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Effect of High Pressure Carbon Dioxide on tomato juice: inactivation kinetics of pectin methylesterase and polygalacturonase and determination of other quality parameters

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Abstract

Tomato juice, *Lycopersicon esculentem* cv. Canario, has been treated by HPCD as non-thermal preservation treatment. The inactivation kinetics for pectinmethylesterase (PME) and polygalacturonase (PG) were determined at different pressures (8.5 to 20 MPa) and temperatures (35 to 55 ºC). At the highest operating pressure and temperature essayed in this work, it was found that PME could be almost completely inactivated, whereas PG resulted to be more HPCD resistant at the working conditions. PME enzyme inactivation curves were properly described by a Weibull type model, while the fractional conversion model was the most appropriate for the PG with a sharp initial decrease in activity. On the contrary, high hydrostatic pressure led to a nearly complete inactivation of PG while PME was very resistant at 600 MPa. It was also found that HPCD treatment led to a smaller particle size distribution of tomato juice.

Keywords: Tomato juice, HPCD, enzyme inactivation, properties, HPP.

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1. Introduction

Tomatoes are usually consumed in a processed form, such as juice and pasta sauce, being viscosity one of the most important quality parameters. Viscosity is influenced by the concentration and type of cell wall polymers in the serum and pulp fraction (Kalamaki et al., 2012). Therefore, enzymes such as pectinmethylesterase (PME) and polygalacturonase (PG), involved in the breakdown of pectins (Anthon et al., 2002), should be inactivated during processing. PME catalyzes pectin demethylation while PG hydrolyses the α-1,4-glycosidic bonds of the polygalacturonic acid chain. In this regard, two different effects of PME action on PG action have been described in the literature. On the one hand, partial demethylated pectin can bind bivalent calcium ions to form insoluble calcium pectate gels leading to a loss of juice cloud in a pH dependent manner (Croak and Corredig, 2006). This cross-linked network also shows a higher resistance to PG attack, increasing viscosity. On the other hand, the lower the degree of esterification of the pectin molecules, the better substrate for PG they are, leading to the depolymerization of cell wall pectin chains and therefore a reduction in viscosity (Andreou et al., 2016; Crelier et al., 2001). Coldbreak and hot break thermal treatments are traditionally used in the tomato industry. Coldbreak treatment, by using temperatures around 60ºC, yields tomato products with a good retention of color and taste, but enzymes such as PME and PG are not completely inactivated and this fact is related to the low viscosity of coldbreak-treated products (Anthon et al., 2002). Hotbreak treatments, by using temperatures around 85-90ºC, get the inactivation of PG and PME. It is applied for the production of tomato products with high viscosity, but it results in loss of flavor, browned color and nutritional degradation (Wu et al., 2008).

In the last years, the food industry is searching for alternative technologies to replace conventional food heat treatments that may affect food quality in terms of nutritional and physicochemical parameters. Among these technologies, high pressure carbon dioxide (HPCD) treatment has been

proposed as an alternative non-thermal pasteurization. Typically, operating pressure does not exceed 50 MPa and temperature ranges between 20 and 50 ºC, below pasteurization temperature (Briongos et al., 2016). HPCD has been mainly applied to liquid foods such as fruit and vegetables juices (Amaral et al., 2018; Briongos et al., 2016; Illera et al., 2018) and dairy products (Amaral et al., 2017).

To our knowledge, no previous studies on HPCD treatment of tomato juice have been found in the literature. Other non-thermal technologies have been applied to inactivate some of the deleterious enzymes in tomato juice. Most of the studies were focused on the use of high pressure processing (HPP), observing a different behavior for both enzymes with pressure. Generally, it was found that PME was more pressure resistant while PG can be inactivated at moderate pressure and temperature by HPP (Andreou et al., 2016; Crelier et al., 2001; Fachin et al., 2003; Hernández and Cano, 1998; Houben et al., 2014; Hsu, 2008; Van Den Broeck et al., 2000). Other studies can be also found in the literature that employ other different non-thermal treatments, such as electric processing, cold plasma, membrane processing, ultrasound and ultraviolet irradiation (Bevilacqua et al., 2018).

The main objective of this work was to study the effect of HPCD on PME and PG inactivation from *Canario* tomato juice on a kinetic basis. Control samples of tomato juice treated in the same temperature range at atmospheric pressure, were studied in parallel. Additionally, enzyme inactivation was compared with results obtained by HPP performed at Hiperbaric (Burgos, Spain). The effect of HPCD on other quality parameters of tomato juice such as particle size distribution (PSD) and ζ potential was also studied.

2. Materials and methods

2.1 Juice preparation

Fresh red tomato, *Lycopersicon esculentem* cv. Canario, from the local market, of uniform size, color and appearance were washed and chopped. Tomatos were subsequently squeezed with a screw juice extractor. The liqueur was filtered through a screen of 1 mm size to remove peel and seeds. The initial pH of the tomato juice was 4.09 ± 0.02.

2.2 HPCD equipment and processing

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 80ºC, respectively (Melgosa et al., 2017). Tomato juice, 40 mL, was charged 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 by using a syringe pump with a pressure controller (ISCO 260 D). CO_2 was bubbled directly into the tomato juice through a sintered stainless steel micro-filter of 10 μm (Briongos et al., 2016; Illera et al., 2018) Experiments were carried out in the temperature range from 35 to 55 ºC and pressure from 8.5 to 20 MPa. Enzyme inactivation kinetics were followed by collecting samples periodically and enzymatic activity was determined.

Particle size distribution, ξ potential and pH were also measured before and right after HPCD treatment for some of the experiments.

2.3 HPP processing

HPP treatment is currently applied to a great variety of juices and smoothies at industrial level. This experimental part was carried out at Hiperbaric España (Burgos). Tomato juice was sealed in plastic bottles of polyethylene terephthalate (PET) and introduced into a vessel subjected to 600 MPa of isostatic pressure transmitted by water. Pressurization rate was 150 MPa/min and final pressure was held for 5 minutes. Depressurization occurred in about 2 s. The initial operating temperature was 17.2 ºC. Physical compression during pressure treatment results in a volume reduction and an increase in temperature and energy, approximately 3ºC per 100 MPa. Therefore,

92 temperature of the juice during treatment was around 35ºC (data provided by Hiperbaric, Burgos,
93 Spain). However, due to fast temperature decrease during decompression, this treatment
94 temperature was only held during treatment time (Yordanov and Angelova, 2010)
95 Three different tomato juice samples were treated by HPP to analyze the effect of the nature of the
96 dissolved gasses into the tomato juice on enzyme inactivation. In two of the samples, air dissolved
97 in the juice was displaced by bubbling CO_2 or N_2 into the juice (HPP-CO_2 and HPP-N_2 samples,
98 respectively) until the O_2 concentration of the juice was below 0.4 mg/L (YSI ProODO optical
99 dissolved oxygen meter). The air dissolved in the third sample was not removed (HPP-Air sample).
100 All the three samples were treated together in the high pressure unit. CO_2 permeability for PET is
101 higher than for O_2 and N_2, coefficients of permeability for PET are 0.05, 0.22 and 1.53 ml mm $cm^{-2}s^{-1}cmHg^{-1}$
102 for N_2, O_2 and CO_2, respectively at 30ºC (Zeman and Kubík, 2007). However, CO_2 loss
103 is expected not be high in the first hour after carbonation when HPP experiments were carried out.

