Effect of freezing on physical properties of whey protein emulsion films

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Abstract

The objective of this work was to study the effect of the freezing process on physical properties of whey protein emulsion films with different beeswax content dried at 5 ºC. Thickness, microstructure, water vapour permeability, solubility in water, sorption isotherms and mechanical properties were measured in Control and Frozen films. The freezing process did not cause fractures or perforations in films, but films with beeswax showed a change in the appearence of the lipids after freezing. Only films with 40% of beeswax showed a significant increase in the water vapour permeability after freezing. The freezing process did not affect film solubility in water but produced small differences in the equilibrium moisture content values. In the puncture test, the freezing process increased puncture strength and deformation of films without beeswax but those parameters were not affected in films with beeswax. In tensile test, tensile strength and elastic modulus decreased, but elongation was not affected by freezing process. Principal component analysis accomplished an adequate condensation of the date grouping samples according to film formulation and treatment (Control and Frozen films). Indeed, the relationships of sample grouping and measured parameters were enlightened by principal component analysis. In conclusion, whey protein emulsion films were resistant to the freezing process (freezing, frozen storage and thawing) and could be a good alternative as a treatment to preserve the quality of frozen foods.

Keywords: whey protein emulsion films; beeswax; freezing; physical properties.

1. Introduction

Moisture loss in frozen foods has important economic consequences and is continuously receiving the attention of food scientists mainly due to the fact that drip loss during thawing, as a result of irreversible tissue damage during the freezing process (freezing, storage, and thawing), leads to reduced visual attraction and nutrient loss (Duan & Zhao, 2011). Weight loss is important not only for the economical impact of sealable weight reduction, but also because moisture loss is strongly related with the preservation of food structure and consequently food texture. In addition, moisture loss can produce freezer burn and a glassy appearance in some food products, caused by the presence of tiny cavities caused by sublimated ice (Pham & Mawson, 1997).

Edible films and coatings could constitute a good alternative for improving the quality of frozen foods, mainly because they can reduce the rate of moisture transfer between the food and the surrounding atmosphere, improve structural integrity of frozen foods during thawing, and slow down the occurrence of freezer burn (Duan, Cherian, & Zhao, 2010).

Edible coatings have been applied on frozen foods, mainly in fish, meat, and poultry. Several authors reported that the rate of moisture loss was reduced, and other quality parameters were either improved or maintained, in comparison with uncoated food samples, when coatings of different biopolymers (whey proteins, chitosan, alginate, collagen, celullose, methylcellulose, etc.), with or without lipids, were applied (Stuchell & Krochta, 1995; Han, Zhao, Leonard & Traber, 2004; Yu, Li, Xu, & Zhou, 2008; Duan & Zhao, 2010). Stuchell and Krochta (1995) described the application of an edible coating based on whey protein isolate and acetylated monoglycerides in salmon fillets that was effective in reducing the rate of moisture loss and to delay the onset of lipid oxidation after 3 weeks of storage at -23 ºC. Another study reported that chitosan

65 coatings incorporated with fish oil reduced the drip loss of frozen lingcod fillets (Duan
66 & Zhao, 2010). Moreover, coatings based on sodium alginate and calcium decreased
67 thawing loss, shear force, and thiobarbituric acid reactive substances, and thus were
68 effective to maintain the quality of frozen pork (Yu et al., 2008). Only one study
69 analyzing the effect of edible coatings to maintain the quality of frozen fruits was found
70 in literature (Han et al., 2004). These authors used edible coatings to improve storability
71 of frozen strawberries and observed that chitosan based coatings were able to reduce
72 drip loss and helped to maintain textural quality of frozen strawberries after thawing.
73 Edible films have been hardly applied in wrapped frozen foods. Only one study
74 analyzing the effect of the freezing process on quality parameters of steak overwrapped
75 with an edible film was reported (Farouk, Price, & Salih, 1990). These authors observed
76 that round steak beef overwrapped with an edible collagen film (Coffi®) exhibited
77 much less fluid exudate after a week of frozen storage at -25 ºC, when compared with
78 standard permeable film overwrap. However, the edible film had no significant effect on
79 colour and lipid oxidation.
80 In the case of heterogeneous foods, edible films with good water barrier properties,
81 placed between two food components with different water activity, can reduce moisture
82 transfers between those components (Duan & Zhao, 2011). This area has also been
83 scarcely studied and only three reports were found in literature in products like bread
84 and tomato-based sauce, simulated sundae ice cream cone, and model system of tomato
85 paste and ground crackers (Kamper & Fennema, 1985; Kester & Fennema, 1989; Rico-
86 Peña & Torres, 1990). An edible lipid-cellulose composite film effectively limited
87 internal transmission of water when situated at the interface between high and low
88 moisture component of a model frozen-food system consisting of bread and a tomato-
89 based sauce after 9 weeks of frozen storage (Kester & Fennema, 1989). Similarly, an

edible bilayer film of methylcellulose and palmitic acid retarded moisture transfer from ice cream to the sugar cone keeping its crispness longer than the commercial storage-life of the uncoated product (Rico-Peña & Torres, 1990).

In addition to moisture migration, other physicochemical properties such as physical integrity, mechanical strength, and plasticity of free-standing films have an influence on the application, particularly in the case of overwrapped products. Thus, it is surprising that the effect of freezing on physical properties of free-standing films had been restricted to the study of moisture migration, and limited to certain film forming materials like cellulose or hydroxypropyl methylcellulose and fatty acids (Kamper & Fennema, 1984; Kamper & Fennema, 1985; Kester & Fennema, 1989).

