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PII: S0260-8774(17)30448-X

DOI: 10.1016/j.jfoodeng.2017.10.017

Reference: JFOE 9050

To appear in: *Journal of Food Engineering*

Please cite this article as: A.E. Illera, M.T. Sanz, S. Beltrán, R. Melgosa, A.G. Solaesa, M.O. Ruiz, Evaluation of HPCD batch treatments on enzyme inactivation kinetics and selected quality characteristics of cloudy juice from *Golden delicious* apples, *Journal of Food Engineering* (2017), doi: 10.1016/j.jfoodeng.2017.10.017

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HPCD inactivation rate of PPO and PME increased with increasing p and T.

Sensitivity to temperature change is similar for HPCD and mild thermal treatment.

PME is more HPCD resistant than PPO.

HPCD treatment decreased particle size of cloudy apple juice.

Colloidal stability and nutritional properties were not affected by HPCD.

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Evaluation of HPCD batch treatments on enzyme inactivation kinetics and selected quality characteristics of cloudy juice from *Golden delicious* apples

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Abstract

Cloudy apple juice has been treated by high pressure carbon dioxide (HPCD) as non-thermal technology to inactive polyphenoloxidase and pectinmethylesterase in batch mode. Stirring speed (from 200 to 600 rpm) induced an increase in the enzyme inactivation rate while a triple cycle of pressurization/depressurization led to the same enzyme inactivation efficiency. Enzyme inactivation kinetics were determined at different temperatures (from 35 to 45 ºC) and pressures (from 10 to 20 MPa). Data were described by the first order kinetic model and the Weibull model. For the first order kinetic model, decimal reduction time for HPCD treatment was found to be smaller than for mild heating, in the same temperature range. The same tendency was observed for the first decimal reduction time in the Weibull model. HPCD treatment resulted in a homogenization effect reflected in the shifting of the particle size distribution towards smaller diameters after treatment. HPCD treatment did not result in a change of water and oxalate soluble pectin content, total phenolic compounds and hidroxymethylfurfural content.

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Keywords: Cloudy apple juice, HPCD, inactivation kinetics parameters, properties

1. Introduction

Consumption of cloudy apple juice seems to be more beneficial than consuming clear apple juice due to changes in phenolic compounds and loss of pectins during clarification process (Markowski et al., 2015). However, color and cloud stability are main challenges in preservation of cloudy apple juice during its processing and storage. Enzymatic browning in cloudy apple juice is due to the action of polyphenol oxidase (PPO) that catalyzes the oxidation of phenolic compounds to quinones. On the other hand, the mechanism of cloud stability is not yet understood. Among the different mechanisms proposed, electrostatic repulsion by negative charges present in the partly demethylated pectin due to galacturonic residues could be responsible of cloud stability avoiding aggregation (Zhao et al., 2008). In this regard, the activity of pectinmethylesterase (PME) causes pectin demethylation that could precipitate with calcium ions present in the juice causing clarification and the loss of turbidity. However, other components with negative charge have been also reported in the literature as stabilizing agents of the cloud in the serum such as phosphatide acids of cloud lipids (Krapfenbauer et al., 2006).

Thermal treatments have been traditionally used for the inactivation of microorganisms and enzymes that affect negatively the quality of foods. However, these methods cause undesirable effects in quality attributes such as flavor and loss of nutritional components. High pressure carbon dioxide (HPCD) treatment has been proposed as an alternative non-thermal pasteurization technique that can inactivate certain microorganisms and enzymes under mild operation conditions. Typically, operating pressure does not exceed 50 MPa and temperature ranges between 20 and 50 ºC, below pasteurization temperature (Manzocco et al., 2016). The mechanism of pressurized CO_2 on enzyme inactivation is not yet fully understood. In the

literature different approaches can be found such as pH lowering due to dissolved CO_2, conformational changes or formation of different complex with CO_2 molecules (Hu et al., 2013).

Some studies have been found in the literature regarding the effect of HPCD on enzymes of cloudy apple juice from *Fuji* apples. Niu et al. (2010) evaluated the quality of cloudy *Fuji* apple juice processed from apple slices previously treated by HPCD. These authors found that PPO could be completely inactivated at 20 MPa for 20 min and at temperature as low as 25 ºC; however, residual activity of 18% was still found for PME after treatment at 65 ºC and 20 MPa for 20 min. Gui at al. (2007) found higher values of residual activity than the work of Niu et al. (2010), for PPO in HPCD-treated cloudy *Fuji* apple juice in a batch mode (40% at 30 MPa and 55 ºC for 60 min). When using a continuous HPCD system, holding times of 10 min were needed at 22 MPa and 60 ºC to achieve complete inactivation of PPO but, at the same working conditions, still PME residual activity of 42% was obtained (Xu et al., 2011).

From previous reported results, it can be clearly observed that there is a great variety in the literature data on enzyme inactivation from cloudy apple. This is a main drawback in the commercialization of this technology and reliable data on juice quality are still needed.

According to Wang et al. (2010) the influence of agitation on enzyme inactivation under high-pressure environment is a key factor in the design of reactors of industrial scale volume. However, the effect of stirring speed on enzyme activity has not been considered in most of the studies. Wang et al. (2010) determined the effect of agitation at 40ºC on isoamylase, obtained from a fermentation broth from *Pseudomonas amyloderamosa* after cell removal, and a commercial β-amylase under supercritical carbon dioxide, at different operating times (up to 120 min) and different operating pressure (from 11.0 to 17.2 MPa), observing that the activity of both enzymes decreased by increasing the speed of agitation.

In this work, the effect of HPCD treatment on some quality characteristics of cloudy apple juice from *Golden delicious* has been studied. The effect of some variables, non-previously studied in literature, on enzyme inactivation from cloudy apple juice, has been considered such as the effect of stirring speed and the number of successive pressurization/depressurization cycles. Enzyme inactivation kinetic data parameters are also necessary to scale-up HPCD process. This regard, enzyme inactivation kinetics of PPO and PME have been determined at different operating pressures and temperatures. Enzyme inactivation kinetic data have been described by the first order kinetic model and the Weibull model as an alternative to the classical first order kinetic model. Some other important quality parameters of cloudy apple juice have been also determined before and right after HPCD treatment, such as pectin content, particle size distribution, color and antioxidant capacity.

2. Materials and methods

2.1 Juice preparation

Golden delicious apples were peeled and cut in cubes and then added to a 0.3 % of L-ascorbic acid solution to avoid enzymatic browning during processing. Apple cubes were wiped and immediately squeezed with a screw juice extractor. The liqueur was filtered with 2 layers of cheesecloth. The pH of the juice obtained was 3.89 ± 0.01 and the soluble solid content was 12.5 ± 0.1 °Brix. The juice was stored frozen at −18 °C until further use.

2.2 HPCD equipment and processing

The HPCD cell was made of stainless steel (SS-316) and had an internal volume of 100 mL with a maximum operating pressure and temperature of 30 MPa and 80 ºC, respectively (Melgosa et al., 2017). For each experiment, 40 mL of apple juice were charged into the high pressure cell that was immersed in a thermostatic water bath at the operating temperature. The magnetic stirring was then connected and the system was pressurized by a using a syringe pump with a

pressure controller (ISCO 260 D) up to the desired pressure. CO_2 was fed into the high pressure cell through a sintered stainless steel micro-filter with a pore size of 10 µm (Briongos et al., 2016). This regard, Ishikawa et al. (1995) showed that the concentration of CO_2 in the sample was influenced by the way CO_2 was fed into the sample, increasing by decreasing the pore size of the filter at 25 MPa and 35ºC.

Samples were collected periodically to follow the inactivation kinetics of the enzymes. Experiments were carried out in the ranges of temperature (T) from 35 to 45 ºC, pressure (p) from 10 to 20 MPa and stirring speed from 200 to 600 rpm. The effect of the sampling procedure and the number of pressurization/depressurization cycles on enzyme activity was also analyzed.

