Effect of high pressure carbon dioxide processing on pectin methylesterase activity and other orange juice properties

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Abstract:

Inactivation of pectinmethylesterase (PME) and quality parameters of orange juice have been studied after high pressure carbon dioxide (HPCD) treatment. The HPCD treatment conditions covered a wide range of temperature from 2 to 40 ºC, far below normal thermal treatment, while operating pressure was varied from 10 to 30 MPa and exposure time from 3 to 60 min. A decrease in PME activity was found, even at the lowest temperature studied in this work, 2 ºC. Different inactivation kinetic models were used to correlate the PME residual activity: the two-fraction model, the fractional-conversion model and the Weibull model. The two-fraction model presents the lowest mean relative deviation. Some quality parameters such as colour, pH, ºBrix, turbidity, ascorbic acid, total acidity and particle size distribution (PSD) were also determined right after HPCD treatment and along storage at 4ºC up to 12 days. PSD shows that HPCD treatment results in a volume increase of small particles and a volume decrease of large particles

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regarding the non-treated orange juice. Calcium content was also determined before and after HPCD treatment to check for insoluble calcium carbonate formation but not significant changes were observed in calcium content after HPCD treatment.

Keywords: Orange juice, HPCD, pectin methylesterase, cloud stability

1 Introduction

Fruit juice and nectars consumption amounted to 9.7 million litres in 2014 in the EU, of which orange juice is one of the most consumed (European Fuit Juice Association, 2015). Cloud loss is a quality defect in orange juice, since cloud particles are involved in the colour, flavour, texture and aroma of orange juice (Klavons, Bennett, & Vannier, 1991). Additionally, consumers associate the cloud loss with spoilage and quality loss. Citrus cloud is a complex mixture of protein, pectin, lipid, hemicellulose, cellulose and other minor components. Cloud particles of citrus juices range from 0.4 to 5 μm, being particles smaller than 2 μm the most stable clouds (Ellerbee & Wicker, 2011). In the literature, one of the most accepted theories of cloud destabilization is based on pectin demethylation by pectinmethylesterase (PME) (EC 3.1.1.11) in a blockwise fashion. The negative charges generated by PME activity allow subsequent formation of insoluble calcium pectate gels with calcium ions present in the juice. These gels can precipitate pulling the cloud with them causing orange juice clarification due to the loss of turbidity (Ellerbee & Wicker, 2011). Thermal treatment of orange juice at 90 ºC for 1 minute is the method currently used to prevent microbial spoilage as well as the inactivation of the PME (Oulé, Dickman, & Arul, 2013). However thermal treatment causes undesirable changes in several quality parameters such as flavour, colour and texture and can also destroy heat-sensitive nutritional components such as vitamins (Hu, Zhou, Xu, Zhang, & Liao, 2013). Non-thermal

technologies have gained interest and acceptance as food processing methods due to the consumer increased demand for fresh-like products. Among them, high pressure carbon dioxide (HPCD) has been proposed as an alternative non-thermal pasteurization technique for foods. HPCD can also cause the inactivation of certain enzymes that affect the quality of some foods such as PME in the orange juice under mild operation conditions (Damar & Balaban, 2006). In HPCD treatments, operating temperatures can range between 5 – 60 ºC and pressures usually below 50 MPa. Some other advantages of using HPCD as non-thermal treatment are that carbon dioxide is nontoxic, nonflammable, inexpensive and readily available. It can also be easily removed after treatment by depressurization.

Some previous studies dealing with the effect of HPCD treatment on orange juice quality can be found in the literature. The first work was carried out by Balaban, Arreola, Marshall, Peplow, Wei, and Cornell (1991), who found 100 % PME inactivation when using a commercial Milton Roy Supercritical X-10 System while only 86 % PME inactivation was achieved when a custom-made supercritical system was used. These authors also found that, when using the custom-made system, cloud significantly increased. Kincal, Hill, Balaban, Portier, Sims, Wei, and Marshall (2006) also reported a high increase in the cloud values (between 446 – 846%) in orange juice, when using a continuous system but a maximum PME inactivation degree of only 46.3%. Recently, Zhou, Bi, Xu, Yang, and Liao (2015) reviewed the effects of HPCD processing on flavour, texture and colour of foods including orange juice. Combined technologies of high power ultrasound assisted SC-CO_2 (HPU-$SCCO_2$) have been also reported to inactivate PME of orange juice (Ortuño, Balaban, & Benedito, 2014). These authors found a lowest residual activity of 10.65 %. Therefore, different inactivation degrees have been reported in the literature when treated freshly squeezed orange juice to HPCD. This regard, in the literature it has been reported

an improvement of inactivation of different enzymes by increasing the CO_2 concentration in the enzyme solutions when CO_2 was fed through a cylindrical filter nozzle (Ishikawa, Shimoda, Kawano, & Osajime, 1995). Unfortunately, in most of the previous studies, no information about the way CO_2 is put in contact with the substrate can be found and comparison is difficult to stablish. Additionally, differences in inactivation levels are related to cultivars, original pH of the juice, isoenzyme forms, total solid content and other processing factors.

