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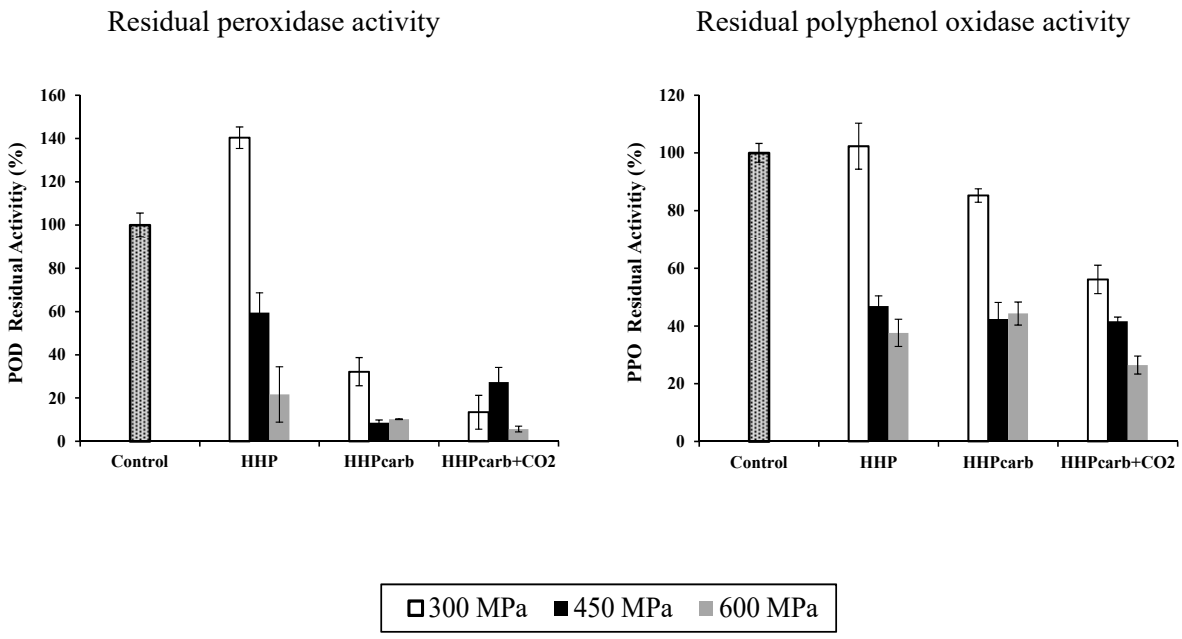
Residual peroxidase activity
POD Residual Activity (%)
160
140
120
100
80
60
40
20
0
Control
HHP
HHPcarb
HHPcarb+CO2
Residual polyphenol oxidase activity
PPO Residual Activity (%)
120
100
80
60
40
20
0
Control
HHP
HHPcarb
HHPcarb+CO2
300 MPa
450 MPa
600 MPa

Highlights

- A combined treatment of HHP and DPCD was used to inactivate feijoa puree enzymes.
- The residual activity of enzymes decreased with increasing pressure in all treatments.
- The addition of CO_2 in the package enhanced the HHP inactivation of POD and PPO.
- Using HHP and DPCD, lower HHP pressures may be used for a given inactivation level.

Combined High Hydrostatic Pressure and Carbon Dioxide Inactivation of Pectin Methylesterase, Polyphenol Oxidase and Peroxidase in Feijoa Puree

Carmen Ortuño[1*], Trang Duong[2], Murat Balaban[2], Jose Benedito[1]

[1]Department of Food Technology, Universitat Politècnica de València, Camí de Vera s/n, E 46022, Valencia, Spain.

[2]Department of Chemical and Materials Engineering, University of Auckland, Auckland 1142, New Zealand.

*Corresponding author: ASPA Group, Food Technology Department, Universitat Politècnica de València, Camí de Vera s/n, E46022, Valencia, Spain.

Tel.: +34-96-3873683 Fax:+34-96-3879839.

E-mail address: carorca1@upvnet.upv.es (Carmen Ortuño)

Abstract

A combined treatment of high hydrostatic pressure (HHP) and dense phase carbon dioxide (DPCD) was investigated to inactivate pectin methylesterase (PME), peroxidase (POD) and polyphenol oxidase (PPO) in feijoa (*Acca sellowiana*) puree. The treatments were HHP (HHP); carbonation and HHP (HHPcarb); carbonation + addition of 8.5 mL CO_2/g puree into the headspace of the package and HHP (HHPcarb+CO_2). The different samples were treated at 300, 450 and 600 MPa, for 5 min.

The residual POD and PPO activity decreased in the order HHP > HHPcarb > HHPcarb+CO_2 at all pressures used. Treatments with HHP at 300 MPa increased POD activity to 140 %. The residual PME activity of HHPcarb and HHPcarb+CO_2 samples at 600 MPa (45-50 %) was significantly ($p<0.05$) lower than for HHP treatment (65 %).

The simultaneous application of HHP and DPCD seems to synergistically enhance the inactivation of the enzymes studied, the CO_2 concentration being a key process factor.

Keywords: High hydrostatic pressure, carbon dioxide, enzymes, residual activity, synergistic effect

1. Introduction

Enzymes and microorganisms in foods cause quality deterioration and spoilage during storage and distribution. In the food industry, non-thermal processing alternatives have been developed in response to an increasing consumer demand for fresh-like and high quality food products. These technologies aim to economically produce safe, nutritious, and tasty foods using less severe processing conditions [1-3].

