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Effect of High Pressure Carbon Dioxide on polyphenoloxidase from *Litopenaeus vannamei*

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Abstract

The extraction of polyphenoloxidase (PPO) from *Litopenaeus vannamei* was studied at different temperatures, pH and ionic strength conditions. Optimum extracts at pH = 7.2 were treated by hiph pressure carbon dioxide (HPCD) technology at 20 MPa and in a temperature range from 35 to 50 ºC. Inactivation kinetics for PPO extracts were determined and fitted to the Weibull model. The effect of HPCD on PPO activity was also evaluated in shrimps in vivo, finding that PPO could be easier inactivated than in the crude PPO extract. Colour of *Litopenaeus vannamei* was visually followed right after HPCD treatment and thermal treatment at the same temperature and during storage at 4 ºC during a week, showing good results when shrimps were treated with HPCD in the absence of water.

Keywords: *Litopenaeus vannamei*, HPCD, solid samples, PPO inactivation.

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19 **1. Introduction**

20 Pacific white shrimp (*Litopenaeus vannamei*) accounts for 90 % of the Western hemisphere
21 aquaculture shrimp production. The most common problem in shrimps during post-harvest
22 storage is melanosis. It is a natural post-mortem process, where the polymerization of phenols
23 into insoluble high molecular mass black pigments, the melanins, takes place. This is a
24 multistage process: polyphenol oxidases (PPO) enzymes oxidize phenols into quinones, which
25 spontaneously polymerize to form melanin (Gonçalves & de Oliveira, 2016). Despite not being
26 harmful, melanosis can affect the sensory properties of the product, reducing its quality.

27 During harvest and storage of shrimps, sulphites, mainly sodium metabisulphite (SMS), are used
28 for preventing melanosis. SMS reduces the *o*-quinones, which are coloured, to diphenols, which
29 do not have any colour. Despite being widely used to inactivate PPO, sulphites exhibit several
30 disadvantages, such an incomplete prevention of melanosis. This fact involves a continued
31 reapplication of SMS on the seafood (Gonçalves & de Oliveira, 2016), which can lead to the
32 formation of unpleasant smells. Moreover, sulphites have potential pathological effects
33 associated to allergies and asthmatic attacks. For that reason, many alternatives to sulphites can
34 be found in the literature with the aim of avoiding browning in shrimps. Pacific white shrimps
35 have been treated with a wide range of natural preservatives to inhibit PPO activity, such as
36 ferulic acid and lead seed (Nirmal & Benjakul, 2009), and extracts from different leaves (Sae-
37 leaw & Benjakul, 2019). However, usually large concentrations of extract or combinations with
38 other technologies are needed.

39 Traditionally, thermal treatments have been applied for inhibiting PPO activity. Verhaeghe,
40 Vlaemynck, Block, Weyenberg, & Hendrickx (2016) probed that after treating brown shrimp
41 PPO extract during 2 min at 65 ºC, the enzyme was completely inactivated. Manheem, Benjakul,
42 Kijroongrojana, & Visessanguan (2012) found out that after treating shrimps at 80 ºC, melanosis

43 formation decreased after 7 d in refrigerated storage. However, thermal treatment produces a big
44 change in food properties (Ferrentino & Spilimbergo, 2011). For that reason, different non-
45 thermal technologies are being investigated as an alternative.
46 One of them is the use of HPCD (High Pressure Carbon Dioxide) technology. HPCD is a cold
47 pasteurization method that affects microorganisms and enzymes by using pressures below
48 50 MPa and temperatures usually lower than 50 ºC. This technology is gaining interest since CO_2
49 is nontoxic, inexpensive, nonflammable, recyclable and after depressurization it doesn´t leave
50 residues in the product (Briongos et al., 2016). Effectiveness of HPCD on PPO inactivation has
51 been extensively reported for different fruits and vegetables juices, such as orange juice
52 (Briongos et al., 2016), apple and tomato juice (Illera, Sanz, Solaesa, Ruiz, & Beltrán, 2018;
53 Illera, Sanz, Trigueros, Beltrán, & Melgosa, 2018), carrot juice (Zhou, Wang, Hu, Wu, & Liao,
54 2009), celery (Marszałek, Krzy, Wo, & Sk, 2016) or beetroot (Marszałek, Krzyżanowska,
55 Woźniak, & Skąpska, 2017) among others. However, compared to liquids, HPCD application to
56 solid foods has been less studied. According to Ferrentino & Spilimbergo (2011), the complexity
57 of the matrix, which can make the CO_2 action more difficult, and the few information available
58 about the inactivation mechanism make the use of this technology on solid foods less attractive.
59 The objective of this work was to study the effect of HPCD at 20 MPa and different temperatures
60 (35-50 ºC) on PPO crude extracts from *Litopenaeus vannamei* on a kinetic basis, by previously
61 studying the optimal conditions for PPO extraction. The fresh product (whole piece), with no
62 addition of any additive, was also treated and, after HPCD treatment of the shrimps, PPO was
63 extracted under the previously selected conditions to determine the effect of the treatment on
64 PPO activity. Color was visually analyzed in treated shrimps right after treatment and during a
65 shelf life study of 7 d at refrigerated storage conditions (4 ºC). A temperature control was

66 prepared by treating the shrimps at the same temperature conditions under atmospheric pressure
67 to distinguish the effect of pressurized CO_2.

68 ## 2. Materials and methods

69 ### 2.1 Raw material

70 Pacific white shrimps (*Litopenaeus vannamei*) with an average size of 40-60 shrimps/kg were
71 kindly donated by "Gamba Natural" (Medina del Campo, Spain), where shrimps were harvested
72 right before being sent to our laboratory without any addition of sulphites or other additives.
73 Shrimps were kept at refrigeration temperature during transport and after arrival until used (not
74 more than 1 h from arrival).