CO_2 was used under supercritical conditions in previous reported HPCD treatments of orange juice. The main objective of this work is to assess the effect of HPCD treatment under supercritical and liquid conditions on PME activity. The effect of HPCD processing on other physical and chemical parameters of orange juice will be also studied.

2 Materials and methods

2.1 HPCD equipment and processing

Valencia oranges were purchased from a local supplier. Oranges were squeezed in an orange squeezer. The experimental apparatus used for the HPCD treatment has been designed in our laboratory with a maximum operating pressure and temperature of 30 MPa and 80 ºC respectively (Melgosa, Sanz, G. Solaesa, Bucio, & Beltrán, 2015). It consists of a CO_2 reservoir, a high pressure syringe pump with a pressure controller (ISCO 260 D) and 3 high pressure cells immersed in a thermostatic water bath. In a typical HPCD experiment, orange juice was charged into the high pressure cell, which was then placed in the thermostatic water bath at the preset temperature. Afterwards, the system was pressurized and maintained at constant temperature and pressure for a pre-established treatment time. CO_2 was fed to the high pressure cell through a sintered stainless steel micro-filter with a pore size of 10 μm to increase the concentration of

85 CO_2 dissolved in the sample. The duration of the pressurization and depressurization was less
86 than 2-3 min and it was not included in the treatment holding time. The high pressure cells were
87 magnetically stirred. Experiments were carried out in a temperature (T) range from 2 to 40 ºC,
88 pressure (p) from 10 to 30 MPa and exposure time (t) from 3 to 60 min. Different pressure cells
89 were arranged in series to carry out experiments at different operating times. After HPCD
90 treatment, the high pressure cells were depressurized and the treated orange juice was analysed
91 (see section 2.2). During depressurization, a temperature decrease of the orange juice was
92 observed due to Joule-Thomson cooling effect depending on applied pressures (Zhou, Zhang,
93 Leng, Liao, & Hu, 2010).

94 PME activity, pH and calcium content were determined before and after HPCD treatment at
95 different operating conditions. To evaluate the effect of HPCD treatment on the self-life of
96 orange juice, a sample of orange juice treated at 30 MPa and 40 ºC for 40 min was stored in the
97 refrigerator (4ºC). Aliquots were taken after 5 and 12 days of storage, and different quality
98 parameters of orange juice were determined and compared with original freshly squeezed orange
99 juice.

100 **2.2 Physico-chemical analysis**

101 **2.2.1 Determination of pectin methylesterase activity.** PME activity was determined by using
102 an automatic titrator system (Metrohm® Titrando). A 1% of pectin solution (Alfa Aesar® Pectin
103 Citrus) prepared in NaCl 0.3 M was used as substrate. 50 mL of pectin solution mixed with 5 mL
104 of orange juice were adjusted to pH 7.5 with NaOH 0.02 N. During hydrolysis at room
105 temperature, pH was maintained at 7.5 by adding NaOH 0.02 N. The amount of NaOH added for
106 30 minutes 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 room temperature. PME activity was calculated according to the following equation:

$$UPE/mL = \frac{(mL\ NaOH) \cdot (Normality\ of\ NaOH) \cdot (factor\ NaOH) \cdot (1000)}{(mL\ juice) \cdot (minutes)} \quad [1]$$

Results are presented as residual PME activity, defined as the relationship between PME activity after and before HPCD treatment:

$$Residual\ PME\ activity\ = \frac{PME\ activity\ after\ HPCD}{PME\ activity\ before\ HPCD} = \frac{A}{A_o} \quad [2]$$

2.2.2 Determination of pH, ºBrix, total acidity, Vitamin C and colour. pH of orange juice was determined with a pH-meter (Crison® pH & Ion-Meter GLP 22). ºBrix were measured with a Milton Roy® refractometer (Model 334610) at 25ºC. Temperature and acidity corrections were made (Kimball, 1999).

Total acidity was determined by using an automatic titrator (Metrohm® Titrando). A sample of 2 mL of orange juice was mixed with 50 mL of distilled water. The mixture was titrated with 0.02 N NaOH. Titrable acidity was expressed as citric acid percentage (g citric acid/100g). Vitamin C was determined with 2,6-dichloroindophenol titrimetric method (Kimball, 1999).

