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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 esculentum 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 $\alpha$-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 60$^\circ$C, 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 85-90$^\circ$C, 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 °C, 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 esculentum* 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 80°C, 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). CO₂ 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 °C 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 °C. Physical compression during pressure treatment results in a volume reduction and an increase in temperature and energy, approximately 3°C per 100 MPa. Therefore,
temperature of the juice during treatment was around 35°C (data provided by Hiperbaric, Burgos, Spain). However, due to fast temperature decrease during decompression, this treatment temperature was only held during treatment time (Yordanov and Angelova, 2010).

Three different tomato juice samples were treated by HPP to analyze the effect of the nature of the dissolved gasses into the tomato juice on enzyme inactivation. In two of the samples, air dissolved in the juice was displaced by bubbling CO$_2$ or N$_2$ into the juice (HPP-CO$_2$ and HPP-N$_2$ samples, respectively) until the O$_2$ concentration of the juice was below 0.4 mg/L (YSI ProODO optical dissolved oxygen meter). The air dissolved in the third sample was not removed (HPP-Air sample).

All the three samples were treated together in the high pressure unit. CO$_2$ permeability for PET is 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$^{-1}$ for N$_2$, O$_2$ and CO$_2$, respectively at 30°C (Zeman and Kubík, 2007). However, CO$_2$ loss is expected not be high in the first hour after carbonation when HPP experiments were carried out.

2.4 Physico-chemical analysis

2.4.1. Determination of enzyme activity

**Pectinmethyllesterase.** Samples were analyzed by using an automatic titrator system (Metrohm ® Titrando) similar to Briongos et al. (Briongos et al., 2016) but with different amount of juice. 50 mL of a 1% pectin solution (Alfa Aesar ® pectin citrus) prepared in NaCl 0.3 M was used as substrate. After the addition of 100 µL of tomato juice, pH was adjusted to 7.5 with NaOH 0.02 N. During pectin hydrolysis at 30 ºC, pH was maintained at 7.5 by adding NaOH 0.02 N. The amount of NaOH added for 15 min was recorded. One PME activity unit (UPE) is defined as the micromoles of carboxylic groups produced per minute and mL of juice at pH 7.5 and 30 ºC.

**Polygalacturonase.** PG activity was determined by following a similar procedure to the one proposed by Anthon et al. (Anthon et al., 2002) and Fachin et al. (Fachin et al., 2003) with some 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 35°C 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 °C.

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, A₀:

\[ \text{Residual activity} = \left( \frac{\text{Enzyme specific activity after treatment}}{\text{Enzyme specific activity of the untreated juice}} \right) \times 100 = \frac{A}{A_0} \times 100 \]  

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: 
  \[ D(3,2) = \frac{\sum n_c d_c^3}{\sum n_c d_c} \]  

- The equivalent volume mean diameter: 
  \[ D(4,3) = \frac{\sum n_c d_c^4}{\sum n_c d_c^3} \]
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 below 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 ($A_{\infty}$) treatment and it can be expressed as (Hu et al., 2013):
\[ A = A_\infty + (A_0 - A_\infty) \exp(-kt) \]  \[4\]

where \( A_0 \) is the initial activity of the enzyme, \( A \) is the residual activity at different treatment times, \( A_\infty \) 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, \( A_\infty \), 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:

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

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

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

\[ \ln\left[\frac{k_1}{k_2}\right] = \frac{E_a}{RT_1}\left[\frac{1}{T_2} - \frac{1}{T_1}\right] \]  \[7\]

\[ \ln\left[\frac{k_1}{k_2}\right] = \frac{V_a}{RT_1}\left[p_2 - p_1\right] \]  \[8\]

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

\[
\log_{A_0} A = - \frac{1}{2.303} \left( \frac{t}{a} \right) ^ \beta 
\]  

where \( \alpha \) is the scale parameter (a characteristic time) and \( \beta \) 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 (Van Boekel, 2002):

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

### 2.6 Statistical analysis

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

To estimate the kinetic parameters for the different models essayed in this work, non-linear regression was performed by using the Marquardt algorithm (Statgraphics X64).