104 **2.4 Physico-chemical analysis**

105 **2.4.1. Determination of enzyme activity**

106 **Pectinmethylesterase.** Samples were analyzed by using an automatic titrator system (Metrohm ®
107 Titrando) similar to Briongos et al. (Briongos et al., 2016) but with different amount of juice.
108 50 mL of a 1% pectin solution (Alfa Aesar ® pectin citrus) prepared in NaCl 0.3 M was used as
109 substrate. After the addition of 100 µL of tomato juice, pH was adjusted to 7.5 with NaOH 0.02 N.
110 During pectin hydrolysis at 30 ºC, pH was maintained at 7.5 by adding NaOH 0.02 N. The amount
111 of NaOH added for 15 min was recorded. One PME activity unit (UPE) is defined as the
112 micromoles of carboxylic groups produced per minute and mL of juice at pH 7.5 and 30 ºC
113 **Polygalacturonase**. PG activity was determined by following a similar procedure to the one
114 proposed by Anthon et al. (Anthon et al., 2002) and Fachin et al. (Fachin et al., 2003) with some
115 modifications. First, PG was extracted from the tomato juice at 4 ºC. 1 mL of tomato juice was

centrifuged at 7500 g for 10 min, the supernatant was replaced by cold distilled water (1:1) adjusted to pH = 3 with 0.1 M HCl and mixed for 30 min. After centrifuging at 9000 g for 20 min, the supernatant was removed and PG was extracted from the pellets with 1.2 M of NaCl (1:1) for 1 h. Subsequently the mixture was centrifuged at 18200 g for 10 min and the supernatant was collected to determine the PG activity. 0.2 mL of the extracted enzyme solution were mixed with 0.6 mL of a 0.2 % polygalacturonic acid solution at 35ºC for 10 min. Polygalacturonic acid was prepared in a acetate buffer solution 0.05 M (pH = 4.5). 4 mL of 0.1 M borate buffer solution (pH = 9) and 0.8 mL of 1% cyanoacetamide were added to the mixture to stop the reaction and boiled in sealed bottles for 10 min. After cooling, absorbance was measured at 276 nm using a Jasco V-750 spectrophotometer equipped with a Peltier thermostated cell holder and a water pump to keep the temperature constant at 30 ºC.

Each enzyme activity was measured at least in duplicate. Relative residual enzyme activities were evaluated as the ratio of the measured activity after treatment, A, and the enzyme activity before treatment, A_o:

$$\text{Residual activity} = \frac{\text{Enzyme specific activity after treatment}}{\text{Enzyme specific activity of the untreated juice}} \cdot 100 = \frac{A}{A_o} \cdot 100 \quad [1]$$

2.4.2 Particle size distribution (PSD)

Particle size distribution of tomato juice was determined by laser diffraction at 750 nm with a Mastersizer 2000 (Malvern® Inst., MA) (Illera et al., 2018). Size distributions (volume fractions against particle size) before and after HPCD treatment were calculated and the weight-average sizes expressed as:

- The equivalent surface area mean diameter: $D(3,2) = \sum n_c d_{lc}^3 / \sum n_c d_{lc}^2$ [2]
- The equivalent volume mean diameter: $D(4,3) = \sum n_c d_{lc}^4 / \sum n_c d_{lc}^3$ [3]

where d_{lc} is the diameter of the particle and n_c is the percentage of particles. Both properties were evaluated since the D[4,3] is highly influenced by large particles, whereas D[3,2] is more influenced by the small ones (Augusto et al., 2013).

Other useful parameters $d_{v,0.9}$, $d_{v,0.5}$ and $d_{v0.1}$ correspond to the particle size bellow which, 90%, 50% and 10% of the particles lie. PSD measurements of the treated samples were carried out immediately after treatment and for the untreated tomato juice PSD was determined right after juice preparation.

ζ-potential was determined with a Zetasizer Nano ZS apparatus, using the Laser Doppler Velocimetry techniques. Samples were diluted 1:50 with deionized water and filtered through 5 μm filter (Illera et al., 2018). ζ-potential gives an indication of the potential stability of a colloidal system and it is a good index of the colloidal electrostatic repulsive forces. Soluble pectin carries negative charge being important to keep a high ζ–potential. Particles with ζ -potentials more positive or negative than 30 mV or -30 mV are usually considered stable (Genovese and Lozano, 2001).

2.5 Kinetic data analysis

Enzyme inactivation by applying pressure and/or temperature has been described in the literature by different models, such as the first order, two-fraction, fractional-conversion and Weibull models. In this work, PG inactivation data were fitted to the fractional conversion model, while the Weibull model described PME inactivation data.

Fractional-conversion model. The fractional-conversion model is a special case of the first order kinetic model that takes into account the non-zero residual activity after prolonged heating and/or pressure (A_∞) treatment and it can be expressed as (Hu et al., 2013):

$$A = A_{\infty} + (A_o - A_{\infty})exp(-kt) \quad [4]$$

where A_o is the initial activity of the enzyme, A is the residual activity at different treatment times, A_∞ is the non-zero activity after prolonged heating and/or pressure treatment, k is the inactivation rate constant of the inactivated fraction at the operating conditions (min^{-1}) and t is the treatment time, min. By plotting A versus treatment time at constant pressure and temperature, the inactivation rate constant, k, and the remaining activity, A_∞, can be estimated by nonlinear regression analysis (Hu et al., 2013).

From the decimal reduction time, D, treatment time needed to achieve a 90 % inactivation of the initial enzyme activity at a certain operating pressure and temperature, z_T and z_P (temperature and pressure increase needed for a 90% reduction of the D value, respectively) were evaluated as the negative reciprocal slope of the regression line of log D as function of T or p respectively:

$$\log\left[\frac{D_1}{D_2}\right] = \frac{T_2 - T_1}{z_T} \quad [5]$$

$$\log\left[\frac{D_1}{D_2}\right] = \frac{p_2 - p_1}{z_p} \quad [6]$$

The dependence of the inactivation rate constant on temperature and pressure can be expressed through the Arrhenius and Eyring equations respectively:

$$\ln\left[\frac{k_1}{k_2}\right] = \frac{E_a}{RT}\left[\frac{1}{T_2} - \frac{1}{T_1}\right] \quad [7]$$

$$\ln\left[\frac{k_1}{k_2}\right] = \frac{V_a}{RT}[p_2 - p_1] \quad [8]$$

where p_2, p_1, T_2 and T_1 are pressures and temperatures corresponding to the decimal reduction times D_1 and D_2 or constants k_1 and k_2, respectively, R is the universal gas constant, E_a, the activation energy (kJ/mol) and V_a, cm^3/mol, is the activation volume.