The application of high hydrostatic pressure (HHP) allows the inactivation of undesirable enzymes [4] in liquid and solid food systems, without altering their quality to the same extent as thermal treatments and with a comparable preservation effect. Park et al. [5] reported that by increasing the pressure in HHP treatments (25 ºC-5min) from 200 to 600 MPa, the residual activity of polyphenol oxidase (PPO), lipoxigenase (LOX) and pectin methylesterase (PME) in carrot juice decreased from 83 %, 78 % and 80 % to 10 %, 30 %, and 45 %, respectively. Nevertheless, some undesirable enzymes, such as PPO and some isozymes of PME, are highly pressure resistant [6]. In this case, higher temperatures are needed to inactivate these enzymes, thereby negating the non-thermal advantages of HHP process.

Similarly, DPCD has been reported to inactivate different microorganisms in liquid foods [2, 7-9] without exposing them to the adverse effects of heat which allows retain their fresh-like physical, nutritional, and sensory properties [10]. Similarly to HHP, DPCD has also been proven effective in inactivating many undesirable enzymes, including PPO [11, 12], peroxidase (POD) [12], and PME [13, 14]. However, in some cases the inactivation level was less than satisfactory [15, 16].

Therefore, there is increasing interest in process intensification, with simultaneous application of different non-thermal technologies, seeking for synergistic effects. In this regard, DPCD could be a good candidate to enhance the effect of HHP processing. It is well known that the effect of HHP is enhanced at lower pH, moreover, it is assumed that CO_2 could dissolve in the hydration layer associated with the enzyme and could decrease the local pH [17], therefore the presence of CO_2 in sample medium might create an acid environment, and positively interact with pressure to destroy or damage the structure of enzymes. Few studies have shown synergistic effects of combining DPCD and HHP process on inactivation of PPO, LOX and PME enzymes in orange [18] and carrot [5] juice. Corwin and Shellhammer [18] first carbonated enzyme preparations at atmospheric pressure, then treated them with HHP. They showed that CO_2 had an additional inactivation effect on PME at 500 MPa. Park et al. [5] reported

that a sequential application of DPCD at 4.9 MPa (5 ºC-5 min) and HHP at 200 MPa (25 ºC-5 min) improved the inactivation of the PPO, LOX and PME enzymes in carrot juice with a residual activity of 35 %, 17 % and 45 %, respectively, compared with the residual activity of DPCD (40 %, 20 % and 50 %, respectively) and HHP (83 %, 78 % and 80 %, respectively) treatments.

The extension of atmospheric carbonation could be to add gaseous CO_2 into the headspace of the packaged liquid food before HHP treatment. The CO_2 in the headspace could dissolve into the sample during the HHP treatment and the CO_2 concentration inside the sample could be higher than in carbonated samples. Therefore, the effect associated to CO_2, like the acidification of sample, could be increased, improving the CO_2 effects compared with only carbonated samples. No references have been found in the literature covering simultaneous application of HHP and DPCD techniques involving additional gases in the package for either enzymatic or microbial inactivation purposes.

Feijoa (*Acca sellowiana*), an exotic fruit in New Zealand, has many desirable nutritional characteristics such as good source of vitamin C, low in calories and high in minerals and fibre, and interesting bioactive components such as high antioxidant activity, high phenolics and phytochemicals content [19]. Therefore, the preservation of feijoa products by non-thermal technologies is advantageous to retain these desirable characteristics.

The objective of this study was to determine the effect of different levels of added carbon dioxide in a package on the efficiency of HHP treatment to inactivate POD, PPO and PME at different pressures in feijoa puree.

2. Material and methods

2.1. Raw material

The feijoa, (*Acca sellowiana*) was supplied by Frans and Tineke de Jong grower, Southern Belle Orchards (Matamata, Waikato), New Zealand. 15 kg of feijoa were stored at room temperature until they started ripening and released a sweet aroma volatile, and then they were put into storage at 4ºC for 2-3 days, time necessary to perform the chemical-physical analyses. The fruit that was not used for the chemical-physical analysis was cleaned, peeled and chopped, put in Ziploc bags and stored at -20 ºC until required for the preparation of samples for the inactivation treatments.

2.2. Chemical-physical analysis of feijoa

For the chemical-physical analysis, 30 feijoa pieces were randomly selected. Color, pH and firmness were determined directly on the fruit. Afterwards, a puree was made using the same feijoa fruits, and the moisture, ºBrix and water activity, were determined.

2.2.1. Color determination

Color assessment was conducted at 25 ºC using a CR400-Chroma Meter Colorimeter (Konica Minolta, USA) in CIE L*a*b* color space system after calibration with the reference tile.

The fruit color was measured in 9 different sites of the fruit (3 readings around each end of fruit and 3 at the equator) and averaged. 10 fruits from the 30 previously selected were measured and a total of 90 readings were done.

2.2.2. pH

The pH was measured directly inside the feijoa fruit at 25 ºC using a digital pH meter (PerpHec LogR meter, model 320, Orion research Inc., USA) and pH was recorded after stabilization, for 30 selected fruit.

2.2.3. Texture analysis

The firmness of fresh feijoa (Table 1) was measured using a universal texture analyzer (TA.XT Plus Texture Analyser, Stable Micro Systems Ltd., UK) linked to a computer for data acquisition and processing (Exponent software, Stable Micro System Ltd., UK), using a small cylindrical probe (10 mm diameter). The maximum force (firmness, N) was measured and computed with a test speed of 0.03 mm/s and travel distance of 5 mm down on the fruit surface, at the centre of its equator and at each side of the fruit (2 punctures per side). 30 pieces of fruit were measured.

