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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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1. Introduction

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

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

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

formation decreased after 7 d in refrigerated storage. However, thermal treatment produces a big change in food properties (Ferrentino & Spilimbergo, 2011). For that reason, different non-thermal technologies are being investigated as an alternative.

One of them is the use of HPCD (High Pressure Carbon Dioxide) technology. HPCD is a cold pasteurization method that affects microorganisms and enzymes by using pressures below 50 MPa and temperatures usually lower than 50 ºC. This technology is gaining interest since CO_2 is nontoxic, inexpensive, nonflammable, recyclable and after depressurization it doesn´t leave residues in the product (Briongos et al., 2016). Effectiveness of HPCD on PPO inactivation has been extensively reported for different fruits and vegetables juices, such as orange juice (Briongos et al., 2016), apple and tomato juice (Illera, Sanz, Solaesa, Ruiz, & Beltrán, 2018; Illera, Sanz, Trigueros, Beltrán, & Melgosa, 2018), carrot juice (Zhou, Wang, Hu, Wu, & Liao, 2009), celery (Marszałek, Krzy, Wo, & Sk, 2016) or beetroot (Marszałek, Krzyżanowska, Woźniak, & Skąpska, 2017) among others. However, compared to liquids, HPCD application to solid foods has been less studied. According to Ferrentino & Spilimbergo (2011), the complexity of the matrix, which can make the CO_2 action more difficult, and the few information available about the inactivation mechanism make the use of this technology on solid foods less attractive.

The objective of this work was to study the effect of HPCD at 20 MPa and different temperatures (35-50 ºC) on PPO crude extracts from *Litopenaeus vannamei* on a kinetic basis, by previously studying the optimal conditions for PPO extraction. The fresh product (whole piece), with no addition of any additive, was also treated and, after HPCD treatment of the shrimps, PPO was extracted under the previously selected conditions to determine the effect of the treatment on PPO activity. Color was visually analyzed in treated shrimps right after treatment and during a shelf life study of 7 d at refrigerated storage conditions (4 ºC). A temperature control was

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

2. Materials and methods

2.1 Raw material

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

2.2 Crude extract preparation

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

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

2.3 HPCD equipment and processing

2.3.1 Treatment of PPO extracts by HPCD

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

2.3.2 Treatment of shrimps by HPCD

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

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

2.4 Thermal treatment

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

2.5 Physico-chemical analysis

2.5.1. Determination of PPO activity.

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

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

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

2.5.2. Protein content in the PPO extract

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

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

2.6 Kinetic data analysis

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

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

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

$$t_d = \alpha\left(-ln(10^{-d})^{\frac{1}{\beta}}\right) \quad [4]$$

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

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

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

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

2.7 Statistical analysis

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

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

3. Results and discussion

3.1 Preparation of crude PPO extract

3.1.1 Effect of the presence of an anion exchange resin.

First, the effect of the anion exchange resin AG2-X8 on PPO activity in the crude extract was evaluated by using a ratio of 1:2 (g resine:g shrimp head). Sodium phosphate 0.05 mol/L at pH = 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 medium (Table 1). It can be observed that there were no significant differences ($p \leq 0.05$) between using or not resin in PPO activity and total protein content in both extracts.

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

3.1.2. Effect of pH.

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

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

3.1.3. Effect of extraction temperature

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

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

3.1.4. Effect of salt concentration (ionic strength)

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

3.2 HPCD inactivation of PPO crude extract

PPO inactivation kinetics in the crude extract were determined at 20 MPa and 35 ºC. Crude PPO 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

Pacific white shrimps by HPCD at 37 ºC and 20 MPa, 10 % of residual activity after 30 min of treatment and total inactivation was reported when increasing pressure to 25 MPa at same temperature and time conditions (Zhang et al., 2011). Chen et al. (1992) achieved total inactivation in shorter treatment time (4 min) when they treated PPO extract from brown shrimp and in only one min when the extract was from Florida spiny lobster, in experiments done at 0.59 MPa and 43 ºC. The higher inactivation degree of PPO reported by Chen et al. (1992) could be attributed to the enzyme source that it is well known to play an important role on the enzyme inactivation degree, since the operation mode was similar to this work, charging 80 mL of the extract in a 100 mL high pressure vessel.