75 ### 2.2 Crude extract preparation

76 The tails of the shrimps were separated and the heads were grounded into a fine powder by using
77 liquid nitrogen in a blender. The obtained powder was kept at -20 ºC until used for the different
78 PPO extraction trials. The extraction procedure of PPO was carried out from the head of Pacific
79 white shrimp (*Litopenaeus vannamei*) according to the method described by Chen, Balaban,
80 Wei, Marshall, & Hsu (1992) with slight modifications. The powder was mixed with the
81 extraction buffer in a 1:3 proportion. The extraction buffer consisted of 0.5 mol/L sodium
82 phosphate containing 0.2 g/100 mL of Brij 35 (Merck KGaA, Darmstad, Germany). Different
83 extraction buffer pH was tested in the extraction: 5.7, 6.5, 7.2 and 8. NaCl concentration effect
84 was also studied, being 0, 0.25, 0.5, 1 and 1.5 mol/L in the different experiments carried out at
85 each different pH conditions. The mixture was stirred continuously during 30 min at 4 ºC, and
86 then centrifuged at 5000 *g* at 4 ºC during 30 min using a refrigerated centrifuge (Thermofisher
87 Waltham-MA, USA, model Sorvall ST 16R). The effect of temperature during the mixing phase
88 on the extraction yield was also studied, varying from 4 to 30 ºC. The supernatant was collected
89 and considered as PPO crude extract. Extracts were kept at -20 ºC until use. The addition of an

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90 anion exchange resin AG2-X8 (Biorad Laboratories, Hércules-CA, USA) in the preparation of
91 the crude extract was also analyzed at pH = 7.2, 1 mol/L NaCl and 0.2 g/100 mL of Brij 35 at
92 4 ºC. Resin AG2-X8 was added in a 1:2 (w/w) proportion to the mix of shrimp powder and
93 extraction buffer before mixing. This anion exchange resin has been shown to improve PPO
94 activity in crude extracts from *Fuji* apples skin due to the adsorption of phenolic compounds
95 during the extraction (Imm & Kim, 2009).
96

97 **2.3 HPCD equipment and processing**

98 **2.3.1 Treatment of PPO extracts by HPCD**

99 HPCD treatment was carried out in a stainless steel (SS-316) cell with an internal volume of
100 100 mL and a maximum operating pressure and temperature of 30 MPa and 80 ºC, respectively
101 (Melgosa et al., 2017). 25 mL of PPO extract were loaded into the cell, which was tightly closed
102 and immersed in a water bath set at the operating temperature. Magnetic stirring was connected
103 and the system was pressurized to the desired pressure by using a syringe pump with a pressure
104 controller (ISCO 260 D, Lincoln-NE, USA). CO_2 was bubbled directly into the extract through a
105 sintered stainless steel micro-filter of 10 μm (Briongos et al., 2016). PPO extracts were treated at
106 20 MPa in the temperature range from 35 to 50 ºC. Enzyme inactivation kinetics of PPO crude
107 extracts were followed by collecting samples periodically for 60 min. Samples were kept at 4 ºC
108 until being analyzed.

109 **2.3.2 Treatment of shrimps by HPCD**

110 Whole shrimps were also exposed to HPCD in vivo. For these treatments, a bigger cell was used
111 (V_{cell} = 500 mL), with a maximum operating pressure of 15 MPa. Operation conditions were
112 12 MPa and 40 ºC during 30 min in a dry treatment (no water added) or shrimps submerged in
113 water (300 mL). After treatment, the cell was slowly depressurized and the shrimps were kept in

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114 an ice batch (Zhang et al., 2011). Right after treatment, PPO was extracted at 4 ºC under the
115 previously selected conditions, to determine the effect of HPCD on enzyme activity when
116 applying it directly on the solid pieces. The color of the pieces was also visually analyzed right
117 after treatment and along storage during 7 d at 4 ºC stored in properly closed food grade bags.

118 **2.4 Thermal treatment**

119 Thermal treatment was applied both to PPO extracts and to whole shrimps in the same
120 temperature conditions as their respective HPCD treatments but at atmospheric pressure. These
121 samples were used as a control to know the real effect of supercritical CO_2 despite of
122 temperature effect. PPO activity and color evolution were also studied in these samples.

123 **2.5 Physico-chemical analysis**

124 **2.5.1. Determination of PPO activity.**

125 PPO activity was determined spectrophotometrically by using a 0.005 mol/L L-3,4-
126 dihidroxifenilalanine (L-DOPA, Sigma Aldrich, San Luis-MO, USA) solution prepared in
127 0.02 mol/L phosphate buffer (pH 6.5) as substrate. PPO activity was analyzed by adding 800 µL
128 of PPO extract into 2.2 mL substrate solution. Oxidation of L-DOPA was determined
129 immediately by the increase in absorbance at 475 nm and 25 ºC by using a V-750
130 spectrophotometer (JASCO, Tokyo, Japan) equipped with a Peltier thermostatted cell holder.
131 The PPO activity was determined as the very first linear part of the reaction curve. One unit of
132 PPO activity was defined as the amount of enzyme required for 0.001/min absorbance increase
133 under the reaction conditions, U.

134 Relative residual activity of PPO after HPCD treatment was evaluated as:

135 $$\text{Residual activity, RA} = \frac{\text{Enzyme specific activity after HPCD treatment}}{\text{Enzyme specific activity in the crude extract}} \cdot 100\% = \frac{A}{A_o} \cdot 100 \quad [1]$$

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136 **2.5.2. Protein content in the PPO extract**

137 Total protein content in the crude extract was determined by using the kit *RC DC*™ (Biorad
138 Laboratories, Hércules-CA, USA), that allows protein determination in the presence of reducing
139 agents and detergents. Bovine serum albumin was used as standard. For each extraction process,
140 the specific PPO activity and total protein ratio was evaluated as:

141 $$U/mg\ protein = \frac{PPO\ specific\ activity}{Total\ protein\ content} = \frac{U/g\ \ shrimp\ head}{mg\ protein/g\ \ shrimp\ head} \qquad [2]$$

142

143 **2.6 Kinetic data analysis**

144 The Weibull model has been used to describe PPO inactivation in the crude extracts. According
145 to Van Boekel (2002), it can be written in the power-law form:

146 $$\log\frac{A}{A_0} = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta} \qquad [3]$$

147 where α is the scale parameter (a characteristic time) and β is the shape parameter. The time
148 required to achieve a number of decimal reductions, d, can be calculated by using the shape and
149 scale parameters:

150 $$t_d = \alpha\left(-ln(10^{-d})^{\frac{1}{\beta}}\right)$$

151 [4]

152 The scale parameter, α, was modelled in a similar way to the classical D value of the first order
153 kinetic model, suggesting a linear dependence of the log α on temperature, considering that the
154 shape parameter, β, did not depend on temperature:

155 $$log\alpha = a_1 - b_1 T \qquad [5]$$

156 A z_T' value was defined as suggested by van Boekel (2002), similar to the z_T evaluated for first
157 order kinetic models (Illera, Sanz, Solaesa, et al., 2018):

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158 $z'_T = \frac{1}{b_1}$ [6]

159

160 **2.7 Statistical analysis**

161 Statistical analyses were conducted using software Statgraphics X64. The results are presented as
162 the mean ± standard deviation of at least three replicates. The significance of the differences was
163 determined based on an analysis of the variance with Fisher's least significant method at p-value
164 ≤ 0.05.

