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1            **Bioactive films based on cuttlefish (*Sepia officinalis*) skin gelatin**  
2            **incorporated with cuttlefish protein hydrolysates: physicochemical**  
3            **characterization and antioxidant properties**  
4

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19

20 **Abstract**

21 The objective of this study was to apply cuttlefish (*Sepia officinalis*) skin protein isolate  
22 (CSPI) and hydrolysates (CSPH), using commercial Savinase® and Purafect® enzymes, as  
23 bioactive additives in the elaboration of gelatin-based films. CSPH and CSPI enriched films  
24 were colored and exhibited a higher UV-barrier properties compared to gelatin film. In  
25 addition, compared to CSPI added film, an increase of the glass transition temperature by  
26 20% and 4%, respectively, for Purafect and Savinase hydrolysates enriched films was noted.  
27 However, elongation at break decreased significantly for CSPH incorporated films by 2.5-  
28 fold. The tensile strength was reduced by 28.2% and 44.4% for Purafect and Savinase  
29 hydrolysates added films, respectively. Furthermore, a decrease of water contact angle by  
30 45% and 51% for films added with Purafect and Savinase hydrolysates, respectively, was  
31 displayed compared to gelatin film. Interestingly, CSPH enriched films also displayed higher  
32 antioxidant potential than control gelatin films evaluated by several *in vitro* assays.

33

34 **Keywords:** Cuttlefish skin proteins and hydrolysates; Edible films; Functional properties;  
35 Antioxidant activity.

36

## 37 **1. Introduction**

38           In recent years, the interest in by-products (viscera, head, trimmings, bones and skin)  
39 from the fishing industry has been gradually increased, now being considered as a potential  
40 source of resources rather than a disposal waste (Alfaro, Balbinot, Weber, Tonial, &  
41 Machado-Lunkes, 2015). In order to valorize fish by-products, several bioactive molecules  
42 can be extracted from the skin of various marine species such as gelatin, protein isolate, etc.  
43 Indeed, fish proteins have advantageous filmogenic properties that can promote the  
44 development of films, such as the ability to form networks, plasticity, elasticity and good  
45 barrier to oxygen (Cortez-Vega, Pizato, de Souza, & Prentice, 2014).

46           Gelatin is an important biopolymer derived by hydrolysis from collagen, the primary  
47 protein component of animal connective tissues, including skin and tendon (Poppe, 1997).  
48 Gelatin is widely used by food, cosmetic and pharmaceutical industries because of its  
49 functional and technological properties. Fish gelatins have been also extensively studied as  
50 biodegradable biopolymers due to their good film forming ability leading to produce  
51 transparent, almost colorless, water-soluble and highly extensible films (Hosseini & Gómez-  
52 Guillén, 2018; Alfaro et al., 2015). Furthermore, these biodegradable films are considered as  
53 ecofriendly packaging reducing thereby plastic wastes (Hoque, Benjakul, & Prodpran, 2011a;  
54 Alinejad, Motamedzadegan, Rezaei, & Regenstein, 2017).

55           Gelatin films could be used as carrier agents for many types of additives such as  
56 antimicrobial agents in order to delay or prevent the growth of microorganisms on the  
57 products surface and thereby extend the shelf life and improve the safety of packaged foods  
58 (Etxabide, Uranga, Guerrero, & de la Caba, 2017). Antioxidants including plant extracts  
59 (Gómez-Guillén, Ihl, Bifani, Silva, & Montero, 2007; Hoque, Benjakul, & Prodpran, 2011b;  
60 Jridi et al., 2017), phenolic compounds (Bao, Xu, & Wang, 2009; Benbettaïeb et al., 2016),  
61 essential oils (Martucci, Gende, Neira, & Ruseckaite, 2015) or polysaccharides (Abdelhedi et

62 al., 2018) are additives often incorporated in fish gelatin films preparation to prevent or delay  
63 food oxidation. Recently, many studies dealt with the elaboration and characterization of  
64 protein hydrolysates from various marine sources. Protein hydrolysates, generally obtained by  
65 autolytic or heterolytic enzymatic hydrolysis process under controlled conditions from marine  
66 sources, are considered as bioactive peptides which are characterized by several biological  
67 activities including antioxidant (Abdelhedi et al., 2016; Nasri et al., 2013), antibacterial  
68 (Beaulieu, Bondu, Doiron, Rioux, & Turgeon, 2015), anti-diabetic (Harnedy et al., 2018),  
69 anti-hypertensive (Lassoued et al., 2015), anti-inflammatory (Ahn, Cho, & Je, 2015),  
70 cholesterol-lowering ability and immunomodulating effects (Nasri, 2017).

71 However, few studies were interested in protein hydrolysates incorporation as  
72 antioxidant agents into gelatin films. In this context, Giménez, Gómez-Estaca, Alemán,  
73 Gómez-Guillén, & Montero (2009) investigated the effect of the incorporation of giant squid  
74 gelatin hydrolysates on the antioxidant property of the gelatin film. Additionally, Alinejad et  
75 al. (2017) studied the influence of adding protein hydrolysates obtained from whitecheek  
76 shark on the physical-mechanical properties and antioxidant activity of bovine gelatin films.  
77 Abdelhedi et al. (2018) reported that bioactive blend and bilayer films based on gelatin and  
78 smooth-hound viscera proteins, incorporated or not with sulfated polysaccharide or smooth-  
79 hound peptides were successfully made and showed interesting antioxidant potential.

80 In a previous work, blend films based on cuttlefish (*Sepia officinalis*) skin gelatin  
81 (CSG) and protein isolate (CSPI) at different ratios were prepared and showed interesting  
82 antioxidant activity which is CSPI content dependent (Kchaou et al., 2017). In the present  
83 research, enzymatic hydrolysis was used in order to produce different protein hydrolysates  
84 (CSPH) from CSPI. Therefore, the aim of this study was to evaluate the effect of CSPH  
85 incorporation on the physical-chemical and antioxidant properties of gelatin films.

## 86 **2. Materials and methods**

### 87 *2.1. Collection and preparation of cuttlefish skin*

88 Cuttlefish (*S. officinalis*) by-products were obtained from the local fish market of Sfax City,  
89 Tunisia. Cuttlefish were collected from February to April at the golf of Gabes. The samples  
90 were packed in polyethylene bags, placed in ice and transported to the research laboratory  
91 within 30 min. Upon arrival, cuttlefish skins were washed several times with tap water to  
92 eliminate residues and dark ink and then stored at -20 °C in plastic bags until used for gelatin  
93 and protein isolate production.

### 94 *2.2. Extraction of gelatin*

95 Gelatin extraction was carried out from cuttlefish skin as described by Jridi et al. (2013a).  
96 Cuttlefish skin was first cut into small pieces (1 cm × 1 cm) and soaked in 0.05 M NaOH  
97 (1:10, w/v). The mixture was stirred for 2 h at room temperature (25±2 °C) and alkaline  
98 solution was changed every 30 min. The alkaline-treated skins were then washed with  
99 distilled water until a neutral pH was obtained. The prepared skins were soaked in 100 mM  
100 glycine-HCl buffer, (pH 2.0) with a solid/solvent ratio of 1:10 (w/v) for 18 h at room  
101 temperature (25±2 °C) (hydrolysis of collagen), and then treated at 50 °C for additional 18 h  
102 to extract the gelatin fractions. The supernatant of the obtained mixture was then freeze-dried  
103 (Moduloyd Freeze dryer, Thermo Fisher, USA) at -50 °C and 121 mbar during 72 h. The  
104 resulting cuttlefish skin gelatin (CSG) was used for film preparation.

### 105 *2.3. Extraction of cuttlefish skin protein isolate*

106 Protein isolate was extracted from cuttlefish skin as reported in our previous work  
107 (Kchaou et al., 2017) using the pH-shifting method. An aqueous dispersion of cuttlefish skin  
108 mince was first prepared, by solubilisation in distilled water. The pH was adjusted at 11.0

109 using 2 M NaOH solution for 30 min. The ratio cuttlefish mince and water was 1:3 (w/v).  
110 Solubilisation was maintained under continuous stirring at room temperature ( $25\pm 2$  °C). The  
111 resulting mixture was centrifuged. The obtained pellet containing the collagen underwent an  
112 acidic treatment with an HCl solution (1 M) at pH 2.0 for 15 minutes, followed by a thermal  
113 treatment at 50 °C for 1 hour to denature the triple helix collagen structure. The resulting  
114 mixture was centrifuged and the resulted supernatant was freeze-dried (at -50 °C and 121  
115 mbar during 72 h) and referred to as cuttlefish skin protein isolate (CSPI).

#### 116 *2.4. Preparation of protein hydrolysates from CSPI*

117 In order to obtain protein hydrolysates, CSPI was first dissolved in distilled water at  
118 50 °C with a solid/solvent ratio of 1:4 (w/v). Then, the pH of the mixture was adjusted to the  
119 optimum value of each enzymatic activity (pH 10.0) by adding 4 N NaOH solution.  
120 Thereafter, protein isolate was subjected to enzymatic hydrolysis, using two exogenous  
121 enzymes, Savinase<sup>®</sup> and Purafect<sup>®</sup>, added at the same enzyme/protein ratio 6/1 (U/mg of  
122 protein) to compare their hydrolytic efficiencies. During the reaction (50 °C), the pH of the  
123 mixture was maintained constant (pH 10.0) by continuous addition of NaOH solution. After  
124 the achievement of the final digestion reaction time (7 h), the reactions were stopped by  
125 heating the different solutions at 95 °C for 20 min to inactivate the enzymes. The  
126 supernatants, corresponding to the different protein hydrolysates, were then collected, freeze-  
127 dried (at -50 °C and 121 mbar during 72 h) and stored at -20 °C for further use. Hydrolysates  
128 prepared using Savinase<sup>®</sup> and Purafect<sup>®</sup> were noted as Savinase and Purafect hydrolysates,  
129 respectively.

