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Additional Information

1 Influence of homogenization conditions on physical properties and antioxidant

2 activity of fully biodegradable pea protein-alpha tocopherol films.

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ABSTRACT

In this work, antioxidant biodegradable films based on pea protein and alpha-tocopherol were successfully developed by solution casting. The effect of homogenization conditions (rotor-stator and microfluizer) and the relative humidity (RH) on the morphology and physico-chemical properties (tensile, oxygen and water barrier properties) of pea protein/alpha-tocopherol based films were evaluated. The addition of alpha-tocopherol produced minimum changes in the optical film properties while the incorporation of alpha-tocopherol provided antioxidant properties to the pea protein films prepared by both homogenization conditions and improved barrier properties of these films at low and intermediate RH. The alpha-tocopherol addition provided more flexible and deformable films, mainly when the microfluidizer was used. These results add a new insight into the potential of pea protein and alpha-tocopherol for the development of fully biodegradable antioxidant films of interest in food packaging.

KEYWORDS: antioxidant capacity, oxygen permeability, water vapor permeability, tensile properties, pea protein.

1. INTRODUCTION

Nowadays, there is an increasing amount of research into developing active packaging or food coatings containing natural, bioactive compounds, such as antioxidants, or antimicrobials to increase the shelf life and safety of food. Direct antioxidant incorporation into foodstuffs is a usual practice in products sensitive to oxidation processes to prevent oxidation of food products and the development of off-flavors or color changes. However, some studies reveal that this practice may not be as effective as expected due to the fact that the oxidation reactions increase rapidly when the equivalent antioxidant finishes (Ozkan, Simsek & Kuleasan, 2007). On the other hand, the amount of antioxidant added to foods is regulated and some studies have reported that the direct addition of high levels of antioxidants may lead to a risk of lipid pro-oxidation, such as occurs for corn oil containing tocopherol in concentrations of up to $500 \mu g \ mL^{-1}$ (Frankel, Huang, Kanner & German, 1994, Huang, Frankel & German, 1994) or purified soybean oil (concentrations of up to 250 ppm) (Jung & Min, 1990). In this sense, active packaging is a good option for the controlled release of antioxidants into the foods, avoiding overconcentration which could provoke pro-oxidation effects (López-de-Dicastillo, Alonso, Catalá, Gavara & Hernández-Muñoz, 2010; Pereira de Abreu, Paseiro Losada, Maroto & Cruz, 2011).

Most of the studies which analyze the effect of alpha-tocopherol addition refer to plastic films such as polypropylene (PP) or low- and high-density polyethylene (LPDE and HDPE, respectively). However, nowadays there is an increasing demand for alternative biodegradable polymers based on proteins, polysaccharides and lipids. Pea protein films show good film manufacture properties in both, wet (casting) and dry process

72 (extrusion), however little information has been reported in the literature about this

73 protein (Choi & Han, 2001, 2002).

74 In this work, the effect of alpha-tocopherol on physical properties (tensile, oxygen and

75 water vapor barrier properties) as well as antioxidant capacity of pea protein films has

76 been studied by the film casting method. Although the casting method is not feasible for

77 the manufacture of commercial films, this work is a preliminary study to demonstrate

78 the potential of pea protein to be used as a protein matrix and future works will deal to

79 compare casting and dry process in order to simulate the realistic manufacture of films

80 with biodegradable materials.

81

82 **2. METHODS AND MATERIALS**

83

84 *2.1 Film preparation*

85 Film forming aqueous dispersions contained 4% (w/w) of isolate pea protein (PP)

86 (Pisane®C9 Cosucra, Warcoing, Belgium) and glycerol (Panreac Quimica, S.A.,

87 Castellar Del Vallés, Barcelona, Spain) as plasticizer, using a hydrocolloid:glycerol

88 mass ratio of 1:0.25 were prepared. The required amount of alpha-tocopherol (> 95.5%

89 of purity, Sigma–Aldrich Chemie GmbH, Steinheim, Germany) (60 and 100 mg alpha-

90 tocopherol ·g protein^{-1}) was added and stirred for 30 minutes. Emulsions were carried

91 out using a rotorestator homogenizer ("T samples") (Ultraturax T25, Janke & Kunkel,

92 Germany) in a first step at 13,500 r.p.m. for 1 min and at 20,500 r.p.m. for 3 min Some

93 of these samples ("M" samples) were homogenized in a second step using a

94 Microfluidizer M-110P (Microfluidics International Corp., Newton, Massachusetts,

95 USA)

96 Finally, they were degasified at 7 mbar with a vacuum pump (Wertheim, Germany).

97 Film-forming dispersions were obtained in flasks protected from light to prevent

98 oxidation.

99 Each film was prepared by weighing the amount of film-forming emulsion containing

100 1.5 g of total solids. This mass of emulsion was spread evenly over a teflon casting plate

101 (15 cm diameter) resting on a level surface, and films were formed by drying for

102 approximately 24 h at 45% RH and 20 ºC. Dry films could be peeled intact from the

103 casting surface.

104 Sample nomenclature was *Ya*, where "*Y*" refers to the homogenization process

105 (Ultraturax –T- or Micrufuidization-M-) and *"a"* is the amount of alpha-tocopherol (0,

106 60 or 100 g alpha-tocopherol ·g protein^{-1}) in the film-forming emulsion.