165 To estimate the kinetic parameters, non-linear regression was performed by using the Marquardt
166 algorithm (Statgraphics X64).

167

168 **3. Results and discussion**

169 **3.1 Preparation of crude PPO extract**

170 **3.1.1 Effect of the presence of an anion exchange resin.**

171 First, the effect of the anion exchange resin AG2-X8 on PPO activity in the crude extract was
172 evaluated by using a ratio of 1:2 (g resine:g shrimp head). Sodium phosphate 0.05 mol/L at pH =
173 7.2 in the presence of 0.2 g/100 mL of Brij 35 and 1 mol/L NaCl at 4 ºC was used as extracting
174 medium (Table 1). It can be observed that there were no significant differences (p≤ 0.05)
175 between using or not resin in PPO activity and total protein content in both extracts.

176 In the study carried out by Imm and Kim (2009), where PPO was extracted from the skin of *Fuji*
177 apples, a significant difference was observed when using resin AG2-X8, improving the enzyme
178 recovery value. In this case, resin did not affect PPO extraction, for that reason, further
179 extractions were carried out without addition of the anion exchange resin.

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180 **3.1.2. Effect of pH.**

181 Fig. 1a and 1b show the effect of pH of the extraction medium on the PPO extract activity and
182 protein content when using 0.2 g/100 mL of Brij 35 and 1 mol/L NaCl at 4 ºC. Four different pH
183 values were tested (5.6, 6.5, 7.2 and 8), showing that by increasing the pH of the medium, PPO
184 activity per g of shrimp head and total protein increased. For example, at pH = 5.6, U/ g shrimp
185 head and U/ mg protein were 91 ± 3 and 1.7 ± 0.1, respectively; while at pH value of 8, those
186 values increased more than double to 212 ± 4 and 4.0 ± 0.4, respectively. There is scarce
187 literature regarding the isoelectric point of PPO enzyme. Ali et al. (1994) found out that it was
188 4.76 ± 0.03 for Florida Spiny Lobster (*Panulirus argus*), and it was around 5.2 in PPO from
189 *Penaeus japonicus* prawns according to (Montero, Ávalos, & Pérez-Mateos, 2001). pH of the
190 extraction buffer used in this work and in the literature are higher than this isoelectric points;
191 therefore, PPO will present a negative charge. On the other hand, when pH is increased, the
192 concentration of dissolved sodium ions increased, what may produce higher electrostatic
193 interactions, and in consequence, a higher protein extraction.

194 Other studies in crustaceans tried to determine the best pH conditions for PPO extraction.
195 Montero et al. (2001) chose pH = 8 as the optimum pH for extracting the enzyme from prawns
196 (*Penaeus japonicus*), and Lv et al. (2018) determined pH = 6.2 as the optimum for extracting it
197 from *Penaeus vannamei*. Nirmal & Benjakul (2012) also worked with Pacific white shrimp and
198 chose pH = 6 as the optimum for PPO extraction.

199 **3.1.3. Effect of extraction temperature**

200 The effect of temperature was studied in an extraction buffer at pH = 8 containing 0.2 g/100 mL
201 of Brij 35 and 1 mol/L NaCl. Three different temperatures were studied, 4, 21 and 30 ºC, and
202 results are compiled in Table 2. As can be observed, there is no significant difference among
203 temperatures in PPO activity or protein content. Therefore, no denaturalization of the enzyme

204 has occurred at the highest temperature assayed (30 ºC). Although there was no difference
205 between extraction temperatures, 4 ºC was chosen as working temperature for further work,
206 since it is the usual temperature employed in the literature (Chen et al., 1992; Manheem et al.,
207 2012; Montero et al., 2001; Pal & Rao, 2017; Verhaeghe et al., 2016; Wanyou et al., 2014).
208 Although, from an economic point of view, room temperature could simplify the process and
209 reduce the processing cost.

210 **3.1.4. Effect of salt concentration (ionic strength)**

211 Fig. 2a and 2b show the effect of salt concentration on PPO extraction in the range from 0 to
212 1.5 mol/L NaCl at different pH values of the extraction buffer (6.5, 7.2 and 8). It can be observed
213 that PPO extraction depends on the ionic strength of the medium, whatever it is the pH. When
214 NaCl concentration increased in the extraction medium its extraction capacity decreased. Best
215 results were obtained when no NaCl was added to the extraction buffers, so further experiments
216 were carried without using NaCl. Verhaeghe et al. (2016) extracted PPO from brown shrimp
217 (*Crangon crangon*) with no NaCl added into the extraction medium; however, in the literature
218 most of the extraction protocols for crustaceans use NaCl for enzymatic extraction. For
219 extracting PPO from *Litopenaeus vannamei*, Nirmal & Benjakul (2012) used 1 mol/L NaCl,
220 meanwhile Wanyou et al. (2014) and Zhang et al. (2011) used 0.5 mol/L. For extraction in other
221 crustaceans, 1 mol/L NaCl was also used (Montero et al., 2001; Pal & Rao, 2017; Zamorano,
222 Martínez-Álvarez, Montero, & Gómez-Guillén, 2009). All the mentioned studies used an
223 existing protocol and they did not test the effect of salt concentration.

224

225 **3.2 HPCD inactivation of PPO crude extract**

226 PPO inactivation kinetics in the crude extract were determined at 20 MPa and 35 ºC. Crude PPO
227 extracts were obtained at two different values of pH, 6.5 and 7.2 with no NaCl added to the

228 extraction medium. Although higher PPO activity was obtained in the extracts at the highest pH
229 studied in this work (pH = 8), inactivation kinetics were determined at pH =6.5 and 7.2 since,
230 according to the literature, fresh prawns have a pH varying between 6.5 and 6.9
231 (Shaikhmahamud & Magar, 1965) and the pH of the carapace of the cephalothorax of *Penaeus*
232 *japonicus* prawns was 7.2.