Colour was evaluated by a Konica Minolta ® CM-2600d colorimeter. The L*, a* and b* values were obtained representing lightness, red to green colour and yellow to blue colour, respectively. Other conditions are illuminant D65 (daylight source) and a 10º standard observer (perception of a human observer) following the CIE recommendations. Changes in colour were expressed as:

$$\Delta E = \sqrt{\left(L^*_{before} - L^*_{after}\right)^2 + \left(a^*_{before} - a^*_{after}\right)^2 + \left(b^*_{before} - b^*_{after}\right)^2} \quad [3]$$

Differences in perceivable colour can be classified analytically as not noticeable (0-0.5) slightly noticeable (0.5-1.5), noticeable (1.5-3) well visible (3.0-6.0) and great (6.0-12.0) (Yuk, Sampedro, Fan, & Geveke, 2014).

Another parameter that can be used to evaluate alterations in colour of a beverage is the chroma, C, which measures colour intensity:

$$C = \sqrt{(a^*)^2 + (b^*)^2} \quad [4]$$

2.2.3 Determination of turbidity and particle size distribution. Cloud quality was determined by using a spectrophotometric method. The orange juice sample was centrifuged at 9000 r.p.m. for 30 min. The supernatant was poured into a quartz cuvette and absorbance at 660 nm was measured in a Hitachi® spectrophotomter (Model U-2000). Distillated water was used as a blank. Percent cloud change was calculated as:

$$Percent\ cloud\ change = \frac{(Final\ cloud\ value - Initial\ cloud\ value)}{Initial\ cloud\ value} \cdot 100 \quad [5]$$

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) and the weight average size expressed as the equivalent surface area mean diameter, D(3,2) and the equivalent volume mean diameter, D(4,3) were calculated before and after HPCD treatment and along storage.

2.2.4 Determination of Calcium content. Calcium in orange juice before and after HPCD treatment was determined by atomic absorption spectrometry (Perkin Elmer 3300). The orange juice was centrifuged (Eppendorf Centrifugue® 5804) at 9000 rpm for 30 minutes (Zhou et al., 2010). The precipitate was discharged and the calcium content of the supernatant was determined. La_2O_3 (Merck®) was added to samples to a final concentration of 0.5% of lanthanum in the medium. The addition of lanthanum avoids the interference of phosphates in the calcium determination. HCl was also added (5% in the sample) to promote dissolution of both calcium and lanthanum in the medium. Calcium content was obtained by calibration with different standard solutions of calcium (Merck *Certipur*®, 1 g/L) by following the same method as with the original freshly squeeze orange juice.

Some experiments were also performed with a McIlvaine buffer solution containing 0.05 M citric acid and 0.1 M disodium hydrogen phosphate, at pH close to the orange juice (pH ≈ 4), to which calcium was added to achieve a content similar to that in orange juice (around 100 ppm - $2.5 \cdot 10^{-3}$ $M_{Ca^{2+}}$) using two types of calcium salts (chloride, citrate). A McIlvaine buffer solution was chosen since this solution had a buffer capacity similar to that of orange juice (Yoshimura, Furutera, Shimoda, Ishikawa, Miyake, Matsumoto, Osajima, & Hayakawa, 2002).

2.3 Kinetic data analysis

Different kinetic models were tested to correlate the inactivation kinetics of PME (Hu et al., 2013) .

Two-fraction kinetic model. This model takes into account the existence of several isoenzymes of PME in orange juice, grouped into two fractions, a labile and a stable fraction. Both enzymes

were considered to be inactivated according to first-order kinetics, but independently of each other:

$$A = A_L exp(-k_L t) + A_S exp(-k_S t) \quad [6]$$

where A_L and A_S (A_S = 1 - A_L) are the activity of the labile and stable fractions respectively and k_L and k_S (min^{-1}) the inactivation rate constants of both the labile and stable fractions respectively.

Fractional-conversion model. A fraction-conversion model is a special case of a first order kinetic model that takes into account the non-zero residual activity after prolonged heating and/or pressure (A_∞) treatment:

$$ln(1 - f) = ln\left[\frac{(A - A_\infty)}{(A_o - A_\infty)}\right] = -kt \quad [7]$$

$$A = A_\infty + (A_o - A_\infty) exp(-kt) \quad [8]$$

Weibull model. This model can be written in the power-law form as (Ortuño et al., 2014):

$$log_{10}\left(\frac{A}{A_o}\right) = -bt^n \quad [9]$$

where b is a non-linear rate parameter and n is the shape factor.