177 ***Weibull model.*** The non-linear Weilbull model can be written in the power-law form (Van Boekel,
178 2002):

179 $$\log\frac{A}{A_o} = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta} \quad [9]$$

180 where α is the scale parameter (a characteristic time) and β is the shape parameter. The time
181 required to achieve a number of decimal reductions, d, can be calculated by using the shape and
182 scale parameters (Van Boekel, 2002):

183 $$t_d = \alpha\left(-ln\left(10^{-d}\right)^{\frac{1}{\beta}}\right) \quad [10]$$

184 **2.6 Statistical analysis**

185 Statistical analyses were conducted using software Statgraphics X64. The results are presented as
186 a mean ± standard deviation of at least three replicates. The significance of the differences was
187 determined based on an analysis of the variance with the Tukey's honestly significant difference
188 (HSD) method at p-value ≤ 0.05.
189 To estimate the kinetic parameters for the different models essayed in this work, non-linear
190 regression was performed by using the Marquardt algorithm (Statgraphics X64).

191 **3. Results and discussion**

192 **3.1 Mild thermal and HPCD inactivation of PME**

193 Figure 1a shows the thermal inactivation curves of PME under mild thermal treatment in the
194 temperature range from 35 to 55 ºC for 120 min heating. No thermal inactivation was observed at
195 the lowest temperatures essayed, 35 and 45 ºC. At 55 ºC, inactivation of tomato PME was observed
196 reaching a lowest residual activity of 70 ± 4% after 120 min.

Different values have been reported in the literature regarding mild thermal treatment of different tomato varieties (T ≤ 60ºC). For tomatoes of Alamanda cv., a PME residual activity of 50% was reported after 60 min heating at 55ºC (Andreou et al., 2016). These authors reported inactivation rate constants of 0.012 ± 0.002 and 0.088 ± 0.005 min^{-1} at 45 and 55ºC, respectively. Temperatures higher than 60 ºC were needed to get some PME inactivation by 5-min thermal treatment of tomatoes cv. Patrona (Houben et al., 2014). Inactivation rate constants for a first order kinetic model of 0.026 ± 0.003 and 0.0097 ±0.0005 min^{-1} were obtained at 60ºC for ripened tomatoes, Heinz 3402 variety and Nema 1401 variety, respectively (Terefe et al., 2009). The different inactivation data reported indicated that tomato variety played an important role on PME inactivation. The fastest PME inactivation kinetics were reported by van de Broeck et al. (Van Den Broeck et al., 2000) with a value of the inactivation rate constant of 0.152 ± 0.004 min^{-1} at 60ºC. However, in this case, tomato PME was not in its natural media but as commercial lyophilized powder dissolved in water, and in general, an enzyme is more stable in an intact tissue or in a homogenate where it is protected by the presence of other materials, such as proteins, carbohydrates and pectins, than in its purified form (Terefe et al., 2009).

PME inactivation kinetics by HPCD are plotted in Figures 2a and 2b. Figure 2a shows the effect of operating pressure in the range from 8.5 to 20 MPa at constant temperature of 45ºC. At this temperature, PME inactivation was observed for all the working pressures, unlike the results obtained at atmospheric pressure in which no PME inactivation was found at 45ºC. A pressure increase led to a faster inactivation rate. At 45ºC, in the pressure range from 8.5 to 20 MPa, CO_2 is in supercritical state, and a pressure increase, results in an increase in CO_2 density from 282 kg/m^3 at 8.5 MPa to 813 kg/m^3 at 20 MPa. In any case, at 45ºC and 20 MPa and after 120 min of treatment, still 42 % of PME residual activity was obtained

220 At constant pressure, 20 MPa, enzyme inactivation rate increased by increasing temperature (from
221 35 to 55 ºC) (Figure 2b). In addition to the intrinsic effect of temperature on enzyme inactivation,
222 by increasing temperature, although CO_2 density decreases, there is an improvement of mass
223 transport properties of CO_2, enhancing CO_2 diffusivity and the number of collisions between the
224 CO_2 and the enzyme. At 55ºC, nearly complete PME inactivation was determined (1.5 ± 0.5 %)
225 after 90 min of treatment at 20 MPa.

226 In the literature, it has been reviewed that enzyme inactivation by HPCD could be due to different
227 causes such as pH lowering, conformational changes of the enzyme and inhibitory effects of
228 molecular CO_2 due to formation of different complexes or decomposition of the enzyme by CO_2
229 (Hu et al., 2013). The lowering of pH it has been attributed to CO_2 dissolution into liquid food,
230 and dissociation into bicarbonate and carbonate, and hydrogen ions. However, in acidic juices,
231 such as tomato juice (pH = 4.09), carbonic acid will hardly dissociate because the dissociation
232 constant of carbonic acid and bicarbonate are $pK_a = 6.57$ and $pK_a = 10.62$, respectively (Zhou et
233 al., 2015). Zhou et al. (Zhou et al., 2009) studied the alteration in activity and structure of PME
234 from peel of Valencia oranges treated by HPCD, founding changes in the secondary and tertiary
235 structures. Conformational changes were also found in other type of enzymes such as lipases (Chen
236 et al., 2013; Melgosa et al., 2015) concluding that a decrease or increase in fluorescence intensity
237 of HPCD treated enzyme is related to its tertiary structure and with changes in enzyme activity.

238 PME inactivation kinetics were fitted to the first order kinetic model and the Weibull model. At
239 atmospheric pressure (Fig 1a), good fitting was obtained for both, the linear and the Weibull
240 model. At atmospheric pressure, the decimal reduction time determined by the first order kinetic
241 model at 55ºC was 807 ± 45 min. Some of the PME inactivation curves by HPCD were not properly
242 described by a first order kinetic model; therefore, only kinetic parameters for the Weibull are

243 listed in Table 1. Scale parameter, α, decreased with pressure and temperature. The shape factor,
244 β, was less than 1 and it was found to increase with temperature. However, there was not clear
245 trend of the shape factor with pressure, being nearly independent on pressure in the range from 10
246 to 20 MPa, and increasing at the lowest pressure studied, 8.5 MPa. Scale, α, and shape, β,
247 parameters were used to calculate the time required to inactivate 1 log ($t_{d=1}$) of PME. According
248 to the values of α and β, $t_{d=1}$ decreased with pressure in the range from 10 to 20 MPa, and an
249 anomalous value of $t_{d=1}$ was obtained at 8.5 MPa. Although, as it was previously described, faster
250 kinetics at the beginning of the treatment were observed by increasing pressure as it was also
251 indicated by the values of the scale parameter. $t_{d=1}$ was found to decrease also with operating
252 temperature at 20 MPa. At 55ºC, a $t_{d=1}$ of 77 min was calculated, being much lower than the value
253 obtained at atmospheric pressure at the same temperature, 1305 min. These results indicated that
254 temperatures higher than 45 ºC, around 55 ºC, are needed in HPCD treatment to get nearly
255 complete PME inactivation at 20 MPa.