233 Fig. 3 shows similar PPO inactivation kinetics at both values of pH, with a minimum residual
234 activity of 35.0 ± 0.7 % after 60 min of treatment. Therefore, the effect of HPCD temperature
235 has been only studied at pH = 7.2, in order to compare with other previously published results,
236 where the extract was also obtained at pH = 7.2 (Chen et al., 1992; Zhang et al., 2011). The
237 similar results between the two pH values can be related to the similar values of CO_2 solubility in
238 the extracts. Solubility of pressurized CO_2 in a phosphate buffer containing 0.2 g/100 mL of Brij
239 35 was determined by using the method and equipment previously reported by Illera, Sanz, &
240 Beltrán (2019). At 20 MPa and 45 ºC, the solubility values were 0.059 ± 0.001 g CO_2 per mL of
241 buffer at pH = 6.5, and 0.056 ± 0.002 at pH = 7.2, what indicated that CO_2 solubility was very
242 similar at both pH values. Although inactivation kinetics of PPO extracts were determined at
243 35ºC and not at 45ºC, based on the results previously reported by Illera et al. (2019) similar trend
244 in CO_2 solubility at 35ºC is expected and no significant changes are expected at both pH values.

245 Inactivation kinetics at 20 MPa in the temperature range from 35 to 50 ºC are plotted in Fig. 4.
246 Enzyme inactivation rate increased significantly by increasing temperature (from 35 to 50 ºC).
247 At 50 ºC, PPO activity was 9 ± 3 % after 60 min of treatment. In this regard, in addition to the
248 intrinsic effect of temperature on enzyme inactivation, an increasing temperature could lead to an
249 improvement of mass transport properties of CO_2, enhancing CO_2 diffusivity and the number of
250 collisions between the CO_2 and the enzyme (Illera, Sanz, Solaesa, et al., 2018). Similar values
251 for the PPO residual activity have been reported in the literature when treating PPO extracts from

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252 Pacific white shrimps by HPCD at 37 ºC and 20 MPa, 10 % of residual activity after 30 min of
253 treatment and total inactivation was reported when increasing pressure to 25 MPa at same
254 temperature and time conditions (Zhang et al., 2011). Chen et al. (1992) achieved total
255 inactivation in shorter treatment time (4 min) when they treated PPO extract from brown shrimp
256 and in only one min when the extract was from Florida spiny lobster, in experiments done at
257 0.59 MPa and 43 ºC. The higher inactivation degree of PPO reported by Chen et al. (1992) could
258 be attributed to the enzyme source that it is well known to play an important role on the enzyme
259 inactivation degree, since the operation mode was similar to this work, charging 80 mL of the
260 extract in a 100 mL high pressure vessel.
261 To assess the effect of pressurized carbon dioxide on enzyme inactivation, PPO crude extracts
262 were also treated in the same temperature range but at atmospheric pressure. Fig. 5 clearly shows
263 that higher inactivation degree was obtained when the PPO crude extract was treated by
264 pressurized CO_2. At 50 ºC, PPO inactivation after 60 min at atmospheric pressure was only
265 around 30 % (residual activity, 70 %) compared to the 90 % after HPCD treatment (residual
266 activity, 10 %). Wanyou et al. (2014) also treated a PPO extract from Pacific white shrimp by
267 thermal treatment, and they determined that a minimum temperature of 60 ºC was necessary for
268 enzymatic inactivation, since they observed no inactivation when treating it at 50 ºC (95.2 %
269 residual activity). This indicates that PPO is not mainly inactivated by the temperature effect, but
270 pressurized CO_2 has an important role in enzymatic inactivation. Furthermore, Zhang et al.
271 (2011), explained that PPO activity is not only affected by pressure, but also by CO_2 molecular
272 effects under pressure.
273 PPO inactivation kinetics were fitted to the Weibull model, since the first order kinetic model
274 was found not to be able to properly describe PPO inactivation kinetics by HPCD. Fitting
275 parameters of the Weibull model are listed in Table 3. The scale parameter, α, decreased with

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276 temperature, however, the shape factor, β, did not show any dependence on temperature, with
277 values lower than the unity. Scale, α, and shape, β, parameters were used to calculate the time
278 required to inactivate 1 log ($t_{d=1}$) of PPO. According to the values of α, $t_{d=1}$ decreased with
279 temperature from 392 to 98 min at 35 and 50 ºC, respectively.

280 The value of the z_T' (Eq.[6]) has been also included in Table 3, with the quality of the fitting of
281 equation 5. The $z_{T'}$ value obtained in this work by HPCD, 25 ± 6 ºC, was slightly higher than the
282 values reported in the literature for thermal treatment. Wanyou et al. (2014), obtained a z_T value
283 of 18.83 ºC after treating Pacific white shrimp from 60 to 100 ºC. An Arrhenius type equation
284 was considered to relate the inverse of the scale parameter, 1/α, with temperature (Illera, Sanz,
285 Benito-Román, et al., 2018). Although 1/α cannot be considered a kinetic constant, the value
286 obtained from the slope was calculated as 77 ± 20 kJ/mol.

287 **3.3 HPCD inactivation of PPO in whole shrimps**

288 Shrimps were treated in vivo by HPCD at 12 MPa and 40 ºC during 30 min in the presence or
289 absence of water. Although faster inactivation kinetics of PPO in the extracts were observed at
290 50 ºC (Fig. 4), 40ºC was chosen to treat shrimp in vivo since similar inactivation degree was
291 reached after 30 min treatment, and, this way, HPCD, as non-thermal technology, could preserve
292 organoleptic and nutritive properties in a greater extent. Operating pressure was fixed taking into
293 account the maximum operating pressure of the vessel (15 MPa). Shrimps were also treated at
294 atmospheric pressure and 40 ºC as control, in order to assess the effect of temperature on PPO
295 inactivation and on visual changes of colour. After treatment, PPO was extracted and activity
296 was determined and compared to a PPO crude extract of non-treated shrimps (Table 4). The PPO
297 inactivation degree obtained when heating the shrimps at 40 ºC for 30 min both in presence and
298 in the absence of water was similar to the inactivation obtained when treating the PPO crude
299 extract by mild heating at 40 ºC for 60 min (Fig. 5). However, no PPO activity was detected,