107

2.2 Barrier properties

2.2.1. Water vapour permeability.

110 A modification of the ASTM E96-95 (McHugh, Avena-Bustillos & Krochta, 1993)

111 gravimetric method for measuring the water vapour permeability (WVP) of flexible

112 films was employed, using Payne permeability cups (Elcometer SPRL, Hermelle /s

113 Argenteau, Bélgica). Deionised water or over-saturated NaCl and $Mg(NO_3)_2$ (Panreac

114 Quimica, SA, Castellar del Vallés, Barcelona) solutions were used inside the testing cup

115 to reach 100, 75 or 53% RH respectively, on one side of the film through a circular

116 opening of 3.5 cm in diameter. Once the films were secured, the cups were placed in

117 pre-equilibrated cabinets fitted with a variable-speed fan to reduce resistance to water

118 vapour transport. The environment within the cabinets was held at a constant RH using

119 over-saturated $MgCl_2$ or $Mg(NO_3)_2$ solutions to reach 33 or 53% RH, respectively. So,

120 the imposed RH gradients were: 100/53, 75/53 and 53/33; During WVP measurement,

121 the side of the film in contact with the Teflon plate was placed in contact with the

122 highest relative humidity. The cabinets were placed at a controlled temperature of 25ºC.

123 The cups were weighed periodically (every two hours for a day) after the steady state

124 was reached using an analytical balance (±0.0001g). Water vapour permeability was

125 determined from the slope obtained from the regression analysis of the weight loss data

126 as a function of time, once the steady state was reached. At least four replicates were

127 obtained from each sample. The method proposed by McHugh *et al*. (1993) to correct

128 the effect of concentration gradients established in the stagnant air gap inside the cup

129 was used.

130

131 *2.2.2. Oxygen permeability.*

132 The oxygen permeation rate of the films was determined (in triplicate) at 33, 53 and

133 75% RH and 25ºC using an OX-TRAN Model 2/21 ML Mocon (Lippke, Neuwied,

134 Germany). Film samples were previously preconditioned for one week in the

135 desiccators at the relative humidity level of the test using over-saturated $MgCl_2$,

136 $Mg(NO_3)$ and NaCl solutions. Each sample were placed in the equipment for analysis

137 and they were conditioned in the cells for 6h, then the transmission values were

138 determined every 20 min until equilibrium was reached. Oxygen permeability was

139 calculated taking into account both the oxygen transmission rate as well as the thickness

140 of the film.

141

142 **2.3 Tensile properties**

143 A universal traction testing machine (TA.XT2 plus model, Stable MicroSystems, Haslemere, England) was used to determine tensile strength (TS), elastic modulus (EM)

144

145 and elongation (E) properties, according to ASTM standard method D882 (ASTM,

146 2001). TS, EM and E properties were determined from stress–strain curves. Two

147 samples of each film were selected for the tensile properties measurements and at least

148 eight replicates of each were tested. The film samples have been cut as rectangular

149 samples of 2.5 cm wide and 10 cm long. Before testing, all samples were equilibrated

150 for one week at 33%, 53% and 75% - RH in a cabinet using over-saturated $MgCl_2$,

151 $Mg(NO_3)$ and NaCl solutions, respectively at 25 ºC. Equilibrated film specimens were

152 mounted in the extension grips of the testing machine and stretched at a rate of 50 mm

153 min^{-1} until breaking.

154

155 *2.4 Antioxidant activity*

156 The antioxidant capacity of films and the pure alpha-tocopherol, were evaluated by

157 means of the Trolox equivalent antioxidant capacity (TEAC), using a modification of

158 the original TEAC method (Re et al., 1999). Trolox (6-hydroxy-2,5,7,8-

159 tetramethylchroman-2-carboxylic acid), a vitamin E analogue, was used as an

160 antioxidant standard. ABTS (2,20 -azinobis(3-ethylbenzothiazoline-6-sulphonic

161 acid)diammonium salt) was dissolved in water to a concentration of 7 mM and allowed

162 to reactwith a 2.45 mM potassium persulphate solution (final concentrations) for 16 h.

163 To this end, ABTS solution was stored overnight in the dark. All three reagents were

164 provided by Sigma (Sigma–Aldrich Chemie, Steinheim, Germany). ABTS radical

165 cation ($ABTS^{\cdot+}$), a blue chromophore, was produced in the reaction. The $ABTS^{\cdot+}$

166 solution was diluted with a water:methanol (2:8) solution to an absorbance of 0.70

167 (±0.02) at 734 nm. All the determinations were carried out in a Beckman Coulter DU

168 730 spectrophotometer, using a water:methanol (2:8) solution as blank. 0.1 g of each

169 film sample was cut into small pieces and hydrated with 2 ml of distilled water for 1 h,

170 when 8 ml of methanol were added in order to favour the extraction of the antioxidant

171 components. These samples were maintained under stirring overnight and centrifuged at

172 4ºC, 5000 rpm for 10 min. The supernatant (film extract) obtained was analyzed for

173 ABTS radical scavenging activity. To this end 10ll of the film extracts were added to 1

174 ml of the ABTS $^{.+}$ solution and absorbance at 734 nm was registered every minute for 6

175 min. For calibration, Trolox standards of different concentrations were prepared and the

176 same procedure was followed. The TEAC of the film samples was determined by

177 comparing the corresponding percentage of absorbance reduction at 6 min with the

178 Trolox concentration–response curve. All the determinations were carried out in

179 triplicate using a Beckman Coulter DU 730 (England) spectrophotometer, with

180 methanol as the blank.

181

182 *2.5 Differential scanning calorimetry*

183 Differential scanning calorimetry (DSC) was carried out using a DSC 220 CU-SSC5200

184 (Seiko Instruments). Samples were prepared by lyophilizing (ioalfa-6, TELSTAR;

185 Terrassa, España) and reconstituting them in a 30% (w/v) with distillated water. Small

186 amounts (25–30 mg) of each sample were placed onto aluminum pans (Seiko

187 Instruments, P/N SSC000C008), hermetically sealed and heated from 25 to 120 ºC at 5

188 ºC/min. An empty aluminum pan was used as reference.

189

190 *2.6 Scanning Electron Microscopy (SEM)*

191 Microstructural analysis of surface and cross-section of the films was carried out by

192 using SEM technique in a JEOL JSSM-5410 (Japan) electron microscope. Pieces of

193 6mm x 6 mm were cut from films, mounted on copper stubs and fixed on the support

194 using double side adhesive tape with an angle of 90 ℃ to the surface to allow

195 observation of cross-section. Samples were gold coated and observed using an

196 accelerating voltage of 10 kV. Measures were taken in triplicate for each sample and

197 three different films of each formulation were used.