In a previous investigation we obtained whey protein emulsion films with good mechanical properties and improved water vapour permeability characteristics when films were dried at 5 ºC compared with 25 ºC (Soazo, Rubiolo, & Verdini, 2011a). Furthermore, we studied the sorption behaviour of whey protein emulsion films and showed the influence of both film formulation and drying temperature on the equilibrium moisture content of films (Soazo, Rubiolo, & Verdini, 2011b).

The objective of this investigation was to study the effect of freezing on microstructure, water vapour permeability, solubility in water, moisture sorption, and mechanical properties of whey protein emulsion films dried at 5 ºC.

2. Materials and methods

2.1. Materials

Whey protein concentrate (WPC) 80% (Arla Food Ingredients S.A., Argentina), beeswax (BW) (yellow, refined, Sigma-Aldrich, USA), Glycerol (Gly) (Cicarelli,

115 Argentina), potassium sorbate (Anedra, Argentina), and Tween 80 (Anedra, Argentina)
116 were used.
117
118 **2.2. Preparation of film-forming solutions**
119 Aqueous solutions of 8% (w/w) WPC (pH=6.2) were prepared as described in Soazo et
120 al. (2011a). Glycerol (in proportion WPC/Gly 3:1 w/w dry solid basis) and potassium
121 sorbate (to obtain a final concentration of 0.12% w/w dry solid basis) were added, and
122 solutions were magnetically stirred during 15 min. Then, BW (at 0, 20 and 40% w/w
123 dry solid basis in the mixture WPC/Gly) and Tween 80 (in proportion BW/Tween 4:1)
124 were incorporated. Tween 80 was used as emulsifier in the solutions containing BW.
125 The amount of distilled water was adjusted to obtain a total solid content of 11.5%. Film
126 forming solutions were heated at 90 ºC for 30 min in a water bath (Dalvo Instruments,
127 Argentina) to achieve whey proteins denaturation. Emulsions were obtained by
128 homogenization in the water bath at 90 °C using a high-shear probe mixer Ultra-Turrax
129 T25 (IKA Werke, Janke & Kunkel GmbH & Co KG, Staufen, Alemania) with a stator
130 diameter of 10 mm, a beaker diameter of 70 mm, and a protein dispersion volume of
131 200 mL, during 5 min at 21500 rpm. After homogenization, the emulsions were placed
132 in an ice bath during 30 min to prevent further denaturation of the whey proteins and to
133 crystallize the lipid particles. The emulsions were degassed at room temperature with a
134 vacuum pump.
135
136 **2.3. Film formation**
137 Eight grams of the degassed emulsion were pipetted on 90 mm diameter disposable
138 polyethylene Petri dishes. Films were dried at 5 °C and 58% relative humidity (RH) on
139 a levelled surface in an environmental chamber Tabai Comstar PR 4GM (Tabai Espec.

Corp., Japan) equipped with a fan that circulates interior air at approximately 60 m/min. Drying was completed after 20±2 h. The films used in the different tests were selected based on the lack of physical defects such as cracks, bubbles, and holes. A group of films was conditioned at 25 °C and 58% RH for 3 days and subsequently employed for the determinations (Control Group). Another group of films was frozen in the environmental chamber at -30 ºC, and then stored in plastic containers at -20±2 ºC for 30 days. Completed this period the films were thawed at 5 °C and conditioned at 25 ºC and 58% RH for 3 days before analysis (Frozen Group).

2.4. Film thickness

Film thickness was measured with a digital micrometer (Schwyz, China). For each film, nine thickness measurements were taken. Films were obtained with an average thickness of 0.156 ± 0.011 mm.

2.5. Scanning electron microscopy

The film samples were cryo-fractured by immersion in liquid air and mounted on bronze stubs perpendicularly to their surface. The portions were coated with gold during 15 min at 70-80 mTorr. Micrographs of films cross-section were taken with a JEOL JSM-35C electron microscope (JEOL, Japan) using an accelerating voltage of 20 kV. Magnification of 400 was used.

2.6. Water vapour permeability

A modification of the (E96-95, ASTM, 2002) gravimetric method for measuring water vapour permeability (WVP) was used. Films were mounted on cups containing 10 mL of distilled water, with a permeation area of 19.6 cm^2, with the film surface which had

been exposed to air during drying facing the external side of the cup, as described in Soazo et al. (2011a). Cups were placed in the environmental chamber at 25 °C and 58% RH and weighted every hour and a half to obtain four successive steady-state measurements. Weight loss-time curves were used to calculate the % RH at the film underside and the resulting WVP as described in McHugh, Avena Bustillos and Krochta (1993). Determinations were performed in quintuplicate.

2.7. Solubility in water

Pieces of films of 15 × 7.5 mm were cut with a scalpel, dried in an oven (Dalvo Instruments, Argentina) at 70 ºC for 24 h and weighed to obtain the initial film dry weight. Each piece of film was placed into a glass tube with 10 mL of distilled water and 0.01% potassium sorbate to prevent microbial growth. Capped tubes were placed on a shaking platform (Vicking, Argentina) for 24 h at 25 ºC. Circular filter papers (qualitative grade, Boeco, Germany) were dried 24 h in the oven at 70 ºC, cooled to room temperature in a desiccator, and weighed to obtain the initial dry filter weight. The solution containing the films was filtered by pouring the contents of the test tube onto a filter paper placed in a Buchner funnel attached to the neck of a 250 mL erlenmeyer connected to a vacuum pump (Soazo et al., 2011a). The remaining solids on the filter were dried in the oven at 70 ºC for 24 h to determine the final filter dry weight. The difference between the final dry filter weight and initial dry filter weight yielded the final dry film weight. Solubility in water, expressed as soluble solids (%), was obtained by subtracting the weight of dry matter not solubilised from the weight of initial dry matter and reported on initial dry weight basis (Sothornvit, & Krochta, 2000). Determinations were performed in quintuplicate.