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300 according to the experimental procedure previously described, when treating the shrimps in vivo
301 by HPCD, both in absence and in the presence of water (Table 4). As previously reported,
302 residual activity in crude PPO extracts treated by HPCD at higher operating pressure, 20 MPa,
303 but at the same temperature, 40 ºC for 30 min, was much higher (38.7 ± 3.2) than the values
304 obtained in the sample in vivo. These results agree with the residual activity of PPO reported by
305 Zhang et al. (2011) after treatment of Pacific white shrimps in vivo at 20-25 MPa at 37 ºC for 10
306 min, with residual activity values lower than 6 %, and total inactivation for treatment time of 30
307 min. Therefore, we can conclude that PPO in vivo was easier to be inactivated than in crude
308 extracts. This was corroborated by Zhang et al. (2011), that attributed this finding to a bigger
309 contact area between PPO and HPCD. Additionally, CO_2 solubility in aqueous media is
310 relatively low (Illera et al., 2019). Dissolved CO_2 could decrease pH of the media; however, the
311 presence of salts of the buffer media could exert a buffer effect. Direct contact of CO_2 with the
312 shrimp could induce a more efficient enzyme inactivation due to better contact between the
313 enzyme and the CO_2. Water could act as a barrier for the direct action of CO_2 on the shrimps.
314 This indicates that enzyme inactivation mechanism could be different when CO_2 is in contact
315 directly with the shrimps that when CO_2 is added to an aqueous solution.
316 Visual control of color was carried out, with shrimps stored in plastic bags at 4 ºC during 7 d.
317 Untreated shrimps were used as controls for color. Pictures from the shrimps during the shelf life
318 study can be seen in Fig. 6. Untreated shrimps and the ones that were only thermally treated
319 showed up browning in the cephalothorax soon, in the 2nd d of storage. HPCD treated shrimps in
320 absence of water presented a similar aspect to cooked shrimps, slightly orange and white
321 appearance, and light browning in cephalothorax appeared in the fifth day of storage. On the
322 other hand, the shrimps that were HPCD treated but in water showed a similar behaviour to the
323 mild thermally treated ones, starting to show browning on the second day of storage, although

324 browning intensity was lower than the control and the thermally treated ones. By the 7th d of
325 storage, melanosis is present in all the samples, being a bit lower in the HPCD treated one in
326 absence of water (Fig. 6d). Zhang et al. (2011) treated whole Pacific white shrimps by HPCD
327 observing their appearance during 3 d of storage at 4 ºC. In that time, no melanosis appeared in
328 HPCD treated shrimps in absence of water, while untreated samples showed advanced browning,
329 as in the present work.

330 Furthermore, color of shrimps after dry HPCD treatment was light orange. Wei, Balaban,
331 Fernando, Peplow, & Florida (1991) also noticed a cooked appearance after HPCD treatment of
332 shrimps. Li, Tian, & Li (2012) observed the same effect in color after treating *Litopenaeus*
333 *vannamei* by HPP. They explained that the change in color is related to protein coagulation,
334 which changes the sample surface properties, increasing light reflection and, therefore, a
335 whitened color. In addition, CO_2 in the supercritical states has drying properties. After studying
336 melanosis in different parts of shrimps, Zamorano et al. (2009) explained that the presence of the
337 cephalothorax was required for melanosis to be initiated, where they observed melanosis in just
338 one day in an untreated shrimp.

339

340 **4. Conclusions**

341 The study of PPO extraction from cephalothorax of Pacific white shrimps concluded that higher
342 pH values improved the extraction, while the presence of NaCl or anionic exchange resin (AG2-
343 X8) did not show any benefit. Extraction yield was no dependent on temperature, in the range
344 from 4 to 30 ºC.

345 HPCD treatment resulted to be effective in PPO inactivation of these extracts. An increase of
346 treatment temperature, in the range from 35 to 50ºC, increased enzymatic inactivation and
347 inactivation rate. HPCD treatment showed better results in PPO inactivation of shrimps in vivo,

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348 where the enzyme was totally inactivated according to our analytical procedure, although
349 melanosis quickly appeared in all samples except in the HPCD treated one in absence of water,
350 which showed good visual aspect during 5 d of storage at 4 ºC. Based on the high inactivation
351 degree reached in shrimps in vivo at 40ºC and 12 MPa, milder conditions could be tried. In
352 conclusion, HPCD technology has been found to be a promising non-thermal technology for the
353 processing of shrimps, and it can be considered as an alternative to the use of sulphites in this
354 product.
355

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360

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Table 1. PPO activity expressed as U per g of shrimp head and mg of protein after extraction with and without resin AG2-X8 (1 g resin: 2 g shrimp head) using a sodium phosphate extraction buffer pH 7.2, 1 mol/L NaCl and 0.2 g/100 mL Brij 35 at 4 ºC.

	With resin	Without resin
U/g shrimp head	190 ± 1[a]	182 ± 12[a]
U/mg protein	3.9 ± 0.2[a]	3.6 ± 0.4[a]

Values with different letters in each row are significantly different when applying the Fisher´s (LSD) test at p-value ≤0.05.

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Table 2. PPO activity expressed as U per g of shrimp head and mg of protein after extraction at different temperatures using a sodium phosphate extraction buffer at pH 8, 1 mol/L NaCl and 0.2 g/100 mL Brij 35.

Temperature	4 ºC	21 ºC	30 ºC
U/g shrimp head	212 ± 4[a]	209 ± 7 [a]	211 ± 3[a]
U/mg protein	4.0 ± 0.4[a]	4.2 ± 0.2[a]	4.3 ± 0.2[a]

Values with different letters in each row are significantly different when applying the Fisher´s (LSD) test at p-value ≤0.05.

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Table 3. Kinetic parameters of the Weibull model (Eq. 3) for the inactivation of PPO extracts by HPCD treatment at 20 MPa. PPO extract was obtained using a sodium phosphate buffer at pH =7.2 with 0.2 g/100 mL Brij 35 and 4 ºC)

T, ºC	α, min	β	R^2	$t_{d=1}$, min
35	69 ± 10	0.48 ± 0.06	0.984	392
40	30 ± 3	0.53 ± 0.07	0.982	145
50	16 ± 2	0.46 ± 0.07	0.984	98

z'_T = 25 ± 6 ºC (R^2 = 0.932)

ln (1/α) vs (1/T): 77 ± 20 kJ/mol (R^2= 0.940)

α =scale parameter; β = shape parameter
t_d = time needed to achieve one decimal reduction; z_T' was evaluated through Eq. 5

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Table 4. PPO residual activity after HPCD and mild heating treatment (MH) at 40 ºC both in presence and in the absence of water. PPO was extracted using a sodium phosphate buffer at pH =7.2 with 0.2 g/100 mL Brij 35 at 4 ºC.