130 The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide  
131 broken to the total number of bonds, was calculated based on the volume of NaOH added  
132 during the reaction, as described by Adler-Nissen (1986) using the following formula:

133 
$$DH (\%) = \frac{(B \times Nb) * 100}{(MP \times \alpha \times h_{tot})}$$

134 where B is the amount of NaOH consumed (mL), Nb is the normality of the base, MP is the  
135 mass (g) of the protein (N = 6.25),  $\alpha$  represents the average degree of dissociation of the  $\alpha$ -  
136  $NH_2$  groups in protein substrate ( $\alpha = \frac{10^{pH-pK}}{1 + 10^{pH-pK}}$ ) and  $h_{tot}$  is the total number of peptide  
137 bonds in the protein substrate and was assumed to be 8.6 meq/g (Alder-Nissen, 1986).

138 CSPI was hydrolyzed with Purafect<sup>®</sup> and Savinase<sup>®</sup> in order to elaborate bioactive  
139 peptides. The hydrolysis kinetic curves (data not shown) displayed the same evolution,  
140 characterized by a high rate of hydrolysis during the first hour, which was subsequently  
141 slowing down with the reaction time and then reached a stationary phase. Regarding the  
142 protease activity, Savinase<sup>®</sup> was more efficient than Purafect<sup>®</sup>. After 30 min of hydrolysis,  
143 DHs values reached 6.92% and 3.84% for Savinase hydrolysate and Purafect hydrolysate,  
144 respectively. After 7 h of hydrolysis, DHs values were 13.52% and 8.87% using Savinase<sup>®</sup>  
145 and Purafect<sup>®</sup>, respectively. Indeed, the difference in DH values between Purafect and  
146 Savinase hydrolysates is essentially due to the difference in the specificity of enzymes used.  
147 During hydrolysis, Savinase<sup>®</sup> and Purafect<sup>®</sup> have different cleavage positions on polypeptide  
148 chains. Savinase<sup>®</sup> and Purafect<sup>®</sup> produce therefore different hydrolysates (Bkhairia et al.,  
149 2016). Typical hydrolysis curves were reported for protein hydrolysates of smooth hound  
150 (*Mustelus mustelus*) (Abdelhedi et al., 2016), thornback ray (*Raja clavata*) (Lassoued et al.,  
151 2015) and Goby (*Zosterisessor ophiocephalus*) (Nasri et al., 2013). In the following work,  
152 we will focus only on hydrolysates obtained after 7 hours (end of hydrolysis).

### 153 2.5. Films preparation

154 CSG film forming solution was prepared by dissolving 4 g of CSG in 100 mL distilled  
155 water. The mixture was heated at 60 °C for 30 min with continuous stirring and the pH was



156 adjusted to 5.5 with NaOH (0.5 M) to ensure fully dissolution and to obtain an homogeneous  
157 colloidal solution of gelatin, that conduct to less crystalline and more homogeneous films.  
158 CSG-enriched films were prepared by incorporating CSPH and CSPI at a concentration of  
159 10% (w/w gelatin) in the film forming solutions. Then, the mixtures were gently stirred at  
160 room temperature ( $25\pm 2$  °C) for 30 min. Glycerol was used as plasticizer at a concentration of  
161 15% (w/w of gelatin). Films were obtained by casting each solution (25 mL) into plastic Petri  
162 dishes (12 cm of side). Control films were made from the CSG film forming solutions without  
163 adding CSPH and CSPI. Drying was then performed in a ventilated climatic chamber (KBF  
164 240 Binder, ODIL, France) at 25 °C and 50% relative humidity (RH) for 24 h. Dried films  
165 were manually peeled off from the surface and equilibrated at 25 °C and relative humidity  
166 (RH) of 50% before analyses.

## 167 *2.6. Physical characterization of the films*

### 168 *2.6.1. Thickness*

169 Films thickness was measured using a digital thickness gauge (PosiTector 6000,  
170 DeFelsko Corporation, USA). Five measurements at different locations were taken from each  
171 film sample peeled from Petri dish, one from the center and four from the perimeter. The  
172 average value was used in the calculation and taken into account for mechanical properties.

### 173 *2.6.2. Color*

174 Color of films was determined using a CIE colorimeter (CR-200; Minolta, Japan). A  
175 white standard color plate ( $L_0^* = 97.5$ ,  $a_0^* = -0.1$ , and  $b_0^* = 2.3$ ) was used as background for  
176 the color measurements of the films. Color of the films was expressed as  $L^*$   
177 (lightness/brightness),  $a^*$  (redness/greenness) and  $b^*$  (yellowness/blueness) values. The

178 difference in color ( $\Delta E^*$ ) for enriched films was calculated referred to the control CSG films  
179 according to the following equation:

$$180 \quad \Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

181 Where  $\Delta L^*$ ,  $\Delta a^*$  and  $\Delta b^*$  are the differences between the color parameters of the enriched  
182 films and those of control CSG films.

### 183 *2.6.3. Light transmission*

184 Film portions (1 cm x 3 cm) were placed in the test cell of a UV-Visible  
185 spectrophotometer (SAFAS UVmc). An empty test cell was used as a reference. UV-vis  
186 absorption spectra were recorded in the wavelength ranging from 200 to 800 nm. Results of  
187 UV-vis absorption spectra were then converted in terms of transmission spectra using the  
188 following formula:

$$189 \quad T(\%) = 10^{(-A)} \times 100$$

190 Where T is the light transmission (%) and A representing the absorbance

### 191 *2.6.4. FTIR spectroscopy*

192 FTIR spectra of films were obtained using a Perkin-Elmer spectrometer (Spectrum 65,  
193 France) equipped with an attenuated total reflectance (ATR) accessory with a ZnSe crystal. 32  
194 scans were collected with 4  $\text{cm}^{-1}$  resolution in the wavenumber range 650-4000  $\text{cm}^{-1}$ .  
195 Calibration was done using background spectrum recorded from the clean and empty cell at  
196 25 °C. The Spectrum Suite ES software was used for FTIR data treatment.

### 197 *2.6.5. Differential scanning calorimetry (DSC)*

198 Thermal properties of films were studied using a differential scanning calorimeter  
199 (DSC Q20, TA Instruments). Films (5 mg) were placed into aluminum pans, sealed and  
200 subjected to a double heating-cooling cycle from -50 °C to 150 °C at a rate of 10 °C/min. The  
201 empty aluminum pan was used as a reference. Nitrogen was used as purge gas at a flow rate  
202 of 25 mL/min. Glass transition temperature (T<sub>g</sub>) for each sample was then determined from  
203 the mid-point of the second heating cycle using TA Universal Analysis 2000 software  
204 (version 4.5 A, TA instruments).

#### 205 *2.6.6. Thermogravimetric analysis (TGA)*

206 Thermogravimetric analysis was carried out to determine the thermal stability of the  
207 film samples. This technique permits the continuous weighing of the film as a function of the  
208 temperature rise in a controlled atmosphere (nitrogen). Thermogravimetric measurements  
209 were performed using a TGA instrument (SDT Q 600). The samples (approximately 10 mg)  
210 were heated from 25 to 600 °C at a heating rate of 5 °C/min under nitrogen atmosphere. Data  
211 analysis was performed using TA Universal Analysis 2000 software (version 4.5 A, TA  
212 instruments).

#### 213 *2.6.7. Observation of film microstructure*

214 The cross-section morphology of film samples was determined using scanning  
215 electron microscopy (SEM) (Hitachi S4800), at an angle of 90° with the surface, using  
216 different magnifications. Prior to imaging the film cross-section, film samples were  
217 cryofractured by immersion in liquid nitrogen and fixed on the SEM support using double  
218 side adhesive tape, and observed under an accelerating voltage of 2.0 kV and an absolute  
219 pressure of 60 Pa, after sputter coating with a 5 nm thick gold.

#### 220 *2.6.8. Mechanical properties*

221 Tensile strength (TS, MPa) and elongation at break (EAB, %) of film samples were  
 222 determined using a texture analyzer (TA. HD plus model, Stable MicroSystems, UK) with a  
 223 300 N load cell, according to the standard method ISO 527-3 (similar to the ASTM D882  
 224 method). Rectangular film samples with dimensions (2.5 cm x 8 cm) were cut using a  
 225 standardized precision cutter (Thwing-Albert JDC Precision Sample Cutter) in order to get  
 226 tensile test piece with an accurate width and parallel sides throughout the entire length. Before  
 227 testing, all the samples were equilibrated for two weeks at 25 °C and 50% RH. Equilibrated  
 228 films samples were then installed vertically in the extension grips of the testing machine and  
 229 stretched uniaxially with a cross-head speed of 50 mm/min until breaking according to the  
 230 ISO standard. The maximum load and the final extension at break were determined from the  
 231 corresponding stress-strain curves and used for the calculation of TS and EAB as follows:

$$232 \quad TS \text{ (MPa)} = \frac{\text{Maximum force}}{t \times w}$$

$$233 \quad EAB(\%) = 100 \times \frac{(l - l_0)}{l_0}$$

234 where, t is the thickness (mm), w the width (mm) of films,  $l_0$  the initial length of the film and  
 235 l is the length of the film when it breaks. Measurements were carried out at room temperature  
 236 ( $25 \pm 2$  °C) and six samples for each film formulation were tested.