2.2.4. Moisture content

The moisture content of fresh feijoa puree was determined using the official method [20] for a vacuum oven. 5 g of fresh feijoa puree were accurately weighed and placed on a ceramic crucible, dried at 70 ºC and 10 mmHg vacuum for 24 h in a vacuum oven (VT 6205, Haraeus Vacutherm, Germany). The vacuum was released slowly and the dried samples were stored in desiccators at ambient temperature prior to weighing by an analytical balance (ED224S, Sartorius Ag, Germany). The moisture analysis was

161 conducted in triplicate. The moisture content (Table 1) of the feijoa was calculated
162 using the following equation:

163 $$\text{Moisture content (\%)} = \frac{\text{Total moisture loss after drying (g)}}{\text{Inital weight (g)}} \text{x} 100 \quad (1)$$

164 2.2.5. ºBrix

165 The ºBrix of fresh feijoa puree (Table 1) was measured in triplicate at 25 ºC using E-
166 Line ATC range 0-18 ºBrix refractometer (Bellingham + Stanley Ltd., UK).

167 2.2.6. Water activity

168 The water activity of fresh feijoa puree was measured in triplicate at 25 ºC using a
169 digital water activity meter (Aqua Lab 4TE, Decagon Devices, USA). The water
170 activity of the fresh feijoa puree was 0.9901±0.0018.

171 *2.3. Sample preparation and storage*

172 The frozen fruit was thawed at 4 ºC for 12-14 h before processing. Thawed feijoa were
173 blended (Laboratory blender, Model 38BL40, Waring Commercial, USA), until well
174 mashed and mixed into a puree. 30 g portions of feijoa puree were poured into plastic
175 bags (155x180x30mm, SURT155180, Cas-Pak Products Ltd., New Zealand), vacuum
176 sealed (Vacutherm, VT 6205, Germany) and stored at -20 ºC until required.

177 *2.4. Sample treatment*

178 2.4.1. CO_2 treatment

179 The frozen feijoa puree was thawed in the bag at 4 ºC for 12-14 h before processing.
180 Three different CO_2 levels were considered in this study. Feijoa puree without CO_2
181 (HHP); carbonation at 1 atm (HHPcarb); carbonation and addition of 8.5 mL CO_2/g
182 puree into the headspace of the package (HHPcarb+CO_2). The carbonation of samples
183 was carried out by bubbling CO_2 at atmospheric pressure at 1.28 L/min from the bottom
184 of the puree for 5 min at 0-3 ºC by placing the bags of puree in an ice water bath and
185 manually and vigorously agitating to facilitate mass transfer. The bags were
186 immediately sealed without gas loss and were placed on ice until HHP treatment.

187 2.4.2. High pressure processing

188 The HPP unit used in this study was Avure 2 L Food Processor (Avure Technologies,
189 Columbus, Ohio, USA). The equipment can operate at a maximum pressure and

temperature of 600 MPa and 90 ºC, respectively. The equipment consists of a cylindrical pressure treatment chamber, a pumping system, water circulation and the control system operated through a personal computer with software supplied by the manufacturer. Water was the working fluid in the pressure chamber where the packaged puree was placed. The temperature history of the water in the chamber was recorded by two thermocouples during processing.

For each pressure run, 3 bags (1 HHP sample, 1 HHPcarb sample and 1 HHPcarb+CO_2 sample) were treated together in the hydrostatic pressure processing unit (HPP). The pressure levels used were 300, 450 and 600 MPa, for 5 min. It is generally agreed that pressures lower than 300 MPa do not have much deactivating effect on enzymes in a process with only HHP [21]. The process time selected was 5 min in order to reduce the cost of the process and to increase its industrial applicability. Pressure come up times were approximately 0.5 min and 1.5 min to reach 300 MPa and 600 MPa, respectively. Depressurization occurred in less than 2 s. The starting temperature of samples was 25 ºC. The maximum temperature reached at 600 MPa runs was 42 ºC.

Two replicates of each run were carried out for each pressure condition tested. The plastic bags were frozen after treatment at -70 ºC and thawed before enzyme analysis.

2.5. Analysis of treated samples

The treated frozen puree was thawed at 4 ºC for 12-14 h before the analysis. Moreover, feijoa puree without CO_2 and HHP treatments was subjected to the same freezing and thawing processes and it was used as a control sample.

2.5.1. pH

The pH of the puree was measured in triplicate in the control sample and in the treated samples before the enzyme analysis. For the samples with CO_2 (HHPcarb and HHPcarb +CO_2) the puree was decarbonated previously to the pH measurement by agitation under vacuum (10 mmHg, 25 ºC).

2.5.2. Color determination

Color assessment was conducted at 25 ºC in CIE L*a*b* color space system after calibration with the reference tile. The color of control puree was measured in triplicate prior to the enzymes analysis (after the freezing and thawing processes). The color of treated samples was measured in triplicated after the treatment, just before to the

enzymes analysis. Chroma (C*) and hue angle (H°), and total color difference (ΔE) (with respect to control sample after the freezing and thawing processes) were also calculated.

$$\Delta E = [(L^* - L^*_0)^2 + (a^* - a^*_0)^2 + (b^* - b^*_0)^2]^{1/2} \quad (2)$$

$$C^* = (a^{*\,2} + b^{*\,2})^{1/2} \quad (3)$$

$$H^o = \arctan (b^*/a^*) \quad (4)$$

where L*: lightness of treated sample at time t; L^*_0: lightness of reference sample; a*: redness of treated sample at time t; a^*_0: redness of reference sample; b*: yellowness of treated sample at time t; and b^*_0: yellowness of reference sample.

2.5.3. PPO and POD assay

The frozen puree was thawed at 4 ºC for 12-14 h before the analysis. 10 g of feijoa puree was homogenized (Laboratory blender, Model 38BL40, Warning Commercial, USA) with 30 mL of 0.05 M potassium phosphate buffer solution, at 13000 rpm, for 2 min. The slurries were centrifuged (SA600 rotor, Sorvall RC28S supraspeed centrifuge, Du Pont Company, USA) at 10000 rpm for 10 min at 4 ºC, and the supernatant was filtered through filter paper (Whatman #2) using a suction flask. The pellet was re-extracted and centrifuged. The filtrates of the two extractions were combined and centrifuged at 10000 rpm for 15 min. The supernatant was used to test enzyme activity.