Treatment	MH (water)	MH	HPCD (water)	HPCD
PPO residual activity	72.5 %	83.3 %	n.d.	n.d.

n.d (Non detected).

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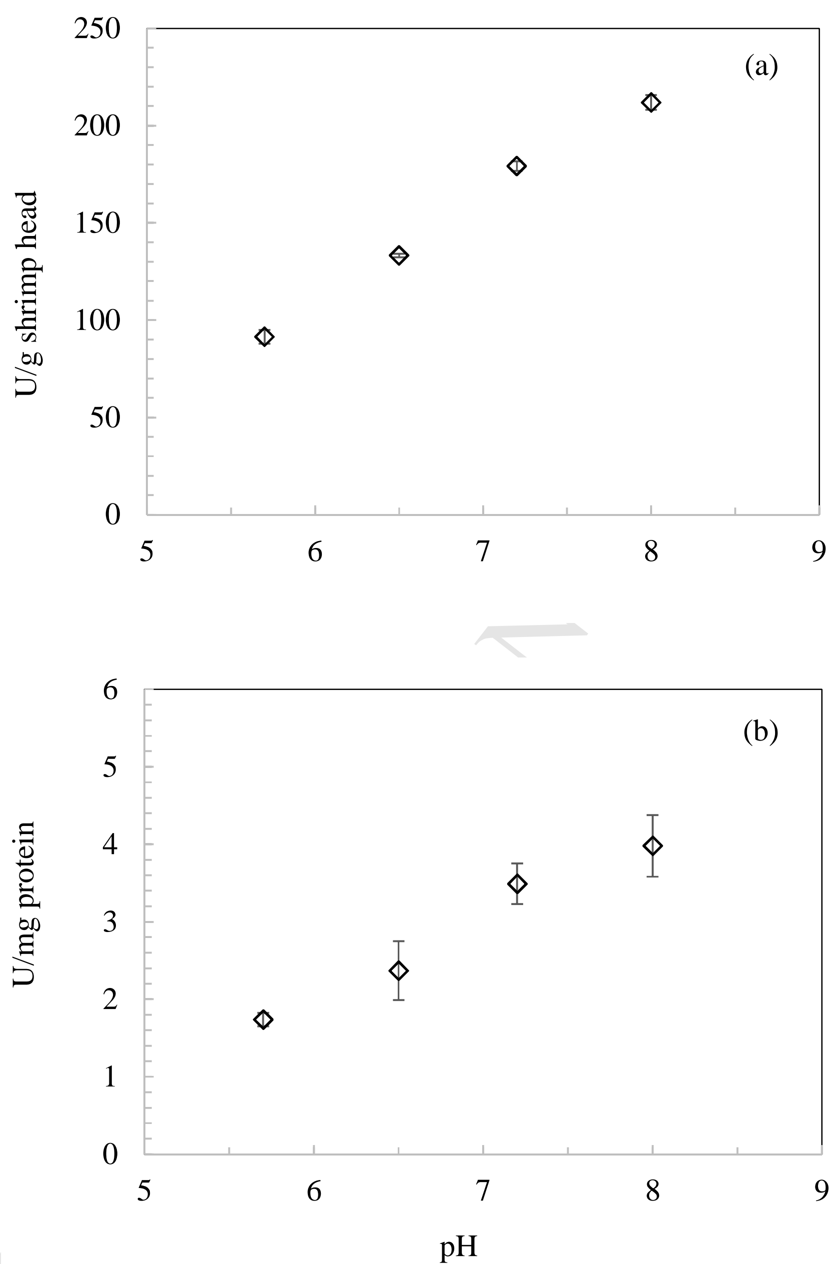

Fig. 1. Effect of pH of the extraction sodium phosphate buffer on (a) PPO activity and (b) extract protein content when using 1 mol/L NaCl and 0.2 g/100 mL of Brij 35 35 at 4 ºC.