To compare the effect of the HPCD treatment on enzyme activity with mild heating treatment, cloudy apple juice was heated at atmospheric pressure in the same temperature range (from 35 to 45 ºC). Samples were taken periodically at different treatment times up to 120 min.

Some other important quality attributes of cloudy apple juice, such as particle size distribution, ξ potential, turbidity, pH, pectin content, total phenolic compounds, non-enzymatic browning and antioxidant capacity were also determined before and right after HPCD treatment.

2.3 Physico-chemical analysis

2.3.1. Determination of PPO activity

The activity of PPO was determined spectrophotometrically by using a 0.05 M catechol (Sigma Aldrich) solution prepared in a 0.1 M phosphate buffer (pH 6.5) as substrate. Samples were analyzed by adding 100 µL of apple juice into 2.9 mL substrate solution. Oxidation of catechol was determined immediately by the increase in absorbance at 420 nm by 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. The PPO activity was taken as the very first linear part of the reaction curve.

2.3.2. Determination of PME activity

PME activity was determined by using an automatic titrator system (Metrohm ® Titrando) by using a 1 % of pectin solution (Alfa Aesar ® pectin citrus) prepared in NaCl 0.3 M as substrate. 50 mL of pectin solution was mixed with 1 mL of cloudy apple juice and 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 (Briongos et al., 2016).

Relative residual activities of PPO and PME were evaluated as:

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

2.3.3. Determination of pectic substances

Pectic substances in cloudy apple juice before and after HPCD treatment have been determined according to Robertson (1979) by progressive extraction of the alcohol insoluble solids by water (high methoxyl pectins are extracted) and ammonium oxalate (low methoxyl pectins are extracted). Water soluble pectins play an important role in the turbidity of the cloudy apple juices acting as colloid stabilizers, while oxalate-soluble fraction can form gels with polyvalent metal ions (Robertson, 1979). Pectic substances are determined spectrophotometrically at 520 nm after total hydrolysis to galacturonic acids by using m-hydroxydiphenil (MHDP) as a chromogenic reagent. Solutions were freshly prepared before analysis: 0.15% solution of m-hydroxydiphenyl (Sigma Aldrich) in 0.5% NaOH and 0.0125 M sodium tetraborate (Panreac) in concentrated sulphuric acid. For pectin determination, 1 mL of water extract and 5 mL of the sodium tetraborate solution were mixed and placed in a water-ice bath. The mixture is then heated in water bath at 80 ºC for 6 min and subsequently cooled again in a water-ice bath.

0.1 mL of the m-hydroxydiphenyl solution was added and after homogenization of the mixture, absorbance at 520 nm was measured as a function of time. According to Ibarz et al. (Ibarz et al., 2006) spectrophotometric data measurements were taken at the highest absorbance instead of at a certain reaction time. A calibration curve was prepared with standard solutions of D-galacturonic acid (Sigma Aldrich) by following the same colorimetric method. A blank was also prepared with no D-galacturonic acid.

2.3.4. Particle size distribution and zeta-potential

Particle size distribution (PSD) was determined by laser diffraction with a Mastersizer 2000 (Malvern® Inst., MA). The system uses a laser light at 750 nm wavelength to size particles from 0.4 to 2000 µm by light diffraction. Particle size distribution was calculated by the Fraunhofer model. 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.

To describe distribution width one common parameter is the Span:

$$\text{Span} = \frac{D_{v,0.9} - D_{v,0.1}}{D_{v,0.5}} \quad [4]$$

where $D_{v,0.9}$, $D_{v,0.1}$ and $D_{v0.5}$ are the particle size bellow which, 90%, 50% and 10% of the particles lies.

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. 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. Particles will repel each other when

having a large negative or positive ζ -potential. 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 normally considered stable (Genovese and Lozano, 2001).

2.3.5. Total phenolic compounds and antioxidant capacity

Total phenolic compounds were determined by using the Folin-Ciocalteau reagent (VWR). First, 100 µL of the juice were mixed with 2.8 mL of water and subsequently 100 µL of the Folin-Ciocalteau reagent, in that order. After that, 2 mL of sodium carbonate 7.5% (w/v) were added and the reaction started. Color was measured after 60 min of reaction at 750 nm at 21 ºC. A blank was also prepared using water instead of juice. A calibration curve was prepared with standard solutions of gallic acid by following the same colorimetric method.

Antioxidant capacity was determined by the ABTS method. The ABTS method is based on the decolorization of the radical cation 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$). This radical is produced by oxidation of a 7 mM solution of ABTS with potassium persulfate (2.45mM), allowing the mixture to stand in the dark at room temperature for 16 h before use (Re et al., 1999). The $ABTS^{\cdot+}$ solution was diluted with ethanol to reach an absorbance close to 0.7 at 734 nm and equilibrated at 21 ºC. Diluted $ABTS^{\cdot+}$ solutions were reduced in the presence of hydrogen-donating antioxidants present in the apple juice. For the antioxidant test, juice samples were diluted 1:10 with deionized water. A ratio of 100 µL/1 mL (diluted cloudy apple juice/$ABTS^{\cdot+}$ solutions) was chosen and absorbance was registered along time at 734 nm, since antioxidant activity depends on the selected time point (Re et al., 1999). All the spectrophotometric measurements have been carried out at 21 ºC in a Jasco (V-750) spectrophotometer with Peltier temperature control. Results were expressed as inhibition percentage of the radical $ABTS^{+}$.

2.3.6. Turbidity, pH, color and non-enzymatic browning

187 Turbidity of cloudy apple juice was measured at room temperature by diluting it 1/20 with
188 distillate water (Xu et al., 2011). A turbidimeter (Eutech Instruments, TN-100) was used and
189 turbidity was expressed as nephelometric turbidity units (NTU). pH of apple juice was
190 determined with a pH-meter (Crison® pH & Ion-Meter GLP 22).

191 The L*, a* and b* values were obtained respectively by a suitable program installed in a
192 Beckman DU-650 spectrophotometer with diode-array of UV–vis (Beckman Instruments),
193 representing brightness, red to green color and yellow to blue color, respectively. Other
194 conditions are illuminant D65 (daylight source) and a 10º standard observer (perception of a
195 human observer) following the CIE recommendations. Changes in color were expressed as:

$$\Delta E = \sqrt{\left(L^*_{before} - L^*_{after}\right)^2 + \left(a^*_{before} - a^*_{after}\right)^2 + \left(b^*_{before} - b^*_{after}\right)^2} \quad [5]$$

196 According to Krapfenbauer et al. (2006) a casual viewer can notice a difference between two
197 colors when ΔE is higher than 2-3.5, although they pointed out that a trained eye is capable of
198 differentiating two colors that differ by $\Delta E < 2$.

199 Non enzymatic browning reactions are important parameters that determine the quality of food
200 products during processing and storage. Non enzymatic browning in the juice is measured as
201 hydroxymethylfurfural (HMF) concentration. 0.7 mL of juice was mixed with 0.7 mL of ethanol
202 in a 1.5 mL Eppendorf tube. The mixture was centrifuged at 12000 *g* for 10 min. After that,
203 1 mL of the supernatant is mixed with 1 mL of a trichloroacetic acid solution (734 mM) and
204 1 mL of a thiobarbituric acid solution (25 mM) in a closed recipient. Samples were incubated at
205 40 ºC during 50 min in an agitated batch and absorbance was measure at 443 nm. A blank was
206 also prepared with distilled water instead of juice. A calibration curve was prepared using
207 different concentrations of HMF from 0.5 mg/L to 10 mg/L.