PPO and POD activities were assayed by the method described by Chen et al., [22] with some modifications. PPO assay medium contained 0.4 mL of the sample and 2.6 mL of substrate solution (1.3 mL 0.05 M sodium phosphate buffer, pH = 6.8, added to 1.3 mL 0.02 M catechol solution); to the blank 0.4 mL of distilled water, instead of sample, was added. POD assay medium contained 0.2 mL of the sample with 3 mL of substrate solution (3 mL of 30 % hydrogen peroxide added to 1.9 mL of liquid guaiacol, made up to 300 mL with 0.2 M sodium phosphate buffer, pH = 6); to the blank 0.2 mL of distilled water, instead of sample, was added.

The increase in absorbance at 420 nm (PPO) or 470 nm (POD) was monitored at intervals of 5 s immediately after the addition of sample to the corresponding substrate solution using an UVmini-1240 spectrophotometer (Shimadzu, Tokyo Japan) at ambient temperature. One unit of specific PPO or POD activity was defined as the change per

min and milliliter of sample in the absorbance measured at 420 nm or 470 nm, respectively. The residual activity of each enzyme was obtained using the following equation:

$$\text{PPO (POD) residual activity} = \frac{\text{Specific activity PPO (POD) after treatment}}{\text{Specific activity PPO (POD) control sample}} \text{x} 100 \quad (5)$$

2.5.4. PME activity measurement

Before the PME activity was evaluated, the puree was decarbonated by agitation under vacuum (10 mmHg, 25 ºC). PME activity was determined as described by Castaldo et al. [23] with some modifications. The substrate solution was prepared by dissolving 10 g of pectin powder (Sigma Chemical Co. St. Louis, MO) in 1 L of 0.15 M NaCl. The NaCl solution was heated to 50-55 ºC and added in the blender while pectin powder was sprinkled on the surface and blended. Pectin solution was stored at 4ºC until required.

The pH of pectin solution was adjusted to 7 prior to each analysis and 4 mL of feijoa puree were added into 12 mL of pectin solution. The pH was quickly adjusted to 7 (1 M NaOH for gross adjustment, 0.05 M NaOH for fine adjustment), and PME activity was measured by recording the decrease of pH every 5 s until pH dropped to 6.5. One unit of specific PME activity was defined as the slope of pH vs time in min. The residual activity of PME was calculated using the following equation:

$$\text{PME residual activity} = \frac{\text{Specific activity PME after treatment}}{\text{Specific activity PME control sample}} \text{x } 100 \quad (6)$$

2.6. Statistical analysis

All treatment conditions were duplicated and analyses triplicated. Using the statistical package Statgraphics Plus (Statistical Graphics Corp. 5.1, Warrenton, USA), simple ANOVA and a two-way ANOVA were carried out and LSD (Least Significant Differences) were identified, in order to evaluate the effect of pressure, CO_2 level and the possible interaction between factors, on the residual PPO, POD and PME activity of treated samples.

A two-way ANOVA was carried out in order to evaluate the effect of pressure and CO_2 level on the pH, and color parameters of the treated samples, compared with the control sample.

3. Results

3.1. POD activity

Figure 1 shows the effect of 3 types of treatments on residual POD activity. In the HHP treatments, at 300 MPa for 5 min the residual POD activity of feijoa puree increased to 140±5 %. With further increase in pressure, the residual POD activity significantly ($p<0.05$) decreased to 60±9 % and 22±13 % at 450 MPa and 600 MPa, respectively. The addition of CO_2 had a significant effect ($p<0.05$) on the residual POD activity for all the pressures tested. At 300 MPa, the residual POD activities in HHPcarb decreased to 32±7 % compared with 140±5 % of HHP alone; in HHPcarb+CO_2 samples the residual activity drooped to a value of 13±8 %. At 450 MPa and 600 MPa, the residual POD activities in HHPcarb samples were 9±1 % and 10±0.02 %, respectively while in HHPcarb+CO_2 samples were 27±7 % and 6±1 %, respectively. In the samples with CO_2 in the headspace of the package (HHPcarb+CO_2), the residual POD values were by 60 and 45 % lower than in HHPcarb samples at 300 and 600 MPa. Moreover, the addition of gaseous CO_2 in the bag resulted in a residual activity at 300 MPa (13±8 %) that could only be obtained at 600 MPa with high pressure alone (22±13 %).

From the two-way AVOVA it was observed that the residual POD activity obtained at the different pressures significantly decreased ($p<0.05$) in the order HHP (78 $\%_{avg}$) > > HHPcarb (45 $\%_{avg}$) > HHPcarb+CO_2 (25 $\%_{avg}$). On the other hand, for the different CO_2 levels, the residual POD activity was significantly lower ($p<0.05$) as pressure increased: 300 MPa (80 $\%_{avg}$) > 450 MPa (29 $\%_{avg}$) > 600 MPa (13 $\%_{avg}$). These results indicate that, the combined HHP and DPCD processing of feijoa puree had a significant effect on the residual POD activity and this effect was higher with increasing treatment pressure and CO_2 level.