256 According to van Boekel (Van Boekel, 2002), the scale parameter, α, could be modelled in a
257 similar way to the classical D value of the first order kinetic model, suggesting a linear dependence
258 of the log α on temperature and considering that the shape parameter, β, did not depend on
259 temperature:

260 $$log\alpha = a_1 - b_1 T \quad [11]$$

261 Although, in this work β was statistically significant dependent on temperature when tested at the
262 95% significance level for a linear relationship, a z_T' value was defined as suggested by van Boekel
263 (Van Boekel, 2002):

264 $$z_T^{'} = \frac{1}{b_1} \quad [12]$$

265 The value of the z_T' is listed in Table 1, together with the quality of the fitting to equation 11. The
266 z'_T value obtained in this work by HPCD is similar to the values reported in the literature for
267 thermal treatment, z_T. Terefe et al. (Terefe et al., 2009) reported a z_T value of 11.4 ºC in the
268 temperature range from 60 to 75 ºC for Heinz 3402 variety. A similar value was reported by
269 Raviyan et al. (Raviyan et al., 2005), with z_T = 12.3 ºC, for tomato cv Roma for thermal inactivation
270 in the temperature range from 50 to 72 ºC. An Arrhenius type equation was considered to relate
271 the inverse of the scale parameter, 1/α, with temperature. Although 1/α cannot be considered a
272 kinetic constant, the value obtained from the slope was calculated as 197 ± 31 kJ/mol. This value
273 was similar to the one reported by Terefe et al. (Terefe et al., 2009) for the inactivation of tomato
274 PME by thermal treatment, 193± 28 kJ/mol, in the temperature range from 60 to 75 ºC. Therefore,
275 similar PME sensitivity to temperature was determined by HPCD and by thermal treatment,
276 although the HPCD temperature range, 35-55 ºC was lower than the temperature range employed
277 by thermal inactivation.
278 In this work, a linear dependence of the log α on pressure was also found, being the shape
279 parameter not statistically significant dependent on pressure, when tested at the 95% significance
280 level for a linear relationship:
281 $$log\alpha = a_2 - b_2 p \qquad [13]$$
282 the inverse of the slope of log α versus p was also evaluated. Analogous to z'_T, z'_p was defined:
283 $$z'_p = -\frac{1}{b_2} \qquad [14]$$
284 z'_p value was evaluated as 43 ± 3 MPa. Comparing z'_T and z'_p values for PME it can be concluded
285 that PME was more sensitive to changes in temperature than in pressure. An Eyring type equation
286 was considered to relate the inverse of the scale parameter, 1/α, with pressure. Although 1/α is not
287 a kinetic constant, an V'_a was evaluated from the slope as -14 ± 1 cc/mol. The negative value of

this parameter indicated that PME inactivation was favored by increasing operating pressure. Although this value is much higher (lower in absolute value) than other V_a reported in the literature for different enzymes in juices treated by HPCD, such as PPO in cloudy apple juice with values of -251 cm^3/mol (Illera et al., 2018).

3.2 Thermal and HPCD inactivation of PG

The inactivation kinetics of PG at atmospheric pressure are shown in Figure 1b in the temperature range from 35 to 55 ºC. The shape of the inactivation curves indicates that some fraction of the PG remained stable even after long treatment in this temperature range. In this regard, the existence of two fractions with different PG activity in tomato fruit has been extensively documented in the literature: a thermolabile (PG2) and a thermostable fraction (PG1) (Fachin et al., 2003). At 35 and 45 ºC, low inactivation degree, with residual activities of 93 ± 5% and 84 ± 3% respectively, was observed. At 55 ºC, higher partial PG inactivation was obtained with residual activities of around 60 ± 2%. As for tomato PME, different values have been reported for PG inactivation under mild thermal treatment of different tomato varieties (T ≤ 60ºC). Andreou et al. (Andreou et al., 2016) determined the thermal inactivation of PG at 55 ºC with a residual activity around 80 % after 60 min of treatment for Alamanda cv.. Terefe et al. (Terefe et al., 2009) found that thermal treatment at 50 ºC did not have a significant effect on the PG activity of tomato Heinz 3402 cv, while residual activity of 37 % was reached at 60 ºC after 60 min of heating. Lower PG residual activity for ripened tomato var. Flandria Prince was obtained being around 20 % at 50 ºC and 60 ºC after 120 min heating (Fachin et al., 2003). At 55 ºC, a residual activity of 80% after 5 min heating was observed for Patrona cv. (Houben et al., 2014). Based on these results, it can be concluded that tomato variety plays an important role in PG inactivation, similar to PME.

Figures 3a and 3b show the PG inactivation kinetics by HPCD at constant temperature, 45ºC, in the pressure range from 8.5 to 20 MPa and at constant pressure, 20 MPa, in the temperature range from 35 to 55ºC, respectively. The shape of the inactivation kinetics curves was similar to that described above for mild heating treatment. This indicated that PG also presented two fractions with different HPCD resistance. Although a higher degree of inactivation was obtained for all HPCD experiments compared to the results obtained for mild heating, a complete inactivation of the enzyme was not obtained in any of the experiments carried out. A pressure increase led to lower residual activity, although based on the inactivation curves at different operating pressures, PG was not very HPCD pressure sensitive in the pressure range from 8.5 to 20 MPa. At 8.5 MPa, the residual activity was 72 ± 2%, and it only decreased down to 55 ± 5% at 20 MPa.

Based on the kinetic curves, the fractional conversion model was found to adequately describe mild thermal and HPCD inactivation curves. The kinetic parameters for tomato PG are presented in Table 2. The inactivation rate constant increased both, with pressure and temperature. The corresponding D values for the labile fraction are also listed in Table 2. Lower D values were obtained by HPCD than under mild heating treatment. In any case, the residual PG activity was still 41 ± 4% at the hardest conditions used in this work, 20 MPa and 55ºC.

In this work, first order kinetic model could not describe satisfactorily PG inactivation kinetics neither at atmospheric pressure nor by HPCD. However, Andreou et al. (Andreou et al., 2016) found that PG inactivation followed a first order kinetic model, in the temperature range from 55 to 75ºC at atmospheric pressure for Alamanda cv., with values of the inactivation rate constant ranging from 0.030 ± 0.005 to 0.137 ± 0.015 min^{-1}. The fractional conversion model has been also used in the literature to describe thermal inactivation kinetics of PG in tomato juice of different varieties such as Heinz 3402 cv (Terefe et al., 2014) and Flandria Prince (Fachin et al., 2003) in

the temperature range from 60 to 75ºC and 55 to 70ºC, respectively. For these two varieties different kinetic parameters (percentage of the stable fraction, A_∞, and inactivation rate constant for the labile fraction, k) have been reported. For instance, at 60ºC, for Heinz 3402 cv, $A_\infty = 37 \pm 6$ % and $k = 0.085 \pm 0.024$ min^{-1} (Terefe et al., 2009) while for Flandria Prince cv, $A_\infty = 14 \pm 4\%$ and lower kinetic reaction constant, $k = 0.048 \pm 0.005$ min^{-1}, were reported for the labile fraction (Fachin et al., 2003).

An Arrhenius type equation was considered to relate the inactivation rate constant of the labile fraction with temperature. From the slope, the activition energy of the labile fraction was calculated as 48 ± 14 kJ/mol and 27 ± 9 kJ/mol at atmospheric pressure and at 20 MPa by HPCD respectively, in the temperature range from 35 to 45 ºC. These values are much lower than the values reported in the literature for the PG labile fraction, 116 ± 25 and 228.35 kJ/mol, for Heinz 3402 cv and Flandria Prince cv respectively (Fachin et al., 2003; Terefe et al., 2009) and also compared with the values obtained for the PME. These differences could be attributed to different tomato varieties and degree of ripening, among other factors, and indicated that the PG labile fraction of Canario cv. is not very temperature sensitive in the temperature range from 35 to 55 ºC. This can be also observed in the values of z_T presented in Table 2, being much higher than z_T values for thermal inactivation of enzymes under pasteurization conditions. Sensitive pressure parameters were evaluated through equations 6 and 8, Va =-3.7 ± 0.8 cc/mol and z_P= 166 ± 38 MPa. These values showed that PG was neither very sensitive to temperature nor to pressure changes by HPCD treatment in the range covered in this work, from 35 to 55 ºC and 8.5 to 20 MPa, respectively.