2.4 Statistical analysis

All 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 tested 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 was also evaluated:

$$MRD = \frac{1}{n}\left(\sum_{all\ samples}\left|\frac{RA_{calc} - RA_{exp}}{RA_{exp}}\right|\right)\cdot 100 \qquad [10]$$

3 Results and discussion

3.1 Effect of HPCD treatment on PME activity

PME activity was determined before and after HPCD treatment at different operating conditions: 10 to 30 MPa, 2 to 40 ºC and for 3 to 60 min. To consider HPCD as an effective non-thermal treatment, temperatures assayed in this work were lower than 40ºC in all the experiments

Figure 1 shows the residual activity of the PME as a function of operating pressure after 20 min of HPCD treatment at two different operating temperatures (2 and 21ºC). At each temperature, PME activity decreases with increasing CO_2 pressure. Even at the lowest temperature essayed in this work, 2 ºC, some PME inactivation degree was reached, especially at the highest operating pressure. From Figure 1, it can be also observed that at a fix operating pressure, the higher the temperature, the higher the inactivation degree. This fact can be also observed in Figure 2 where a simple exponential function of residual activity with temperature has been found:

$$A/A_o = (0.99\pm0.03)\cdot\exp((-0.060\pm0.004)\cdot T) \quad R^2 = 0.993 \qquad [11]$$

where T is the temperature in Celsius degrees. In addition to the intrinsic effect of temperature on enzyme inactivation, high temperatures can stimulate the diffusivity of CO_2 and also accelerate the molecular collisions between CO_2 and the enzyme (Hu et al., 2013).

Figure 3 shows the effect of operating time at different operating conditions. In all cases, a sharp decrease of PME activity is observed at the beginning of the process, while longer operation times do not involve further substantial enzyme inactivation. This behaviour may indicate that HPCD-labile and HPCD-stable PME fractions coexist in the Valence orange juice. Multiple isoenzymes have been also observed for PME extracts from other sources such as carrot, peach and apple (Zhi, Zhang, Hu, Wu, & Iao, 2008) (Zhou, Zhang, Hu, Liao, & He, 2009b) after HPCD treatment.

Table 1 presents previous results found in the literature on the inactivation of PME in orange juice after HPCD treatment. Balaban et al. (1991) reached a 100% of inactivation in a Milton Roy System after 4 h of treatment at 29 MPa and 50 ºC. The inactivation percentage decreased down to 82 % when using a static custom made supercritical system. In this work, similar inactivation degrees were reached when working at similar operating pressure (30 MPa) but lower operating temperatures (40 ºC), and shorter treatment times (less than 60 min). The faster inactivation reached in our work could be due to the way CO_2 was fed into the sample through CO_2-microbubbles, which helps to maximizes the interface area for the orange juice and the CO_2. Inactivation percentages slightly higher than 50 % were reached when using a continuous HPCD system (Kincal et al., 2006). Table 1 also presents the inactivation degree of PME in orange juice when using a combined technology of high power ultrasound-assisted supercritical carbon dioxide (HPU-$SCCO_2$). The lowest residual activity reported by HPU-SCO_2 is similar to the maximum inactivation degree reached in this work (Table 1) by using a microfilter. In both

cases, an increase in the solubilisation rate of pressurized CO_2 in the orange juice could accelerate the inactivation rate. Table 1 also presents the degree of inactivation of PME in orange juice after heat pasteurization at 90ºC for 10 s and 20 s as recently reported by Agcam, Akyıldız, and Evrendilek (2014).

Different factors have been reported to affect the inactivation of PME by thermal and non-thermal treatments. Bull, Zerdin, Howe, Goicoechea, Paramanandhan, Stockman, Sellahewa, Szabo, Johson, and Steward (2004) studied the influence of natural variations of pH in orange juice due to the harvesting season on PME inactivation by High Pressure Processing, HPP, (pH range: 3.6-4.3). These authors found that low values of pH (pH=3.6) help to the inactivation of PME by HPP. However, based on the results presented in Table 1, no correlation can be established between pH of orange juice and the inactivation degree reached by HPCD. For instance, the value of pH of Valence orange juice reported by Balaban et al. (1991) was 3.8 while in this work, pH was found to be around 4.1 but similar degree of PME inactivation was reached in both cases. In this regard, in the literature, there are some studies about pH lowering induced by contact to pressurized CO_2. Meysammi, Balaban, and Teixeira (1992) reported that the pH of orange juice remained essentially constant when adding CO_2 in the pressure range 0.1-5.5 MPa. However, Balaban et al. (1991) measured the pH of orange juice in contact to pressurized CO_2 at higher operating pressures, 31 MPa and 27 MPa, observing that pH was lowered by about 0.7 pH units (from 3.6 to 2.9 and from 3.8 to 3.1). In any case, these authors explained that, for substantial PME inactivation, pH must be lowered to 2.4. Therefore, different factors seem to determine PME inactivation.

Some studies on fluorescence intensity of PME treated with HPCD showed modifications in the tertiary structure of PME (Hu et al., 2013) due to relocation of tryptophan residues. Other

suggested enzyme inactivation mechanisms in food processing by HPCD, such as formation of different complex with CO_2 molecules or decomposition of the enzyme by CO_2 have been also recently reviewed by Hu et al. (2013).