### 237 2.6.9. Surface properties

238 The surface tension of films ( $\gamma_{\text{film}}$ ) and its polar ( $\gamma_{\text{film}}^{\text{P}}$ ) and dispersive ( $\gamma_{\text{film}}^{\text{D}}$ )  
 239 components were determined using the Owens & Wendt (1969) method, using water ( $\gamma_{\text{Liq}} =$   
 240 72.8 mN/m ;  $\gamma_{\text{Liq}}^{\text{D}} = 21.8$  mN/m ;  $\gamma_{\text{Liq}}^{\text{P}} = 51$  mN/m), ethylene glycol ( $\gamma_{\text{Liq}} = 47.7$  mN/m ;  $\gamma_{\text{Liq}}^{\text{D}}$   
 241 = 30.9 mN/m;  $\gamma_{\text{Liq}}^{\text{P}} = 16.8$  mN/m) and diiodomethane ( $\gamma_{\text{Liq}} = 50.8$  mN/m ;  $\gamma_{\text{Liq}}^{\text{D}} = 50.8$  mN/m  
 242 ;  $\gamma_{\text{Liq}}^{\text{P}} = 0$  mN/m) according the following equations:

243

$$\gamma_S = \gamma_S^D + \gamma_S^P$$

244

245

$$\gamma_{\text{Liq}}(1 + \cos\theta) = 2\left(\sqrt{\gamma_{\text{film}}^D \times \gamma_{\text{Liq}}^D} + \sqrt{\gamma_{\text{film}}^P \times \gamma_{\text{Liq}}^P}\right)$$

246

247

248

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250

251

Where  $\theta$ ,  $\gamma_{\text{Liq}}$ ,  $\gamma_{\text{Liq}}^D$  and  $\gamma_{\text{Liq}}^P$  are respectively the contact angle, the surface tension, the dispersive and the polar components of the surface tension of the tested liquid;  $\gamma_{\text{film}}^P$  and  $\gamma_{\text{film}}^D$  are the polar and dispersive components of the surface tension of the film surface tested. The contact angle is expressed in degree and all the surface tension parameters are expressed in  $\text{mN}\cdot\text{m}^{-1}$ .

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Three liquids (water, ethylene glycol and diiodomethane), with well-known polar  $\gamma_{\text{Liq}}^P$  and dispersive  $\gamma_{\text{Liq}}^D$  contributions, were used. The contact angle measurements were carried out using the sessile drop method on a goniometer (Drop Shape Analyzer 30 from KrussGmbH), equipped with an image analysis software (ADVANCE). First, a droplet of each liquid ( $\sim 2 \mu\text{L}$ ) was deposited on the film surface with a precision syringe. The method is based on image processing and curve fitting for contact angle measurement from a theoretical meridian drop profile, determining contact angle between the baseline of the water drop and the tangent at the drop boundary. Then, the contact angle was measured at 0 time ( $< 2 \text{ s}$ ) and at 30 s on both sides of the drop and averaged. Five measurements per film were carried out. All the tests were conducted in an environmental chamber with a constant environment at a temperature of  $25 \pm 2 \text{ }^\circ\text{C}$  and a relative humidity of  $50 \pm 1\%$ .

263

## 2.7. *In vitro* antioxidant activity

264

### 2.7.1. Reducing power assay

265 The ability of CSPI, CSPH and films to reduce iron (III) was determined according to  
266 the method of Yıldırım, Mavi, & Kara (2001). The hydrolysates and the protein isolate were  
267 tested alone or in films with a concentration of 4.4 mg/mL. For this, a volume of 0.5 mL of  
268 each sample or small pieces of each film (10 mg), was mixed with 1.25 mL of 0.2 M  
269 phosphate buffer (pH 6.6) and 1.25 mL of 1% (w/v) potassium ferricyanide. The mixtures  
270 were then incubated for 30 min (3 h for the films) at 50 °C. After incubation, 1.25 mL of 10%  
271 (w/v) trichloroacetic acid was added to the mixtures which were centrifuged for 10 min at  
272 10,000g. Finally, 1.25 mL of the supernatant solution of each sample mixture was mixed with  
273 1.25 mL of distilled water and 0.25 mL of 0.1% (w/v) ferric chloride. After 10 min reaction  
274 time, the absorbance of the resulting solutions was measured at 700 nm using polystyrene  
275 spectrophotometry cuvettes. Higher absorbance of the reaction mixture indicated higher  
276 reducing power. The values are presented as the means of triplicate analyses.

#### 277 2.7.2. DPPH free radical-scavenging activity

278 The DPPH free radical-scavenging activity of CSPH, CSPI and films was determined  
279 as described by Bersuder, Hole, & Smith (1998) with some modifications. 500µL of each  
280 sample or small pieces of each film (10 mg) were added to 375 µL of ethanol solution and  
281 125 µL of 0.02 mM DPPH in ethanol. The mixtures were then incubated for 1 h at room  
282 temperature in the dark. Control tubes were assessed in the same manner without film  
283 samples. The reduction of DPPH radical was measured at 517 nm, using a UV-visible  
284 spectrophotometer.

285 The free radical-scavenging activity was calculated as follows:

$$286 \quad DPPHscavenging(\%) = \frac{A_c - (A_s - A_b)}{A_c} \times 100$$

287 where  $A_c$  is the absorbance of DPPH solution without addition of the films,  $A_s$  is the  
288 absorbance of DPPH solution containing the film samples and  $A_b$  is the absorbance of blank  
289 tubes containing film samples without addition of the DPPH solution.

290 A lower absorbance of the reaction mixture indicated a higher radical-scavenging activity.  
291 The test was carried out in triplicate.

### 292 2.7.3. $\beta$ -carotene-linoleate bleaching assay

293 The ability of CSPH, CSPI and films to prevent  $\beta$ -carotene bleaching was determined  
294 according to the method of Koleva, van Beek, Linssen, de Groot, & Evstatieva (2002). 0.5 mg  
295  $\beta$ -carotene in 1 mL chloroform was mixed with 25  $\mu$ L of linoleic acid and 200  $\mu$ L of Tween-  
296 40. The chloroform was completely evaporated under vacuum in a rotator evaporator at 40  
297  $^{\circ}$ C, then 100 mL of double distilled water were added and the resulting mixture was  
298 vigorously stirred. The emulsion obtained was freshly prepared before each experiment.  
299 Aliquots (2.5 mL) of the  $\beta$ -carotene-linoleic acid emulsion were transferred into test tubes  
300 containing 0.5 mL from each sample or small pieces of each film (10 mg). The tubes were  
301 immediately placed in a water bath and incubated at 50  $^{\circ}$ C for 2 h. Thereafter, the absorbance  
302 of each sample was measured at 470 nm using polystyrene spectrophotometry cuvettes. The  
303 control tube was prepared in the same conditions by adding 0.5 mL of distilled water instead  
304 of the sample solution. The antioxidant activity was evaluated in terms of  $\beta$ -carotene  
305 bleaching inhibition using the following formula:

$$306 \quad \beta - \text{carotene bleaching inhibition (\%)} = \left( 1 - \left( \frac{A_{\text{sample}}^0 - A_{\text{sample}}^{120}}{A_{\text{control}}^0 - A_{\text{control}}^{120}} \right) \right) \times 100$$

307 where  $A^0$ : absorbance at  $t=0$  min,  $A^{120}$ : absorbance at  $t=120$  min. The test was carried out in  
308 triplicate.

309 *2.8. Statistical analysis*

310 Statistical analyses were performed with SPSS ver. 17.0, professional edition using ANOVA  
311 analysis at a p level < 0.05. Duncan's multiple range test (p-value < 0.05) was used to detect  
312 differences among mean values of all the parameters analyzed for the different films. A  
313 standard deviation at the 90% confidence level was used to compare the DSC data for the  
314 different films.

315 **3. Results and discussion**

316 *3.1. Functional properties of films*

317 *3.1.1. Color of films*

318 The color data of CSG films and those enriched by CSPI and CSPH are given in  
319 Table 1. The highest L\* and lowest b\* values were detected with control films. Decreases in  
320 L\*-values and increases in a\* and b\*-values were observed in films, when CSPI and CSPH  
321 were incorporated, indicating a decrease in lightness and an increase in browning color.  
322 Enriched films are slightly brown compared to control films. The color difference was  
323 confirmed by the calculation of  $\Delta E^*$  taking the gelatin film as reference. The obtained  $\Delta E^*$ -  
324 values ranged from 5.47 to 6.94. Indeed, at the final moment of the enzymatic hydrolysis,  
325 more colored peptides are generated. According to Dong et al. (2008), the longer hydrolysis  
326 time probably accelerated the pigments oxidation and Maillard reaction. This may explain the  
327 darkening and browning color of CSPH. Similarly, Nuanmano, Prodpran, & Benjakul (2015)  
328 reported that the addition of fish gelatin hydrolysates with higher DH (95%) to fish  
329 myofibrillar protein films leads to the same behaviour. Indeed, the yellowness may be due to  
330 the amino groups (-NH<sub>2</sub>) of the hydrolysate, which may interact with the carbonyl groups  
331 (C=O) of lipid oxidation products in the polymeric matrix via the Maillard reaction,



332 particularly during drying of the film (Nuanmano et al., 2015; Rocha et al., 2018). Hasanzati  
333 Rostami, Motamedzadegan, Hosseini, Rezaei, & Kamali (2017) indicated a rise of the  
334 yellowish (b-values) and  $\Delta E^*$  values in gelatin films with the silver carp protein hydrolysate  
335 content. Furthermore, Lin et al. (2018) attributed the increase of yellowness to the higher  
336 content of lysine and histidine amino acids incorporated in the gelatin film matrix. Regarding  
337 the increase in redness with the addition of CSPH to gelatin films, this fact could be due to the  
338 initial colored compounds existing in CSPI (undigested protein) as it has been reported in  
339 previous work (Kchaou et al., 2017). Indeed, as a function of hydrolysis time, more peptides  
340 were generated, which may explain the darker color of CSPH.