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2.4 Kinetic data analysis

The knowledge of the kinetic parameters that describe the time course of the enzyme inactivation is of great importance for a correct design of a HPCD process. Gui et al. (2007) obtained good results by fitting the inactivation kinetics of PPO in cloudy apple juice to a linear kinetic model. In this work, the inactivation kinetic data at different operating temperatures and pressures were described by using two different models: (1) a first-order kinetic model, and (2) the Weibull model.

The first-order kinetic model can be described as:

$$\log \frac{A}{A_o} = -kt \quad [6]$$

where A_o is the initial activity of the enzyme, A is the residual activity at different treatment times, k is the inactivation rate constant at the operating conditions (min^{-1}) and t is the treatment time (min).

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:

$$\log\left[\frac{D_1}{D_2}\right] = \frac{T_2 - T_1}{z_T} \quad [7]$$

$$\log\left[\frac{D_1}{D_2}\right] = \frac{p_2 - p_1}{z_p} \quad [8]$$

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

$$\ln\left[\frac{k_1}{k_2}\right] = \frac{E_a}{RT}\left[\frac{1}{T_2} - \frac{1}{T_1}\right] \quad [9]$$

$$\ln\left[\frac{k_1}{k_2}\right] = \frac{V_a}{RT}[p_2 - p_1] \quad [10]$$

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

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

$$\log \frac{A}{A_o} = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta} \quad [11]$$

where α is the scale parameter (a characteristic time) and β is the shape parameter. When n < 1 indicates upward concavity and n >1 downward concavity of the enzyme inactivation curve. The time required to achieve a number of decimal reductions, d, can be calculated by using the shape and scale parameters (Van Boekel, 2002):

$$t_d = \alpha\left(-ln(10^{-d})^{\frac{1}{\beta}}\right) \quad [12]$$

2.5 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 Fisher's least significant 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). The mean relative deviation (MRD) between experimental and calculated residual activities (RA) was also evaluated:

$$\mathrm{MRD} = \frac{1}{n}\left(\sum_{\text{all samples}}\left|\frac{RA_{calc} - RA_{exp}}{RA_{exp}}\right|\right)\cdot 100 \quad [13]$$

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3. Results and discussion

3.1 Agitation speed

The effect of agitation on the inactivation of PPO from cloudy apple juice has been evaluated at 20 MPa and 45 ºC, at three stirring speeds, 200, 400 and 600 rpm (Figure 1a). When working at higher stirring speeds, the magnetic system of agitation did not work properly in our experimental equipment; therefore stirring speeds higher than 600 rpm were not included in this work. At any of the stirring speeds, the inactivation degree increased with increasing time. From Figure 1a, it can be also observed that an increase in the stirring speed led to a higher inactivation degree. Agitation speed enhanced mass transfer rate of CO_2 in the medium, facilitating CO_2 solubilization and therefore the contact between CO_2 and the enzyme (Garcia-Gonzalez et al., 2009). In this regard, Wang et al. (2010) also pointed out that an increase in the agitation speed can create a larger amount of bubbles that help to enzyme inactivation. These authors deduced an expression for enzyme inactivation as a function of N·t (stirred speed·time) considering an equilibrium reaction between the native and the denatured enzyme and the consequently irreversible aggregation between the denatured enzymes:

$$(A_o/A)=1+cte(N\cdot t) \quad [14]$$

By plotting the inverse of the residual enzyme activity versus N·t a straight line should be obtained with the intercept equal to 1. Figure 1.b shows that for PPO inactivation in cloudy apple juice, the ratio (A_o/A) is proportional to N·t for operation times shorter than 60 min:

$$(A_o/A)=1+1.704\cdot 10^{-4}\,(N\cdot t)\ (R^2=0.940). \quad [15]$$

At longer treatment times, residual activities for all the three stirring speeds became nearly equal and were not included.

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3.2 Sampling procedure and number of pressurization/depressurization cycles

PPO activity after HPCD treatment was compared when sample was obtained after system depressurization or removed while the system was under pressure for a single cycle pressurization. HPCD treatment was carried out at 45 ºC and 20 MPa for 15 min. No significant differences have been determined among sample RA means of different sampling procedure when applying the Fisher's least significant method at p-value ≤ 0.05, with PPO RA values of 38 ± 4 % and 33 ± 4 % when sampling was withdrawn while the system was under pressure or after total depressurization, respectively.

The effect of a single cycle has been compared with a triple cycle for a total treatment time of 15 min, that is 5 min of pressure-treatment each cycle, respectively ($t_{total,\ treatment} = t_{per\ cycle}$·× number of cycles). The same effectiveness for PPO inactivation was determined when applying the Fisher's least significant method at p-value ≤ 0.05, with values of 38 ± 4 % and 33 ± 3 % for one single cycle and a triple cycle for a total treatment time of 15 min, respectively.

Primo et al. (2007) studied the effect of successive cycles of pressurization/depressurization in HPCD treatment on inactivation of PPO from mate tea leaves extracts. They observed that PPO presented a continuous loss of activity with the number of cycles at 7.05 MPa, 30 ºC and 1 h each cycle. However, to distinguish between the effect of the number of pressurization/depressurization cycles and treatment time, comparison should be have done at the same pressure and temperature conditions but for a total treatment time equal to $t_{per\ cycle}$·× number of cycles, since time plays also an important role in batch enzyme inactivation. In any case, as pointed out by Hu et al (2013) , the effect of pressurization/depressurization on the activity of enzymes depends on the type of enzyme, its environment, pressure, temperature and exposure time, being difficult to establish any rule. No other studies have been found in the

291 literature about the effect of the number of cycles on enzyme inactivation by HPCD on fresh
292 juices.

293 **3.3 Effect of mild heating on PPO inactivation**

294 PPO inactivation kinetics were determined at atmospheric pressure in the temperature range
295 from 35 to 45 ºC (same temperature range as for HPCD treatment, Figure 2). The maximum
296 reduction of PPO activity was nearly 40 % after 2 h of heating at 45 ºC indicating that PPO of
297 cloudy *Golden delicious* apple juice was rather stable under mild temperatures. Similar
298 inactivation degrees of PPO from cloudy *Fuji* apple juice were obtained by Gui et al. (2007).
299 After 1 hour of mild heating, these authors reached a maximum reduction of PPO activity of
300 20% at 45 ºC. However, Niu et al. (2010) found that when subjecting apple slices to mild heating
301 treatment from 25 to 45 ºC for 20 min and then processing them into cloudy apple juice, PPO
302 activity increased by 22-51%. Buckow et al. (2009) also found that at 45 ºC, PPO activity from
303 cloudy apple juice increased 30%, attributing this behavior to the release of latent PPO.
304 Enzyme inactivation kinetic data under mild heating conditions and at atmospheric pressure were
305 described by the first-order model. Parameters are collected in Table 1. Inactivation rate
306 constant, k, increased with the operating temperature, therefore the corresponding D values
307 decreased with temperature. The temperature sensitivity parameter, z_T, and the activation energy
308 were evaluated as 36 ºC and 53 kJ/mol, respectively (see Table 1). Values of the same order
309 were reported by Gui et al. (2007) for cloudy *Fuji* apple juice, 27.0 ºC for z_T and 72.0 kJ/mol, for
310 Ea. Thermal stability of extracted apple PPO from *Golden delicious* was studied in the
311 temperature range from 68 to 78 ºC by Yemenicigly et al. (1997). These authors reported a z_T
312 value of 9.9 ºC. The higher value obtained in this work for z_T in the temperature range from 35 to
313 45 ºC might indicate a lower susceptibility to temperature change at temperatures lower than the

one observed at pasteurization temperatures. In any case, the values of the activation energy were similar in both temperature ranges.