3.1.1 Kinetic analysis

In this work, as expected from the shape of PME residual activity (see Fig. 3), inactivation curves were not well fitted to the first order kinetic model ($r^2 \leq 0.71$), although Balaban et al. (1991) found good correlation of the inactivation kinetics with a first order model at 31 MPa and 55ºC (D = 20.9 min). The kinetic parameters of the different models tested in this work are listed in Table 2. This Table also includes the decimal reduction time (D value), defined as the treatment time needed for a 10-fold reduction of the initial enzyme activity at a given condition, the statistical parameters for the fit of the kinetic models, r^2 and the mean relative deviation (MRD) between experimental and calculated residual activities (Eq. 10). For the two-fraction model, A_L was higher than A_S and k_L was 50-70 times higher than k_S indicating that there is a fast inactivation period followed by a decelerated decay. Therefore, the corresponding D_L and D_S followed the opposite trend. k_L and A_L from the two-fraction model increased with increasing pressure. The same tendency was found for k and b for the fractional-conversion and the Weibull models, respectively; while the residual activity A_∞ and the exponent, n, decreased with increasing pressure. The lowest MRD was obtained for the two-fraction model.

3.2 Effect of HPCD treatment on quality parameters and storage study

The highest PME inactivation degree was obtained at 30 MPa and 40ºC (see Figure 3). Therefore, to carry out the storage study, samples were treated by HPCD at 30 MPa and 40ºC for 40 min. Orange juice was characterized before and immediately after HPCD treatment and

evaluated along 12 days of storage at 4 ºC. PME activity and other quality parameters in orange juice, such as PSD, turbidity, colour, ºBrix, total acidity and ascorbic acid, were recorded as a function of storage time.

3.2.1 PME activity. Figure 4 shows that PME recovered some activity during storage at 4 ºC. This result suggests that PME inactivation by HPCD could be somehow reversible. Similar results were obtained by Arreola et al. (1991) and Niu et al. (2010) after HPCD treatment of orange juice. The increased PME activity along storage has been attributed to isoenzymes arising during the storage of orange juice (Agcam et al., 2014). On the contrary, when using a commercial PME lyophilized power produced from the peel of Valencia oranges no recovery in PME activity was observed during 7 day-storage at 4ºC after HPCD treatment (8-30 MPa, 55ºC for 10 min), (Zhou, Wu, Hu, Zhi, & Liao, 2009). In any case, an extracted enzyme suspended in a buffer solution can give different inactivation results from those obtained in an original juice.

Different results have been found in the literature on the activity of other enzymes after HPCD treatment during storage. Horseradish peroxidase treated at 55ºC and 8 – 22 MPa recovered activity after storage for 7 and 21 days at 4ºC; however this was not obvious when treated at 30 MPa (Gui, Chen, Wu, Wang, Liao, & Hu, 2006). PPO from potato recovered 28% of its original activity during the first two weeks of frozen storage, and then its activity slightly decreased with storage time; however no restoration activity was found for PPOs from lobster and brown (Chen, Balaban, Wei, Marshall, & Hsu, 1992). Therefore, different behaviour has been observed regarding the enzyme and the source of the enzyme.

3.2.2 Cloud. Changes in cloud values (Eq. 5) after HPCD treatment and the corresponding values along storage are presented in Figure 4. After HPCD treatment cloud was improved, increasing nearly a 30% compared to the freshly squeezed orange juice. During storage cloud

283 value decreased, but even after 12 days cloud enhancement remained 18% higher than the
284 original orange juice. Kincal et al. (2006) reported a cloud increase higher than 600%, with little
285 influence of operating pressure when orange juice was treated in continuous HPCD equipment.
286 Arreola et al. (1991) found that cloud increased from 27% to 400% regardless temperature or
287 treatment time. Niu et al. (2010) also found an increase of cloud values around 100%, with little
288 effect of operating time. In this work, values of cloud enhancement were close to the lowest
289 value reported by Arreola et al. (1991). These authors also found that cloud enhancement was
290 less in orange juice drained after depressurization of the system compared to orange juice
291 samples withdrawn while the system was under pressure. This could explain the values of cloud
292 enhancement obtained in this work, especially when comparing to a continuous HPCD system.

293 Taking into account these results, cloud seems to be stabilized after HPCD in a non-enzymatic
294 way, since some PME is still active. Kincal et al. (2006) suggested that HPCD treatment could
295 lead to precipitation of calcium ions present in the orange juice due to the formation of insoluble
296 calcium carbonate. It has been described that dissolved CO_2 could form carbonic acid that
297 dissociates into bicarbonate that could be converted to carbonate when the pressure is released
298 (Kincal et al., 2006) (Yuk et al., 2014). To study the role of formation of insoluble calcium
299 carbonate in cloud stabilization, calcium content was determined before and after HPCD.