### 341 3.1.2. UV and light barrier efficacy

342 Transmission of UV and visible light of gelatin films and those enriched with CSPI  
343 and CSPH was determined at selected wavelengths from 200 to 800 nm. Fig. 1 illustrated that  
344 prepared films have a high UV-barrier property in the range of (200-280 nm). This is  
345 attributed to the presence of some aromatic amino acids such as phenylalanine and tyrosine in  
346 the gelatin that absorb UV light (Jongjareonrak, Benjakul, Visessanguan, Prodpran, & Tanaka,  
347 2006). Hoque et al. (2011a) reported similarly a very low transmission (0.01%) at 200 nm for  
348 cuttlefish (*Sepia pharaonis*) gelatin films. At 350 nm, light transmission decreases remarkably  
349 by about 56% for both hydrolysates incorporated films, respectively. These finding scould be  
350 explained by the fact that CSPH could contain more aromatic amino acids than the gelatin. In  
351 the visible range, control CSG film was the most transparent ( $\approx 80\%$  transmission). The light  
352 transmission decreased with the incorporation of CSPI and CSPH in the UV (200-400 nm)  
353 and the visible (400-800 nm) ranges. Enriched films provided slighter barrier against light  
354 incidence and could be used as barrier packaging to protect packaged foods against light  
355 oxidative deterioration.

### 356 3.1.3. FTIR spectra

357 The infrared spectroscopy was used in this study in order to assess and determine the  
358 interactions established between gelatin and CSPH or CSPI in the film matrix. Fig. 2 showed  
359 the infrared spectra of gelatin film and those enriched with CSPI and protein hydrolysates.  
360 Prepared films displayed similar spectra in the range of 700-1800  $\text{cm}^{-1}$ . The main  
361 characteristic absorption bands in gelatin films are located at 1560-1680  $\text{cm}^{-1}$  (representing  
362 C=C and C=O stretching of primary and secondary amine N-H band of amide-I), 1540-1610  
363  $\text{cm}^{-1}$  (assigned to NH of amide-II) and 1230-1340  $\text{cm}^{-1}$  (assigned to aromatic primary amine,  
364 C-N and N-H stretch of amide-III or vibrations of  $\text{CH}_2$  groups of glycine) (Hoque et al.,  
365 2011a). Moreover, all spectra of gelatin films showed major bands at approximately 3300-  
366 3500  $\text{cm}^{-1}$  and 2920-2945  $\text{cm}^{-1}$ , corresponding to amide A (NH-stretching coupled with  
367 hydrogen bonding) and amide B (asymmetric stretching vibration of =C-H and  $-\text{NH}_3^+$ ). In  
368 addition, a band located at 1040-1080  $\text{cm}^{-1}$  was found in all film samples, corresponding to  
369 the glycerol (-OH group) added as a plasticizer (Bergo & Sobral, 2007). The spectra did not  
370 show significant difference in the position of the amides I, II and III. In addition, all the  
371 samples of gelatins, protein isolates and protein hydrolysates derived from the same raw  
372 material (cuttlefish skin). Thus, the added protein hydrolysates did not generate or suppress  
373 the overall interactions present initially in gelatin films. However, for the amide A region, a  
374 shift to lower wavenumbers was detected with the enriched films compared to gelatin film.  
375 Indeed, amide A shifted from 3320  $\text{cm}^{-1}$  to 3314  $\text{cm}^{-1}$ , 3313  $\text{cm}^{-1}$  and 3317  $\text{cm}^{-1}$  with the  
376 addition of CSPI, Purafect and Savinase hydrolysates, respectively. Generally, the decrease in  
377 vibrational wavenumber and broadening of the OH and NH vibration bands could be linked to  
378 the water content changes and water-biopolymer interactions via hydrogen bonding, which  
379 could affect the network organization (Arfat, Benjakul, Prodpran & Osako, 2014; Kchaou et

380 al., 2017). This strengthening of the matrix by hydrogen bond is often revealed by a higher  
381 thermal stability or Tg

#### 382 *3.1.4. Thermal properties by DSC and TGA analyses*

383 The thermal properties of gelatin films and those enriched with CSPI and CSPH were  
384 examined by DSC and the glass transition temperature (Tg) was determined from the second  
385 cycle of heating. The glass transition is associated with the molecular segmental motion of  
386 disordered (amorphous phase) structure, which undergoes from a brittle glassy solid state to a  
387 rubbery state (Nilsuwan, Benjakul, & Prodpran, 2018). As shown in Table 1, Tg value of  
388 control gelatin film was 58.4 °C and increased gradually to 59.5 °C and 61.8 °C with the  
389 addition of CSPI and Savinase hydrolysate, respectively. Tg value of control gelatin film  
390 (58.4 °C) was higher than that reported by Nilsuwan et al. (2018) for tilapia skin gelatin based  
391 films (45.5 °C) and lower than that stated by Jridi, Abdelhedi, Zouari, Fakhfakh, & Nasri  
392 (2019a) for films based on grey triggerfish skin gelatin (71.3 °C). The difference on Tg values  
393 for gelatin-based films depends on gelatin sources, compositions of film and process used  
394 (Tongnuanchan, Benjakul, Prodpran, & Nilsuwan, 2015).

395 Interestingly, Purafect hydrolysate incorporated films showed the highest Tg values  
396 which reached 71.4 °C. The increase in Tg values with the incorporation of CSPH could be  
397 explained by the establishment of interactions between hydrogen bonds of CSG and CSPH in  
398 the film matrix as displayed from FTIR experiments. An increase of the Tg value was also  
399 reported by Lin et al. (2018) with the addition of amino acids (lysine, arginine and histidine).  
400 Therefore the thermal stability of gelatin films was improved. However, Hasanzati Rostami et  
401 al. (2017) stated a decrease of Tg values with the addition of silver carp protein hydrolysates  
402 to fish gelatin films. The authors suggest that this decrease of Tg might be due to the lower  
403 molecular weight of protein hydrolysates which can position between protein chains  
404 themselves. Protein hydrolysates can also interfere with the protein-protein interaction, which

405 led to increasing the free volume between the polymer chains and the mobility of molecules  
406 *i.e.* a plasticizing mechanism (Giménez et al., 2009).

407 The thermal stability of films was assessed by TGA at temperatures ranging from 25  
408 to 600 °C. The TGA is a technique in which the mass change of a substance is measured  
409 when it is subjected to a controlled temperature program. The thermal degradation  
410 temperature, the weight loss ( $\Delta w$ ) and the residue of films are presented in Table 1. From the  
411 TGA curves (supplementary data), two main stages of weight loss were observed. The first  
412 step of transformation starts from the ambient temperature until around 175 °C. This weight  
413 loss ( $\Delta w1$ ) step corresponds to the loss of free and bound water in the films (above 100 °C)  
414 and varied from 11% to 14%. The second stage of transformation is related to the thermal  
415 degradation or the decomposition of the gelatin chains. The degradation temperatures ( $T_{max}$ )  
416 were ranging from 296.0 °C to 310.7 °C. In this stage, the weight loss ( $\Delta w2$ ) of films is  
417 greater and ranged from 64.9% for gelatin films to around 60% for enriched films. The  
418 residual mass at 600 °C, rose from 19% to about 23 and 25% when the protein hydrolysates  
419 and CSPI were incorporated in gelatin films. The increase in  $T_{max}$  and residual mass values  
420 suggest that the addition of CSPI and protein hydrolysates limited the thermal degradation of  
421 gelatin films. The interactions between CSPH or CSPI and CSG in the film matrix, as  
422 previously demonstrated by FTIR and DSC results, mostly yielded the stronger film network,  
423 leading to higher heat resistance of enriched films than that of the CSG films (Arfat et al.,  
424 2014). Their interactions mainly determine the thermal stability of enriched gelatin films by  
425 hydrogen bonds (de Morais Lima et al., 2017).

### 426 *3.1.5. Microstructure*

427 Scanning Electron Microscopy observations were conducted in order to better  
428 understand the microscopic structure of enriched films. Fig. 3 illustrates the scanning electron

429 micrographs of the cross-section of control gelatin film and those containing CSPI and  
430 Purafect hydrolysate. The cross-section micrographs allow not only the observation of film  
431 internal microstructures but they also contribute to a better knowledge of the film-forming  
432 behavior of polymers. The micrographs revealed homogenous and uniform structure of  
433 control and CSPI enriched films, suggesting therefore that the polymer and the additives  
434 interacted well with each other. This allowed to form a cohesive and continuous matrix (de  
435 Morais Lima et al., 2017). However, the micrograph of Purafect hydrolysate added film  
436 displayed a relatively heterogeneous structure.

### 437 *3.1.6. Mechanical properties*

438 Results of tensile strength (TS) and elongation at break (EAB) of gelatin films and  
439 those enriched with CSPI and CSPH are shown in Table 1. Among the different films, control  
440 gelatin film showed the highest TS (22.67 MPa) and EAB (32.83%) values, followed by CSPI  
441 enriched film, 22.09 MPa and 26.26%, respectively. The CSPH incorporation leads to a  
442 significant decrease in the mechanical properties of gelatin films. Indeed, TS decreased by  
443 30.1% and 45.8% for films added with Purafect and Savinase hydrolysates, respectively. The  
444 EAB were around three-fold lower for CSPH enriched films compared to control film. The  
445 decrease in both TS and EAB for CSPH incorporated films revealed the fragility of these  
446 films, which are mechanically weaker and less deformable compared to control film. The  
447 small peptides could be easily inserted in the protein network and establish hydrogen  
448 bondings with the gelatin chains, which is detrimental for the chain–chain interactions. These  
449 tend to decrease the density of intermolecular interactions and to increase the free volume  
450 between gelatin chains (Giménez et al., 2009).