3.4. Effect of HPCD treatment on PPO inactivation

Enzyme inactivation kinetics for PPO of cloudy *Golden deliciuos* apple juice have been determined at 10 MPa in the temperature range from 35 to 45 ºC (Figure 3a). By increasing temperature, enzyme inactivation rate also increased. In addition to the intrinsic effect of temperature on enzyme inactivation, by increasing temperature, although CO_2 density decreased, the diffusivity of CO_2 can be improved and accelerate the molecular collisions between CO_2 molecules and the enzyme. Figure 3b shows the inactivation kinetics at 45 ºC in the pressure range from 10 to 20 MPa. Higher operating pressures led to faster inactivation rates, but the fraction of the resistant form of the enzyme was nearly independent of the operating pressure, being around 6 %. This value was lower than the minimal residual activity of PPO from cloudy *Fuji* apple juice reported by Gui et al. (2007), 37%, at 30 MPa and 55 ºC for 60 min. In any case, comparison with other HPCD systems is difficult to establish since the inactivation reached would depend not only on the type and source of the enzyme and the experimental conditions such as pressure, temperature and treatment time but also on the agitation of the system and the ratio sample volume/cell volume since this last parameter determines the amount of CO_2 injected to the system. Unfortunately in most of the studies these parameters are not detailed. PPO from cloudy *Fuji* apple juice was completely inactivated by a continuous HPCD system at 22 MPa and 60 ºC for 10 min (Xu et al., 2011). This could suggest a higher efficiency of a continuous system due to the turbulent energy, comparable with a good agitation in a discontinuous system. However, 60 ºC cannot be considered as non-thermal and residual activity as high as 47% was obtained in the continuous system at 43 ºC and 25 MPa for 2 min. (Xu et al., 2011).

Inactivation kinetic data by HPCD were fitted to the first order kinetic model and the Weibull model (Table 2). Although the Weibull model yielded the best fitting quality, the first order kinetic model described the inactivation curve quite well and therefore, kinetic parameters for the linear regression model have been also listed in Table 2 for comparison with data reported in the literature.

For the first order kinetic model, inactivation rate constant, k, increased with temperature and pressure. Consequently, D values decreased with increasing pressure and temperature. At each operating temperature, D values were smaller for HPCD treatment (Table 2) than the corresponding values for mild heating treatment (Table 1) proving that HPCD treatment was more effective than thermal treatment alone. For example D was equal to 515 min at 45 ºC and atmospheric pressure and decreased to 104 min at 45 ºC and 10 MPa.

z_T and activation energy values were determined as 29 ºC and 64 kJ/mol, respectively, for the first order kinetic model (see Table 2). z_T and activation energy values were of the same order as those listed for mild heating treatment (Table 1). This meant that the sensitivity to temperature change was similar for HPCD as for mild heating treatment in the temperature range covered in this work. The same tendency in z_T and E_a was found by Liu et al. (2008) in the inactivation of PPO of red beet extracts. These authors found that under mild heating treatment at atmospheric pressure (35-55 ºC), z_T and E_a were equal to 34 ºC and 57 kJ/mol, respectively. After HPCD treatment at 37.5 MPa, in the same temperature range, they obtained values of 36 ºC and 54 kJ/mol for z_T and E_a, respectively. However, Gui et al. (2007) found for PPO of cloudy *Fuji* apple juice that z_T increased from 27 to 108 ºC and activation energy decreased from 72 to 18 kJ/mol under mild heating treatment (35-55 ºC) and HPCD at 30 MPa in the same temperature range, respectively. This increase in z_T and decrease in Ea found by Gui et al. (2007) would indicate that the enzyme inactivation become less temperature dependent under

pressurized CO_2. This behavior is difficult to understand and it has not been observed in our work. As it has been previously mentioned, in addition to the intrinsic effect of temperature on enzyme inactivation, temperature has two different effects on important properties of CO_2. An increase in temperature involves an improvement of mass transport properties enhancing CO_2 diffusivity and the number of collisions between the CO_2 and the enzyme, but on the other hand, CO_2 density and therefore CO_2 solubility in the aqueous medium decreases. It seems that these effects play both an important role, since similar sensitivity to temperature was found in mild heat treatment and in HPCD.

By using equations 8 and 10 z_P and V_a have been determined as 25 MPa and -251 cm^3/mol, respectively for the first order kinetic model. The negative value of the activation volume indicated that PPO inactivation was favored by increasing operating pressure. Other values of activation volumes reported in the literature for PPO inactivation from different sources are of the same order, -271.7 and -307.9 cm^3/mol for the labile and stable fraction of PPO of water melon at 50 ºC in the pressure range of 8-30 MPa (Liu et al., 2013) or -94.31 cm^3/mol for PPO from cloudy *Fuji* apple juice at 55ºC in the pressure range from 8 to 30 MPa (Gui et al., 2007).

The Weibull parameters are also listed in Table 2. Scale, α, and shape, β, parameters were used to calculate the time require to inactivate 1 log ($t_{d=1}$) of PPO. Similar to the D-values for the first order kinetic model, $t_{d=1}$ decreased with operating pressure and temperature. As suggested by van Boekel (2002) is important to determine the effect of temperature, and in the present work of pressure, on both parameters of the Weibull model. Scale parameter was statistically significant dependent on temperature and pressure, when tested at the 95% significance level for a linear relationship, decreasing with pressure and temperature. The shape factor, n, was less than 1 except for 10 MPa and 35 ºC. The concavity shape of the inactivation curve could be understood as a faster inactivation of the labile form of the enzyme and the presence of a resistant form of

the enzyme to HPCD treatment (Figures 3a and 3b). The shape parameter, β, was found to be statistically significant dependent on pressure but although there seemed to be a trend in temperature dependence of β, it was not statistically significant, when tested at the 95% significance level for a linear relationship. In any case, it must be highlighted that only three temperatures have been studied to determine the effect of temperature. According to van Boekel (2002), the scale parameter, α, 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 α on temperature and considering that β did not depend on temperature:

$$log\alpha = a_1 - b_1 T \quad [16]$$

A z_T' value can be also defined:

$$z'_T = \frac{1}{b_1} \quad [17]$$

The value of the z_T' is listed in Table 2, together with the quality of the fitting. Although the concept of z_T and z'_T is different since z_T is obtained from the linear part, whereas z'_T takes also into account the nonlinear part (Van Boekel, 2002), similar values were obtained for both parameters (z_T = 29 ºC and z'_T = 27 ºC).

In this work, it was also found a linear dependence of the log α on pressure:

$$log\alpha = a_2 - b_2 p \quad [18]$$

Although it was found a statistically significant linear relationship between β and p, the inverse of the slope of log α versus p was also evaluated. Analogous to z'_T it was defined a z'_p

$$z'_p = -\frac{1}{b_2} \quad [19]$$

This value is reported in Table 2 and quite similar values can be observed for z_p and z_p'.

3.5. Effect of HPCD on PME

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To compare the HPCD resistance of PME and PPO, PME inactivation kinetics were determined for the experiments performed at 45 ºC and 10 MPa and 20 MPa (Figure 4). PME inactivation rate was also determined at atmospheric pressure and 45 ºC. At atmospheric pressure, it was found that residual PME activity was still 90% after 120 min at 45ºC (Figure 4). Teleszko et al. (2016) studied thermal treatment of different apple cultivars, founding that PME from cloudy *Golden deliciuous* apple juice was one of the apple cultivars that presented strongest thermostability (30% of residual activity after heating 5 min at 100 ºC). Niu et al. (2010) found that the RA of PME from cloudy *Fuji* apple juice from apple slices previously preheated for 20 min at the corresponding working temperature, varied between 82 to 72 % at 35 and 45ºC, respectively, but with error bars higher than 10% of RA at each temperature (graphical lecture). After HPCD treatment, higher inactivation rates were observed than for mild heating treatment at the same temperature. Although, residual activity was still around 60 % at 20 MPa and 45 ºC after 60 min. This indicates that PME from cloudy *Golden delicious* apple juice is rather stable to HPCD at operating temperatures considered as non-thermal treatment. Different HPCD inactivation values of PME from cloudy apple juice have been reported in the literature. Niu et al. (2010) reported a minimal residual activity of 18% for PME of cloudy *Fuji* apple juice from apple slices treated by HPCD at 20 MPa and 65 ºC for 20 min. In a continuous system, PME from cloudy *Fuji* apple juice still presented a residual activity of 40% at 22 MPa and 60ºC for 10 min (Xu et al., 2011). HPCD inactivation of PME extracts from apple juice was found to be more effectively inactivated at 30 MPa for 60 min, reaching 5% of residual activity at 55 ºC (Zhi et al., 2008). However, in this case, results are different to compare since an extracted enzyme suspended in a buffer solution can give different inactivation results from those obtained in an original juice.