190 **2.8. Moisture sorption isotherms**

191 Films were cut in portions of 400 mg, placed in glass bottles previously weighed, and
192 pre-dried in desiccators containing drierite (a_w=0) during 10 days. Then, the bottles
193 were placed in hermetically sealed glass jars containing different desiccants as
194 described in Soazo et al. (2011b). Ten saturated salt solutions: LiCl, $KC_2H_3O_2$,
195 $MgCl_2 \cdot 6H_2O$, K_2CO_3, $Mg(NO_3)_2 \cdot 6H_2O$, NaBr, $SrCl \cdot 6H_2O$, NaCl, KCl, and $BaCl_2 \cdot 2H_2O$
196 were used to obtained the respective RH/100 conditions: 0.11, 0.22, 0.33, 0.43, 0.53,
197 0.58, 0.71, 0.75, 0.84, and 0.90 (Jowitt & Wagstaffe, 1989). Film portions were
198 equilibrated in contact with each salt solution during 10 days at 25 ºC. After that, the
199 bottles were weighed to obtain the sample weight at equilibrium and then dried in the
200 oven at 105 ºC during 4 hours to obtain the weight of the dry sample. The analyses were
201 made in quintuplicate. The equilibrium moisture content (EMC) was calculated using
202 Equation 1:
203

$$EMC = \frac{(w_2 - w_3)}{(w_3 - w_1)} 100 \quad (1)$$

204 where w_1 is the weight of the empty glass bottle, w_2 is the weight of the sample at
205 equilibrium plus the weight of the empty glass bottle and w_3 is the weight of the dry
206 sample plus the weight of the empty glass bottle.
207

208 In a previous work we studied the effect of drying temperature and beeswax content on
209 moisture isotherms of whey protein emulsion films and observed that the Guggenheim-
210 Anderson-De Boer (GAB) model was the most appropriate to describe the moisture
211 sorption of the films (Soazo et al., 2011b). Consequently, the EMC values were fitted
212 using the GAB model as followed:
213

$$EMC = \frac{m_0 C k a_w}{(1 - k a_w)(1 - k a_w + C k a_w)} \quad (2)$$

where a_w is the water activity, m_0 is the monolayer moisture content (g H_2O/g solids), C is the surface heat constant and k is an additional constant.

Experimental data were fitted to Equation 2 using an algorithm written in Matlab 6.5.1 (MathWorks Inc, USA). The parameters were calculated minimizing the error function as described by Coupland, Shaw, Monahan, O'Riordan and O'Sullivan (2000):

$$error = \frac{1}{n} \sum_{a_w=0}^{n} \left| \frac{EMC_{measured\ (a_w)} - EMC_{modeled\ (a_w)}}{EMC_{measured\ (a_w)}} \right| \quad (3)$$

2.9. Mechanical properties

Tensile and puncture tests were performed to evaluate the mechanical strength of the films using a single column Universal Testing Machine Instron, Series 3340 (Instron, USA) with a 10 N load cell. Films strips of 7 mm wide and 60 mm length were used for the tensile tests, while discs of 90 mm of diameter were used for the puncture test. Samples were equilibrated to the testing environment for 2 h at 22 ºC and 50% RH on average. For each mechanical test ten replications were performed.

Strips for the tensile test were cut using a scalpel. The ends were mounted with a double sided tape and 30 mm squares made of cardstock. These cardstock pads were placed on the film strips ends to prevent tearing and slippage in the testing device (Shellhammer & Krochta, 1997). The exposed film strip length between the cardstock was 30 mm. Crosshead speed was 0.05 mm/s. The parameters obtained from this test were tensile strength (TS), elongation (E), and elastic modulus (EM). TS was calculated dividing the

peak load by the cross sectional area of the initial film (thickness of film × 7 mm), E was obtained as the percentile of the change in the length of specimen to the original distance between the grips and EM was determined from the initial slope of the stress-strain curve (Han, Seo, Park, Kim, & Lee, 2006).

For the puncture test film discs were fixed to a support with a circular opening of 50 mm in diameter and 30 mm in depth. A cylindrical probe of 2 mm diameter was moved perpendicularly to the film surface at a constant speed of 0.8 mm/s until it passed through the film. Puncture strength (PS) and deformation (D) at the puncture point were obtained from force-distance curves (Chen & Lai, 2008).

2.10. Statistical analysis

A full factorial design was performed. Two factors (Freezing and BW content) in two and three levels, respectively, were studied (Frozen and Controls films, and 0, 20 y 40% of BW). Analysis of variance was used and when the effect of the factors was significant ($p < 0.05$), the Tukey multiple ranks honestly significant difference (HSD) test was applied (95% of confidence level).

Principal component analysis (PCA) was used to reduce the dimensionality of the data obtained in the determinations and to show the relationships between treatments (Johnson & Wichern, 1998). The principal components (PCs) are numbered in order of the amount of variation in the original data set. Consequently, the first principal component accounts for the most variation, and each subsequent principal component accounts for as much of the remaining variation. An adequate condensation of the information is achieved when no more than two or three PCs can explain at least 80–90% of the total variability (Verdini, Zorrilla, Rubiolo, & Nakai, 2007).

Because the input variables differed in magnitude, all values of a given variable were scaled from 0 to 100 respect to the range between the smallest and the largest variable value before performing PCA analysis (Verdini et al., 2007). The statistical analysis was performed using Minitab 13.20 (Minitab Inc., USA).