3.3. Effect of HPCD treatment on particle size distribution

As described in the literature, tomato juice consists of suspended particles greater than 150 μm in diameter in colloidal serum (Wu et al., 2008). Figure 4 shows the PSD of the untreated tomato juice and HPCD treated samples at 45 °C for 60 min at different operating pressures in the range from 10 to 20 MPa. A bimodal PSD was obtained for all samples, both in the untreated juice and in HPCD treated samples. HPCD treatment resulted in a reduction of the particle size diameter of the tomato juice particles. On the one hand, the maximum peak corresponding to the larger particles moved from values of 416.9 μm to values of 182.0 μm for the juice treated at 10 MPa and 120 μm for the juice treated at 15 and 20 MPa. On the other hand, the peak corresponding to the smallest particles remains constant in all samples and around 0.7-0.8 μm, but, the number of particles with this size increased after HPCD treatment with operating pressure. For the untreated tomato juice, the total volume of all particles with diameter less than 10 μm represented 7% of the total particles volume, while this number increased up to 11, 37 and 49 % at 10, 15 and 20 MPa, respectively. This trend can be also observed in the values of D[4,3], D[3,2] and $d_v(0.1)$, $d_v(0.5)$ and $d_v(0.9)$ that progressively decreased by increasing operating pressure (Table 3).

This fact has been explained in terms of the homogenization effect caused by HPCD treatment due to several reasons, such as high internal stress surpassing the tensile strength of the particles when CO_2 is removed from the vessel (Niu et al., 2010). This effect has been also observed in other juices treated by HPCD such as orange or apple juice (Briongos et al., 2016; Illera et al., 2018).

The PSD changes of tomato juice caused by HPCD could involve modifications of the properties of particles and serum due to cell disruption and subsequent fragmentation. The suspended particles in tomato juice include intact or broken cells, long-chain polymers of cellulose, lignin, hemicellulose and water insoluble pectic materials (Wu et al., 2008). Cell fragmentation and released cell wall constituents, such as pectins and proteins, caused by HPCD might modified particle–particle interactions and hence juice stability. ξ potential was determined as an indicator

of the colloidal stability of the juice before and after HPCD treatment. Untreated tomato juice presented a ξ potential value of -16.8 ±1.6 mV. This negative value indicated that the juice particles were negatively charged. After HPCD treatment, slightly lower values of ξ potential were obtained with values of -18.4 ± 0.6, -15.9 ±1.7, -20.5 ±1.5 and -18.6 ± 0.5 mV at 8.5, 10, 15 and 20 MPa, respectively. However, this increase is not very important and it can be concluded that colloid stability is not modified after HPCD treatment.

3.4 Enzyme inactivation by HPP.

Enzyme inactivation data obtained by HPCD were compared with data obtained by using high hydrostatic pressure treatment (Figure 5). At the standard operating conditions at Hipebaric, PME activity of tomato juices (HPP-Air sample) was not affected by HPP, with a residual activity of 104 ± 7%. These results agree with the literature that reported tomato PME was very resistant to pressure up to 800 MPa (Crelier et al., 2001; Tangwongchai et al., 2000). Other authors even found an increase on PME activity with pressure in the range from 335 to 500 MPa, with a higher efficiency for PME inactivation of low pressure/mild temperature treatments (150 MPa/30ºC) (Hernández and Cano, 1998).

In the literature, it has been described a positive interaction of pressure with the presence of CO_2 in the sample, which could destroy or damage the structure of the enzyme (Ortuño et al., 2013). However, at the working conditions of 600 MPa, the presence of CO_2 did not bring an important additional inactivation of tomato PME, with a PME residual activity of the carbonate sample of 96 ± 4 %. This indicated that there was no significant interaction between HPP and CO_2. Slightly lower PME residual activity was obtained in the sample HPP-N_2. However, this difference might be considered practically unimportant since still high residual activity was observed. Corwin and Shellhammer (Corwin and Shellhammer, 2002) followed a similar experimental procedure, these

authors first carbonated enzyme preparation at atmospheric pressure and then the samples were treated by HPP, observing that CO_2 had an additional inactivation effect on PME from orange juice at 500 MPa and 25ºC for 3 min, but not at 800 MPa and 25ºC for 1 min. Ortuño et al. (Ortuño et al., 2013) applied HPP in carbonated samples and also a combination of carbonated samples and addition of gaseous CO_2 in the headspace of the packaged liquid food before HPP treatment. According to these authors, gaseous CO_2 into the headspace could dissolve into the sample during the HPP treatment and the CO_2 concentration inside the sample could be higher than in carbonated samples. These authors found that, on average, the addition of CO_2 did not improve the inactivation of PME of feijoa puree in a HPP process in the pressure range from 300 to 600 MPa.

Regarding PG inactivation by HPP, almost complete inactivation at 600 MPa for 5 min was obtained for all samples, although still a 3-4% of HPP resistant PG fraction was observed. In the literature it has been also described that PG in tomato juice can be totally inactivated at some temperature/pressure combination from pressure above 500 MPa and temperature around 20ºC (Crelier et al., 2001; Tangwongchai et al., 2000).

4. Conclusions

HPCD treatment has been found as a promising non-thermal technology to process tomato juice being more effective than mild thermal treatment in the same temperature range. Nearly complete PME inactivation was reached at 55ºC and 20 MPa while PG was found to be more HPCD resistant. The non-linear Weibull model and the fractional-conversion model fitted PME and PG inactivation kinetics, respectively.

HPP processing was also applied to tomato juices obtaining a different behavior in enzyme inactivation, reaching a nearly complete inactivation of PG and no PME inactivation.

HPCD induced a homogenization effect on tomato juice since particle size distribution was shifted to smaller particle size and colloidal stability was not affected by HPCD treatment

Based on these results regarding the enzyme inactivation it can be concluded that a selective inactivation of either PME or PG can be achieved by combining different non-thermal treatments. Further research should be done to elucidate the mechanism for enzyme inactivation by HPCD as well as to develop industrial applications for food preservation by HPCD.