Results obtained in this work, indicate that PME from cloudy apple juice was more resistant to HPCD than PPO in the pressure range covered in this work. In the literature, as for thermal treatment, the higher stability has been attributed to the more complex structure of PPO with three or four subunits, in higher plants, than PME, with one subunit. This structure, makes it more susceptible to HPCD treatment (Niu et al., 2010). In addition, PME in fruit juices is more difficult to be effectively inactivated, since PME is bound to the plant cell wall, which contains natural stabilizing factors (Zhou et al., 2010).

PME inactivation kinetic data were described by the first order model at atmospheric pressure (Table 1). The decimal reduction time at 45 ºC and atmospheric pressure was found to be much higher than for PPO at the same conditions. HPCD inactivation data at 45ºC and 10 MPa and 20 MPa were fitted to the first order kinetic model and the Weibull model (Table 2). The Weibull model yielded the best fitting quality, but still an acceptable good fitting for the first order kinetic model was obtained. For the first order model, inactivation rate constant increased with pressure, but lower values than for PPO inactivation were obtained and therefore higher D values. Regarding the Weibull model, the scale and shape parameters, both, decreased with operating pressure at 45ºC. These parameters were used to calculate the time needed to inactivate 1 log ($t_{d=1}$) of PME at the two pressures studied. The $t_{d=1}$ calculated by the Weibull model is much higher than the corresponding D values by the first order kinetic model. Therefore, in this case, the use of the first order kinetic model could lead to an underprocessing estimation for PME inactivation.

3.6. Effect of HPCD on different quality parameters of cloudy apple juice

Some quality parameters have been determined right after HPCD treatment at 45 ºC, 20 MPa and 60 min of treatment time.

453 **pH.** Initial pH of cloudy apple juice was 3.89 ± 0.01 and immediately after HPCD treatment, pH
454 dropped 0.15 units to 3.74 ± 0.01. Significant differences have been determined among sample
455 pH averages before and after HPCD treatment when applying the Fisher's least significant
456 method at p-value ≤ 0.05 (Table 3). Gui et al (2007) reported a drop of 0.3 units from 3.95 to
457 3.66 ± 0.03 for *Fuji* cloudy apple juice after HPCD treatment at 22 MPa and 55 ºC for 60 min.
458 According to Meysammi et al. (1992), juices and beverages can be considered as an aqueous
459 electrolyte system of multiple ions. Under pressurized CO_2 a number of dissociation reactions
460 can take place liberating some of them H^+ ions that could reduce the pH of the media. In any
461 case, after 2 h after depressurization pH returned to a value close to its initial value (pH = 3.87 ±
462 0.01).

463 **Colour parameters.** Table 3 lists the L*, a*, b* parameters of cloudy apple juice before and
464 after HPCD treatment. During browning, the L* values decrease, the juice's colour becomes
465 darker, while a* and b* values increase, with more red and yellow components (Krapfenbauer et
466 al., 2006). After HPCD treatment, lightness (L*) presented slightly, but significant, lower values;
467 however, 99% of the lightness was still retained. No significant difference was observed in the
468 redness component but a significant increase of the yellowness (b*) value has been observed
469 indicating more yellow components right after HPCD treatment. The change in colour, ΔE (Eq.
470 5) is also presented in Table 3. Slightly noticeable differences in color after HPCD treatment
471 have been determined, ΔE ≈ 0.51 ± 0.05 (Yuk et al., 2014). Other authors found that L*, a* and
472 b* values remained almost constant after HPCD treatment at 55 ºC and different operating
473 pressures (8-30 MPa) for 60 min (Gui et al., 2006).

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474 **Total polyphenolic compounds**. Total polyphenols remained unchanged after HPCD. Gasperi
475 et al. (2009) also found no significant differences in polyphenols content after HPCD treatment
476 of a blend of *Golden delicious* and *Granny Smith* apple at 10 MPa and 36 ºC for 10 min.
477 **Antioxidant capacity**. Antioxidant capacity of Golden cloudy apple juice has been determined
478 by the ABTS tests (Table 3). Slightly higher values were obtained after HPCD treatment
479 although differences before and after treatment were found to be no significant ($p \leq 0.05$).
480 **Hydroxymethylfurfural.** Table 3 presents the HMF content before and after HPCD treatment.
481 From these results, it can be concluded that non enzymatic browning reactions were not
482 significant during HPCD processing, probably due to the mild temperatures employed in HPCD
483 treatments.
484 **Particle size distribution.** The particle size distribution of untreated cloudy apple juice ranged
485 from 0.275 μm to 416.87 μm with three maxima at 0.63, 19.96 and 208.93 μm (see Figure 5).
486 After HPCD treatment, the particle range shifted to smaller particle sizes from 0.04 μm to
487 104.71 μm with one main maximum at 0.182 μm. Table 3 lists values of D[3,2] and D[4,3] of
488 freshly cloudy apple juice and after HPCD treatment. The values after HPCD treatment were
489 significantly lower than those of freshly cloudy apple juice. The homogenization effect of HPCD
490 can be also observed in the values of d(0.1), d(0.5) and d(0.9), which correspond to the size of
491 particle below which 10%, 50% and 90% of the sample lies, respectively. This homogenization
492 effect induced by HPCD could be attributed to the explosive action and the bubbling of CO_2
493 from the juice during decompression (Zhou et al., 2010).
494 For freshly cloudy apple juice, the total volume of all particles with diameter less than 5 μm
495 represented 56.5% of the total particles volume, while this number increased up to 96% after

HPCD treatment. This fact is also reflected in the Span values (Table 3) that shows a higher degree of uniformity of the cloudy apple juice after HPCD treatment.

Turbidity. The turbidity of the cloudy apple juice after HPCD is considerably higher than that of freshly cloudy apple juice (Table 3). It seems that there was a correlation between juice's turbidity and particle size distribution However, no correlation was found with PME residual activity since, after HPCD treatment, PME activity was still more than 60%.

Pectin content. In this work, soluble pectin presented slightly higher values after HPCD (Table 4). HPCD treatment could result in a more dissolution of pectin in fruit cell into the juice, increasing the value of soluble pectin. In addition, according to the PSD previously reported, HPCD induced an homogenization with lower particle size diameters, which could result in a higher solubility of the pectin; since, in the literature, it has been reported that pectin solubility in water increases by decreasing its polymer size (Sila et al., 2009) however no significant differences have been found at $p \leq 0.05$. No significant differences have been also determined for the oxalate-soluble pectin.

In the literature, Zhou et al. (2010) reported no change in water soluble pectin when treating peach juice by HPCD at 30 MPa and 55 ºC at different treatment times, although these authors observed an increase of the large particle after HPCD treatment. However, Yu et al (2013) observed that soluble pectin of HPCD-treated banana pulp at 20 MPa, during 30 min at different temperatures in the range from 45 to 60 ºC, was significantly lower than the untreated pulp. These authors attributed this behavior due to interaction of pectin and protein or gelling of pectin that may occur during HPCD treatment (Yu et al., 2013).

