase was also observed by other authors using mixtures of gelatin and soy protein (Weng and Zheng 2015).

Colour, light transmission and transparency

The colour values of the films tested are shown in Table 1. For all films tested the L* (lightness) and a* (red-dish/greenish) parameters were found to be only slightly different and have values similar to those for the white standard. However, the b* parameter showed high values in the egg yolk films with respect to the gelatin and caseinate films. This yellowish colour of the films was produced by the fat-soluble carotenoids (Li-Chan and Kim 2008) which were not totally removed during the ethanol treatment. Although after the delipidation process a dry powder with a high content in proteins (87 ± 1%) was obtained, it must be expected that part of the non-protein content is composed of these coloured compounds. Finally, it is noteworthy that the transglutaminase does not produce any change in the colour of these films.

The light transmission of the granules, GTT and caseinate films in the UV range was very low, this being a desirable property in order to protect food items high in lipid, since UV radiation is an important starter for their oxidative degradation (Coupland and McClements 1996). In the case of the gelatin film, it showed a decreased light barrier capacity at 280 nm in comparison with the other tested films. In broad terms, the protein-based edible films have a high capacity to prevent UV damage due to the natural presence of aromatic amino acids in their composition. However, the gelatin does not have tryptophan in its composition and it is low in phenylalanine and tyrosine

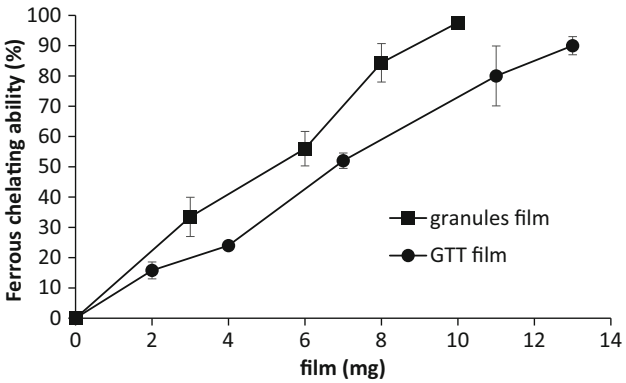

Fig. 3 Ferrous chelating ability of the granules and GTT films

(Nhari et al. 2011). This resulted in lower light barrier properties than in the other films tested.

Regarding the wavelengths in the visible region, the tested films showed good properties for food packaging, since they allow easy visual examination of the product covered. This was confirmed by the transparency index, which was very low for all of them.

Ferrous ion chelating assay

Phosvitin is the most phosphorylated protein found in nature, and its chelating properties have been broadly studied. In the granular fraction, the phosvitin content corresponds to 16% of the whole protein content, and as indicated by the electrophoresis results presented previously, phosvitin can be found as part of the protein obtained after the delipidation process. In Fig. 3 the ferrous chelating property of the egg yolk protein-based films is shown. In this case, the caseinate film could not be tested due to its solubilisation in the aqueous medium during the