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553 **Table 1.** Kinetic parameters of the Weibull model for the inactivation of tomato PME by thermal
554 and HPCD treatment

p, MPa	T, ºC	α, min	β	R^2	$t_{d=1}$, min
0.1	55	472 ± 95	0.82 ± 0.09	0.9794	1305
20	35	3082 ± 1298	0.40 ± 0.04	0.9830	24795
20	45	148 ± 12	0.53 ± 0.03	0.9930	714
20	55	29 ± 3	0.85 ± 0.09	0.9822	77
		z'_T = 10 ± 2 ºC (R^2 = 0.9706) ln (1/α) vs (1/T): 197 ± 31 kJ/mol (R^2= 0.9764)			
8.5	45	273 ± 31	0.72 ± 0.06	0.9894	869
10	45	265 ± 40	0.58 ± 0.06	0.9878	1116
15	45	205 ± 45	0.55 ± 0.09	0.9611	934
		z'_P = 43 ± 3 MPa (R^2 = 0.9877) ln (1/α) vs (p): -14 ± 1 cc/mol (R^2= 0.9877)			

555

556 **Table 2.** Kinetic parameters of the fractional model for the inactivation of tomato PG by thermal
557 and HPCD treatment

p, MPa	T, ºC	A_∞	k, min^{-1}	R^2	D, min
0.1	35	92.0 ± 0.5	0.14 ± 0.05	0.9236	16.5
0.1	45	84.4 ± 0.6	0.19 ± 0.04	0.9594	12.1
0.1	55	60 ± 1	0.44 ± 0.15	0.9637	5.3
		z_T = 40 ± 11 ºC; R^2= 0.9334			
		E_a = 48 ± 14 kJ/mol; R_2= 0.9607			
20	35	83 ± 2	0.18 ± 0.09	0.8373	12.8
20	45	59 ± 1	0.30 ± 0.06	0.9751	7.7
20	55	46 ± 2	0.34 ± 0.07	0.9764	6.8
		z_T = 72 ± 25 ºC; (R^2 = 0.8908)			
		E_a = 27 ± 9 kJ/mol; (R^2= 0.9019)			
8.5	45	72.8 ± 0.2	0.25 ± 0.01	0.9978	9.2
10	45	68.9 ± 0.8	0.27 ± 0.05	0.9809	8.5
15	45	64 ± 1	0.28 ± 0.06	0.9734	8.2
		z_P = 166 ± 38 MPa (R^2 = 0.9074)			
		ln (1/α) vs (p): -3.7 ± 0.8 cc/mol (R^2 = 0.9074)			

558

559 **Table 3.** Effect of operating pressure of HPCD treatment on the particle size distribution (μm) of tomato
560 juice.

Time	D[3,2]	D[4,3]	$d_v(0.1)$	$d_v(0.5)$	$d_v(0.9)$
Untreated	10.7 ± 0.2^{d}	355.7 ± 6.5^{d}	27.65 ± 0.23^{d}	284.9 ± 3.9	762.9 ± 1.7^{d}
10 MPa	6.9 ± 0.2^{c}	181.2 ± 3.2^{c}	14.44 ± 0.22^{c}	153.6 ± 1.2^{b}	370.5 ± 1.9^{c}
15 MPa	2.5 ± 0.1^{b}	90.9 ± 1.8^{b}	0.73 ± 0.03^{b}	54.3 ± 0.4^{a}	245.3 ± 2.8^{b}
20 MPa	1.8 ± 0.1^{a}	71.6 ± 1.2^{a}	0.63 ± 0.01^{a}	16.2 ± 0.8^{a}	211.8 ± 1.2^{a}

561 Data: mean ± SD (n=3). Different letters in a column indicate significant differences by the Tukey's
562 honestly significant difference (HSD) method at p-value ≤ 0.05.

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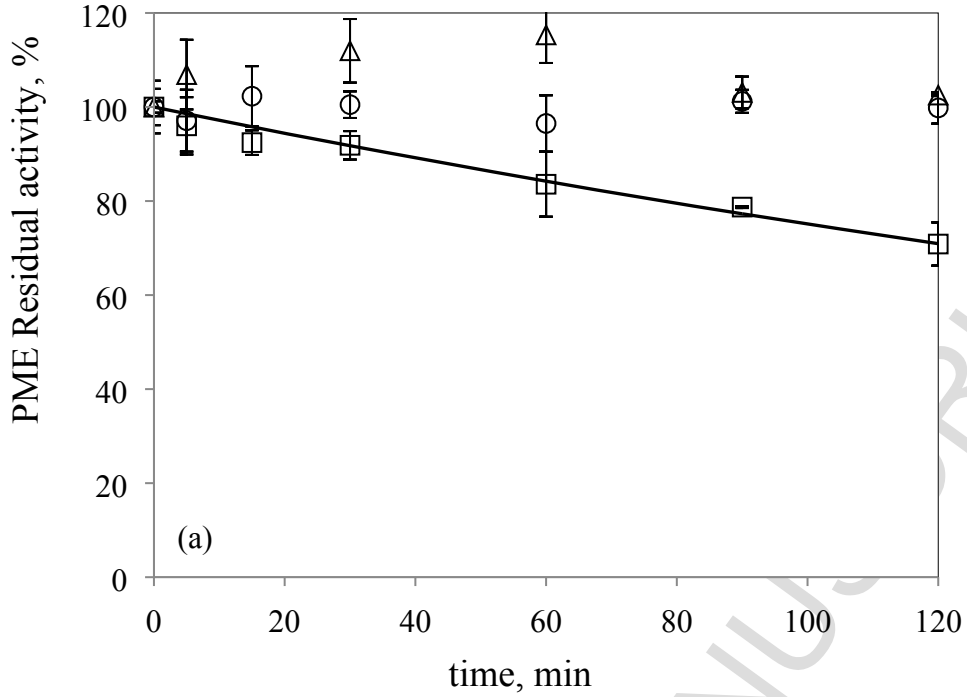

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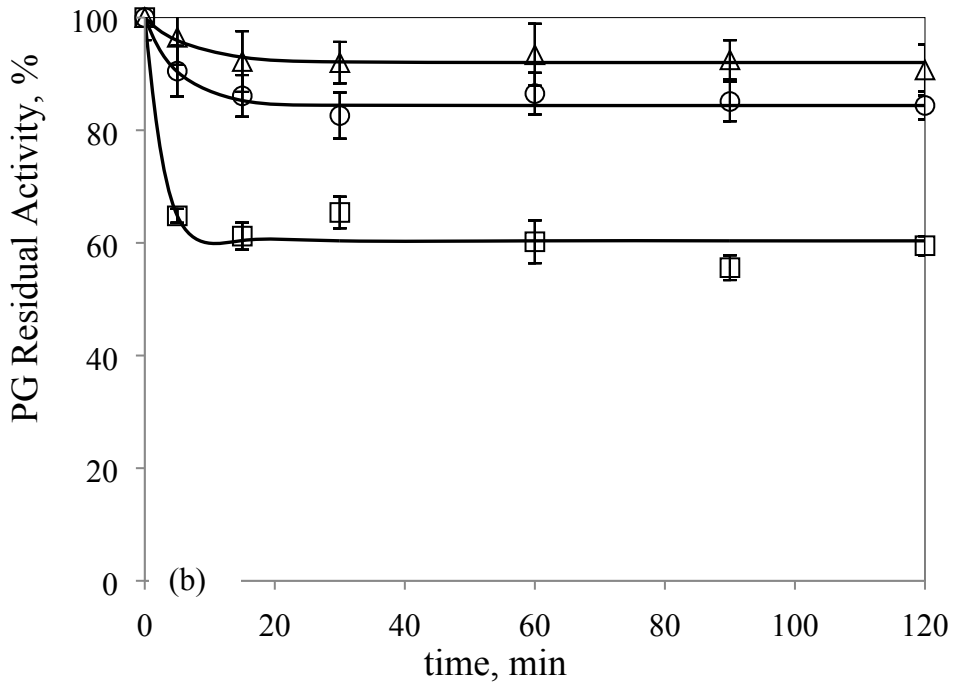