3. Results and discussion

3.1. Scanning electron microscopy

Figure 1 shows characteristic images of transversal sections of Frozen and Control emulsion films. The upper side (A) was exposed to air and the lower side (B) was in contact with the Petri dish surface during drying.

After the freezing process, no fractures or perforations were observed in Frozen films. Frozen and Control films without BW showed a continuous and homogeneous cross-section. The homogeneous cross-section was also observed by other authors in unfrozen films based on casein, amaranth flour and soy protein (Chick & Hernandez, 2002; Tapia-Blácido, Sobral & Menegalli; 2005; Denavi, Tapia-Blácido, Añón, Sobral, Mauri & Menegalli, 2009).

Both Frozen and Control films with BW showed a preferential location of the wax on the film side exposed to air during drying. This phenomenon, may be related to the fact that film forming solutions with BW suffered destabilization during drying, and was more evident as BW content increased. However, the destabilizing phenomenon taking place during the drying step is dependent on the interactions between the components of the film. Prodpran, Chinabhark and Benjakul (2005) showed evidence of emulsion destabilization during drying in surimi protein based edible films. Atarés, Bonilla and Chiralt (2010) reported that the final microstructure of unfrozen caseinate based films

with ginger oil had more discontinuities as the ginger oil content increased. On the other hand, the authors found that unfrozen caseinate based films with cinnamon oil showed no apparent differences in their microstructural aspect, probably due to the fact that the small size of the particles in the emulsions was not modified during the drying process progress, and cinnamon oil stayed homogeneously distributed in the dry protein matrix. Finally, lipids located on the surface of Frozen films looked flattened compared with those of Control films.

3.2. Water vapour permeability

Table 1 shows the WVP of Frozen and Control emulsion films with different BW contents. An increasing tendency in WVP values after 30 days of frozen storage was observed. However, this increase was statistically significant ($p<0.05$) only in films with 40% of BW.

In agreement, Kester and Fennema (1989) who studied the effect of low temperature storage on barrier characteristics of wax-laminated films of lipids and cellulose ethers stored at -40 ºC for 3 and 9 weeks, found a small increase in WVP possibly due to slight imperfections that could develop because of fluctuating storage temperatures and an accompanying contraction and expansion of the lipids. If major cracking or fracture had occurred, a dramatic elevation in permeability would be anticipated.

In the present work, cracking or fractures were not observed. But, the scanning electron microscopy analysis of Frozen films revealed that the morphology of lipids located on the surface showed a flattened appearance. This phenomenon may be related to the slight differences observed in the WVP between Frozen and Control films. In agreement, Kester and Fennema (1989) showed that the lipid morphology was related to moisture transfer resistance of the cellulose based edible films.

312 Analyzing the addition of BW in Frozen and Control films, the lipid addition decreased
313 WVP in both groups, but the decrease was higher for Control films. In agreement, the
314 micrographs showed a high proportion of the film surface exposed to air during drying
315 covered by lipids; this bilayer like structure could explain the low WVP values.
316
317 **3.3. Solubility in water**
318 Table 1 shows the solubility in water of Frozen and Control WPC emulsion films with
319 different BW contents. Both groups were partially soluble (solubility between 22.4 and
320 39.4%) maintaining their integrity during immersion in water.
321 Solubility of whey protein films without BW was not affected by the freezing process.
322 Films with BW showed an increasing tendency in solubility that was not statistically
323 significant ($p>0.05$). These results are in accordance with the microscopic images that
324 showed no signs of altered matrix porosity that could modify the solubility of the
325 hydrophilic compounds included in the film formulation.
326 In reference to the addition of BW, Frozen and Control films showed a tendency to
327 decrease solubility with the corresponding BW addition, although this effect was
328 statistically significant ($p<0.05$) only in the Control films group. Similar measurements
329 were reported in unfrozen whey protein based edible films (Kim & Ustunol, 2001;
330 Ozdemir & Floros, 2008). These authors considered that, because the total solids level
331 remained constant in the formulation, the incorporation of BW reduced the soluble
332 matter present in the films, and consequently the solubility.
333
334 **3.4. Moisture sorption isotherms**
335 Experimental data of EMC for Frozen and Control emulsion films with different BW
336 contents are shown in Figure 2. A slow increase in the EMC until 0.58 a_w, followed by

337 an abrupt increase in the EMC was observed. Such sigmoidal water sorption isotherms
338 are characteristic of materials rich in hydrophilic polymers (Zinoviadou, Koutsoumanis,
339 & Biliaderis, 2009). The effect of the freezing process on the EMC was dependant on
340 the a_w region. Although the freezing process produced slight differences in the EMC
341 values, those differences were more notorious at higher a_w values.
342 Analyzing the parameters of the GAB model (m_0, k and C), parameter C was more
343 sensible to freezing, showing an increase in films without BW and with 40% of BW;
344 and a decrease in films with 20% of BW (Table 2). Although parameter m_0 slightly
345 changed, a clear tendency was not found. On the other hand, parameter k remained
346 almost invariable.
347 In reference to the effect of the addition of BW, a decreasing tendency in parameter m_0
348 was observed. The incorporation of lipids reduces the water sorption capacity of the
349 film due to the fact that lipids correspond to a fraction of solids with a low water uptake
350 capacity, especially beeswax, which is very hydrophobic (Fabra, Talens, Gavara, &
351 Chiralt, 2002). Thus, the decrease in the monolayer moisture content was also reported
352 by Kim and Ustunol (2001) in the case of WPI/candelilla wax and WPI/butterfat. These
353 authors also found a decrease in C and k parameters when lipids were present in the
354 formulations. In our investigation, a clear effect of the addition of BW on parameter C
355 was not found, and lipid addition did not affect parameter k.
356
357 **3.5. Mechanical properties**
358 Tables 3 and 4 show the puncture strength (PS) and deformation (D) calculated from
359 force-distance and the tensile strength (TS), elongation (E) and elastic modulus (EM)
360 derived from stress-strain curves, respectively.