ξ potential. ξ potential is an indication of colloidal stability of the juice. Both, untreated and treated apple juice presented negative values of -22.0 mV and -22.3 mV, respectively. These values were within the range reported by Corak and Corredig (2006) of ξ potential as a function

of pH for orange juice. Negative values of ξ-potential indicated that juice particles were negatively charged. The extent of this negative surface charge before and after HPCD treatment involves electrostatic charge repulsion which has been established as one of the mechanisms of cloud stabilization (Corak and Corredig, 2006). Since no significant differences at p-values ≤ 0.05 were found before and after treatment, it indicates that colloid stability is kept after HPCD treatment.

Conclusions

HPCD treatment is a valid alternative technology to process *Golden delicious* cloudy apple juice being more effective than mild thermal treatment in the same temperature range.

PPO and PME inactivation rate increased whit operating pressure and temperature, although PME was found to be more HPCD resistant compared to PPO. The non linear Weibull performed a better fitting of the enzyme inactivation kinetics by HPCD than the classical first order kinetic model.

However, further research is needed to have reliable data on HPCD as cold pasteurization method, since different results are found in the literature for the same fruit variety. Important variables in enzyme inactivation are usually not described in the research articles such as the way the CO_2 is fed into the sample, agitation speed or the ratio juice/volume of the cell that in fact determined the ratio juice/CO_2. In this work, it was observed that the stirring speed was an important factor to take into account for enzyme inactivation, due to an improvement in CO_2 diffusivity

HPCD induced a homogenization effect on cloudy apple juice since particle size distribution was shifted to smaller particle size. Colloidal stability is not affect by HPCD treatment as well as the pectic content substances. Slightly noticeable differences in color after HPCD treatment have

been determined ($\Delta E \approx 0.5 \pm 0.1$). Other quality parameters in cloudy apple juice, such total polyphenolic compounds, antioxidant capacity or hydroxymethylfurfural content did not change after HPCD treatment. Other important aspects need also to be addressed for an industrial application such as sensory quality, shelf life studies and economic aspects.

ACKNOWLEDGMENTS

To the Spanish Government (MINECO) and the European Regional Development Fund (ERDF) for financial support of project CTQ2015-64396-R and AEI's contract. To MINECO for RM's pre-doctoral contract (BES-2013-063937). To University of Burgos for AGS's pre-doctoral contract.

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657 Table 1. Kinetic parameters at 0.1 MPa for the first order kinetic model for PPO inactivation by
658 mild thermal heating (35-45ºC) and PME at 45 ºC.

PPO	First order kinetic model			
T, ºC	k, min^{-1}	D, min	R^2	MRD
35	0.00102 ± 0.00003	980	0.9814	1.6
40	0.00166 ± 0.00005	602	0.9791	1.1
45	0.00194 ± 0.00003	515	0.9993	1.1
	z_T = 36 ± 11 ºC (R^2 = 0.9188) E_a = 53 ± 14 kJ/mol (R^2 = 0.9248)			
PME, 45ºC	0.00038 ± 0.00003	2632	0.9799	0.8

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659 Table 2. Kinetic parameters for the Weibull and the first order kinetic models for PPO and PME inactivation by HPCD.

PPO		Weibull model					First order kinetic model			
p, MPa	T, ºC	α, min	β	$t_{d=1}$, min	R^2	MRD	k, min^{-1}	D, min	R^2	MRD
10	35	98 ± 2	1.13 ± 0.06	205	0.9940	2.3	0.0044 ± 0.0001	227	0.9891	3.4
10	40	72 ± 2	0.93 ± 0.04	177	0.9939	3.4	0.0060 ± 0.0001	167	0.992	3.9
10	45	41 ± 2	0.89 ± 0.05	105	0.9960	4.9	0.0096 ± 0.0002	104	0.992	6.8
		z'_T = 27 ± 4 ºC; (R^2 = 0.9726)					z_T = 29 ± 3 ºC; (R^2 = 0.9897) E_a = 64 ± 8 kJ/mol; (R^2 = 0.9834)			
12.5	45	30 ± 1	0.74 ± 0.02	93	0.9983	2.9	0.015 ± 0.001	67	0.966	23.6
15	45	23.7 ± 0.4	0.70 ± 0.02	78	0.9995	1.2	0.019 ± 0.001	53	0.953	15.6
20	45	17.2 ± 0.9	0.59 ± 0.02	71	0.9981	5.9	0.026 ± 0.004	38	0.965	44.3
		z'_P = 27 ± 3 MPa; (R^2 = 0.9729)					z_P = 25 ± 5 MPa; (R^2 = 0.9226) Va=- 251 ± 48 cm^3/mol; (R^2 = 0.9332)			
PME										
p, MPa	T, ºC	α, min	β	$t_{d=1}$, min	R^2	MRD	k, min^{-1}	D, min	R^2	MRD
10	45	785 ± 294	0.55 ± 0.09	3576	0.9587	2.3	0.0015 ± 0.0001	667	0.9424	5.2
20	45	373 ± 74	0.45 ± 0.04	2380	0.9940	1.1	0.0037 ± 0.0006	270	0.9181	8.6

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663 Table 3. Some quality parameters before and right after HPCD treatment of cloudy apple juice at
664 20 MPa and 45 ºC after 60 min.

Parameter	Before HPCD	After HPCD
pH	3.89 ± 0.006^{a}	3.74 ± 0.006^{b}
Color $\Delta E = 0.51 \pm 0.05$	$L^* = 37.30 \pm 0.03^{a}$	$L^* = 36.98 \pm 0.01^{b}$
	$a^* = 1.6 \pm 0.1^{a}$	$a^* = 1.59 \pm 0.08^{a}$
	$b^* = 7.90 \pm 0.03^{a}$	$b^* = 8.26 \pm 0.07^{b}$
Total polyphenols, mg galic/L	456.1 ± 13.1^{a}	455.4 ± 4.3^{a}
ABTS, % of inhibition	20 min, 49 ± 2^{a}	20 min, 52 ± 3^{a}
	60 min, 65 ± 2^{a}	60 min, 68 ± 4^{a}
	120 min, 81 ± 3^{a}	120 min, 85 ± 4^{a}
Non-enzimatic browning, (mg HMF/L)	2 ± 0.3^{a}	1.70 ± 0.09^{a}
PSD	$D(3,2) = 1.7 \pm 0.07\ \mu m^{a}$	$D(3,2) = 0.21 \pm 0.01\ \mu m^{b}$
	$D(4,3) = 109 \pm 3\ \mu m^{a}$	$D(4,3) = 2.4 \pm 0.2\ \mu m^{b}$
	$d(0,1) = 0.59 \pm 0.01\ \mu m^{a}$	$d(0,1) = 0.099 \pm 0.001\ \mu m^{b}$
	$d(0,5) = 13 \pm 1\ \mu m^{a}$	$d(0,5) = 0.265 \pm 0.006\ \mu m^{b}$
	$d(0,9) = 337 \pm 7\ \mu m^{a}$	$d(0,9) = 1.63 \pm 0.07\ \mu m^{b}$
	$Span = 26 \pm 7\ \mu m^{a}$	$Span = 5.8 \pm 0.1\ \mu m^{b}$
Turbidity, NTU	105 ± 2^{a}	168 ± 3^{b}
Water soluble pectin	22 ± 2^{a}	28 ± 3^{a}
Oxalate soluble pectin (mg galacturonic/L)	23 ± 4^{a}	23 ± 3^{a}
ξ-potential	-22.0 ± 0.3^{a}	-22.3 ± 0.4^{a}

665 Values with different letters in **each row** (a, b) are significantly different when applying the Fisher's least significant
666 method at p-value ≤ 0.05.
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668 **List of Figure captions**

669 **Figure 1. (a)** Effect of stirring speed on PPO inactivation from cloudy apple juice by HPCD at
670 20 MPa and 45 ºC (◇ 200 rpm, Δ 400 rpm, ○ 600 rpm). **(b)** Inverse of residual activity of PPO
671 versus the product of number of revolutions per time and treatment time (N·t) at 20 MPa and 45
672 ºC (stirring speed range: 200 to 600 rpm). The continuous line corresponds to the linear fitting
673 (Eq. 15).