### 3. Results and discussion

#### 3.1 Mild thermal and HPCD inactivation of PME

Figure 1a shows the thermal inactivation curves of PME under mild thermal treatment in the temperature range from 35 to 55 ºC for 120 min heating. No thermal inactivation was observed at the lowest temperatures essayed, 35 and 45 ºC. At 55 ºC, inactivation of tomato PME was observed 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 \((T \leq 60^\circ C)\). For tomatoes of Alamanda cv., a PME residual activity of 50% was reported after 60 min heating at \(55^\circ C\) (Andreou et al., 2016). These authors reported inactivation rate constants of \(0.012 \pm 0.002\) and \(0.088 \pm 0.005\ \text{min}^{-1}\) at 45 and \(55^\circ C\), respectively. Temperatures higher than \(60^\circ C\) 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 \(0.026 \pm 0.003\) and \(0.0097 \pm 0.0005\ \text{min}^{-1}\) were obtained at \(60^\circ C\) 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 \(0.152 \pm 0.004\ \text{min}^{-1}\) at \(60^\circ C\).

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 \(45^\circ C\). 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 \(45^\circ C\). A pressure increase led to a faster inactivation rate. At \(45^\circ C\), 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 \(45^\circ C\) and 20 MPa and after 120 min of treatment, still 42% of PME residual activity was obtained.
At constant pressure, 20 MPa, enzyme inactivation rate increased by increasing temperature (from 35 to 55 ºC) (Figure 2b). In addition to the intrinsic effect of temperature on enzyme inactivation, by increasing temperature, although CO$_2$ density decreases, there is an improvement of mass transport properties of CO$_2$, enhancing CO$_2$ diffusivity and the number of collisions between the CO$_2$ and the enzyme. At 55ºC, nearly complete PME inactivation was determined (1.5 ± 0.5 %) after 90 min of treatment at 20 MPa.

In the literature, it has been reviewed that enzyme inactivation by HPCD could be due to different causes such as pH lowering, conformational changes of the enzyme and inhibitory effects of molecular CO$_2$ due to formation of different complexes or decomposition of the enzyme by CO$_2$ (Hu et al., 2013). The lowering of pH it has been attributed to CO$_2$ dissolution into liquid food, and dissociation into bicarbonate and carbonate, and hydrogen ions. However, in acidic juices, such as tomato juice (pH = 4.09), carbonic acid will hardly dissociate because the dissociation constant of carbonic acid and bicarbonate are pK$_{a}$ = 6.57 and pK$_{a}$ = 10.62, respectively (Zhou et al., 2015). Zhou et al. (Zhou et al., 2009) studied the alteration in activity and structure of PME from peel of Valencia oranges treated by HPCD, founding changes in the secondary and tertiary structures. Conformational changes were also found in other type of enzymes such as lipases (Chen et al., 2013; Melgosa et al., 2015) concluding that a decrease or increase in fluorescence intensity of HPCD treated enzyme is related to its tertiary structure and with changes in enzyme activity.

PME inactivation kinetics were fitted to the first order kinetic model and the Weibull model. At atmospheric pressure (Fig 1a), good fitting was obtained for both, the linear and the Weibull model. At atmospheric pressure, the decimal reduction time determined by the first order kinetic model at 55ºC was 807 ± 45 min. Some of the PME inactivation curves by HPCD were not properly described by a first order kinetic model; therefore, only kinetic parameters for the Weibull are
listed in Table 1. Scale parameter, $\alpha$, decreased with pressure and temperature. The shape factor, $\beta$, was less than 1 and it was found to increase with temperature. However, there was not clear trend of the shape factor with pressure, being nearly independent on pressure in the range from 10 to 20 MPa, and increasing at the lowest pressure studied, 8.5 MPa. Scale, $\alpha$, and shape, $\beta$, parameters were used to calculate the time required to inactivate 1 log ($t_{d=1}$) of PME. According to the values of $\alpha$ and $\beta$, $t_{d=1}$ decreased with pressure in the range from 10 to 20 MPa, and an anomalous value of $t_{d=1}$ was obtained at 8.5 MPa. Although, as it was previously described, faster kinetics at the beginning of the treatment were observed by increasing pressure as it was also indicated by the values of the scale parameter. $t_{d=1}$ was found to decrease also with operating temperature at 20 MPa. At 55°C, a $t_{d=1}$ of 77 min was calculated, being much lower than the value obtained at atmospheric pressure at the same temperature, 1305 min. These results indicated that temperatures higher than 45 °C, around 55 °C, are needed in HPCD treatment to get nearly complete PME inactivation at 20 MPa.