300 ***Effect of HPCD treatment on Calcium content.***

301 Table 3 presents the residual calcium content, defined as the percentage relationship between the
302 calcium content after and before HPCD treatment, for calcium solutions in a McIlvaine buffer at
303 pH close to the orange juice. It can be observed that calcium content did not change significantly
304 after HPCD treatment. Table 3 also shows the residual calcium content after HPCD treatment at
305 different operating conditions in the fresh orange juice. Although calcium content presented

slightly lower values after HPCD treatment than in buffer solutions, no significant differences have been determined among sample means of buffer and orange juices when applying the Tukey's HSD method.

In this regard, the effect of different experimental variables on $CaCO_3$ solubility has been recently reported in the literature (Coto, Martos, Peña, Rodríguez, & Pastor, 2012). $CaCO_3$ solubility increased with operating pressure (pressurized CO_2) and decreased with temperature and pH of the medium. For instance, at 40ºC $CaCO_3$ solubility in water at 1 bar and 40 bar is about $4.2 \cdot 10^{-4}$ $M_{Ca^{2+}}$ and $2.3 \cdot 10^{-2}$ $M_{Ca^{2+}}$, respectively. Although $CaCO_3$ solubility at atmospheric pressure is very small, depends strongly on pH increasing as pH decreases (for instance, at 25 ºC, at pH = 7 $M_{Ca^{2+}}=2 \cdot 10^{-3}$ and at pH = 6 $M_{Ca^{2+}}=9 \cdot 10^{-3}$). Therefore, taking into account that at the low pH of orange juice, only a small amount of dissolved CO_2 is converted into bicarbonate dissociating into free hydrogen ions (Zhou et al., 2015) and that calcium carbonate solubility increases by decreasing pH, calcium content in orange juice was in fact not expected to change much after HPCD processing. To our knowledge, the only measurement of calcium content before and after HPCD treatment, was reported by Zhou et al. (2010), who found no significant effects on the calcium content of peach juice, which is also an acidic juice (pH ~ 3.8), after HPCD treatment.

To explain the cloud enhancement after HPCD treatment, the possible effect of homogenization induced by gas expansion during the depressurization step has been studied by determining the particle size distribution, PSD.

3.2.3 Particle size distribution. PSD of orange juice before and after HPCD treatment has been represented in Figure 5. Two maximums around 0.8 µm and 850 µm can be observed. The size of stable cloud particle has been reported to be in the range of 0.4-5 µm, with the most stable

329 cloud having particle sizes of 2 μm and smaller (Ellerbee & Wicker, 2011). The larger particle
330 size in Figure 5 is due to the presence of some settling pulp. In this regard, it must be emphasized
331 that laser diffraction methods generates a volume distribution. The total volume of all particles
332 with diameters less than 5 μm represents 24.5 % of the total volume of particles but represents
333 100 % in number distribution. That is, although the number of bigger particles is very small
334 represents a high volume distribution when compared to cloud particles. Clarification of juice
335 takes place when stable cloud showed aggregation by shifting the PSD distribution to larger
336 diameters (Corredig, Kerr, & Wicker, 2001). However, HPCD treatment results in an increase of
337 the volume peak of the smaller particles and a decrease of large particles (Figure 5). This
338 behaviour of orange juice PDS helps to understand the cloud enhancement after HPCD
339 treatment. This fact has been explained in terms of the effect of the homogenization caused by
340 HPCD treatment due to several reasons, such as high internal stress surpassing the tensile
341 strength of the particles when CO_2 is removed from the vessel (Niu et al., 2010). Figure 5 also
342 presents PSD of treated orange juice after 5 and 12 days storage. It can be observed that the
343 volume peak of the small particles increased during the storage, while the volume peak of the
344 large particles decreased. This behaviour could be attributed to the remained active PME that
345 could decompose the high molecular weight compound and then reduce the size of particles.

346 Values of D[3,2] and D[4,3] of freshly squeezed juice, after treatment and after 5 and 12 days
347 storage are presented in Table 4. According to Figure 5, the values after HPCD treatment were
348 lower than those of freshly squeezed orange juice and a continuous decrease was observed with
349 increasing storage time, but this decrease was not significant different along storage (Table 4). In
350 any, case, no shift of PSD to larger diameters can be observed. This tendency can be also

observed in the values of d(0.1), d(0.5) and d(0.9) which correspond to the size of particle below which 10%, 50% and 90% of the sample lies, respectively.

3.2.4 pH, total acidity, ºBrix, ascorbic acid and colour.

pH, ºBrix and total acidity did not change significantly in orange juice after HPCD treatment and remained essentially constant during storage (Table 5). The low pH of the original orange juice made difficult the dissociation of the carbonic acid, formed by dissolved CO_2 in the juice, into H^+ ions. However, in some studies, a decrease in the pH of orange juice has been reported during HPCD treatment (Oulé et al., 2013; Balaban et al., 1991). The pH decrease has been thus associated with the state and density of CO_2 in the juice during the treatment (Oulé et al., 2013). Further investigations should be performed to analyse the pH of the sample during treatment, due to different results found in the literature (see section 3.1).