To assess the effect of pressurized carbon dioxide on enzyme inactivation, PPO crude extracts were also treated in the same temperature range but at atmospheric pressure. Fig. 5 clearly shows that higher inactivation degree was obtained when the PPO crude extract was treated by pressurized CO_2. At 50 ºC, PPO inactivation after 60 min at atmospheric pressure was only around 30 % (residual activity, 70 %) compared to the 90 % after HPCD treatment (residual activity, 10 %). Wanyou et al. (2014) also treated a PPO extract from Pacific white shrimp by thermal treatment, and they determined that a minimum temperature of 60 ºC was necessary for enzymatic inactivation, since they observed no inactivation when treating it at 50 ºC (95.2 % residual activity). This indicates that PPO is not mainly inactivated by the temperature effect, but pressurized CO_2 has an important role in enzymatic inactivation. Furthermore, Zhang et al. (2011), explained that PPO activity is not only affected by pressure, but also by CO_2 molecular effects under pressure.

PPO inactivation kinetics were fitted to the Weibull model, since the first order kinetic model was found not to be able to properly describe PPO inactivation kinetics by HPCD. Fitting parameters of the Weibull model are listed in Table 3. The scale parameter, α, decreased with

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

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

3.3 HPCD inactivation of PPO in whole shrimps

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

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

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

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

4. Conclusions

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

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

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

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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.

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.

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

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).