674 **Figure 2.** Inactivation of PPO in cloudy apple juice at atmospheric pressure at different mild
675 temperatures (35 ºC ◇, 40 ºC ○, 45 ºC □). The continuous lines represent the first order model
676 (Table 1).

677 **Figure 3.** Inactivation of PPO in cloudy apple juice by HPCD **(a)** 10 MPa and different
678 temperatures (35 ºC ◇, 40 ºC ○, 45 ºC □) **(b)** 45ºC and different pressures (10 MPa □, 12.5 MPa
679 Δ, 15 MPa ◇, 20 MPa ○). The continuous lines represent the Weibull model (Table 2).

680 **Figure 4**. Inactivation of PME in cloudy apple juice under mild heating treatment at 45ºC (Δ
681 atmospheric pressure) and by HPCD at 45 ºC (10 MPa □, 20 MPa ○). The continuous lines at
682 atmospheric pressure represent the first order kinetic model (Table 1), while in HPCD treatment
683 represent the Weibull model (Table 2).

684 **Figure 5.** Particle Size Distribution (PSD) of cloudy apple juice before treatment (─) and after
685 HPCD treatment at 45 ºC, 20 MPa for 60 min (-·-·).

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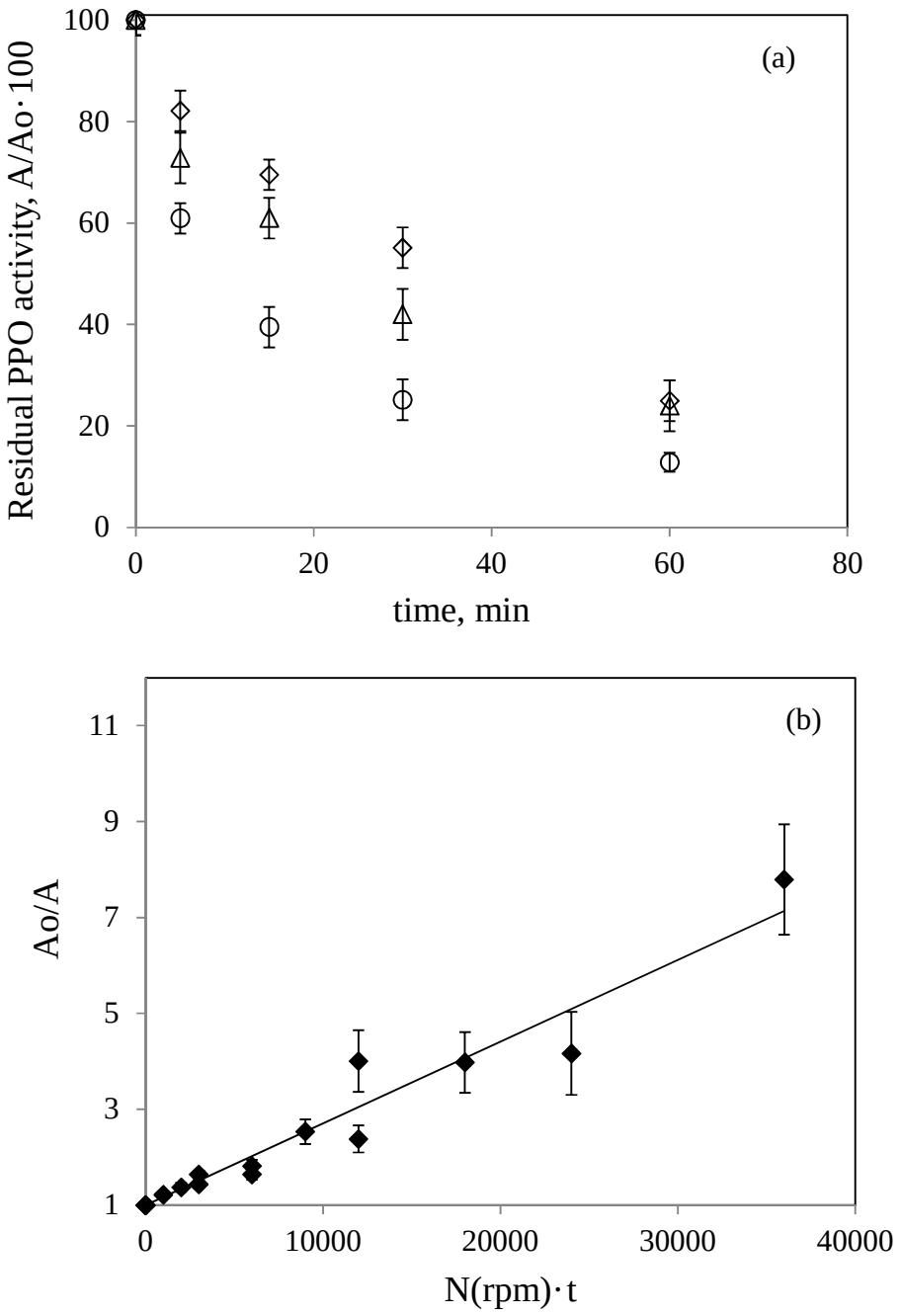

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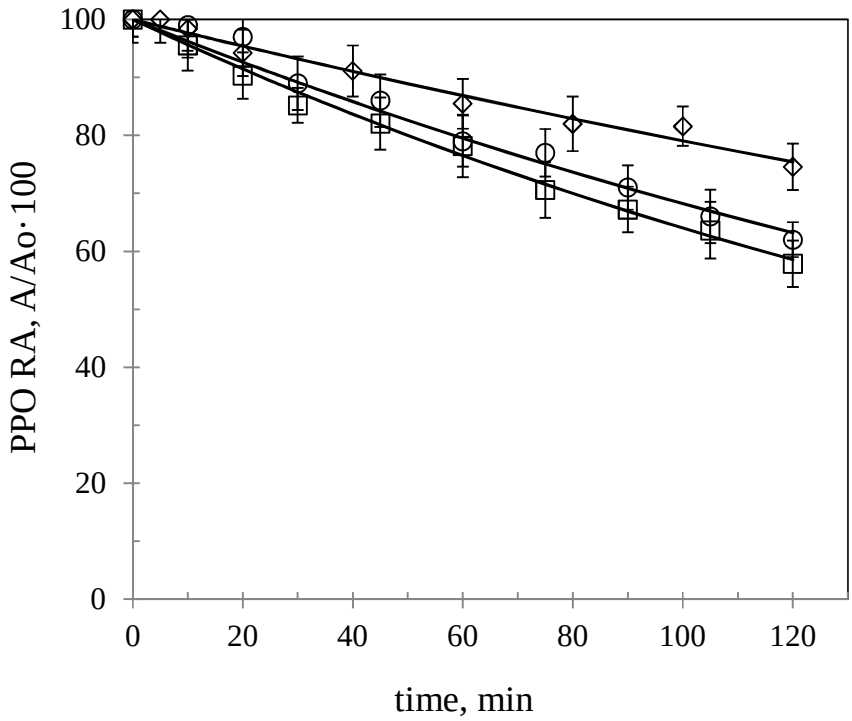