361 The freezing process increased PS and D of films without BW, but did not affect the
362 response to puncture of films with BW. The addition of BW decreased the PS and D of
363 Control and Frozen films. This could be explained because of the expected weakening
364 and lubricating effect of lipids on the whey protein films (Banerjee & Chen, 1995).
365 In tensile test, the freezing process decreased both TS and EM, in films without BW as
366 well as in films with BW. In general E was not affected by freezing. The addition of
367 BW decreased all parameters of Control and Frozen films. These results may be related
368 to the fact that BW causes the disruption of the continuous matrix and induces the
369 development of a heterogeneous film structure (Navarro-Tarazaga, Sothornvit & Pérez-
370 Gago, 2008). Our results were similar to the reports of other authors in unfrozen whey
371 protein emulsion films (Shellhammer & Krochta, 1997; Talens & Krochta, 2005).
372
373 **3.6. Principal component analysis**
374 PCA was applied to visualize the distribution of film samples according to film
375 formulation and treatment (Control and Frozen films) and to achieve an adequate
376 condensation of the information. PC1 and PC2 explained together the 82.2% of the
377 variance, with PC1 and PC2 explaining the 72.2% and 10% of the variance,
378 respectively.
379 Figure 3A shows PC1 versus PC2 score plot displaying the relationship between the
380 samples in the new coordinate (PC defined) space and assembling film samples
381 according to their composition and treatment (Control or Frozen). The score plot
382 illustrated the separation of the samples in four groups: Control 0% BW, Frozen 0%
383 BW, Control 20 and 40% BW and Frozen 20 and 40% BW. PC1 separated the samples
384 according to the presence of BW in the film formulation, and PC2 spreaded the samples
385 from down to up according to treatment.

Figure 3B shows the PC1 and PC2 loadings plot illustrating the relationship between the original variables and the principal components and helping to identify the most important parameters of the discrimination observed in the PC score plot (Henrique, Teófilo, Sabino, Ferreira, & Cereda, 2007). Variables with higher absolute values of PC1 loadings (PS and D) explained the sample separation according to the presence of BW in the film formulation. On the other hand, variables with higher absolute values of PC2 loadings (WVP, Solubility and EM) explained the sample arrangement according to treatment (Frozen and Control films). The EMC at different a_w did not show any clear effect on the sample distribution.

PCA results allowed to sumarize the information showing that freezing increased WVP and solubility but decreased the elastic modulus of whey protein films, both in films with and without BW. On the other hand, freezing increased puncture parameters only in formulations without BW, being this property the most affected by the film formulation.

4. Conclusions

Whey protein emulsion films probed to be resistant to the freezing process (freezing, frozen storage and thawing) maintaining their physical integrity. Frozen films were as efficient as Control films with regards to moisture sorption. In reference to the effect of the freezing process on the mechanical properties, puncture resistance was maintained and in some formulations was even improved while tensile resistance was negatively affected. In summary, whey protein emulsion films could constitute a good alternative as a treatment to preserve the quality of frozen foods; however optimization of the formulations should be performed.

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558 **Table 1.** Effect of freezing on water vapour permeability (WVP) and solubility of whey
559 protein concentrate emulsion films.

Group	Beeswax (%)	WVP (gmm/m^2hkPa)	Solubility (%)
Frozen	0	3.76 ± 0.19^{a}	38.4 ± 5.1^{a}
Control	0	3.48 ± 0.17^{ab}	39.4 ± 5.0^{a}
Frozen	20	3.29 ± 0.15^{bc}	34.8 ± 6.3^{ab}
Control	20	3.07 ± 0.14^{c}	27.1 ± 3.4^{bc}
Frozen	40	3.38 ± 0.15^{b}	31.1 ± 4.1^{abc}
Control	40	2.56 ± 0.08^{d}	22.4 ± 1.7^{c}

560

561 Data corresponds to mean values and standard deviations of five samples.

562 Values with different letters in each column are significantly different ($p<0.05$),
563 according to Tukey's test.

564

Table 2. Parameters obtained by fitting of equilibrium moisture content of whey protein concentrate emulsion films to the GAB model: m_0 (monolayer moisture content), C (surface heat constant) and k (additional constant).

Group	Beeswax (%)	m_0	C	k	R^2	Error[a] (%)
Frozen	0	10.54	25.56	0.93	0.99	3.98
Control	0	10.78	22.37	0.93	1.00	5.16
Frozen	20	8.34	15.27	0.93	1.00	2.81
Control	20	7.40	25.52	0.93	0.99	5.54
Frozen	40	8.18	98.75	0.90	1.00	2.44
Control	40	8.27	25.61	0.93	0.99	5.43

[a] Calculated according to Coupland et al. (2000).

570 **Table 3.** Effect of freezing on parameters derived from the puncture test of whey
571 protein concentrate emulsion films: PS (puncture strength) and D (deformation).