451 Our findings were in accordance with those of Jridi et al. (2013b) who indicated that  
452 both TS and EAB values of CSG films decreased with the increase of pepsin used for gelatin

453 extraction (or the extent of gelatin hydrolysis). Moreover, Hasanzati Rostami et al. (2017)  
454 reported that the mechanical strength was significantly reduced for gelatin films with the  
455 addition of fish protein hydrolysate obtained from silver carp. In addition, Giménez et al.  
456 (2009) reported that increasing the content of gelatin hydrolysates in the squid skin gelatin  
457 films leads to a decrease of the mechanical resistance (puncture force) coupled to an increase  
458 of the distensibility (puncture deformation) revealing a plasticization process. Furthermore,  
459 microstructure results displayed a heterogeneous structure for Purafect hydrolysate enriched  
460 films. This result correlates with the decrease in tensile strength and elongation at break for  
461 CSPH incorporated films.

### 462 *3.1.7. Surface properties*

463 Surface properties of gelatin film and those enriched with CSPI and CSPH were  
464 determined firstly by measuring their water contact angles (WCA) at 0 and 30 s as shown in  
465 Fig. 4A. CSPI enriched film showed the highest initial water contact angle (WCA=114°)  
466 followed by the gelatin film (88°). The higher WCA of CSPI added films could be explained  
467 by the fact that CSPI contains more hydrophobic amino acids (leucine, isoleucine, valine,  
468 methionine, tyrosine and phenylalanine, which represents 191.6 residues per 1000 residues)  
469 compared to CSG that contains 80.4 residues per 1000 residue of hydrophobic acids (Kchaou  
470 et al., 2017). The incorporation of the CSPH leads to a significant decrease in WCA values  
471 which were in the range of 56-63°. Such results could be the consequence of the hydrophilic  
472 character of the CSPH, which has shorter protein chains that contain polar amino acids able to  
473 be re-oriented at the surface of the films. This provides a higher hydrophilicity. In this  
474 context, Hoque et al. (2011a) indicated that protein hydrolysis could expose more carboxylic  
475 group and amino group to the surface, which might then form hydrogen bonds with the water  
476 molecules and lead to the higher hydrophilicity of the resulting films. Moreover, several  
477 studies have shown that fish protein hydrolysates have excellent water holding capacity

478 favored by the presence of polar groups such as COOH and NH<sub>2</sub> generated by the enzymatic  
479 hydrolysis. These polar groups have a substantial effect on the water absorption and  
480 hydrophilicity (Wasswa, Tang, Gu, & Yuan, 2007; Kristinsson & Rasco, 2000). A decrease of  
481 WCA values has been similarly indicated by Hasanzati Rostami et al. (2017) for gelatin films  
482 incorporated with silver carp protein hydrolysates because of its high hydrophilic character.  
483 Abdelhedi et al. (2018) reported that the smaller WCA obtained for gelatin films added with  
484 smooth-hound peptides revealed their sensitivity against moisture. After 30 s, a slight  
485 decrease of WCA was revealed for control gelatin films and those containing CSPI and  
486 Purafect hydrolysate due to exclusively evaporation of the solvent in the surrounding  
487 atmosphere that was not saturated with the liquid vapor (25 °C, 50% RH). For Savinase  
488 hydrolysate enriched films, a higher decrease of WCA measured at 30 s was noted which  
489 explain the faster absorption of the water droplet into the film surface.

490 In order to better understand the effect of CSPH incorporation on the gelatin films  
491 surface properties, the surface tension, besides its polar and dispersive components, were  
492 determined using two other liquids (ethylene glycol and diiodomethane) and results are given  
493 in Fig. 4B. The shape of droplets deposited at the films surface are shown in Fig. 4C. Relative  
494 contact angles values between the film surface and the solvent remained approximately  
495 constant during 30 s (data not shown). Results presented in Fig. 4B displayed that control  
496 gelatin films showed the highest dispersive component (35.8 mN/m) and the lowest polar  
497 component (2.17 mN/m). Similarly, CSPI enriched films presented similarly a low polar  
498 component (2.35 mN/m) but lower dispersive component (20.67 mN/m) compared to gelatin  
499 films. After CSPH incorporation, the surface tension of incorporated films showed  
500 modification due to the concomitant increase of polar component (16.04-22.55 mN/m) and  
501 the decrease of dispersive component (20.61-23.91 mN/m). Thus, the CSPH addition  
502 increased the wettability of gelatin films.

### 503 3.2. Antioxidant activity of films

504 The antioxidant activity was generally determined by different techniques that  
505 involved direct or indirect measurement of the rate/extent of formation/decay of free radicals  
506 (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002). Indeed, the different assays  
507 used for measuring the antioxidant activity are based on the fact that oxidation is largely  
508 inhibited by the capture of initiating or propagating free radicals in the autoxidation process.  
509 Therefore, they focus on monitoring the capacity of additives for radical capture or inhibition  
510 of radical formation rather than on monitoring the actual oxidation itself (Benbettaïeb,  
511 Debeaufort, & Karbowiak, 2018).

512 Three assays were conducted in order to evaluate the effect of CSPH and CSPI  
513 incorporation on the antioxidant potential of gelatin films and to define the different  
514 mechanisms of action of these additives (Fig. 5): reducing power, free radical-scavenging  
515 activity (DPPH) and  $\beta$ -carotene bleaching inhibition.

516 First, the ability of CSPI and CSPH enriched films to reduce ferric ion ( $\text{Fe}^{3+}$ ) was  
517 investigated and data displayed that incorporated films exhibited higher activity than control  
518 gelatin film ( $\text{OD}_{700\text{nm}} = 0.30$ ) (Fig. 5A). The slight increase of the reducing power regarding  
519 the enriched films was found to be more significant for those incorporated with CSPH.  
520 However, the reducing activity of CSPH and CSPI in their free form was more important than  
521 enriched films. These results could be explained either by the delayed release of the active  
522 molecules from the gelatin film matrix or by the interactions established between the gelatin  
523 and the active molecules in the film, which limited their release. Similarly, Giménez et al.  
524 (2009) reported that squid gelatin hydrolysates showed lower antioxidant capacity in the  
525 gelatin films than in the free form at the same amount added into the filmogenic solution  
526 probably due to interactions between the peptides and gelatin film matrix formed via  
527 hydrogen bonding.



528           Moreover, the antioxidant activity of gelatin films was highlighted by the DPPH free  
529 radical-scavenging assay (Fig. 5B). Control gelatin film showed the lowest antioxidant  
530 activity (39.88%). Whereas, the addition of CSPH and CSPI interestingly increased the  
531 antioxidant capacity of gelatin films. Savinase and Purafect hydrolysates enriched films  
532 displayed the highest radical scavenging activity (75.01% and 68.66%, respectively),  
533 followed by CSPI enriched films (61.48%). This difference in the antioxidant activity  
534 between enriched films could be ascribed to differences in film pore size which could affect  
535 the amount of released compounds. In addition, it has been reported that the release of active  
536 compounds from polymeric matrices is influenced mainly by the properties of both the  
537 polymer and the active compound (López-de-Dicastillo et al., 2011). Moreover, the nature of  
538 films seems to have as well a significant effect on films bioactivity and the blend film was  
539 found to accelerate the release of the bioactive molecules from the film matrix (Abdelhedi et  
540 al., 2018). In the free form, CSPI displayed the highest radical scavenging activity followed  
541 by Savinase and Purafect hydrolysates. Indeed, the difference in protein hydrolysates and  
542 isolate activity may be related to the difference in their molecular weight and to their  
543 solubility in ethanol solution. CSPH showed higher antioxidant activity in the film matrix  
544 than that in the free form. Indeed, the formation of protein-protein interactions or hydrogen  
545 bonding between the film network and the added peptides may affect the antioxidant activity  
546 of CSPH enriched films (Giménez et al., 2009). As the DPPH-radical scavenging assay is  
547 based on the electron donating and hydrogen-bond donor properties, both of the CSPH  
548 molecules and the presence of hydrogen bonding in CSPH enriched films could explain the  
549 higher antioxidant activity of CSPH added films compared to their respective free form  
550 (Benbettaïeb, Debeaufort, & Karbowski, 2018).

551           Furthermore, the  $\beta$ -carotene-linoleate bleaching assay, which is based on the  
552 disappearance of  $\beta$ -carotene color under thermally-induced oxidation (50 °C), was used to

553 evaluate the lipid peroxidation inhibitory activity of gelatin films. As shown in Fig. 5C, all  
554 gelatin films prevent  $\beta$ -carotene bleaching by donating hydrogen atoms to peroxy radicals of  
555 linoleic acid. Control gelatin films exhibited the lowest antioxidant activity (29.42%) which  
556 increased significantly and reached 52.75%, 48.63% and 44.12% with the addition of CSPI,  
557 Savinase and Purafect hydrolysates, respectively. A low  $\beta$ -carotene bleaching inhibition  
558 activity (20.35%) was also reported by Jridi et al.(2019b) in the case of grey triggerfish skin  
559 gelatin films. Regarding the free form, CSPI, Savinase and Purafect hydrolysates displayed  
560 high  $\beta$ -carotene bleaching with percentages of inhibition of  $96.79\pm 0.55\%$ ,  $95.03\pm 0.34\%$  and  
561  $90.08\pm 1.14\%$ , respectively. Thus, CSPH and CSPI contain probably hydrogen or electrons  
562 donating peptides that are able to stabilize the free radicals. However, the higher antioxidant  
563 activity of active molecules in the free form suggest that it will be better to use CSPH or CSPI  
564 directly in foods rather than to incorporate them into packaging due to their delayed release.