The content of ascorbic acid decreased after HPCD treatment (around 14%) and continued decreasing during storage. However the decrease of the ascorbic content after HPCD treatment is lower than after pasteurization treatments. Oule et al. (2013) reported a decrease of the vitamin C content of 13% after HPCD treatment (25 MPa, 40ºC) while after pasteurization vitamin C decreased 43% (90ºC and 60 s). This difference is attributed not only to the lower temperatures employed in HPCD treatments but also to the O_2-free environment and the low solubility of vitamin C in SC-CO_2. In this work, higher loss of vitamin C has been observed during storage (10% and 24% after 5 and 12 days respectively) than other values found in the literature during storage. For instance, Oulé et al. (2013) found around 5% vitamin C loss after 56 days storage at 4 ºC. This high percentage loss could be due to the presence of O_2 during storage, but also to the pH of the orange juice used in this work (4.11-4.12), since it is known that more acidic conditions stabilized ascorbic acid (Bull et al., 2004).

Table 6 lists the L*, a*, b* parameters of freshly squeezed orange juice, after HPCD treatment and during storage. Lightness (L*) and yellowness (b*) significantly decreased indicating the darkening of the orange juice and less yellow and more blue colour after HPCD processing. On the contrary, redness (a*) was not significant different in the untreated and HPCD processed orange juice. In the literature, differences have been reported for the lightness, redness and yellowness in HPCD-treated orange juice (Zhou et al., 2015). During storage, lightness (L*), redness (a*) and yellow (b*) did not change significantly after 5 days storage; however redness and yellowness decreased significantly after 12 days storage. According to Zhou et al. (2015) the colour of foods can be influenced by biochemical or chemical reaction as well as physical effects induced by HPCD. Among other mechanisms, oxidation of ascorbic acid could also lead to the colour change (Zhou et al., 2015). This would agree with the results reported in Table 5 of ascorbic acid content. The change in colour, ΔE (Eq 3) is also presented in Table 6 and visible differences in colour after HPCD treatment have been determined ($\Delta E \approx 5$). Kincal et al. (2006) also reported ΔE values as high as 13.83, at 72 MPa and a ratio of 0.64 CO_2/juice (w/w). Chroma values listed in Table 6 show that HPCD treatment results in a significant lower colour intensity juice after processing but no significance differences have been observed along storage.

4. Conclusions

Freshly squeezed orange juice has been treated by HPCD under different operating conditions. PME in orange juice was effectively inactivated by HPCD showing a fast initial decrease that remained nearly constant after prolonged HPCD treatment. The inactivation degree increased with pressure and temperature. Different inactivation kinetic models were used to correlate the residual PME activity, being the two-fraction model the best with the lowest mean relative deviation. Inactivation of PME in orange juice after HPCD treatment seems to be reversible

since its activity is slightly recovered along storage at 4 ºC. PSD shows an increase of the volume peak of the smaller particles (0.3-5 μm) and a decrease of large particles after HPCD treatment, supporting the cloud enhancement observed. Calcium content does not change significantly after HPCD treatment, proving that insoluble calcium content was not formed. Further investigation should be done to analyse the effect of cloud enhancement after HPCD treatment.

ACKNOWLEDGMENTS

To Hyperbaric. To the Spanish Government through MINECO (CTQ2015-64396-R). R. Melgosa acknowledges MINECO for a grant (BES-2013-063937). A. G. Solaesa acknowledges the Burgos University for a pre-doctoral fellowship.

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Table 1. PME inactivation values in orange juice after HPCD and other non-thermal treatments

System	pH	p, MPa	T, ºC	t, min	Inactivation	Reference
Milton Roy System	3.8	29	50	240	100%	(Balaban et al., 1991)
Custom made system	3.8	29	45	120	84%	(Balaban et al., 1991)
Static	3.9	40	55	10	36%	(Niu et al., 2010)
Static	3.9	40	55	60	95%	(Niu et al., 2010)
Continuous	3.7	72	24	10*	56%	(Kincal et al., 2006)
Static	4.12	30	40	20-60	90-92 %	This work
HPU-$SCCO_2$	3.8	23	41	10	89 %	(Ortuño et al., 2014)
Pasteurization	3.5	0.1	90	10 - 20 s	93 – 96 %	(Agcam et al., 2014)

(*) residence time; HPU-SCO_2 = high power ultrasound assisted supercritical CO_2.