565

566 **Figure 1**: Inactivation kinetic data by mild heating treatment (a) PME (b) PG at different
567 temperatures: 35 ºC (Δ), 45ºC (○) and 55ºC (□). Continuous lines represent the Weibull model
568 (Figure 1a) and the fractional-conversion model (Figure 1b)

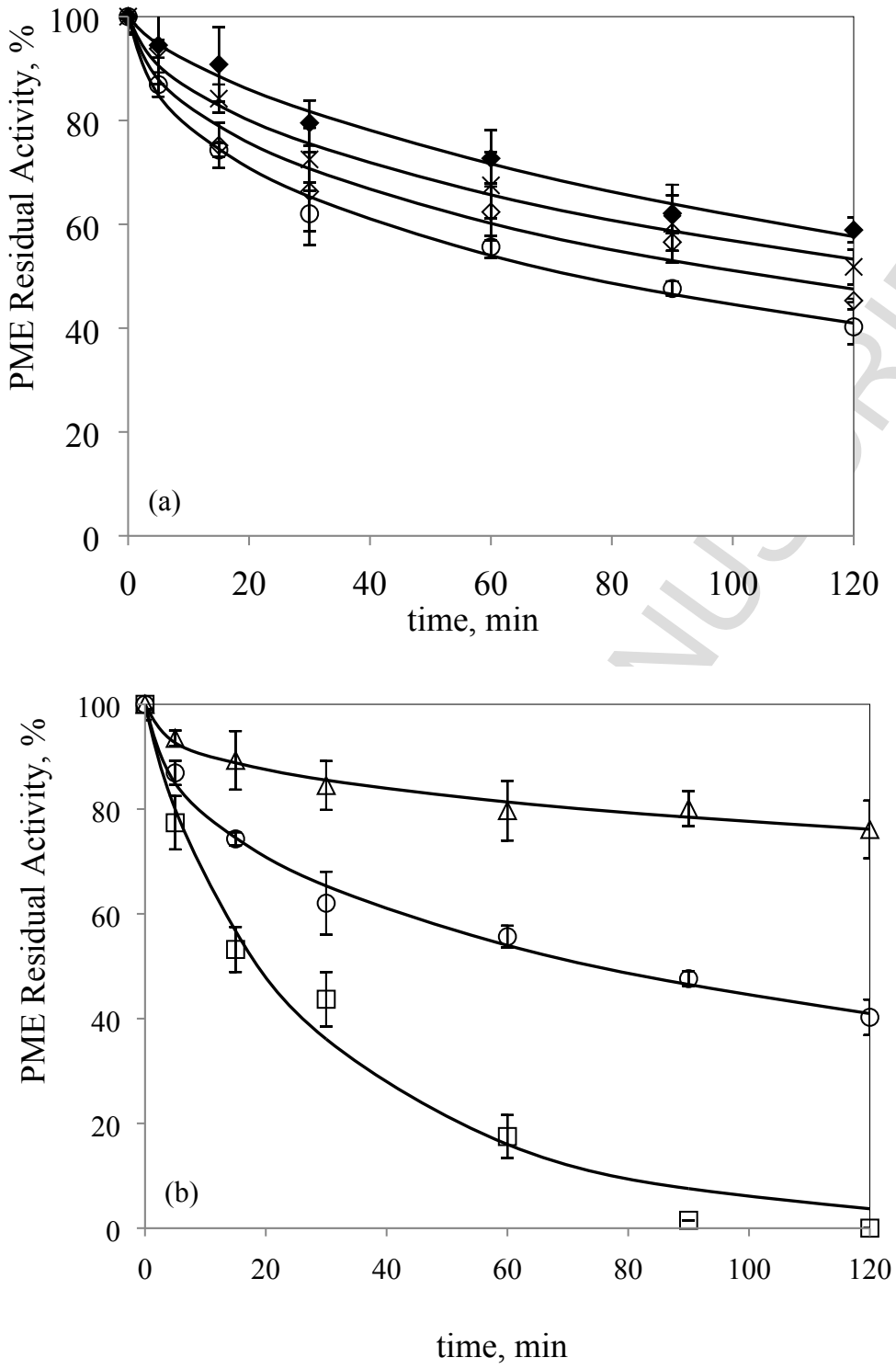
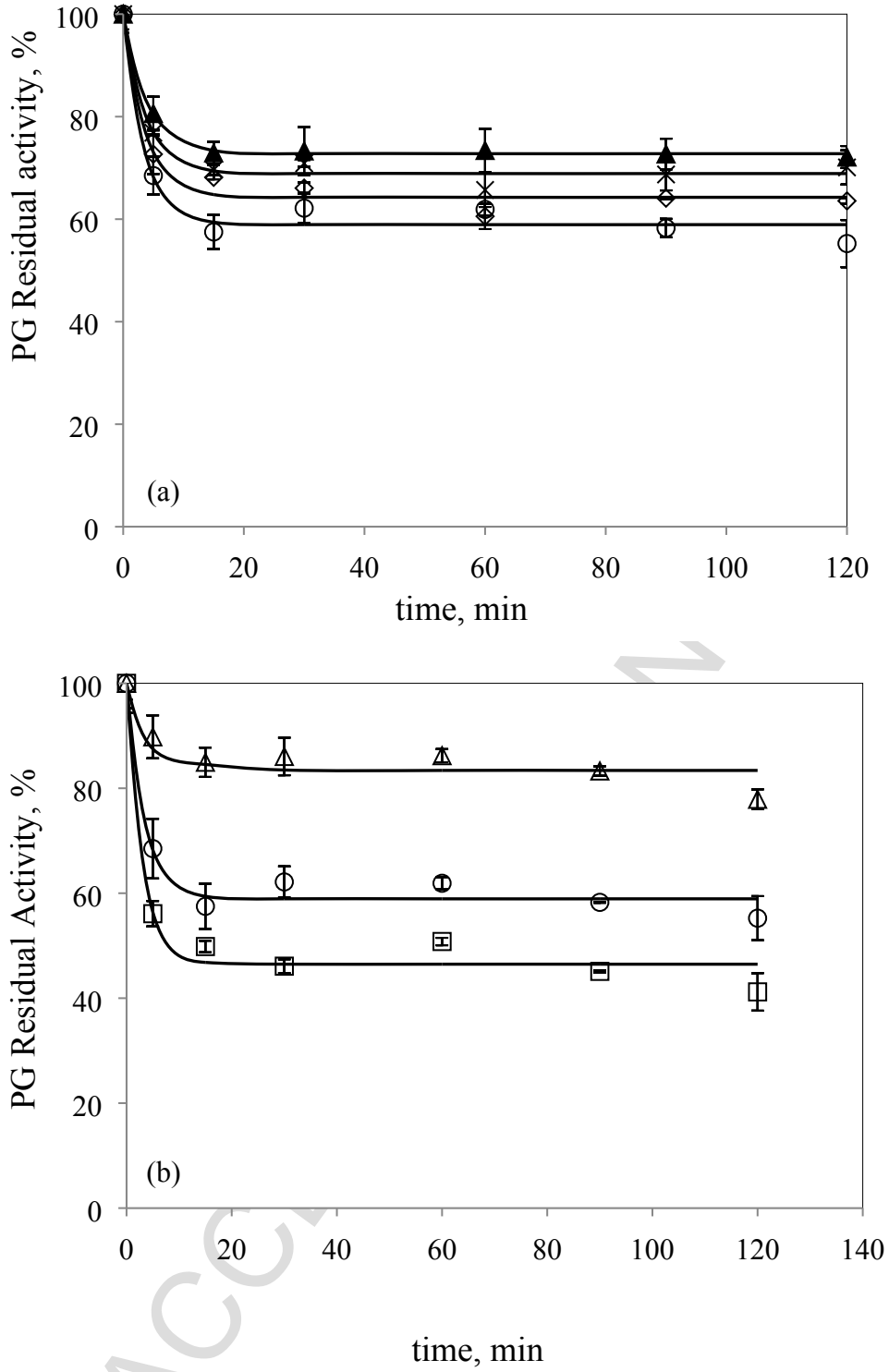