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

$$\log \alpha = a_1 - b_1 T$$

[11]

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

$$z_T = \frac{1}{b_1}$$

[12]
The value of the $z'_T$ is listed in Table 1, together with the quality of the fitting to equation 11. The $z'_T$ value obtained in this work by HPCD is similar to the values reported in the literature for thermal treatment, $z_T$. Terefe et al. (Terefe et al., 2009) reported a $z_T$ value of 11.4 °C in the temperature range from 60 to 75 °C for Heinz 3402 variety. A similar value was reported by Raviyan et al. (Raviyan et al., 2005), with $z_T = 12.3$ °C, for tomato cv Roma for thermal inactivation in the temperature range from 50 to 72 °C. An Arrhenius type equation was considered to relate the inverse of the scale parameter, $1/\alpha$, with temperature. Although $1/\alpha$ cannot be considered a kinetic constant, the value obtained from the slope was calculated as $197 \pm 31$ kJ/mol. This value was similar to the one reported by Terefe et al. (Terefe et al., 2009) for the inactivation of tomato PME by thermal treatment, $193 \pm 28$ kJ/mol, in the temperature range from 60 to 75 °C. Therefore, similar PME sensitivity to temperature was determined by HPCD and by thermal treatment, although the HPCD temperature range, 35-55 °C was lower than the temperature range employed by thermal inactivation.

In this work, a linear dependence of the log $\alpha$ on pressure was also found, being the shape parameter not statistically significant dependent on pressure, when tested at the 95% significance level for a linear relationship:

$$\log \alpha = a_2 - b_2 p$$  \[13\]

the inverse of the slope of log $\alpha$ versus $p$ was also evaluated. Analogous to $z'_T$, $z'_p$ was defined:

$$z'_p = -\frac{1}{b_2}$$  \[14\]

$z'_p$ value was evaluated as $43 \pm 3$ MPa. Comparing $z'_T$ and $z'_p$ values for PME it can be concluded that PME was more sensitive to changes in temperature than in pressure. An Eyring type equation was considered to relate the inverse of the scale parameter, $1/\alpha$, with pressure. Although $1/\alpha$ is not a kinetic constant, an $V'_a$ was evaluated from the slope as $-14 \pm 1$ 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 ($T \leq 60$ºC). 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 75°C and 55 to 70°C, respectively. For these two varieties different kinetic parameters (percentage of the stable fraction, $A_\infty$, and inactivation rate constant for the labile fraction, $k$) have been reported. For instance, at 60°C, for Heinz 3402 cv, $A_\infty = 37 \pm 6 \%$ and $k = 0.085 \pm 0.024 \text{ min}^{-1}$ (Terefe et al., 2009) while for Flandria Prince cv, $A_\infty = 14 \pm 4\%$ and lower kinetic reaction constant, $k = 0.048 \pm 0.005 \text{ min}^{-1}$, 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 activation energy of the labile fraction was calculated as $48 \pm 14 \text{ kJ/mol}$ and $27 \pm 9 \text{ kJ/mol}$ at atmospheric pressure and at 20 MPa by HPCD respectively, in the temperature range from 35 to 45°C. 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°C. 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, $V_a = -3.7 \pm 0.8 \text{ cc/mol}$ and $z_p = 166 \pm 38 \text{ MPa}$. 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°C 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂. Slightly lower PME residual activity was obtained in the sample HPP-N₂. 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 CO₂ had an additional inactivation effect on PME from orange juice at 500 MPa and 25°C for 3 min, but not at 800 MPa and 25°C 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 CO₂ in the headspace of the packaged liquid food before HPP treatment. According to these authors, gaseous CO₂ into the headspace could dissolve into the sample during the HPP treatment and the CO₂ concentration inside the sample could be higher than in carbonated samples. These authors found that, on average, the addition of CO₂ 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 20°C (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 55°C 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.