198

199 *2.7 Optical properties*

200 The film transparency was determined through the surface reflectance in a

201 spectrocolorimeter CM-3600d (Minolta Co, Tokyo, Japan). Measurements were taken

202 from three films of each formulation by using both a white and a black background.

203 Internal transmittance (Ti) was determined by applying the Kubelka-Munk theory

204 (Hutchings, 1999) for multiple scattering to the reflection spectra.

205

206 *2.8 Statistical analysis*

207 The statistical analysis of the data was performed through an analysis of variance

208 (ANOVA) using Statgraphics Plus (Manugistics Corp., Rockville, MD). Fisher's least

209 significant difference (LSD) procedure was used.

210

211 **3. RESULTS**

212 *3.4 Microstructure and optical properties.*

213 Microstructure images give relevant information about the components arrangement in

214 the dried films as well as the interactions produced between them, after the drying

215 process. Internal structure and surface appearance of the films were characterized

216 qualitatively by scanning electron microscopy (SEM). Figures 1 and 2 show surface

217 and cross-section images of films prepared with rotor-stator ("T" films) and with

218 microfluidizer ("M" films). Generally, "T" and "M" control films (without alpha-

219 tocopherol) showed a homogeneous and smooth surface. Curiously, several white spots

220 can be identified on the surface image of non-microfluidized control films ("T") which

221 could be related to some protein that is not well-integrated in the film since these spots

222 disappeared in microfluidized films. As expected, films prepared with alpha-tocopherol

223 showed structural discontinuities according to the lack of miscibility of film

224 components. Surface images show clear differences in the component arrangement in

225 the film when the homogenization energy input increased (comparing microfluidized

226 and non-microfluidized films). For films containing 60 mg alpha-tocopherol· g protein^{-1},

227 antioxidant droplets were clearly identified on the top of the surface micrographs, much

228 more finely arranged when higher energy input was applied during the homogenization

229 process ("M" films). The aggregation of alpha-tocopherol droplets during film drying is

230 evident in films containing the highest amount of tocopherol (100 mg alpha-tocopherol ·

231 g protein^{-1}) since irregular lipid masses embedded in the protein network was observed

232 whatever the homogenization done. Cross-section images gives complementary

233 information, showing a greater phase separation in non-microfluidized films, probably

234 due to the higher particle size of the antioxidant which favors its coalesce and creaming

235 processes which contributes to the alpha-tocopherol migration near to the surface due to

236 it lower density.

237 Previous studies show that optical properties are directly related with the

238 microstructure achieved in the films (Fabra, Talens & Chiralt, 2009; Souza, Cerqueira,

239 Casariego, Lima, Teixeira & Vicente, 2009; Jiménez, Fabra, Talens & Chiralt, 2010).

240 Table 1 shows the internal transmittance (Ti) of studied films, measured at 550nm.

241 According to the Kubelka-Munk theory, an increase in Ti values is associated with an

242 increase in the films' homogeneity and with more transparent films. Microfluidized

243 control films showed the highest Ti values corresponding with a more homogeneous

244 structure. Generally, no significant differences on this parameter was observed, though

245 there is a tend to slightly increase Ti values with the relative humidity and in

246 microfluidized films due to the smaller particle size provides greater homogeneity and

247 so, higher Ti values. Moreover, the microfluidization implies higher energy input

248 during the homogenization process which favors the better integration of alpha-

249 tocopherol in the protein matrix through tocopherol-protein interactions and hence,

250 obtaining a less heterogeneous matrix than their counterparts "non-microfluidized".

251 Similarly, Fabra *et al*. (2010, 2011) reported that microfluidized sodium caseinate-oleic

252 acid or stearic acid films were slightly more transparent and the addition of solid

253 compounds (saturated fatty acids or waxes) greater modify film´s transparency-opacity

254 than components in liquid state such as oleic acid or alpha-tocopherol. This is due to the

255 light scattering phenomena provoked by the presence of particles with a different

256 refractive index distributed throughout the film network

257

3.2 Barrier properties.

259 The analysis of the water vapor permeability gives information about the film´s

260 suitability to be applied as a coating or a film. In this work, three different relative

261 humidities gradients (100/53, 75/53 and 53/33 % RH) were studied in order to simulate

262   the driving force existing in a food product with a high, medium or low water activity

263   exposed to an atmosphere of 53% RH

264   Figure 3 shows water vapour permeability values of films prepared with rotor-stator

265   (films "T") or with rotor stator plus microfluidizer (films "M"), for both alpha-

266   tocopherol concentrations: 60 and 100 mg alpha-tocopherol g protein^{-1}.

267   Alpha-tocopherol addition significantly reduced ($p<0.05$) water vapour permeability

268   values of films analyzed at 53/33 and 75/53% RH gradients, regardless of the

269   homogenization ("T" and "M" films) conditions. At these RH gradients, the amount of

270   the alpha-tocopherol was not decisive in the non-microfludized films ("T" films) and no

271   significant differences ($p>0.05$) were observed between films prepared with 60 and 100

272   mg alpha-tocopherol g protein^{-1}. However, in microfludized films ("M" films) the

273   amount of tocopherol greatly contributes to decrease water vapor permeability of these

274   films, being better barriers those prepared with the highest amount of tocopherol (100

275   mg alpha-tocopherol g protein^{-1}). This could be attributed to the hydrophobic nature of

276   the antioxidant used and the better integration of the alpha-tocopherol into the polymer

277   matrix due to the mechanical effort provided by the microfluidizer. Similar effects have

278   been also observed in sodium caseinate-alpha-tocopherol films (Fabra, Hambleton,

279   Talens, Debeaufort & Chiralt, 2011).

280   Contrary, the effect of the alpha-tocopherol on WVP values of pea protein films

281   analyzed at 100/53% RH gradient produced adverse effects depending on the

282   homogenization done ("T" and "M" films). On the one hand, "T" films showed a

283   similar pattern to that reported for films analyzed at 75/53 and 53/33 % RH gradients,

284   decreasing WVP values, regardless of the concentration used. However, water vapor

285   barrier properties were not improve in microfluidized films ("M" films) in which the

286 tocopherol addition produced an increase in the WVP values of films tested at 100/53%

287 RH gradient. This increase could be due to structural changes that take place in the

288 protein matrix due to mechanical pressures exerted by the microfludizer, which would

289 induce a reorganization of the protein brands, favoring the formations of new bonds and

290 interactions between aminoacids. So, a structure with more stretched protein chains

291 would result. This new reorganization of the protein could lead to greater interchain

292 spaces, facilitating the mobility of water molecules throughout the pea protein matrix.