565 Natural antioxidant activity was similarly reported for fish gelatin based films from  
566 different species (sole, catfish or cuttlefish), which has been mainly attributed to the peptide  
567 fraction of such protein, probably elaborated during the gelatin extraction process (Gómez-  
568 Estaca, Giménez, Montero, & Gómez-Guillén, 2009; Jridi et al., 2017).

#### 569 **4. Conclusion**

570 This study investigates the effect of CSPH incorporation in gelatin films properties.  
571 The addition of CSPH led to colored films with lower homogenous microstructure, higher  
572 UV-barrier property and Tg values, compared to CSPI enriched film. However, mechanical  
573 properties and hydrophobicity decreased for CSPH added films compared to gelatin and CSPI  
574 enriched films. Furthermore, the antioxidant activity of the resulting enriched films was  
575 enhanced, suggesting their possible potential use as active packaging against packaged foods  
576 oxidation.

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588

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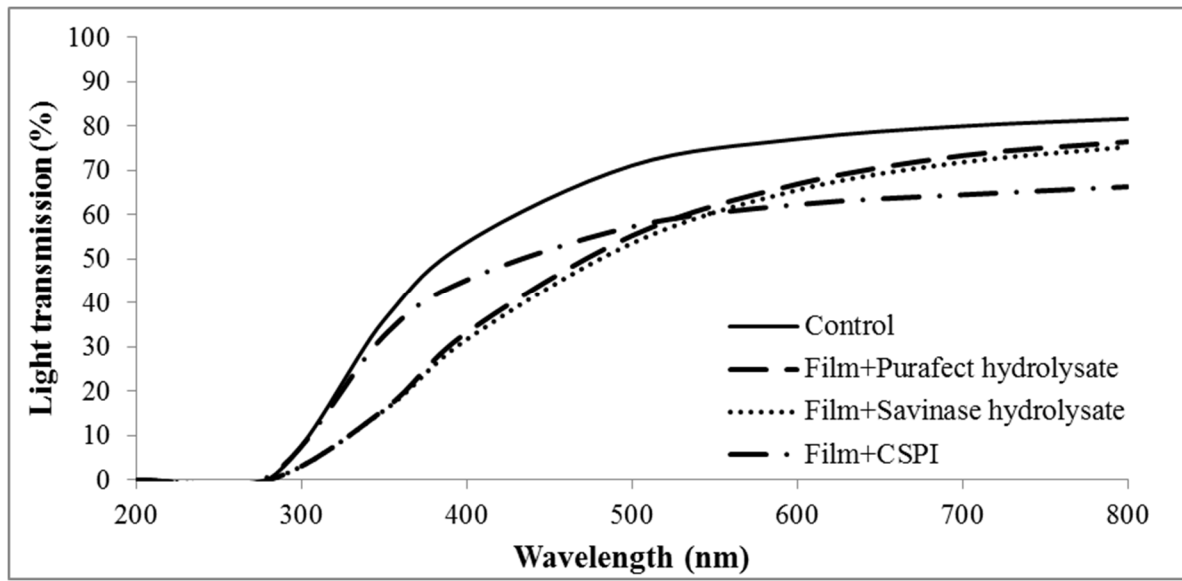
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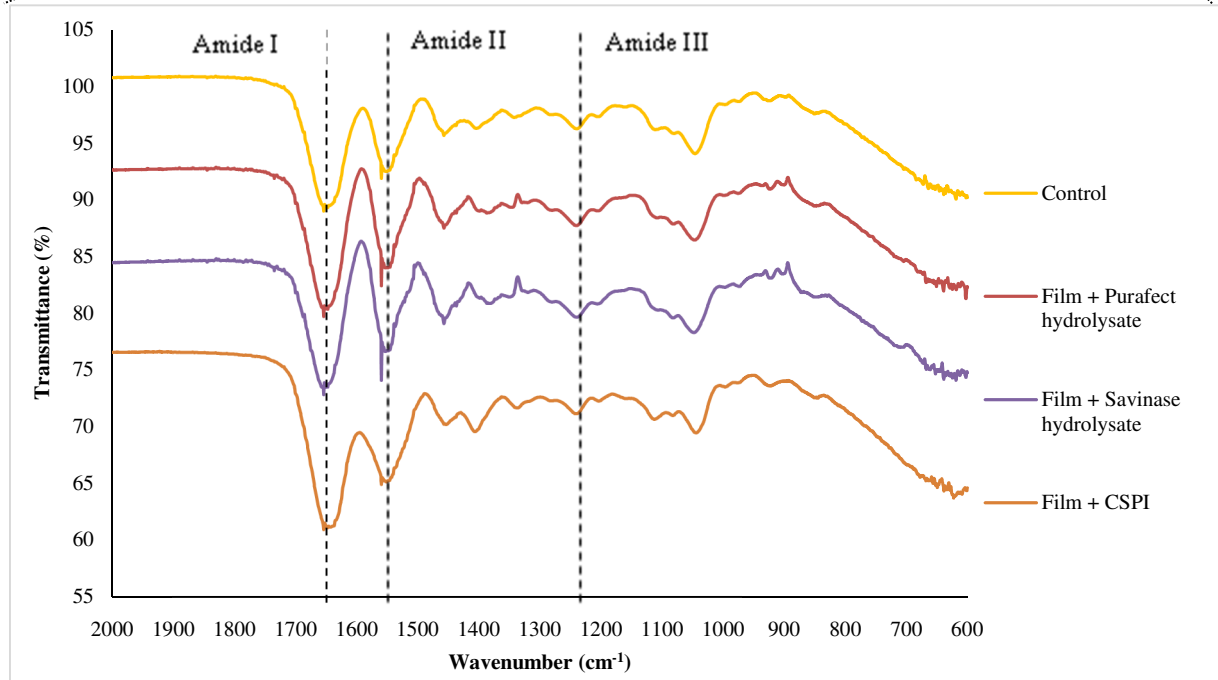
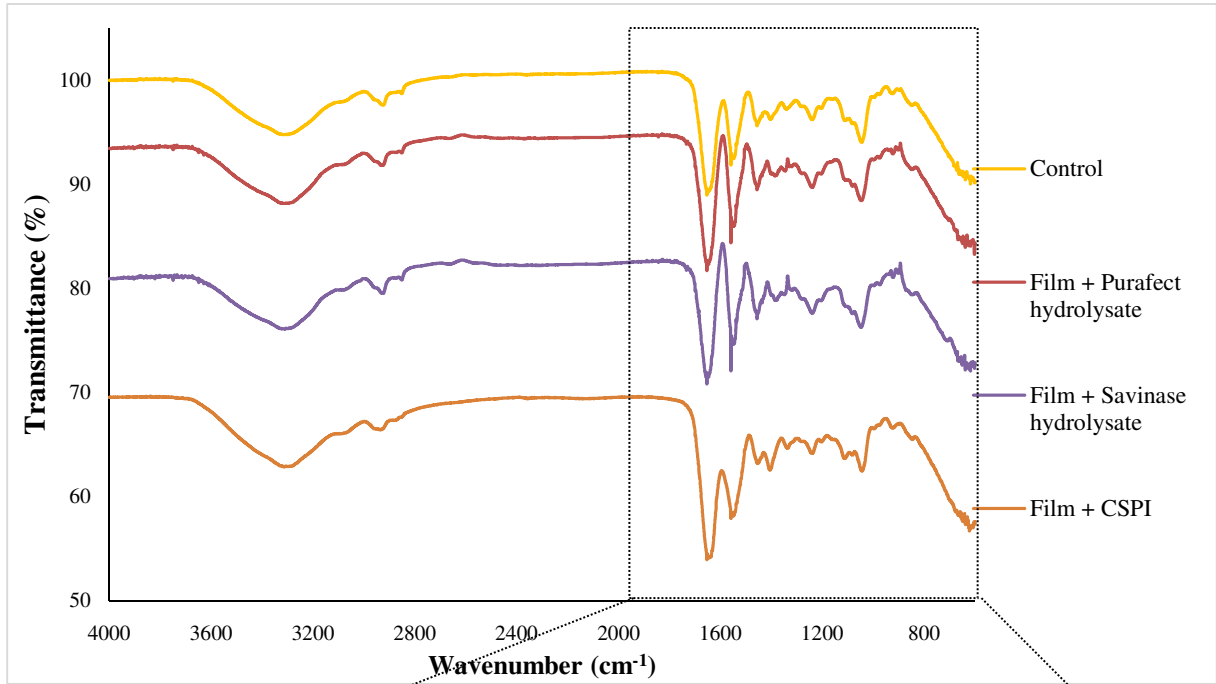
1 **Fig. 1**