No references have been found in the literature regarding the inactivation of POD in feijoa puree with HHP or HHP+DPCD. Garcia-Palazon et al. [24] observed residual POD activity in strawberry puree in the range of 11-35 % after 15 min of HHP treatment (600 MPa) at ambient temperature. In another study, no significant inactivation of strawberry POD was observed after 15 min HHP treatment of the puree at pressures ranging from 50 to 400 MPa and temperatures ranging from 20 to 60 °C [15]. DPCD treatment of red beet extract at 37.5 MPa (55 ºC, 60 min) resulted in a reduction of POD activities by approximately 76% [12]. Other studies suggest that

311 DPCD treatment increases or slightly reduces the POD activity in crude vegetable
312 enzymatic extracts [25, 26]. However, in the present study an increase of POD activity
313 was only observed after 5 min of HHP at 300 MPa, and all HHP+DPCD treatments
314 resulted in a decrease of the POD activity. Based on the results of this study, the
315 addition of CO_2 in the sample allows lower pressures and shorter process times to
316 obtain similar residual POD activities either with HHP or DPCD alone.

317 *3.2. PPO activity*

318 The inactivation of PPO in feijoa puree subjected to HHP, HHPcarb and HHPcarb+CO_2
319 treatments at different pressures is illustrated in Figure 2.

320 The residual PPO activity for HHP, HHPcarb and HHPcarb+CO_2 samples treated at:
321 300 MPa were 102±8 %, 85±2 % and 56±5 %, respectively; 450 MPa were 47±4 %,
322 42±6 % and 42±1 %, respectively; 600 MPa were 38±5 %, 44±4 % and 26±3 %,
323 respectively.

324 On average, the residual PPO activity obtained at different pressures showed a
325 significantly ($p<0.05$) lower value in the HHPcarb+CO_2 samples (52 $\%_{avg}$), compared to
326 HHP (68 $\%_{avg}$) and HHPcarb (62 $\%_{avg}$) between which no significant differences
327 ($p>0.05$) were found. For the different CO_2 levels, on average, the residual PPO activity
328 was significantly lower ($p<0.05$) as pressure increased in the order 300 MPa (81 $\%_{avg}$) >
329 450 MPa (45 $\%_{avg}$) > 600 MPa (36 $\%_{avg}$). Therefore, similar to the POD, the addition of
330 CO_2 into the headspace of the package allows obtaining higher inactivation levels of
331 PPO when HHP is applied, for all the pressures studied, compared with only HHP or
332 with HHPcarb treated samples.

333 No treatment combination could fully inactivate PPO. This result was similar to that
334 obtained by Park et al. [5] using HHP alone, who observed that the residual PPO
335 activity of carrot juice decreased from 83 % to 10 % as pressure increased from 200 to
336 600 MPa (25 ºC, 5 min). In a sequential application of DPCD (4.9 MPa, 25 ºC, 5 min)
337 and HHP (200 MPa, 5 min) the residual PPO activity in carrot juice decreased to 35 %,
338 compared with 83 % using HHP only [5]. Corwin and Shellhammer [18] reported that
339 the percent residual PPO activity in carbonated 0.1 M phosphate buffer (pH = 6.5)
340 treated by HHP (500 MPa, 25 ºC, 3 min) was 59.8 %, compared with 98.5 % after HHP
341 alone. Using carbonated 0.1 M phosphate buffer and HHP at 800 MPa, 25ºC for 1 min,

342 the remaining PPO activity was 21.7 % [18], similar to residual activity obtained in this
343 study in HHPcarb+CO_2 at 600 MPa for 5 min, 26±3 %.

344 *3.3. PME activity*

345 In the inactivation of PME, pressure showed different effects for the different treatments
346 (Figure 3). The residual PME activity of HHP samples was not significantly different
347 ($p>0.05$) with increasing pressure. In the HHPcarb samples, the remaining PME activity
348 at 600 MPa (44±4 %) was significantly lower ($p<0.05$) than at 300 (83±2 %) and 450
349 MPa (78±3 %), between which there were no significant differences ($p>0.05$). For
350 HHPcarb+CO_2 treated samples, only significant differences ($p<0.05$) were observed
351 between 300 MPa (73±14 %) and 600 MPa (53±3 %).

352 From the two-way ANOVA, it was observed that the residual PME activity of the
353 different treated samples significantly ($p<0.05$) decreased as pressure increased in the
354 order 300 MPa (78 $\%_{avg}$) > 450 MPa (58 $\%_{avg}$) > 600 MPa (52 $\%_{avg}$). However, no
355 significant differences ($p>0.05$) were found between the different levels of CO_2 studied.
356 In this case, on average, the addition of CO_2 did not improve the inactivation of PME in
357 a HHP process. The enhancing effect of CO_2 addition to the HHP inactivation process
358 of PME in feijoa puree was only observed at 600 MPa (Figure 3).

359 A portion of PME can be inactivated easily by pressure, but an isozyme of PME
360 remains active even after pressurization at 900 MPa [27]. The lowest remaining PME
361 activity resulting from this study, achieved after HHPcarb treatment (600 MPa, 5 min)
362 was 44±11 %, and no treatment could fully inactivate PME. Similarly, in a sequential
363 application of DPCD (4.9 MPa, 25 ºC, 5 min) and HHP (600 MPa, 5 min) using carrot
364 juice, the lowest residual PME activity was 35 % [5]. Park et al. [5] observed that the
365 residual PME activity in carrot juice decreased from 80 % to 45 % by increasing
366 pressure from 200 to 600 MPa (25 ºC, 5 min). More significant inactivation of PME
367 was found by many authors using orange juice. Corwin and Shellhammer [18] reported
368 that the lowest remaining PME activity in carbonated orange juice was 6.8 %, achieved
369 at 25 ºC, 800 MPa for 1 min.

370 *3.4. pH*

371 The value of pH directly measured in feijoa fruit was 3.30 (Table 1) while the pH of the
372 control sample, after the freezing and thawing process was 3.45 (Table 2). The

comparison of means shows that the blending, freezing and thawing processing had a significant ($p<0.05$) effect on the pH of feijoa, before applying CO_2 or HHP.