293 Thus, an increase in the WVP values could be expected, mainly at higher relative

294 humidies in which a more plasticized structure could be obtained.

295 This phenomenon could also explain that a given alpha-tocopherol concentration, non-

296 microfluidized films showed lower WVP values than microfluidized films, though this

297 effect was less marked when higher driving forces were applied (100/53% RH gradient)

298 probably due to the plasticizing effect which increase molecular mobility whatever the

299 protein structure reached in the film.

300 Finally, water barrier efficiency significantly ($p<0.05$) decreased when higher relative

301 humidies gradients were applied due to the plasticizing effect of water. Assuming that

302 each side of the film was equilibrated at the respective a_w of the gas phase in contact, an

303 a_w gradient (linear profile) in the film´s thickness was established during the water

304 vapor determination. So, films analyzed at 100/53% RH had higher water content than

305 those studied at 53/33% RH and an increase in the water content of the film structure

306 implies both an increase in molecular mobility, due to the plasticization effect, and also

307 in transport properties (Roos, 1995). Similarly, Mauer, Smith & Labuza (2000) reported

308 that β-casein films showed higher WVP values when a RH gradient of 53/76 % was

309 applied, as compared with the lower values obtained for a RH gradient of 53/11 %. The

310 water plasticizing effect could be enhanced with alfa-tocoferol addition which could

311 interact between polymer brands, increasing molecular inter-chains and favouring the

312 mobility of the structural components. Similar effects have been reported by Fabra,

313 Talens & Chiralt (2010) working with sodium caseinate - oleic acid films.

314 To sump up, though pea-protein-tocopherol based films showed good water barrier

315 properties at low relative humidity gradients, they were lost when films were

316 conditioned at higher relative humidities.

317 Oxygen permeability values of microfluidized and non-microfludized films are shown

318 in Table 3. Concerning to the RH effect, a similar pattern was observed to that reported

319 for WVP values, decreasing the oxygen barrier efficiency when films were tested at

320 higher relative humidities.

321 At 33% RH, both microfluidized ("M") and non-microfluidized ("T") films improved

322 oxygen permeability with the alpha-tocopherol addition, mainly when higher amounts

323 of tocopherol were used. However, at intermediate (53 %RH) and high (75 %RH)

324 relative humidities, oxygen barrier properties depend on the homogenization done. At

325 53% RH, the tocopherol addition produced a significant ($p < 0.05$) decrease in oxygen

326 permeability values of non-microfluidized films, though this reduction was not affected

327 by the amount of the tocopherol used. Contrary, oxygen permeability values did not

328 vary significantly ($p < 0.05$) when they were tested at 75 %RH which could be attributed

329 to the plasticizing effect of water.

330 In microfluidized films, oxygen permeability increased with the tocopherol addition (in

331 films tested at 53 and 75 %RH) when the expected behavior would be that the higher

332 molecular mobility of the protein matrix favors the tocopherol-oxygen reactions,

333 preventing and retarding oxygen transport throughout the films by the chemical action.

334 The obtained results suggest that such action does not occur and is likely due to the

335 pressure effect, which favors protein-tocopherol interactions, reducing protein inter-

336 chain bonds and leading a less compact matrix throughout the oxygen and water

337 molecules can be easily diffused. Similarly, Byun, Kim & Whiteside (2010) reported

338 that oxygen permeability of polylactic acid (PLA) - butylated hydroxytoluene (BHT)

339 films were increased by the addition of alpha-tocopherol probably due to its

340 hydrophobic nature which facilitates oxygen transfer (Bertan, Tanada-Palmu, Siani &

341 Grosso, 2005).

342

3.2 Mechanical properties.

344 Figure 4 shows mechanical parameters (EM:elastic modulus, TS: tensile strength and E.

345 elongation at break) of microfluidized ("M") and non-microfluidized ("T") films,

346 conditioned at three different relative humidities (33, 53 and 75% RH). EM and TS

347 values were greatly influenced by the amount of the alpha-tocopherol used, showing a

348 significant decrease in both "T" and "M" pea-protein films. Alpha-tocopherol addition

349 could induce the development of a heterogeneous structure with a number of

350 discontinuities in the polymer network. Moreover, tocopherol could interact with the

351 pea protein matrix by modifying the intermolecular interactions between protein chains,

352 weakening the polymer structure and, therefore, producing a similar effect as

353 plasticizers. This implies that films containing tocopherol were more flexible and less

354 deformable than pea protein based films. Similar effects have been reported by Byun *et*

355 *al*. (2010). However, the reduction in EM and TS values due to tocopherol addition was

356 less marked in films equilibrated at high relative humidities (75 %RH), probably due to

357 this effect is less relevant in plasticized films.

358   Concerning to the homogenization type, microfluidized films showed lower EM and TS

359   values. The elongation at break increased in microfluidized samples, producing more

360   extensible films (cf. Figure 4). This effect may be attributed to the promotion of

361   protein–lipid interactions when homogenization conditions were more intense, and

362   some small lipid particles became embedded in the protein network, which seemed to

363   have some plasticizing effect on the films. The mechanical effort produced by the

364   microfluidizer during the homogenization process may cause the elongation of the

365   protein chains, favoring protein-tocopherol interactions which lead to weaker inter-

366   chain forces. Similar results were also observed by Ma et al., 2012 in gelatin-olive oil

367   composite films. They observed that the elongation at break increased as the

368   microfluidization pressure or cycle increased, producing more extensible films.