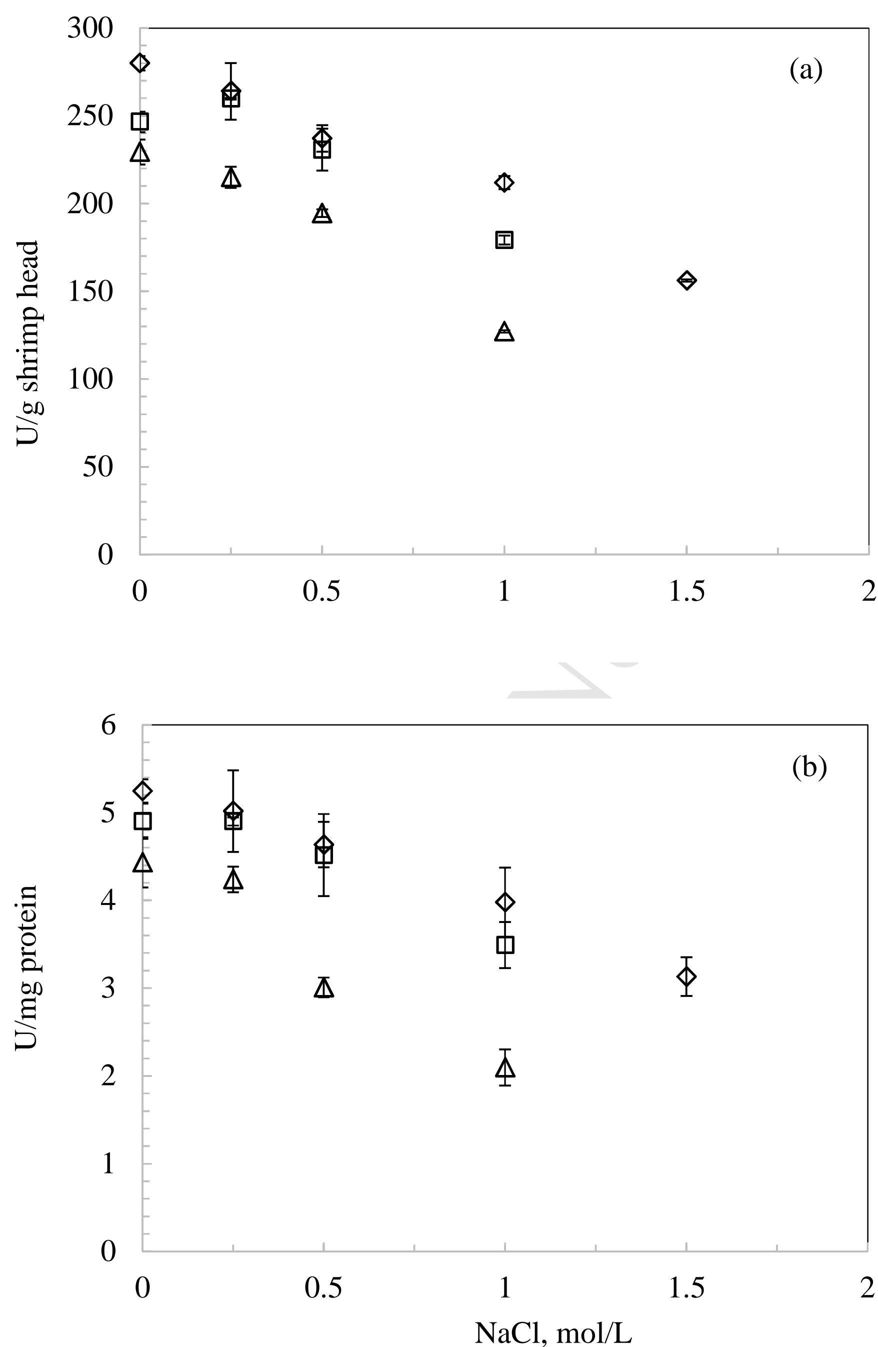

Fig. 2. Effect of extraction sodium phosphate buffer ionic concentration (expressed as NaCl, mol/L) on (a) extraction yield and (b) extract protein content when using buffer at different pH values (◊ pH 8, □ pH 7.2 and Δ pH 6.5) with 0.2 g/100 mL Brij 35 at 4 ºC.

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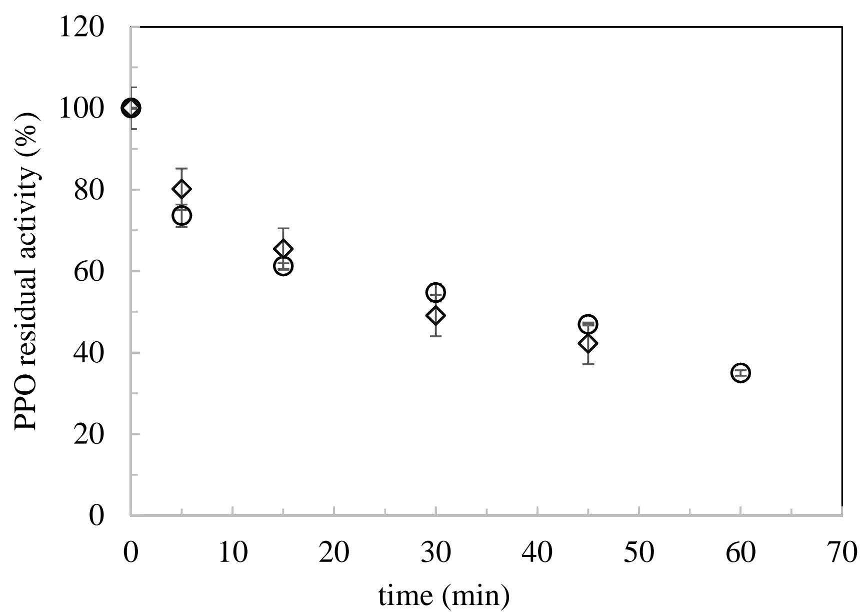

Fig. 3. Inactivation kinetic of PPO by HPCD treatment at 20 MPa and 35 ºC in PPO extract obtained at pH = 6.5 (◊) and pH = 7.2 (○). PPO was extracted with a sodium phosphate buffer with 0.2 g/100 mL Brij 35 at 4º C.

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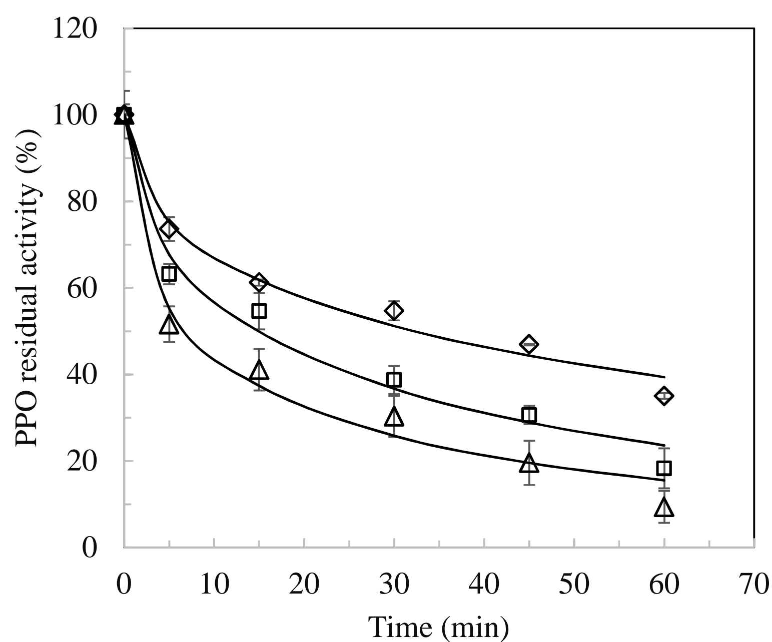

Fig. 4. Inactivation kinetics of PPO extracts by HPCD treatment at 20 MPa and different temperatures (◊ 35 ºC, □ 40 ºC and Δ 50 ºC). PPO was extracted using phosphate buffer at pH = 7.2 with 0.2 g/100 mL Brij 35 at 4 ºC. Symbols represent the experimental results, and lines represent the calculated values for the Weibull model (Eq. 3).