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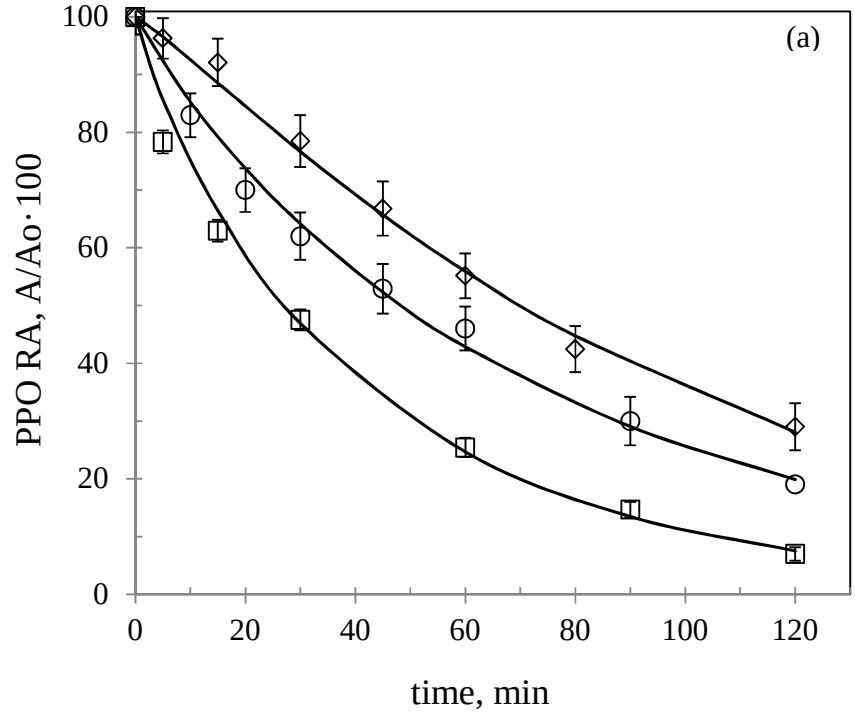

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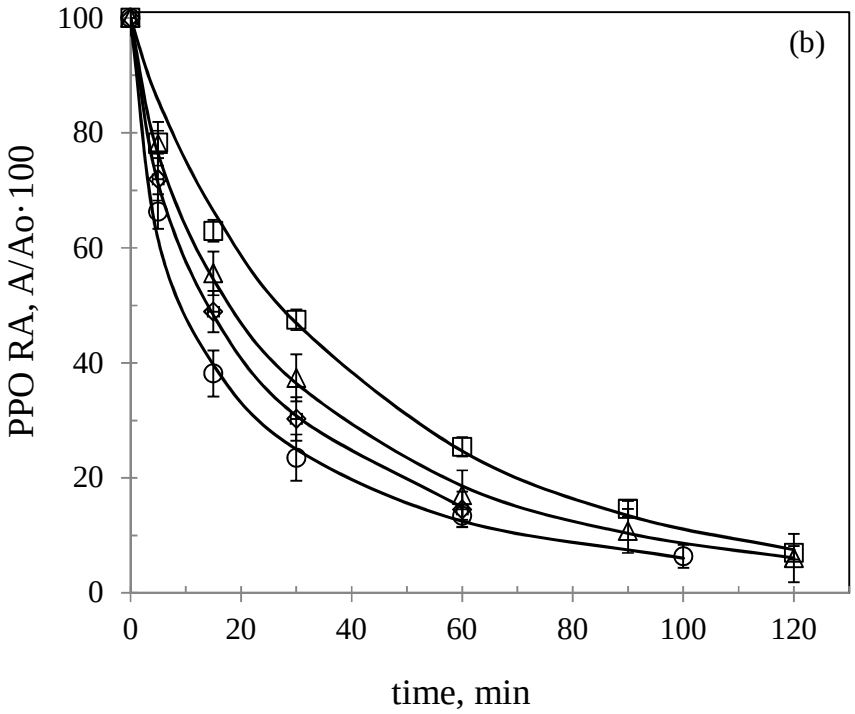

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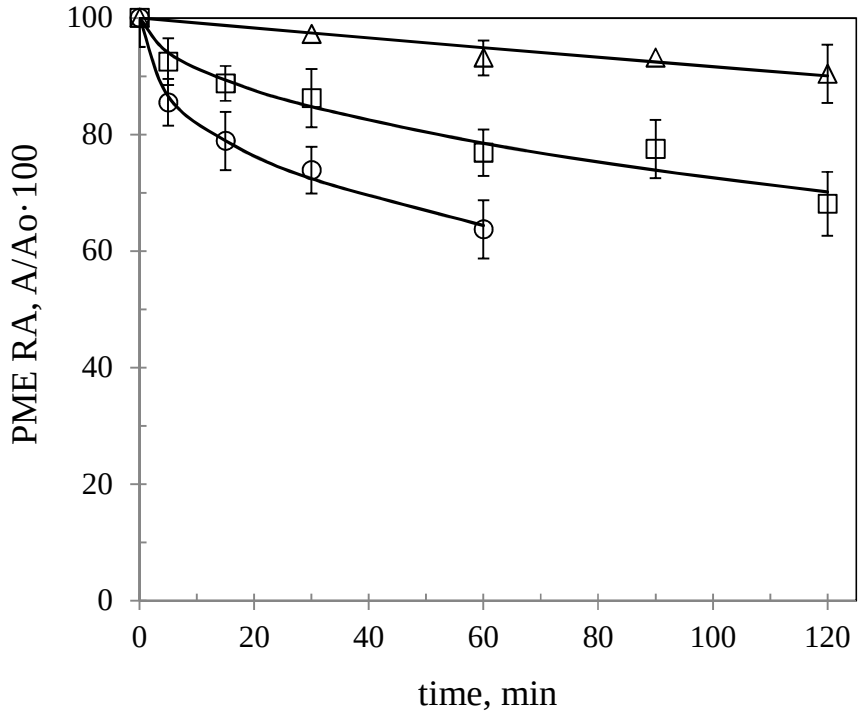

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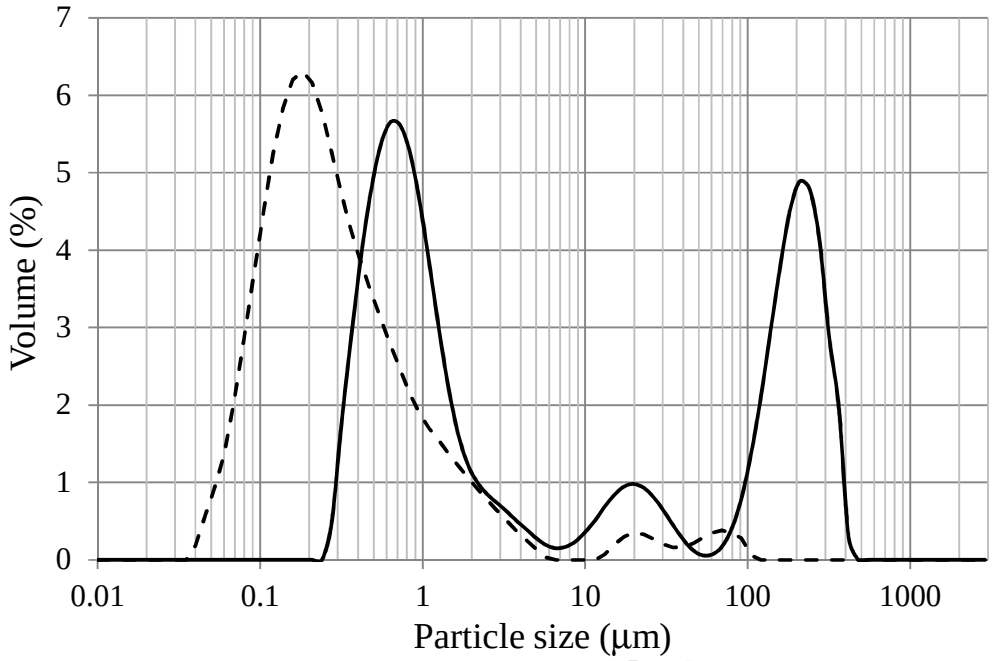

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