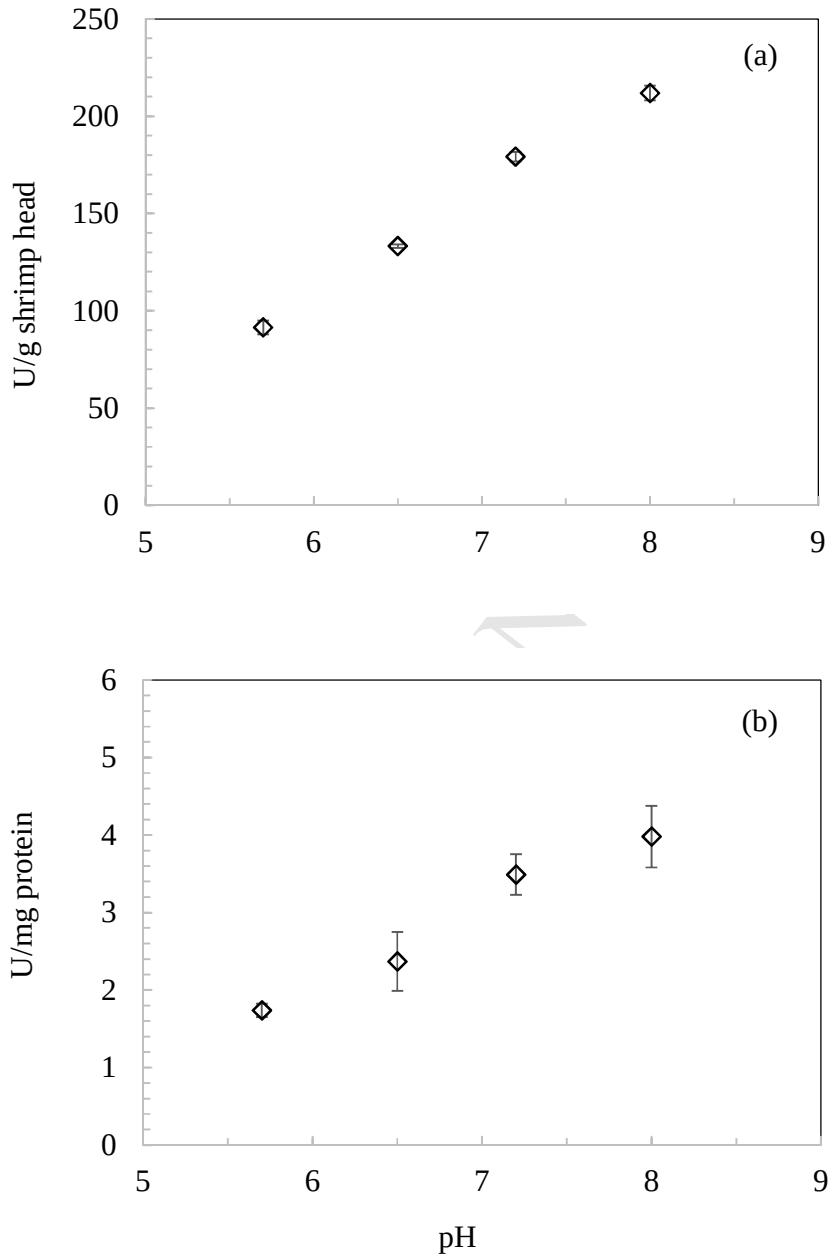

Fig. 1. Effect of pH of the extraction sodium phosphate buffer on (a) PPO activity and (b) extract protein content when using 1 mol/L NaCl and 0.2 g/100 mL of Brij 35 35 at 4 ºC.