**ACKNOWLEDGMENTS**

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

<table>
<thead>
<tr>
<th>p, MPa</th>
<th>T, ºC</th>
<th>α, min</th>
<th>β</th>
<th>R²</th>
<th>t_{d=1}, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>55</td>
<td>472 ± 95</td>
<td>0.82 ± 0.09</td>
<td>0.9794</td>
<td>1305</td>
</tr>
<tr>
<td>20</td>
<td>35</td>
<td>3082 ± 1298</td>
<td>0.40 ± 0.04</td>
<td>0.9830</td>
<td>24795</td>
</tr>
<tr>
<td>20</td>
<td>45</td>
<td>148 ± 12</td>
<td>0.53 ± 0.03</td>
<td>0.9930</td>
<td>714</td>
</tr>
<tr>
<td>20</td>
<td>55</td>
<td>29 ± 3</td>
<td>0.85 ± 0.09</td>
<td>0.9822</td>
<td>77</td>
</tr>
</tbody>
</table>

z'_{T} = 10 ± 2 ºC (R² = 0.9706)
ln (1/α) vs (1/T): 197 ± 31 kJ/mol (R² = 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 (R² = 0.9877)
ln (1/α) vs (p): -14 ± 1 cc/mol (R² = 0.9877)
Table 2. Kinetic parameters of the fractional model for the inactivation of tomato PG by thermal and HPCD treatment

<table>
<thead>
<tr>
<th>p, MPa</th>
<th>T, ºC</th>
<th>$A_\infty$</th>
<th>k, min$^{-1}$</th>
<th>$R^2$</th>
<th>D, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>35</td>
<td>92.0 ± 0.5</td>
<td>0.14 ± 0.05</td>
<td>0.9236</td>
<td>16.5</td>
</tr>
<tr>
<td>0.1</td>
<td>45</td>
<td>84.4 ± 0.6</td>
<td>0.19 ± 0.04</td>
<td>0.9594</td>
<td>12.1</td>
</tr>
<tr>
<td>0.1</td>
<td>55</td>
<td>60 ± 1</td>
<td>0.44 ± 0.15</td>
<td>0.9637</td>
<td>5.3</td>
</tr>
</tbody>
</table>

$z_T = 40 ± 11 ºC; R^2 = 0.9334$

$E_a = 48 ± 14 kJ/mol; R^2 = 0.9607$

| 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   |

$z_P = 166 ± 38 MPa (R^2 = 0.9074)$

$\ln (1/\alpha)$ vs (p): -3.7 ± 0.8 cc/mol (R$^2 = 0.9074$)
Table 3. Effect of operating pressure of HPCD treatment on the particle size distribution (μm) of tomato juice.

<table>
<thead>
<tr>
<th>Time</th>
<th>D[3,2]</th>
<th>D[4,3]</th>
<th>d_{v}(0.1)</th>
<th>d_{v}(0.5)</th>
<th>d_{v}(0.9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>10.7 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>355.7 ± 6.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>27.65 ± 0.23&lt;sup&gt;d&lt;/sup&gt;</td>
<td>284.9 ± 3.9</td>
<td>762.9 ± 1.7&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 MPa</td>
<td>6.9 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>181.2 ± 3.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.44 ± 0.22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>153.6 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>370.5 ± 1.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>15 MPa</td>
<td>2.5 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>90.9 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.73 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54.3 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>245.3 ± 2.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20 MPa</td>
<td>1.8 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.6 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.63 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.2 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>211.8 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data: mean ± SD (n=3). Different letters in a column indicate significant differences by the Tukey’s Honestly Significant Difference (HSD) method at p-value ≤ 0.05.
Figure 1: Inactivation kinetic data by mild heating treatment (a) PME (b) PG at different temperatures: 35 °C (Δ), 45°C (○) and 55°C (□). Continuous lines represent the Weibull model (Figure 1a) and the fractional-conversion model (Figure 1b)
Figure 2: PME inactivation kinetic data by HPCD (a) At constant temperature (45 °C) 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 °C (Δ), 45°C (○) and 55°C (□). Continuous lines represent the Weibull model.
Figure 3: PG inactivation kinetic data by HPCD (a) At constant temperature (45 °C) 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 °C (Δ), 45°C (○) and 55°C (□). Continuous lines represent the fractional-conversion model.
Figure 4 Particle size distribution of fresh tomato juice (-----) and treated by HPCD at different operating pressures: 10 MPa, (-- -- --), 15 MPa (-----), 20 MPa (-----) (T = 45°C, treatment time = 60 min)
Figure 5. Residual PME (grey color) and PG (white color) activity in tomato juice after HPP with different dissolved gases in the tomato juice. Data: mean ± SD (n=3). Different letters for each enzyme indicate significant differences by the Tukey’s honestly significant difference (HSD) method at p-value ≤ 0.05.
Tomato PME was nearly complete inactivated by HPCD at 55°C 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.