Group	Beeswax (%)	PS (N)	D (mm)
Frozen	0	2.20±0.28[a]	1.92±0.19[a]
Control	0	1.86±0.34[b]	1.75±0.14[b]
Frozen	20	0.25±0.07[c]	0.55±0.11[c]
Control	20	0.21±0.03[c]	0.50±0.09[c]
Frozen	40	0.15±0.03[c]	0.45±0.06[c]
Control	40	0.19±0.04[c]	0.44±0.07[c]

572

573 Data corresponds to mean values and standard deviations of ten samples.

574 Values with different letters in each column are significantly different ($p<0.05$),
575 according to Tukey's test.

576

577 **Table 4.** Effect of freezing on parameters derived from the tensile test of whey protein
578 concentrate emulsion films: TS (tensile strength), E (elongation) and EM (elastic
579 modulus).

Group	Beeswax (%)	TS (MPa)	E (%)	EM (MPa)
Frozen	0	3.69±0.43^{b}	4.99±0.59^{a}	154±9^{c}
Control	0	4.92±0.78^{a}	3.50±0.98^{b}	229±22^{a}
Frozen	20	1.40±0.10^{d}	2.30±0.23^{c}	109±9^{d}
Control	20	2.63±0.36^{c}	2.38±0.36^{c}	185±12^{b}
Frozen	40	1.09±0.22^{d}	1.89±0.35^{c}	103±9^{d}
Control	40	1.64±0.33^{d}	1.80±0.42^{c}	149±12^{c}

580

581 Data corresponds to mean values and standard deviations of ten samples.
582 Values with different letters in each column are significantly different ($p<0.05$),
583 according to Tukey's test.
584

585 **Figure captions**

586

587 **Figure 1.** Scanning electron micrographs of Frozen and Control WPC emulsion films:
588 In all pictures the evaporation surface of the film is on the top. Micrographs labelled (A)
589 showed the cross section of the film and the surface exposed to air during drying.
590 Micrographs labelled (B) showed the film cross section and the surface in contact with
591 the Petri dish during drying. The comparison between (A) and (B) illustrates the
592 occurrence of the destabilization during drying of whey protein films containing BW.

593

594 **Figure 2.** Moisture sorption isotherms of Frozen and Control WPC emulsion films.
595 Bars are based on standard deviations (n=5).

596

597 **Figure 3.** Plots of the first two principal components (PC1 vs. PC2): (a) PC scores plot
598 of Control and Frozen WPC emulsion films. (b) PC loadings plot. WVP: water vapour
599 permeability; PS: puncture strength; D: deformation; TS: tensile strength; E: elongation;
600 EM: elastic modulus. EMC: equilibrium moisture content (at each a_w value).