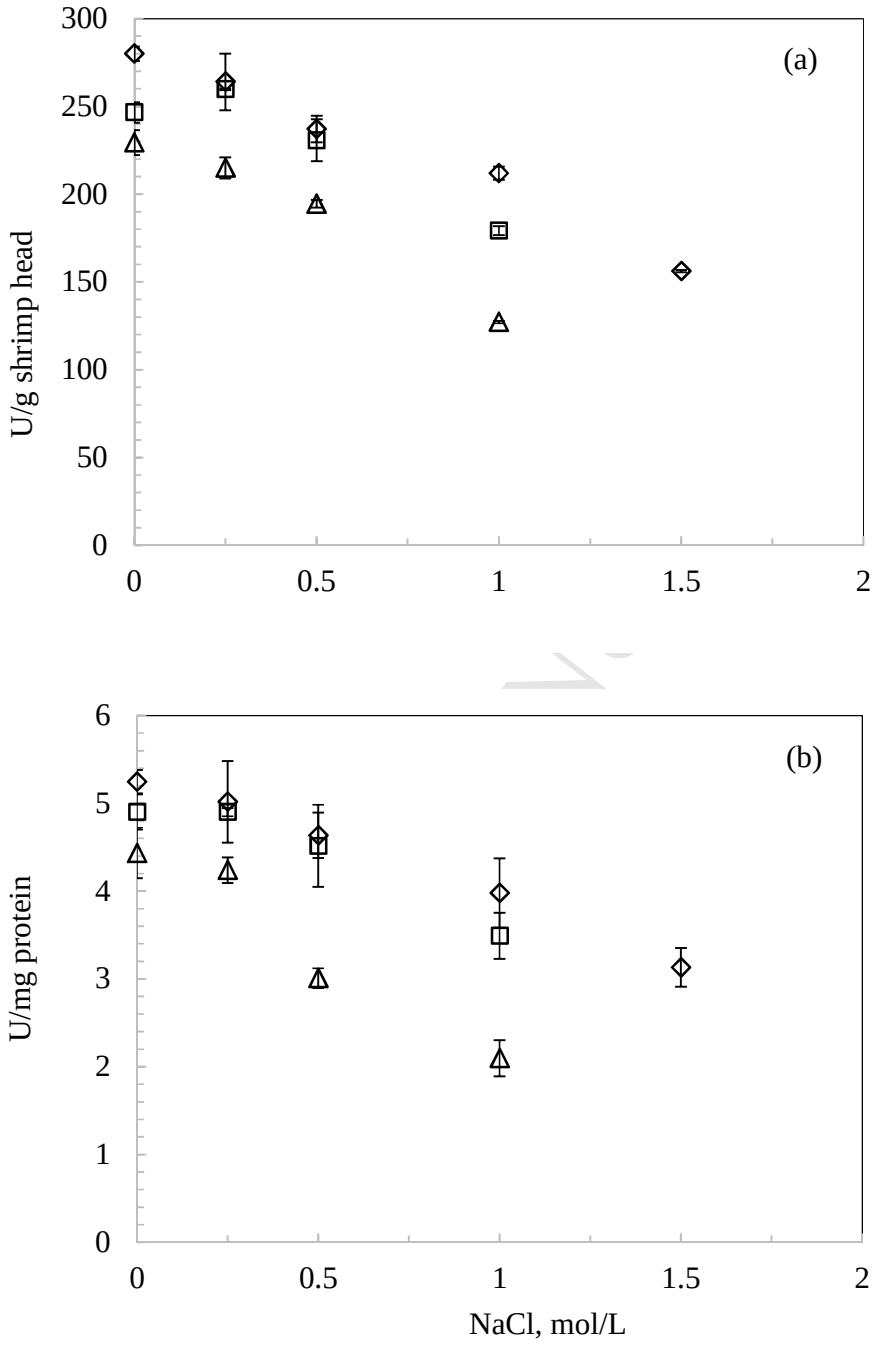

Fig. 2. Effect of extraction sodium phosphate buffer ionic concentration (expressed as NaCl, mol/L) on (a) extraction yield and (b) extract protein content when using buffer at different pH values (◊ pH 8, □ pH 7.2 and Δ pH 6.5) with 0.2 g/100 mL Brij 35 at 4 ºC.

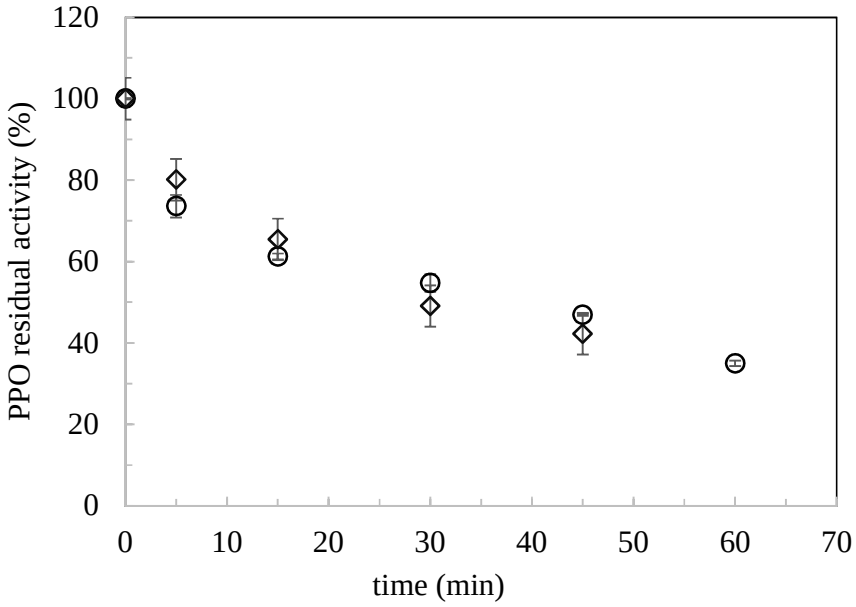

Fig. 3. Inactivation kinetic of PPO by HPCD treatment at 20 MPa and 35 ºC in PPO extract obtained at pH = 6.5 (◊) and pH = 7.2 (○). PPO was extracted with a sodium phosphate buffer with 0.2 g/100 mL Brij 35 at 4º C.