369   Moreover, alpha-tocopherol is liquid at the temperature at which the test is performed

370   and, can flow during the tensile assay favoring the film´s stretchability. This could be

371   enhanced when the particle became smaller and are better distributed into the polymer

372   matrix, its mean, in "M" films. Therefore, in microfluidized films, protein chains could

373   be easily displaced during the tensile test which would explain the increased in E values

374   and the worst barrier properties of these films. The increase in E values of

375   microfluidized films is accentuated when the polymer was greater plasticized, its mean,

376   when films were equilibrated at high relative humidities. As shown in Figure 4, the

377   deformability of the films increased with the relative humidity due to the decrease in the

378   protein inter-chain forces associated with the plasticizing effect of water.

379

380   *3.3 Antioxidant activity.*

381 Antioxidant capacity (measured by the ABTS method) of films is shown in Table 2,

382 expressed as mg equivalent tocopherol · g protein^{-1}. Table 5 shows the TEAC of the dry

383 films, which is defined as the amount (mg) of sample that produces the same

384 absorbance reduction as 1 mM Trolox solution. The TEAC values of the alpha-

385 tocopherol incorporated in the dried films have been also estimated by considering its

386 mass fraction in the dry films. "M" and "T" control films (without alpha-tocopherol) did

387 not show antioxidant activity. However, it has been reported that protein based films

388 could be considered as materials naturally activated with antioxidant properties

389 (Salgado, Molina Ortiz, Petruccelli & Mauri, 2010; Salgado, Fernández, Drago &

390 Mauri, 2011). These authors found that soybean and sunflower protein based films had

391 antioxidant activity, which could be attributed to residual content of phenolic

392 compounds or flavonoids present in sunflower or soybean seeds remaining in the

393 protein isolation. Gómez-Estaca, Giménez, Montero & Gómez-Guillén (2009) also

394 observed some antioxidant activity in skin gelatin and commercial fish gelatin based

395 films which were mainly attributable to the peptide fraction of gelatin (Samaranayaka &

396 Li-Chan, 2008).

397 The Trolox Equivalent Antioxidant Capacity (TEAC) of alpha-tocopherol, expressed as

398 mg of sample that show the same activity as 1 mM of Trolox, was 0.58 ± 0.09 mg.

399 Films showed lower antioxidant capacity (higher TEAC values) than the pure

400 antioxidant since its mass fraction in the film solids is relatively low. The TEAC values

401 estimated for the alpha-tocopherol in the films were greatly increased with respect to

402 those obtained for the pure compound (reduction of the antioxidant activity). This

403 reduction can be attributed to the loss of alpha-tocopherol activity during film-formation

404 (the pressure effect provided by the microfluidizer provokes an increase in the

405 temperature of film-forming emulsions), to the uncompleted extraction of the

406 antioxidant encapsulated in the polymer matrix or to interactions with the protein matrix

407 which retain the antioxidant, avoiding the complete antioxidant extraction.

408 As it is shown in Table 3, the homogenization condition had an important role in the

409 antioxidant capacity of the films. For a given alpha-tocopherol concentration,

410 microfluidized films showed higher antioxidant activity values (lower TEAC values)

411 which could be attributed to the spatial arrangement of the protein chains and the

412 retention of tocopherol between them. As abovementioned, an increase in the

413 homogenization pressure would favor the formation of new bonds and the development

414 of interactions, leading to a network of protein chains most stretched, so that the steric

415 hindrance in the mobility of tocopherol molecules is lower, resulting in a more effective

416 tocopherol extraction.

417 To corroborate if the supplied pea protein was not completely denaturized and if the

418 microfluidizer provokes changes in the protein structure, a differential scanning

419 calorimetric (DSC) assay was done. Figure 3 showed the thermogram of microfluidized

420 and non-microfluidized control films in which no endothermic peak in any of the two

421 films were observed. This suggests that the pea protein is originally denatured, though

422 this not implies that changes in the protein arrangement and structure could be produced

423 by the mechanical treatment provided by the microfluidizer. This could contribute to the

424 lengthening protein chain in a greater degree.

425

4. CONCLUSION

427 Barriers, tensile and optical properties, as well as the antioxidant capacity of pea

428 protein-tocopherol films were influenced by the homogenization conditions of the film-

429 forming emulsions, the amount of the alpha-tocopherol initially added into protein

430 matrix and the relative humidity at which films were stored. At low relative humidities,

431 alpha-tocopherol addition improved oxygen and water vapor barrier properties,

432 whatever the homogenization used. However, that not occurs at high relative humidity

433 probably due to the plasticizing effect of water. These results have demonstrated the

434 potential of pea protein for the development of biodegradable based films in which the

435 addition of alpha-tocopherol provides antioxidant films with greater water and oxygen

436 barrier properties in films stored at low or intermediate RH. Furthermore, more flexible

437 and deformable films were obtained with alpha-tocopherol addition with minimum

438 changes in the optical properties.

439

445

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544 **Table 1.** Internal transmittance (Ti) values measured at 550nm of pea protein films

545 containing 0, 60 or 100 mg alpha-tocopherol · g protein^{-1}, stored at three different

546 relative humidities.