The pH of the treated samples was compared with the pH of control puree subjected to the same temperature changes (Table 2). The pH values of samples with CO_2 inside the bag were measured after degassing by pulling vacuum. Overall, the pH values of all treated samples at different pressures significantly increased ($p<0.05$) compared to the control sample puree, but no significant ($p>0.05$) effect of pressure on the final pH reached in the puree was found. For the different CO_2 levels, on average, the pH values obtained at different pressures significantly decreased ($p<0.05$) in the order HHP > HHPcarb > HHPcarb+CO_2. This cannot be explained by the possibility of residual CO_2 remaining in the juice, since vacuum was pulled to remove the CO_2 from samples before pH measurement.

3.5. Color

The ΔE values (taking the control puree color as reference) are shown in Figure 4 while the L*, a*, b*, Chroma and Hue angle values of the control and treated puree are shown in Table 2.

The ΔE values, on average for the three CO_2 levels studied, were significantly higher ($p<0.05$) in the samples treated at 600 MPa (2.74) compared to samples treated at 300 (2.02) and 450 MPa (2.05). The ΔE values are dependent on L*, a* and b*, and from the two-way ANOVA analysis of these parameters it was observed that pressure also had a significant ($p<0.05$) effect on all of them. The lightness and the yellowness of the samples significantly decreased ($p<0.05$) as pressure increased, while the redness significantly increased ($p<0.05$) as pressure increased. From the two-way ANOVA of Chroma and Hue angle, it was observed that the pressure had a significant ($p<0.05$) effect on them, decreasing their values with increasing pressure.

Regarding the different CO_2 levels, on average for the different pressures studied, in the HHPcarb samples the calculated ΔE value was significantly higher than for HHP and HHPcarb+CO_2 samples, between which no significant differences were found. From the two-way ANOVA, the different CO_2 levels had a significant ($p<0.05$) effect on L* and a* values. Therefore the lightness and redness of the samples treated with CO_2 was significantly lower ($p<0.05$) than samples treated only with HHP. However, the yellowness did not change with the addition of CO_2 into the package compared with

405 only HHP. On the other hand, the CO_2 level had a significant ($p<0.05$) effect on
406 Chroma values, but not on Hue angle values.

407 As a rule, a ΔE value of 1.6 or less is considered as an imperceptible difference to the
408 human eye [28]. From Figure 4 it can be seen that the ΔE values are above this
409 threshold, except for HHPcarb at 450 MPa (0.74), therefore the treatments caused a
410 perceptible color change. The feijoa puree changed from bright yellow tones to shades
411 of brown with lower brightness, after all types of treatments. However, the addition of
412 CO_2 into the headspace of the package did not increase the color change of the samples
413 compared with the samples treated only with HHP.

414 **4. Discussion**

415 The mechanisms associated with the inactivation of enzymes are similar to those
416 associated to the denaturation of proteins because enzymes share the structure and
417 properties of the proteins. Enzymes are folded into a three dimensional state, determined
418 by covalent, hydrophobic and ionic intra-molecular connections [29]. The inactivation
419 of enzymes is caused by the fragmentation or modification of their secondary and
420 tertiary structure; therefore, all the mechanisms that affect the structure of enzymes can
421 be responsible of their denaturation.

422 The application of HHP causes structural rearrangements in the protein, shifting the
423 system equilibrium toward the state occupying the smallest volume and increasing the
424 degree of ordering of molecules of a given substance [29]. The volume decrease can
425 perturb the balance of intramolecular and solvent-protein interactions and can, therefore,
426 lead to structural changes of the proteins [21]. A reduction in the pH of suspending
427 media as a result of the pressure-induced transient pH shift leads to a greater enzyme
428 inactivation by HHP, and this has also been reported for food borne vegetative cells
429 [29].

430 The inactivation of enzymes exposed to DPCD treatment can be explained by different
431 effects such as pH lowering, the inhibitory effect of molecular CO_2 on enzyme activity
432 and the fact that DPCD causes conformational changes [10]. Treatments with high
433 pressure CO_2 are accompanied by a lowering of pH because of the formation of
434 carbonic acid from the dissolution of carbon dioxide in water and under a lower pH
435 environment, protein bound arginine can easily interact with CO_2, forming a
436 bicarbonate complex [29]. Therefore, in addition to its pH-lowering effect, CO_2 may

directly bind to the enzyme and cause loss in activity. Moreover, the inactivation of enzymes exposed to DPCD treatment can be explained by the fact that DPCD causes conformational changes in the secondary and tertiary structure.

The present study is the first work where HPP and DPCD have been simultaneously applied in feijoa puree, and where a modified atmosphere of CO_2 has been considered in the treatment of its puree to preserve the nutritional properties of this product.

As a result, the addition of carbon dioxide into the headspace of the package treated with HHP enhanced the inactivation mechanisms of the enzymes POD, PPO and PME, compared with HHP and the HHPcarb samples. This could be explained because pressure increases the CO_2 solubilization, therefore in the HHPcarb+CO_2 samples, the amount of dissolved CO_2 should be higher than in HHPcarb samples, and it is the first step in the inactivation mechanisms of CO_2 from which other mechanisms follow (decrease of pH, alteration of ionic equilibrium and inactivation of enzymes) [8, 9].
In addition, the CO_2 dissolved into the puree during the HHP treatment, could generate a significant and sudden bubbling during the fast depressurization of the process (2 s), that could contribute to conformational changes responsible for the inactivation of enzymes. The effect associated to the sudden depressurization would be more intense as pressure drop increases; suggesting that the conformational changes would be higher after treatment at 600 MPa than at 300 MPa. Therefore, various depressurization rates should also be investigated.
The same level of inactivation of POD and PPO was obtained at 600 MPa without CO_2, and at 300 MPa with added CO_2. However, to observe the enhanced HHP inactivation of PME in feijoa puree by the addition of CO_2 it is necessary to use 600 MPa.
The addition of CO_2 significantly improved the inactivation of some enzymes in the HHP process, compared with only HHP. Moreover, CO_2 did not affect the color of the puree, compared with puree treated with only HHP. These results are encouraging to apply this combined technique to other foods systems.
It is recommended that more research be conducted to study the effect of the different CO_2 levels in the bags and to elucidate the mode of enzyme inactivation by the simultaneous HHP and DPCD treatments. Kinetics of inactivation should be measured under this combined method. This typically requires treatments using a series of dwell times. Additional studies regarding the effect of simultaneous HHP+DPCD on physico-chemical properties and consumer acceptance of juices and purees would also bring this