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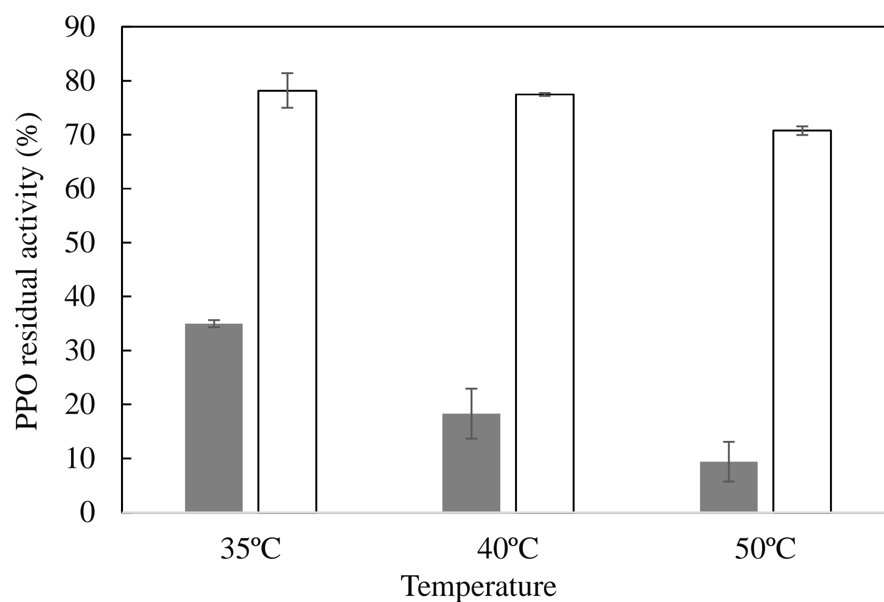

Fig. 5. PPO residual activity of PPO extracts after 60 min of HPCD treatment at 20 MPa (■) or mild heating treatment (□) at different temperatures. PPO was extracted using phosphate buffer pH = 7.2 with 0.2g/100 mL Brij 35 at 4 ºC.

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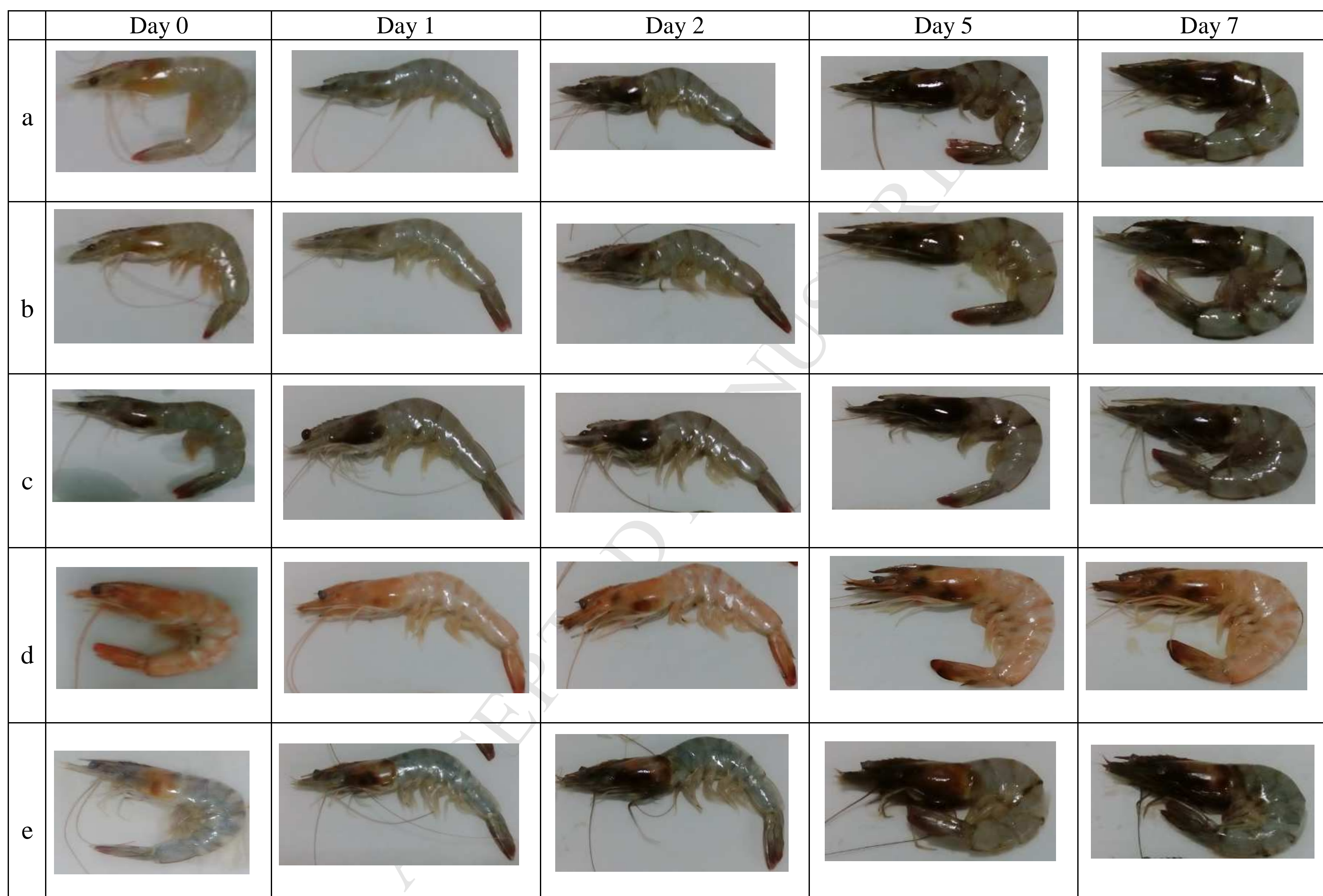

Fig. 6. Appearance of untreated shrimps **(a)** and treated ones at atmospheric pressure, 40 ºC in absence **(b)** and in presence of water **(c)** and by HPCD treatment at 12 MPa, 40 ºC in absence **(d)** and in presence of water **(e)** during 7 d of storage at 4 ºC.

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Extraction conditions have been optimized for higher PPO activity in crude extracts.

The Weibull model fit well HPCD inactivation of PPO extracts from shrimps.

Higher PPO inactivation degree was obtained in vivo than in crude extracts.

Visual aspect was good when treated whole pieces by HPCD in dry medium.

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