Sample	Ti (550nm) 33%	Ti (550nm) 53%	Ti (550nm) 75%
T0	78.7 (0.1)[a1]	77.9 (0.7)[a1]	78.9 (0.5)[a1]
T60	78.0 (0.6)[a1]	78.1 (0.4)[a1]	78.3 (0.2)[a1]
T100	78.1 (0.5)[a1]	79.7 (0.5)[b1.2]	79.6 (0.6)[ab2]
M0	77.7 (0.7)[a1]	81.6 (0.1)[b2]	80.1 (0.8)[ab2]
M60	77.7 (0.5)[a1]	79.4 (0.5)[b2]	80.4 (0.3)[ab2]
M100	79.2 (0.8)[a1]	79.3 (0.1)[b1]	81.6 (0.3)[b2]

547 [a-b]: Different superscripts within a column indicate significant different among formulations
548 [1-2] Different superscripts within a file indicate significant different among formulations
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551 **Table 2.** Oxygen permeability values of pea protein films containing 0, 60 and 100 mg

552 alpha-tocopherol · g protein^{-1} stored at 33, 53 and 75 %RH.

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Film	OP (cm^3 m^{-1} $s^{-1}kPa^{-1}$)*10^{10}		
	33%	53%	75%
T0	0.58 (0.01)[a]	3.16 (0.06)[a]	21.4 (0.9)[a]
T60	0.52 (0.01)[b]	2.79 (0.09)[b]	23.3 (1.3)[a]
T100	0.43 (0.07)[c]	2.67 (0.07)[b]	22.4 (1.3)[a]
M0	0.41 (0.02)[c]	2.64 (0.12)[b]	25.0 (0.8)[a]
M60	0.35 (0.04)[c]	2.91 (0.02)[b]	24.3 (0.2)[a]
M100	0.29 (0.01)[d]	3.72 (0.07)[c]	28.3 (0.4)[b]

554 [a-c]: Different superscripts within a column indicate significant different among formulations.
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562 **Table 3.** Trolox equivalent antioxidant capacity (TEAC) expressed as the amount (mg)

563 of the dry film or compound which gives the same absorbance reduction as 1 mM

564 Trolox solution.

Film	TEAC (mg)	
	Dry film	Antioxidant in the dry film
T60	245 (25)[a]	11.3
T100	198 (16)[b]	14.7
M60	223 (15)[ab]	10.2
M100	160 (15)[c]	11.8

[a-c:] Different superscripts within a column indicate significant different among formulations.

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584 **LIST OF FIGURES**

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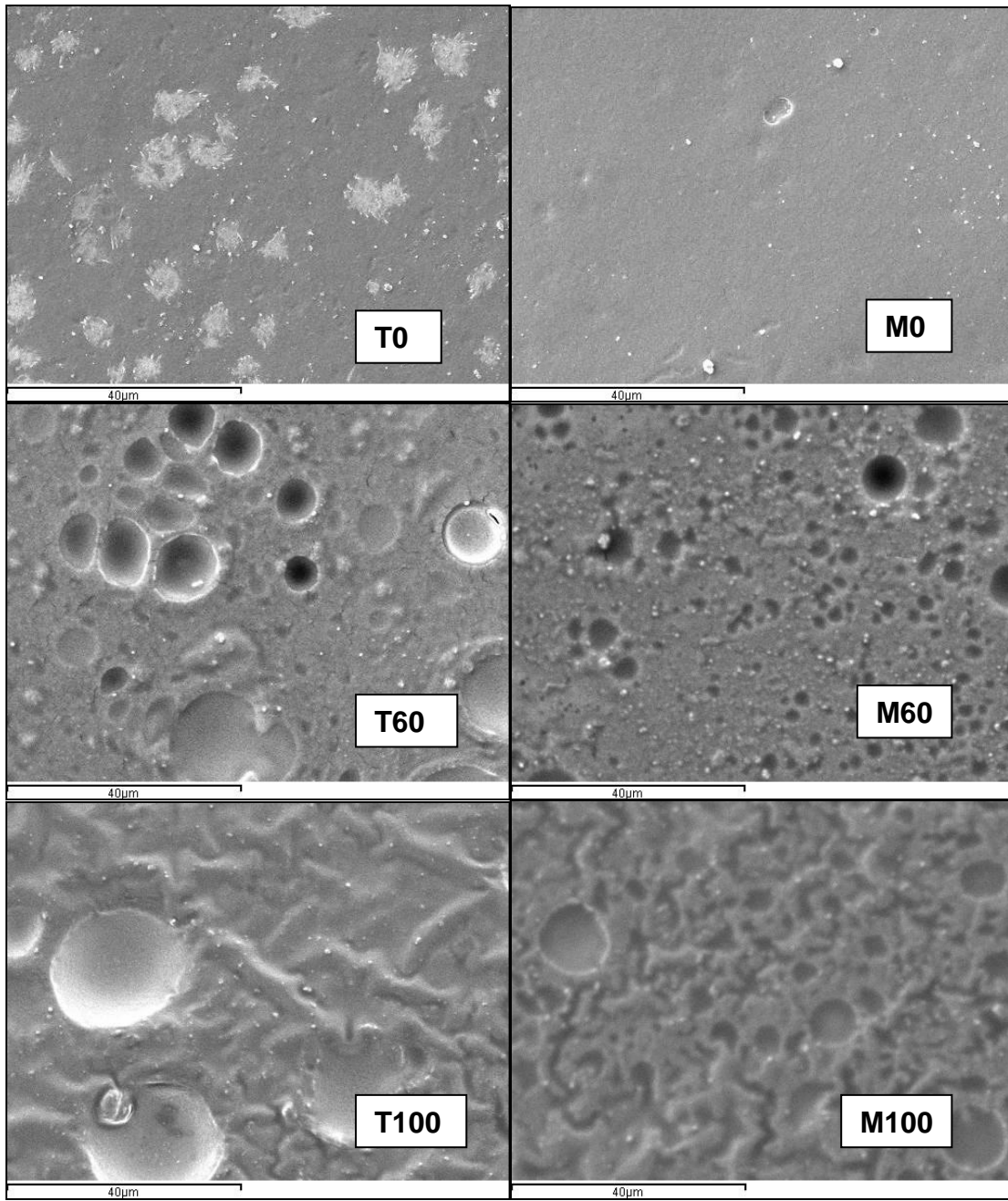
Figure 1