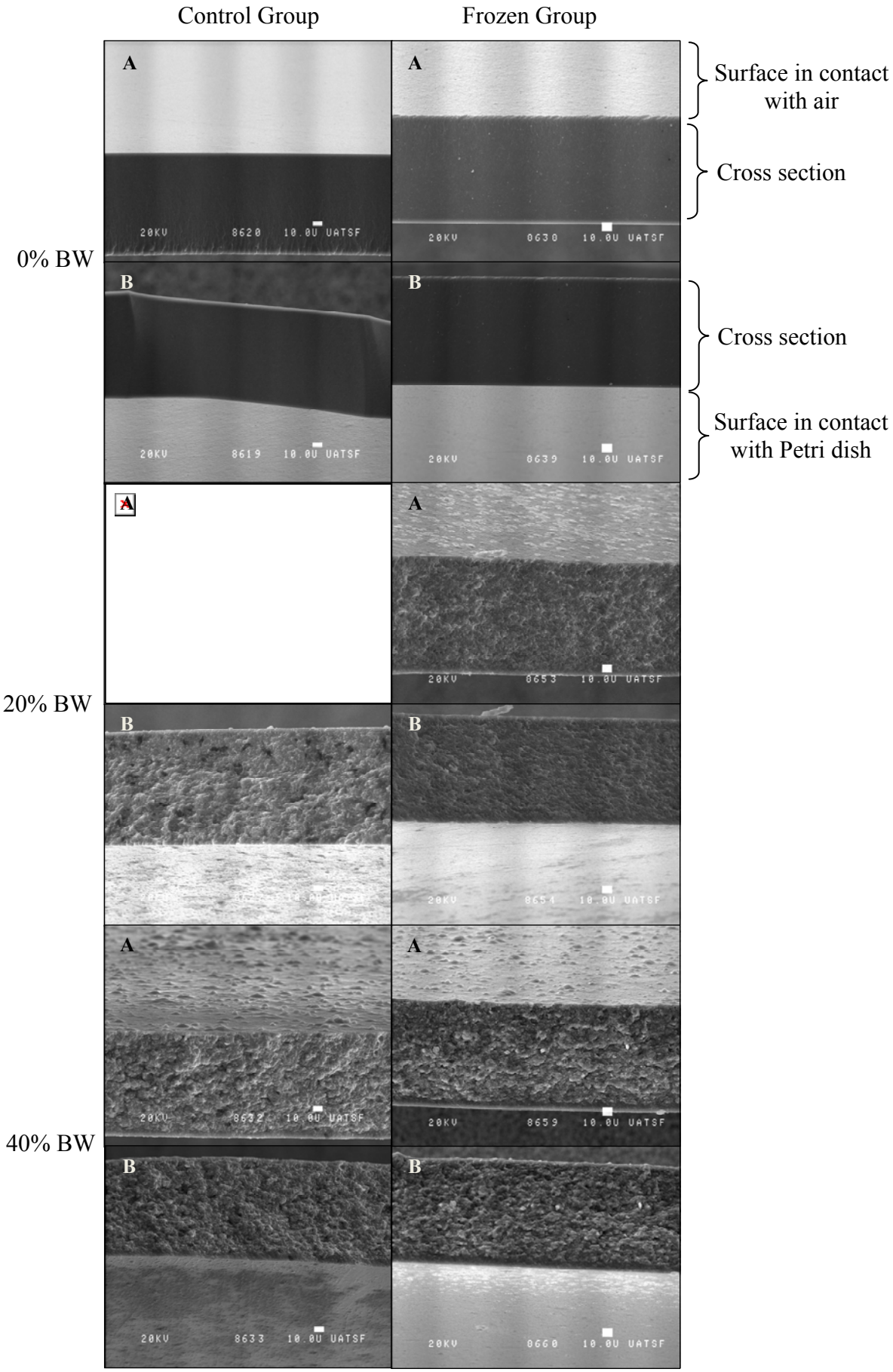

Control Group
Frozen Group
Surface in contact with air
Cross section
Cross section
Surface in contact with Petri dish
0% BW
20% BW
40% BW
A
B

Figure 1

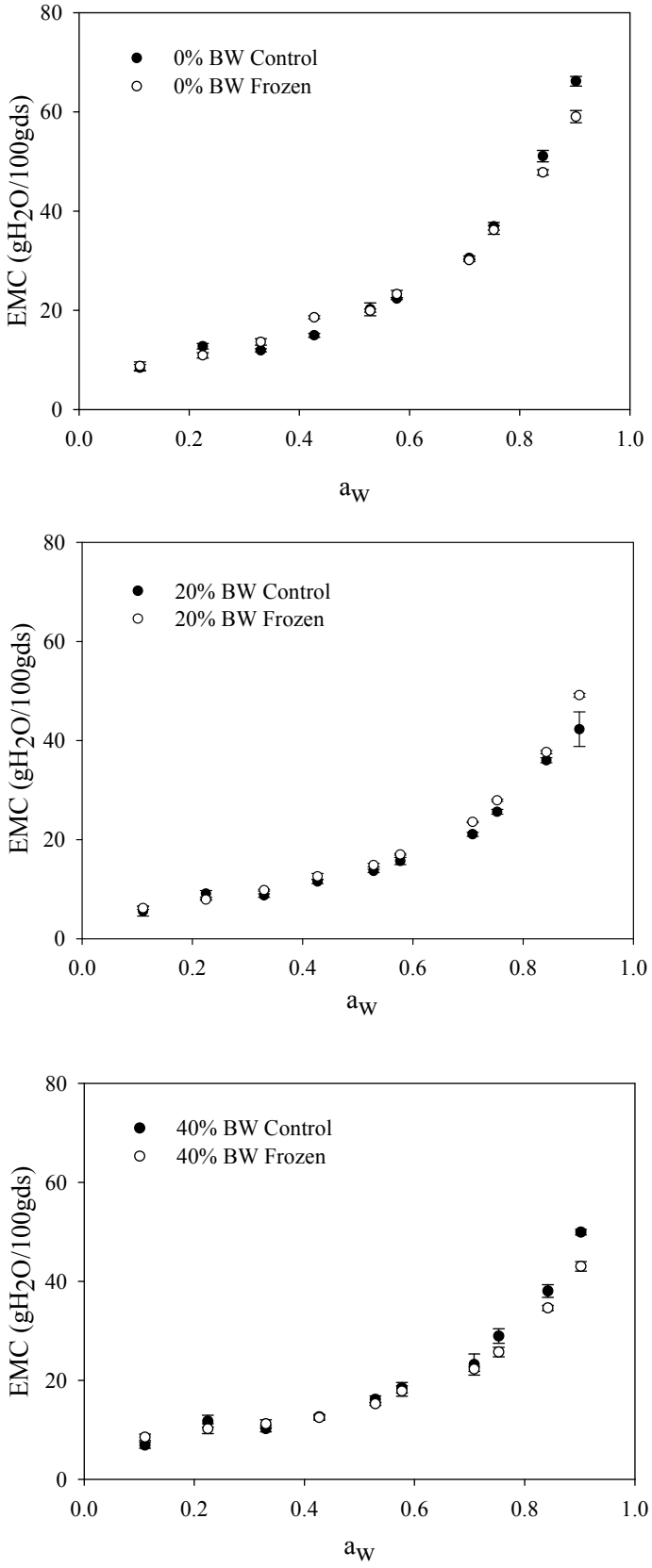
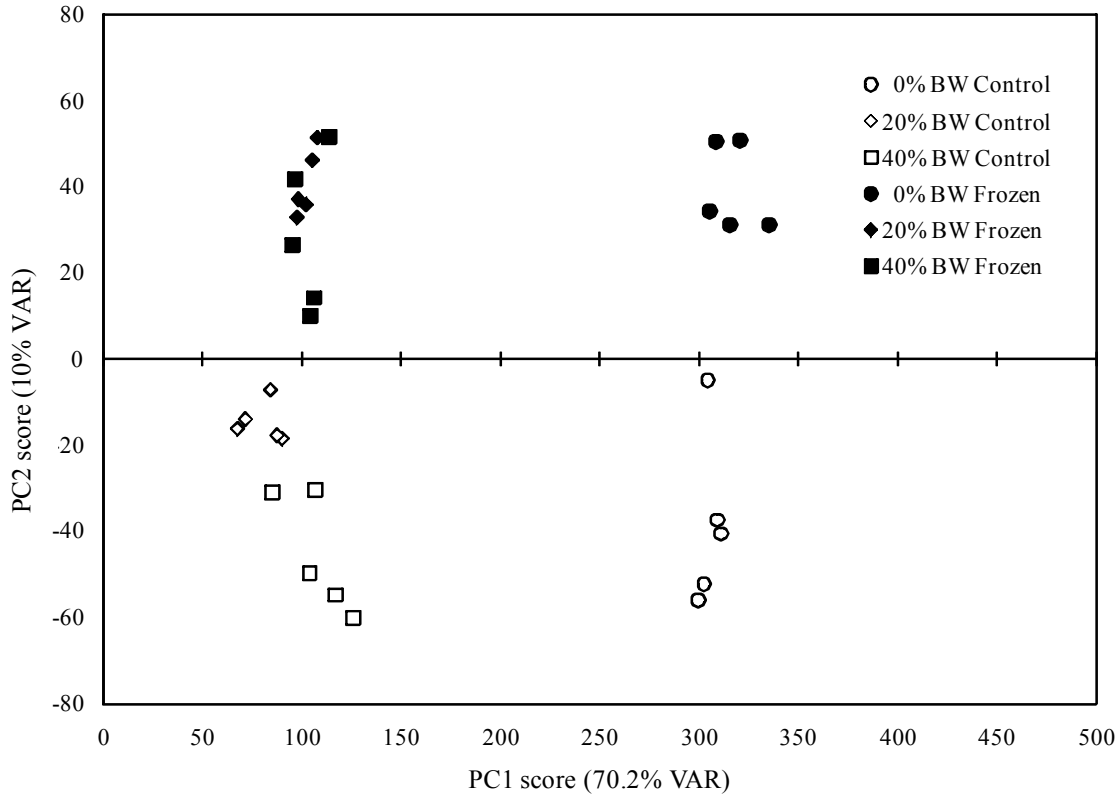