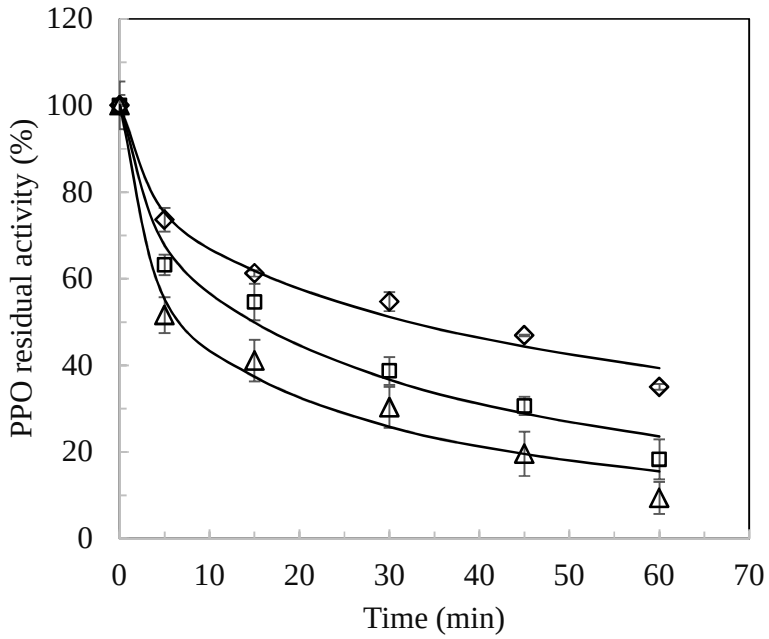

Fig. 4. Inactivation kinetics of PPO extracts by HPCD treatment at 20 MPa and different temperatures (◊ 35 ºC, □ 40 ºC and Δ 50 ºC). PPO was extracted using phosphate buffer at pH = 7.2 with 0.2 g/100 mL Brij 35 at 4 ºC. Symbols represent the experimental results, and lines represent the calculated values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

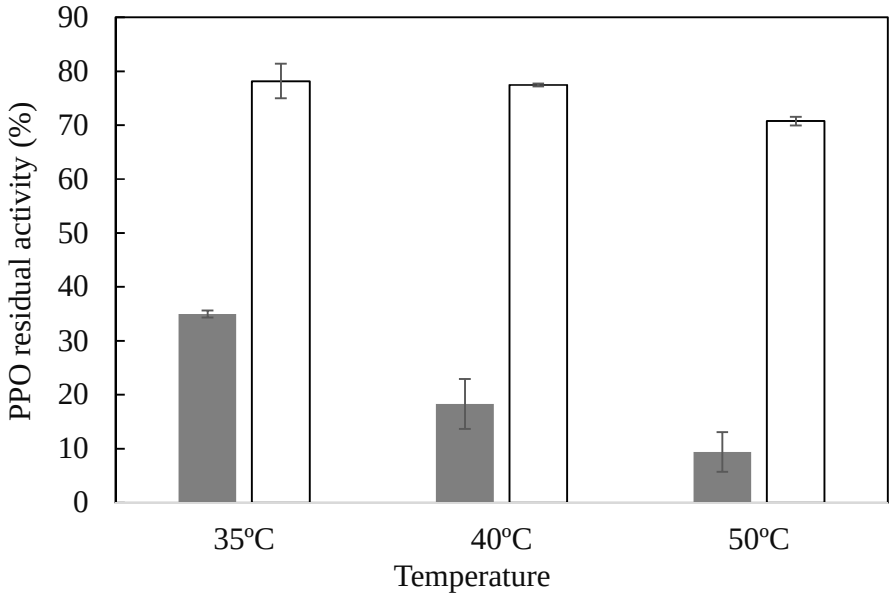

Fig. 5. PPO residual activity of PPO extracts after 60 min of HPCD treatment at 20 MPa (■) or mild heating treatment (□) at different temperatures. PPO was extracted using phosphate buffer pH = 7.2 with 0.2g/100 mL Brij 35 at 4 ºC.