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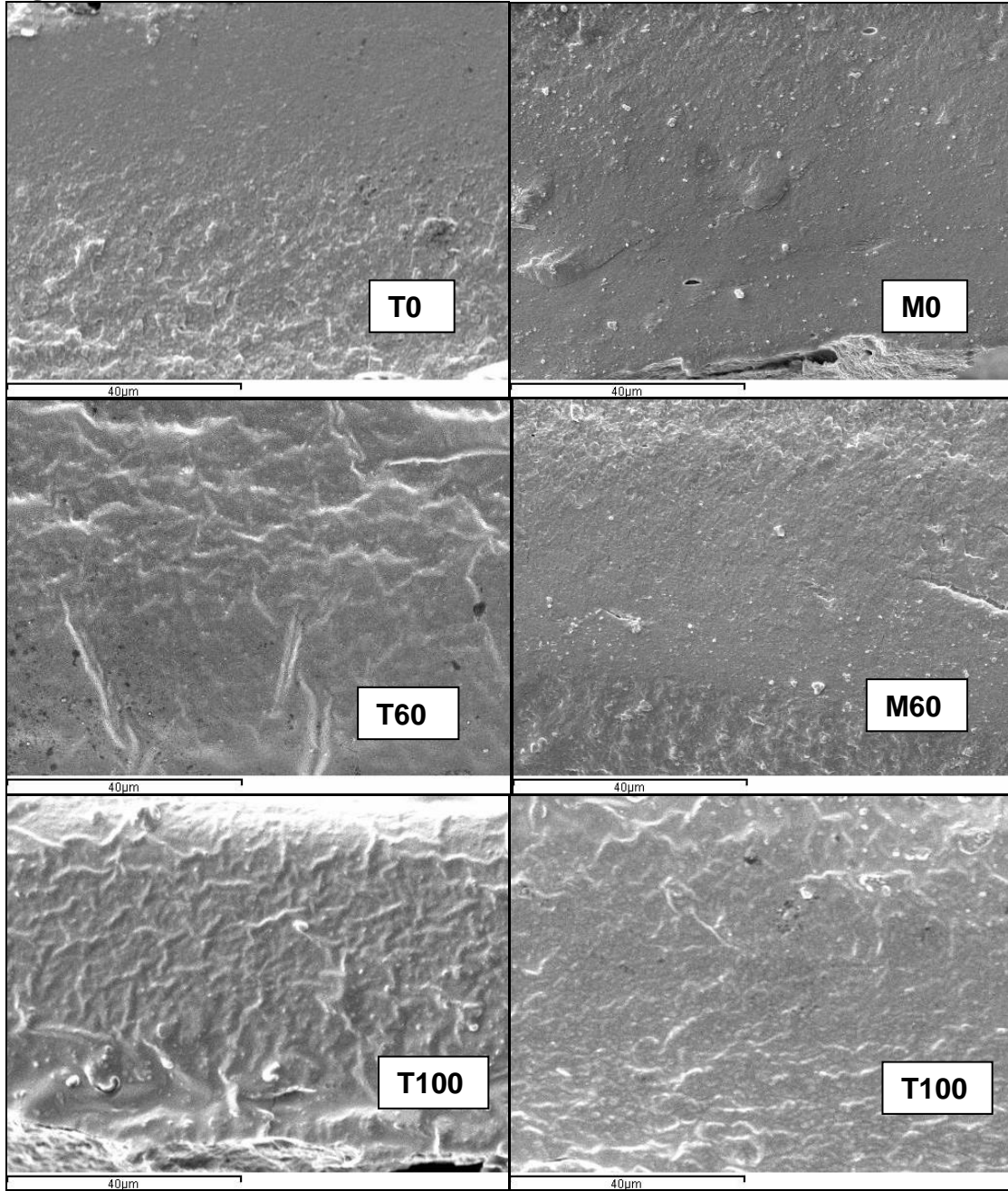
Figure 2