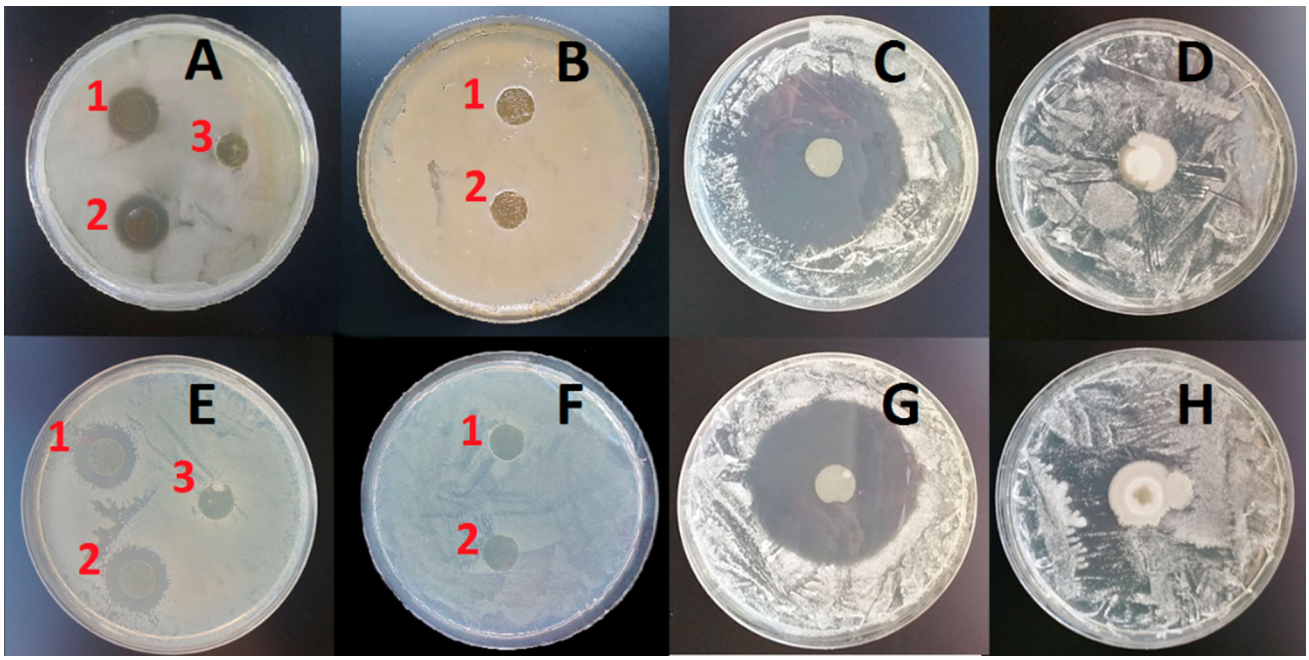

Fig. 4 **a** (*S. aureus*) and **e** (*P. taetrolens*): GTT film with thymol 30% (1), granules film with thymol 30% (2) and granules film control (3). **b** (*S. aureus*) and **f** (*P. taetrolens*): GTT film control (1) and granules film control (2). **c** (*P. roqueforti*): GTT film with natamycin 5%. **g** (*P. roqueforti*): granules film with natamycin 5%. **d** and **h** (*P. roqueforti*): GTT film and granules film control respectively

assay, and the gelatin film, as was expected, does not show any chelating property.

The ferrous chelating ability of both the granules and GTT films showed dose-dependent behaviour with a strong capacity to chelate ferrous ion. The higher chelating capacity of the granules film could be due in part to the dilution of the egg yolk protein in the case of the GTT film. In the GTT films, the addition of transglutaminase decreases by 10% the amount of phosvitin and other egg yolk proteins per gram of film, in agreement with the preparation method reported in the materials and methods section. Furthermore, the fibrous film matrix produced by the action of transglutaminase could have developed a tighter net of fibres which might hinder the interactions between the aqueous solvent and the phosvitin. In any case, this antioxidant activity could be of interest to protect foods containing iron which are rich in lipids, as for example meat-based products, since ferrous iron is involved in the oxidation of lipids (Love and Pearson 1971).

Films with thymol and natamycin added

The antimicrobial and antifungal properties of the egg yolk protein-based films, with and without adding thymol (30% w/w of protein) and natamycin (5% w/w of protein), were tested (Fig. 4). In the case of the control films, bacterial growth in the area under the granules and GTT films was impeded both in the case of *Pseudomonas taetrolens* and *Staphylococcus aureus*. This could be due to the presence in the films of phosvitin, which can remove the iron in the medium, showing bactericidal properties (Zhou et al. 2014). In the case of *Penicillium roqueforti*, the fungi were able to grow under the film without any problem, not being affected by the presence of the egg yolk proteins.

In the case of the films with thymol, an inhibition area greater than the film surface was observed in both *Staphylococcus aureus* and *Pseudomonas taetrolens* agar medium. To produce this inhibition zone, the thymol contained in the films must diffuse from the film to the culture medium, confirming in this way that the addition of an antibacterial agent to improve the functionality of these films is possible. Thymol has been used previously by other authors with a similar purpose (González and Igarzabal 2013). As in the case of thymol, the films with natamycin produced a large inhibition area in the *Penicillium roqueforti* culture medium. In these experiments, the large inhibition area shown in Fig. 4 is explained by the fact that the films' formulation was modified with a high concentration of natamycin (5% w/w egg yolk protein), but lower concentrations of this active agent produced a smaller inhibition zone (data not shown).

Conclusion

The delipidated egg yolk protein-based edible films prepared in this study showed optical properties which are desirable for the food industry, but their low solubility and

their ferrous ion chelating capacity could be considered the main characteristics of these packaging materials. Their low solubility probably derives from the fact that the egg yolk granules are highly insoluble in distilled water, but could also be due to the structural protein modifications which occur during lipid extraction. Furthermore, ferrous ion chelating capacity is seen due to the presence of phosvitin in the granular fraction. So, the addition of phosvitin to other edible films, thus allowing them to acquire this property, is a possibility, although it would increase the cost of the packaging material. Finally, the treatment with transglutaminase produced a noticeable increase in the strength of the film obtained, but the other film parameters were barely affected, so in this case, the transglutaminase treatment would be useful only if the mechanical properties of the non-treated film were considered to be insufficient.

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