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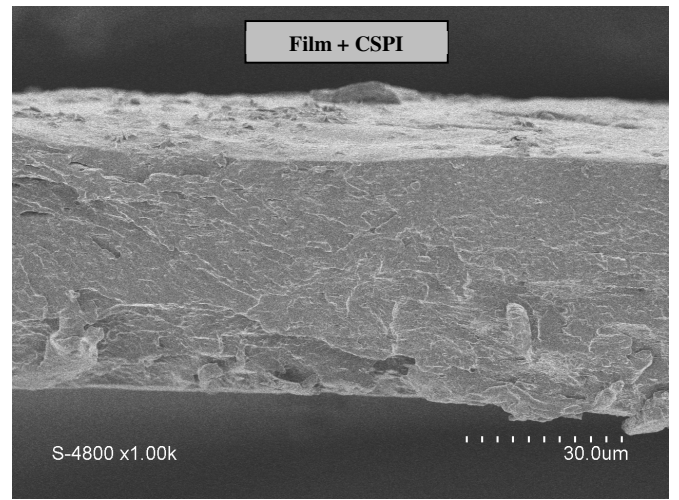
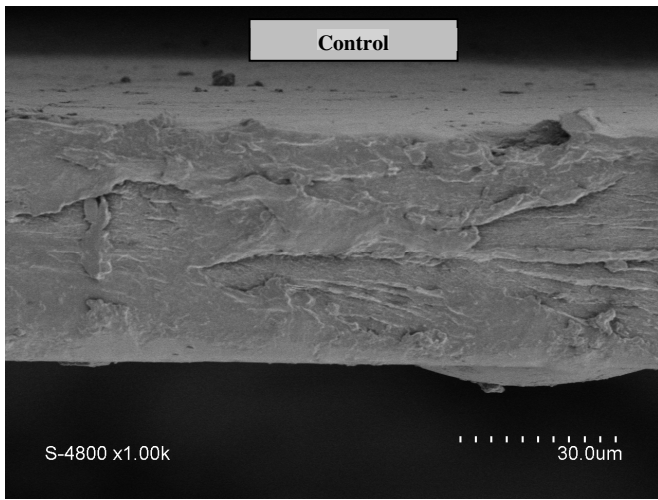
1 **Fig. 2**

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1 **Fig. 3**

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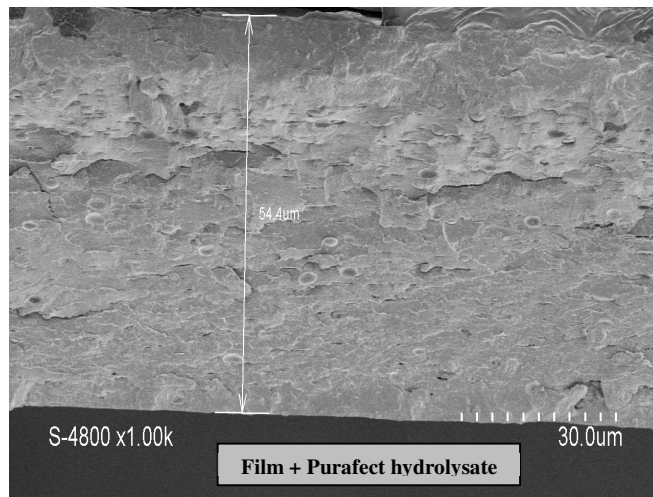
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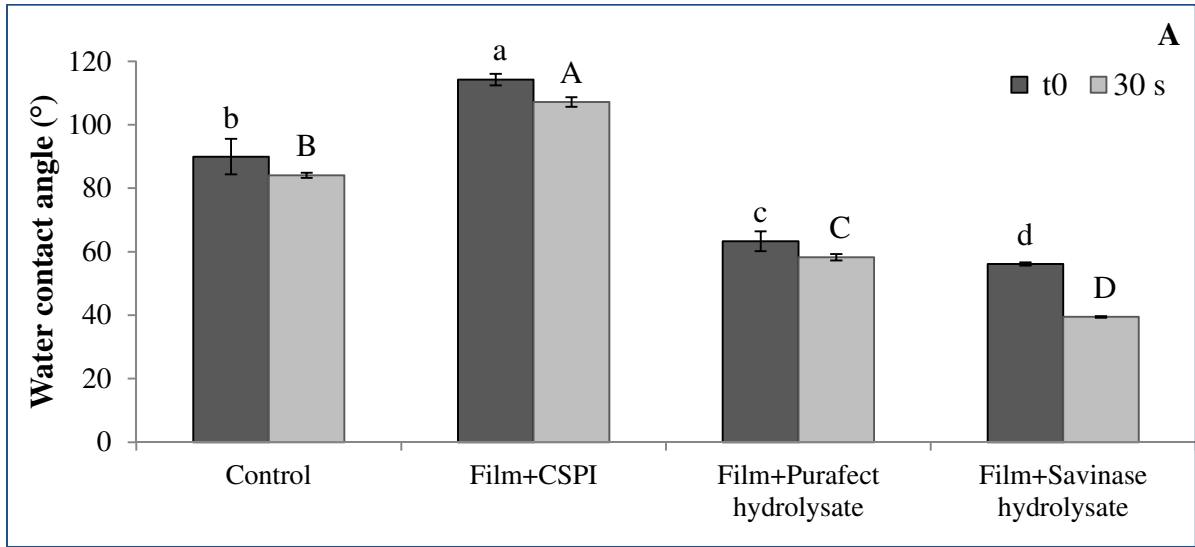
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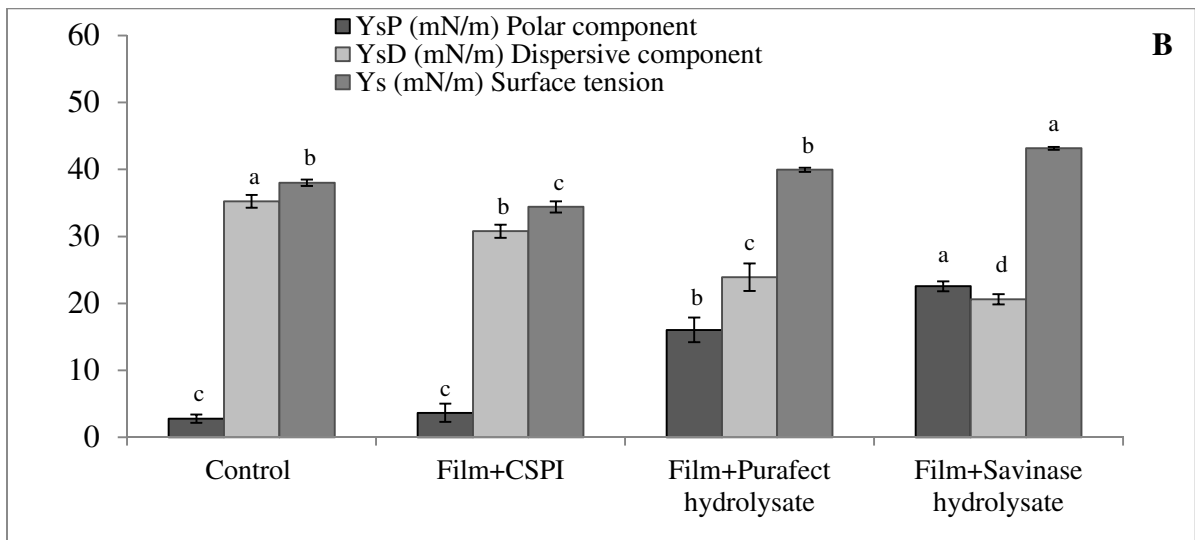
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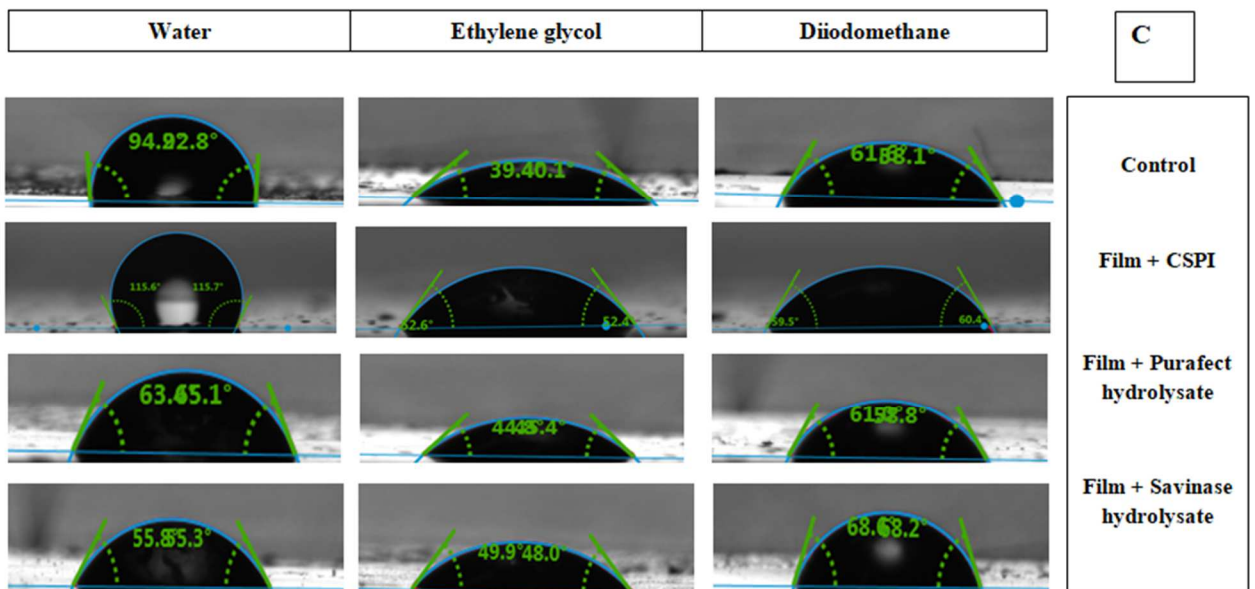
1 **Fig. 4**