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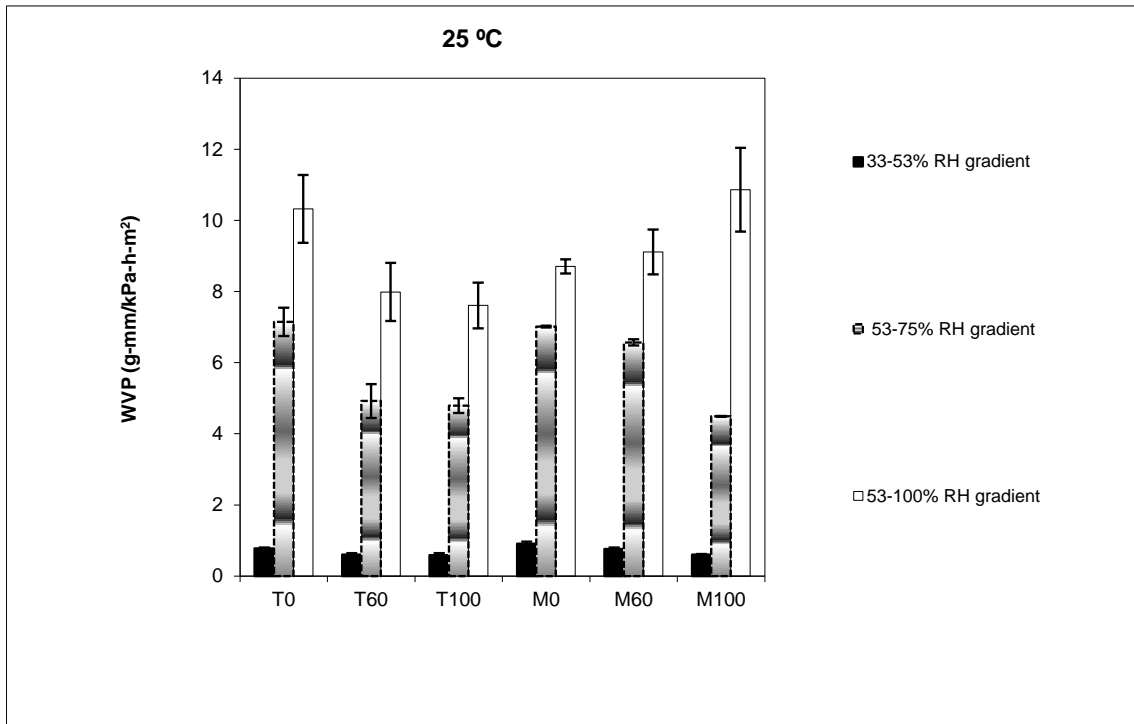
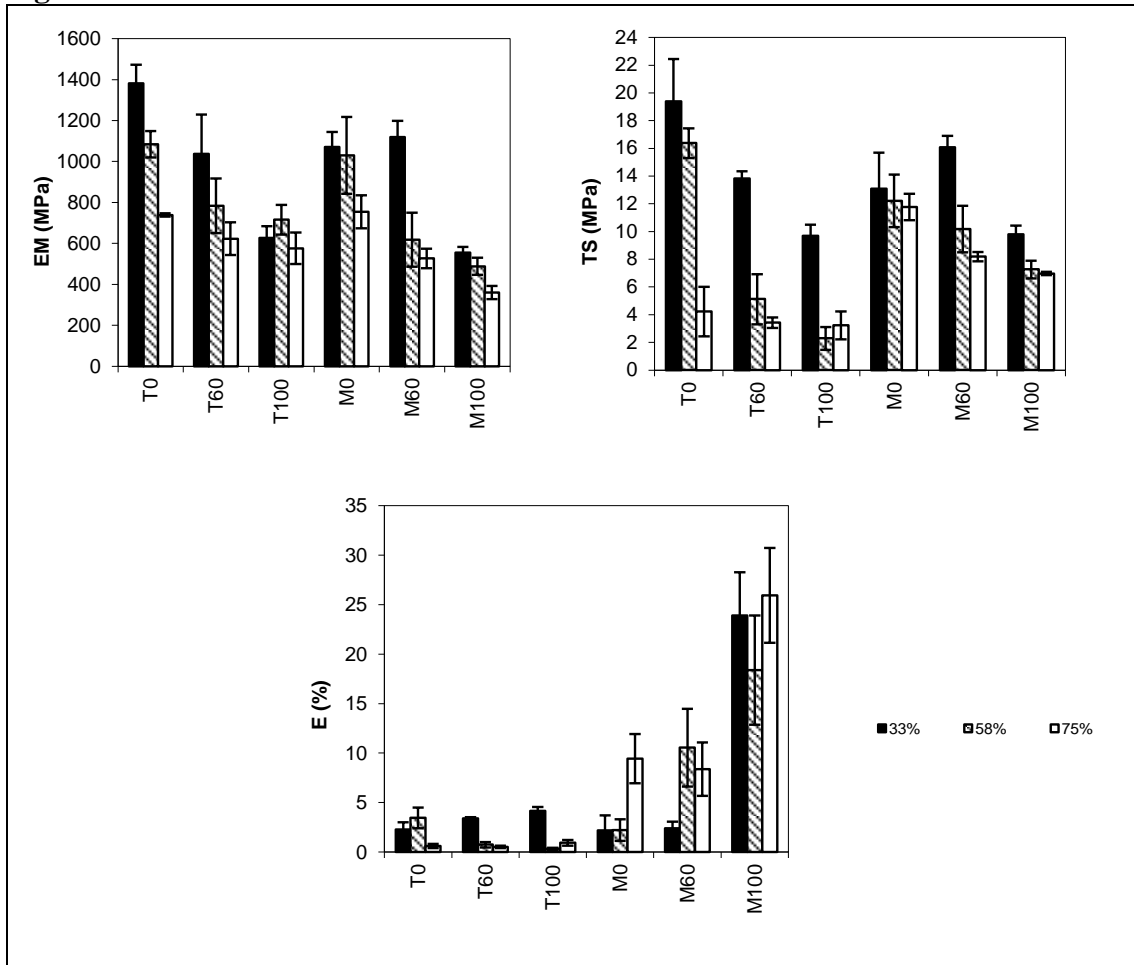**Figure 3**

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Figure 4
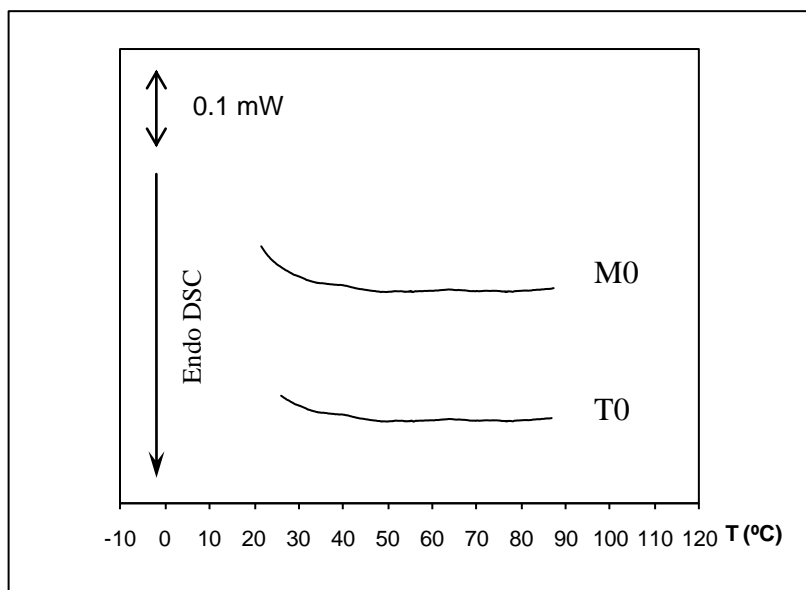

EM: Elastic Modulus; TS: Tensile Strength and E (%) elongation at break

669   **Figure 5**

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