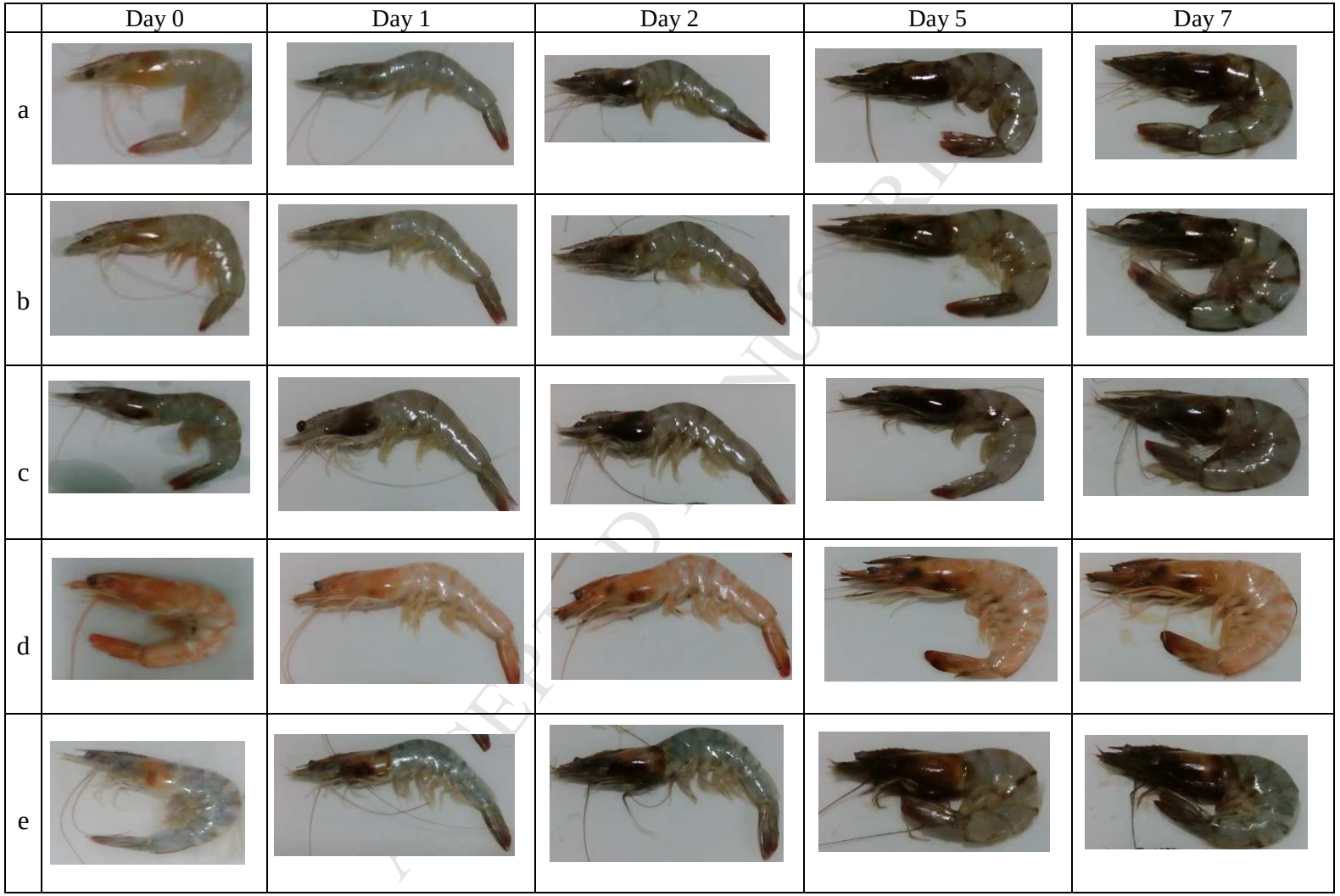

Fig. 6. Appearance of untreated shrimps **(a)** and treated ones at atmospheric pressure, 40 ºC in absence **(b)** and in presence of water **(c)** and by HPCD treatment at 12 MPa, 40 ºC in absence **(d)** and in presence of water **(e)** during 7 d of storage at 4 ºC.

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.