method closer to commercial applications.

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Figure Captions

Figure 1. Residual POD activity in feijoa puree after HHP, HHPcarb and HHPcarb+CO_2 treatments at different pressures (initially at room temperature, 5 min). All data shown are means±SD.

Figure 2. Residual PPO activity in feijoa puree after HHP, HHPcarb and HHPcarb+CO_2 treatments at different pressures (initially at room temperature, 5 min). All data shown are means±SD.

Figure 3. Residual PME activity in feijoa puree after HHP, HHPcarb and HHPcarb+CO_2 treatments at different pressures (initially at room temperature, 5 min). All data shown are means±SD.

Figure 4. Total color difference of feijoa puree after HHP, HHPcarb and HHPcarb+CO_2 treatments at different pressures. All data shown are means±SD.

Figure 1

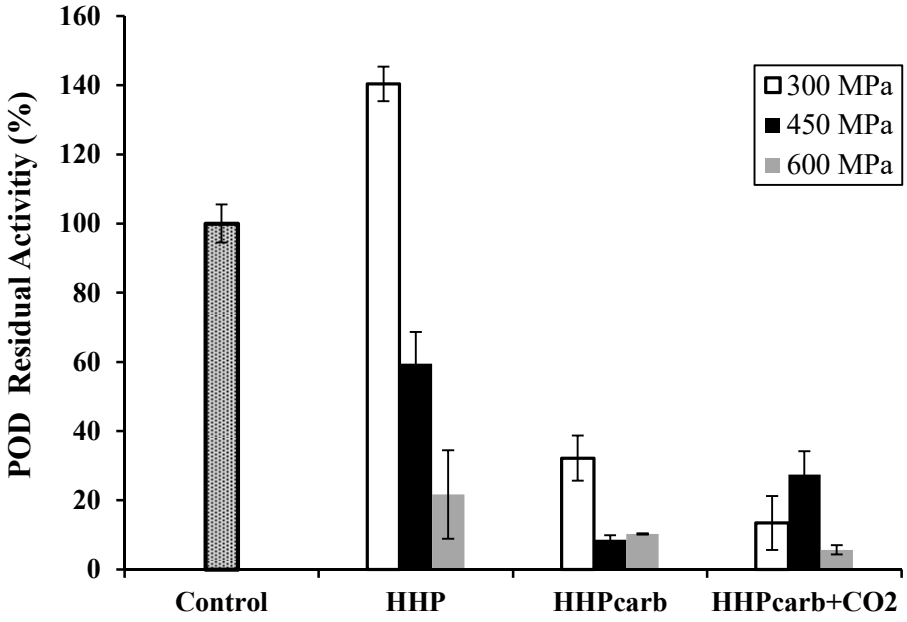
160
140
120
100
80
60
40
20
0
POD Residual Activitiy (%)
300 MPa
450 MPa
600 MPa
Control
HHP
HHPcarb
HHPcarb+CO2

Figure 2

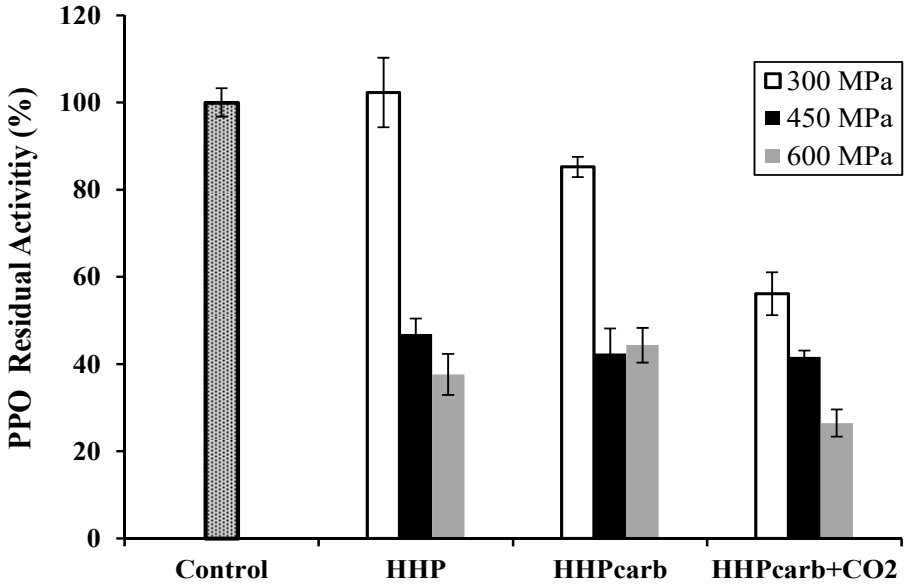
120
100
80
60
40
20
0
PPO Residual Activitiy (%)
300 MPa
450 MPa
600 MPa
Control
HHP
HHPcarb
HHPcarb+CO2