Figure 2: PME inactivation kinetic data by HPCD (a) At constant temperature (45 ºC) and different operating pressures: 20 MPa (○) 15 MPa (◊) 10 MPa (×) and 8.5 MPa (♦) (b) At constant pressure (20 MPa) and different operating temperature: 35 ºC (Δ), 45ºC (○) and 55ºC (□). Continuous lines represent the Weibull model



576

577

578 **Figure 3**: PG inactivation kinetic data by HPCD (a) At constant temperature (45 ºC) and different
579 operating pressures: 20 MPa (○) 15 MPa (◊) 10 MPa (×) and 8.5 MPa (♦) (b) At constant pressure
580 (20 MPa) and different operating temperature: 35 ºC (Δ), 45ºC (○) and 55ºC (□). Continuous lines
581 represent the fractional-conversion model.

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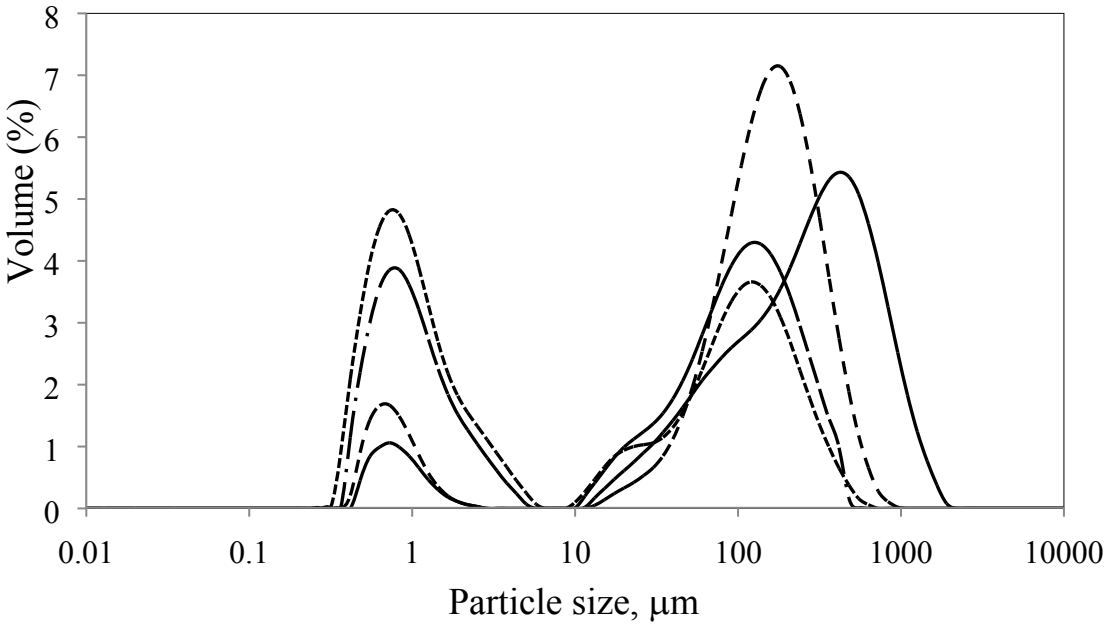

584

585 **Figure 4** Particle size distribution of fresh tomato juice (——) and treated by HPCD at different
586 operating pressures: 10 MPa, (− − − −), 15 MPa (·−·−·−·), 20 MPa (– – –) (T = 45ºC, treatment
587 time = 60 min)

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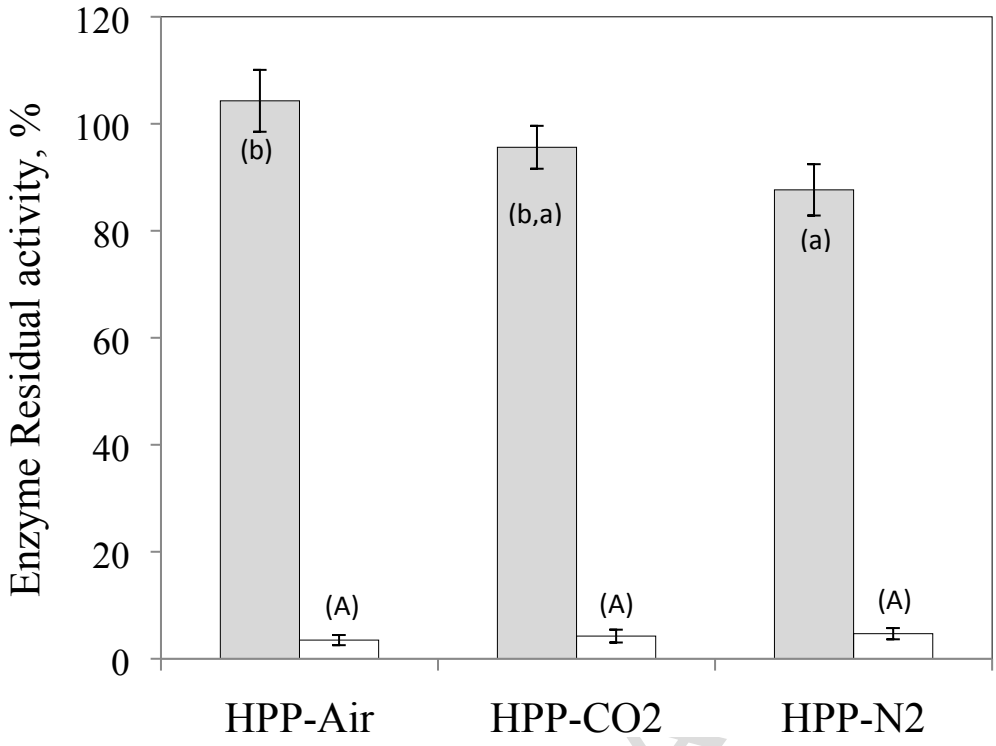

590

591 **Figure 5.** Residual PME (grey color) and PG (white color) activity in tomato juice after HPP with
592 different dissolved gases in the tomato juice. Data: mean ± SD (n=3). Different letters for each
593 enzyme indicate significant differences by the Tukey's honestly significant difference (HSD)
594 method at p-value ≤ 0.05.

595

Tomato PME was nearly complete inactivated by HPCD at 55ºC and 20 MPa

Tomato PG was found to be more HPCD resistant while it was inactivated by HPP

Selective enzyme inactivation could be reached combining HPCD and HPP treatments

HPCD decreased particle size of tomato juice but colloidal stability was not affected