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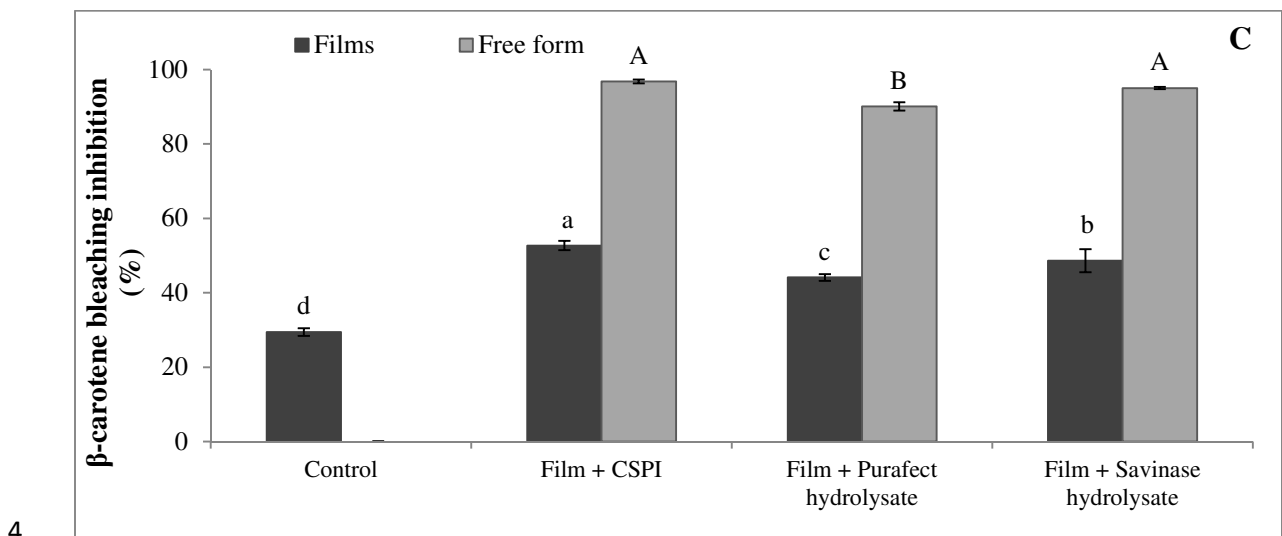
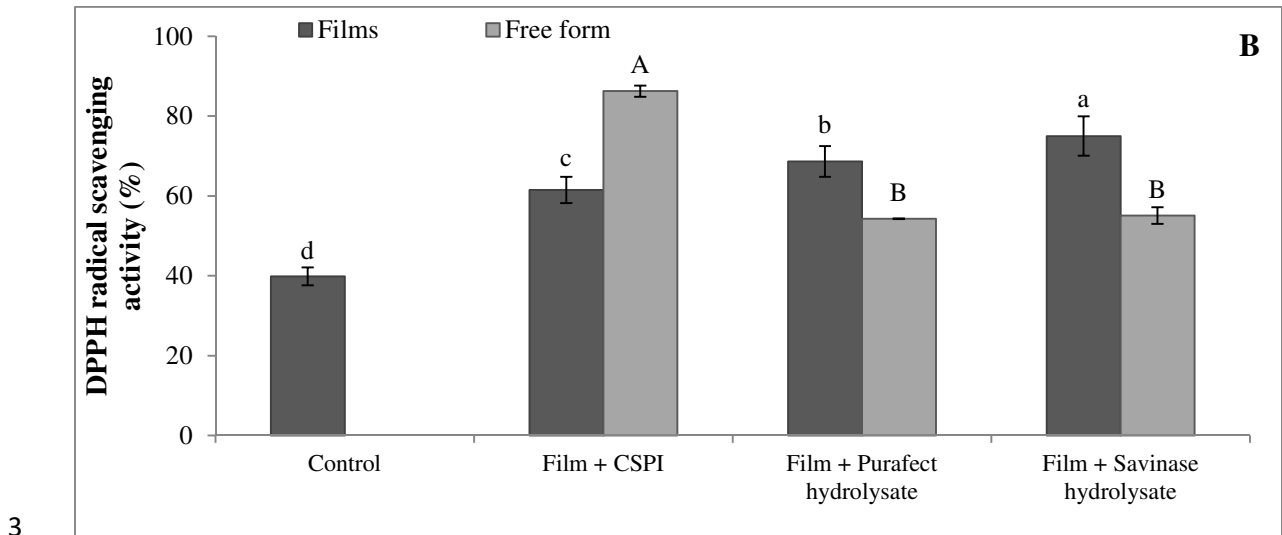
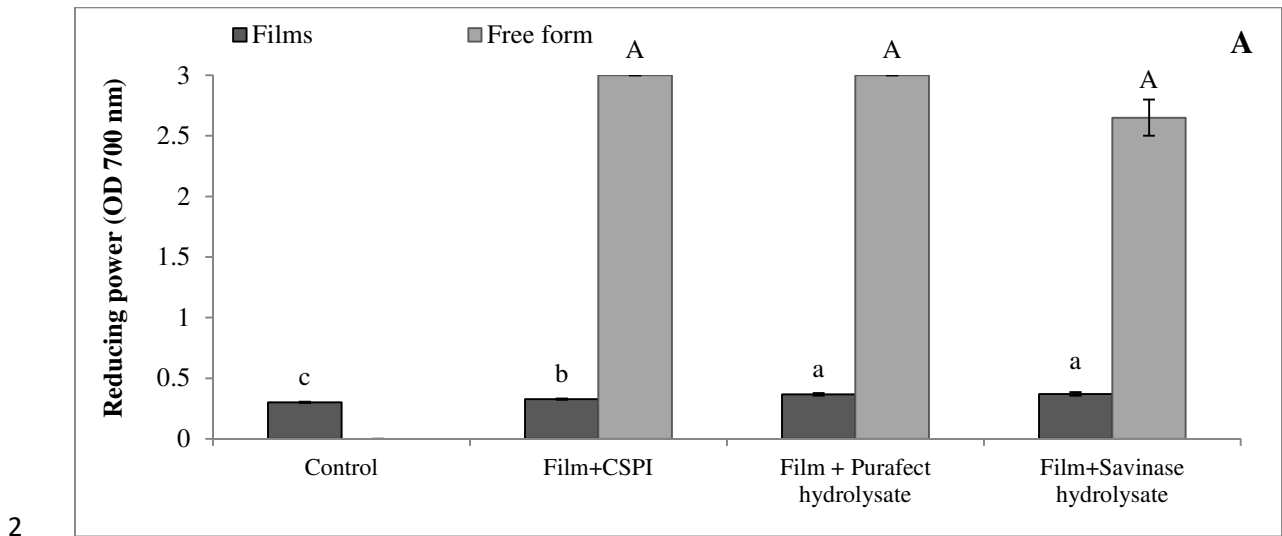


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1 **Fig. 5**



1 **Table 1:** Thickness, color parameters ( $L^*$ ,  $a^*$ ,  $b^*$ ,  $\Delta E^*$ ), thermal properties (glass transition  
2 temperature  $T_g$ , weight loss  $\Delta w$ , thermal degradation temperature  $T_{max}$  and residual mass),  
3 mechanical properties (Tensile strength TS and elongation at break EAB) of CSG films and  
4 those enriched by CSPI and CSPH. All films were previously stored at 25 °C and 50% RH for  
5 the determination of the mechanical properties.

Film characterizations		Control	Film + CSPI	Film + Purafect hydrolysate	Film + Savinase hydrolysate
<b>Thickness (<math>\mu\text{m}</math>)</b>		84.22 $\pm$ 4.01 <sup>a</sup>	79.34 $\pm$ 6.07 <sup>a</sup>	75.62 $\pm$ 1.56 <sup>a</sup>	77.45 $\pm$ 1.34 <sup>a</sup>
<b>Color properties</b>	<b>L*</b>	89.63 $\pm$ 0.12 <sup>a</sup>	85.73 $\pm$ 0.42 <sup>bc</sup>	86.27 $\pm$ 0.25 <sup>b</sup>	85.43 $\pm$ 0.32 <sup>c</sup>
	<b>a*</b>	0.37 $\pm$ 0.12 <sup>d</sup>	1.97 $\pm$ 1.97 <sup>c</sup>	2.20 $\pm$ 0.10 <sup>b</sup>	2.63 $\pm$ 0.15 <sup>a</sup>
	<b>b*</b>	3.63 $\pm$ 0.23 <sup>c</sup>	7.37 $\pm$ 0.45 <sup>b</sup>	7.40 $\pm$ 0.10 <sup>b</sup>	8.53 $\pm$ 0.21 <sup>a</sup>
	<b><math>\Delta E^*</math></b>	/	5.72 $\pm$ 0.57 <sup>b</sup>	5.47 $\pm$ 0.11 <sup>b</sup>	6.94 $\pm$ 0.07 <sup>a</sup>
<b>Thermal properties *</b>	<b><math>T_g</math> (°C)</b>	58.4 <sup>b</sup>	59.5 <sup>ab</sup>	71.4 <sup>a</sup>	61.8 <sup>ab</sup>
	<b><math>\Delta w_1</math> (%)</b>	14.3	10.9	12.8	12.4
	<b><math>\Delta w_2</math> (%)</b>	64.9	60.5	62.1	62.2
	<b><math>T_{max}</math> (°C)</b>	296.0	310.7	301.7	297.3
	<b>Residue (%)</b>	18.9	25.7	23.2	23.6
<b>Mechanical properties</b>	<b>TS (MPa)</b>	22.67 $\pm$ 2.95 <sup>a</sup>	22.09 $\pm$ 0.46 <sup>a</sup>	15.85 $\pm$ 1.50 <sup>b</sup>	12.29 $\pm$ 0.47 <sup>c</sup>
	<b>EAB (%)</b>	32.83 $\pm$ 1.97 <sup>a</sup>	26.26 $\pm$ 3.51 <sup>b</sup>	10.57 $\pm$ 1.28 <sup>c</sup>	10.32 $\pm$ 1.39 <sup>c</sup>

6 Values are given as mean  $\pm$  standard deviation. Means with different superscripts (a-d) within  
7 a same row indicate significant difference ( $p < 0.05$ ) in terms of films samples.

8 \* The average relative error on TGA data is lower than 5%