Figure 3

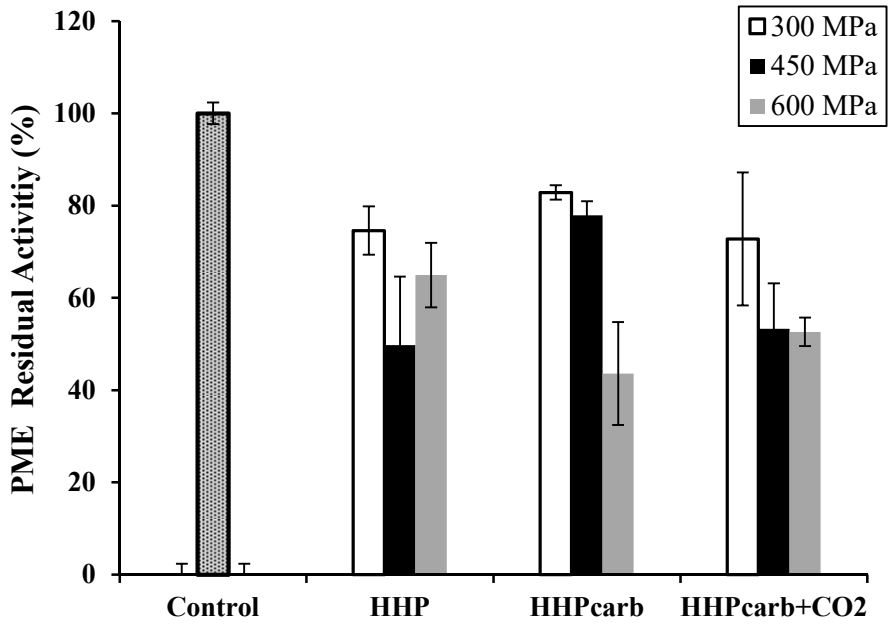
120
100
80
60
40
20
0
PME Residual Activitiy (%)
300 MPa
450 MPa
600 MPa
Control
HHP
HHPcarb
HHPcarb+CO2

Figure 4

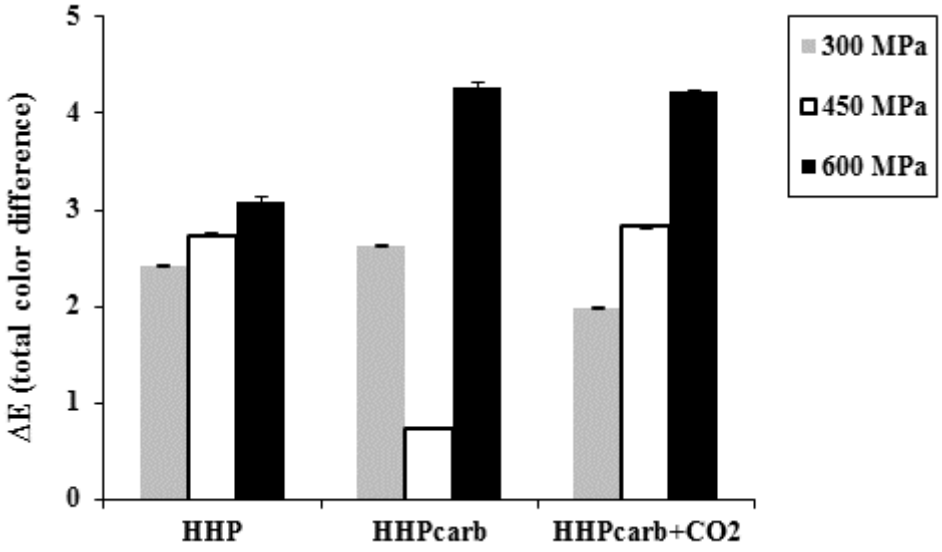
ΔE (total color difference)
5
4
3
2
1
0
HHP
HHPcarb
HHPcarb+CO2
300 MPa
450 MPa
600 MPa

Table 1. Moisture content, ºBrix, pH, firmness, and color of fresh feijoa.

	% Moisture	º Brix	pH	Firmness (N)	Colour		
					L*	a*	b*
Fresh Feijoa	83.33±0.30	11.8±0.8	3.30±0.02	20.67±3.87	54.56±2.56	-8.32±2.07	14.01±3.26

All data shown are means±SD.

Table 2. Values of pH and color of feijoa puree for control and treated samples.

		pH	L*	a*	b*	Chroma	Hue angle
CONTROL		3.45±0.03	55.13±0.86	3.16±0.55	20.76±1.69	21.08±0.76	1.42±0.02
HHP	300	3.63±0.02	55.78±0.53	3.93±0.20	18.6 ±1.07	19.08±0.72	1.36±1.09
	450	3.68±0.01	54.24±0.92	4.43±0.10	18.57±0.17	19.09±0.48	1.34±0.14
	600	3.64±0.01	54.63±0.16	3.97±0.22	17.84±0.65	18.28±0.58	1.35±0.68
HHPcarb	300	3.61±0.02	53.76±0.60	4.30±0.78	18.83±0.82	19.33±0.62	1.35±0.31
	450	3.50±0.01	54.88±0.65	3.61±0.12	21.04±0.09	21.35±0.18	1.40±0.07
	600	3.55±0.03	52.30±0.18	4.58±0.30	17.90±0.16	18.48±0.09	1.32±0.08
HHPcarb+CO_2	300	3.46±0.04	54.84±0.38	2.57±0.80	19.06±0.53	19.25±0.28	1.44±0.42
	450	3.56±0.03	54.58±0.85	3.93±0.20	18.09±0.31	18.52±0.85	1.36±1.85
	600	3.51±0.01	53.68±0.02	4.20±0.09	16.93±0.23	17.44±0.20	1.33±0.25

All data shown are means±SD.