0% BW Control
0% BW Frozen
20% BW Control
20% BW Frozen
40% BW Control
40% BW Frozen
EMC (gH2O/100gds)
aW
0
20
40
60
80
0.0
0.2
0.4
0.6
0.8
1.0

Figure 2


0% BW Control
20% BW Control
40% BW Control
0% BW Frozen
20% BW Frozen
40% BW Frozen
PC2 score (10% VAR)
PC1 score (70.2% VAR)
80
60
40
20
0
-40
-60
-80
0
50
100
150
200
250
300
350
400
450
500

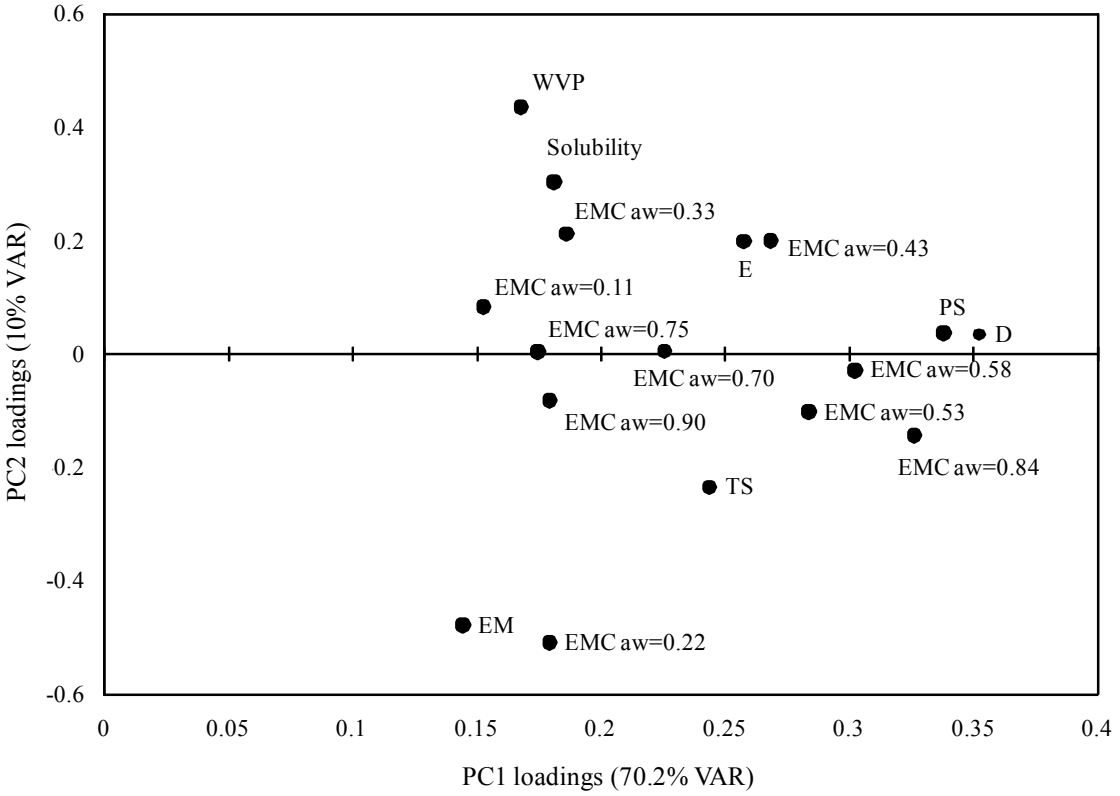

WVP
Solubility
EMC aw=0.33
EMC aw=0.43
E
EMC aw=0.11
PS
D
EMC aw=0.75
EMC aw=0.70
EMC aw=0.58
EMC aw=0.90
EMC aw=0.53
EMC aw=0.84
TS
EM
EMC aw=0.22
PC2 loadings (10% VAR)
PC1 loadings (70.2% VAR)
0.6
0.4
0.2
0
0.2
-0.4
-0.6
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4

Figure 3