Table 2. Estimated kinetic parameters of orange juice PME inactivation at different operating conditions, for different kinetic models

Kinetic model	T, ºC	p, MPa	Model parameters		D value, min	MRD	r^2 ($p<0.05$)
Two-fraction	40	30	$k_L = 0.74 \pm 0.07$	$A_L = 0.85 \pm 0.02$	$D_L = 3.3 \pm 0.3$	5.8	0.999
			$k_S = 0.010 \pm 0.003$	$A_S = 0.15 \pm 0.02$	$D_S = 230 \pm 69$		
	21	20	$k_L = 0.24 \pm 0.01$	$A_L = 0.652 \pm 0.008$	$D_L = 9.6 \pm 0.4$	1.4	0.999
			$k_S = 0.0048 \pm 0.0005$	$A_S = 0.348 \pm 0.007$	$D_S = 480 \pm 69$		
	21	10	$k_L = 0.123 \pm 0.003$	$A_L = 0.628 \pm 0.009$	$D_L = 18.7 \pm 0.5$	1.2	0.999
			$k_S = 0.0020 \pm 0.0005$	$A_S = 0.372 \pm 0.009$	$D_S = 1152 \pm 288$		
Fractional-conversion	40	30	$k = 0.63 \pm 0.06$	$A_\infty = 0.11 \pm 0.01$	$D = 3.7 \pm 0.3$	11.4	0.996
	21	20	$k = 0.18 \pm 0.02$	$A_\infty = 0.28 \pm 0.01$	$D = 13 \pm 1$	4.8	0.997
	21	10	$k = 0.112 \pm 0.003$	$A_\infty = 0.336 \pm 0.004$	$D = 20.6 \pm 0.6$	1.4	0.999
Weibull	40	30	$b = 0.58 \pm 0.03$	$n = 0.15 \pm 0.02$	$D = 1.7 \pm 0.1$	5.8	0.990
	21	20	$b = 0.27 \pm 0.02$	$n = 0.19 \pm 0.02$	$D = 3.7 \pm 0.3$	2.8	0.998
	21	10	$b = 0.14 \pm 0.03$	$n = 0.31 \pm 0.06$	$D = 7 \pm 2$	5.3	0.985

MRD: mean relative deviation (Eq. 10)

Table 3. pH and Calcium content of different McIlvaine buffer solutions and orange juice after HPCD treatment.

System	pH before HPCD	pH after HPCD	HPCD treatment			Residual Ca^{2+}, %
			p, MPa	T, °C	t, min	
McIlvaine buffer 101 ppm Ca (Ca_3Cit_2)	3.92	3.86	20	21	20	95 ± 2^a
McIlvaine buffer…… 100 ppm Ca ($CaCl_2$)	3.97	3.97	20	21	20	96 ± 5^a
Orange juice	4.18	4.18	10	21	20	91 ± 2^a
Orange juice	4.16	4.12	10	40	20	96 ± 5^a
Orange juice	4.17	4.13	30	21	20	91 ± 2^a
Orange juice	4.16	4.15	30	40	20	93 ± 4^a
Orange juice	4.17	4.14	10	40	40	90 ± 5^a

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.

Table 4. Effect of HPCD treatment on the particle size distribution of orange juice after HPCD treatment and during storage.

Time	D[3,2]	D[4,3]	d(0.1)	d(0.5)	d(0.9)
Fresh orange juice	3.6 ± 0.3^{a}	523 ± 37^{a}	0.84 ± 0.04^{a}	424 ± 62^{a}	1270 ± 47^{a}
After treatment	2.6 ± 0.2^{b}	438 ± 26^{ab}	0.71 ± 0.02^{b}	272 ± 48^{b}	1186 ± 40^{a}
5 days	2.5 ± 0.1^{b}	413 ± 38^{b}	0.70 ± 0.01^{b}	219 ± 52^{b}	1161 ± 70^{a}
12 days	2.6 ± 0.2^{b}	400 ± 43^{b}	0.73 ± 0.02^{b}	196 ± 59^{b}	1130 ± 75^{a}

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.

Table 5. Changes in orange juice pH, ºBrix, total acidity and ascorbic acid.

Time	pH	ºBrix(1)	Total acidity, g citric acid/100mL[1]	Ascorbic acid mg/100 mL
Fresh orange juice	4.11 ± 0.05^{a}	11.50 ± 0.08^{a}	0.52 ± 0.05^{a}	50 ± 1^{a}
After treatment	4.09 ± 0.05^{a}	11.35 ± 0.05^{a}	0.53 ± 0.04^{a}	43 ± 1^{b}
5 days	4.11 ± 0.05^{a}	11.45 ± 0.06^{a}	0.50 ± 0.03^{a}	39 ± 2^{c}
12 days	4.12 ± 0.05^{a}	11.45 ± 0.06^{a}	0.49 ± 0.04^{a}	33 ± 1^{d}

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.

(1) values corrected by acidity and temperature

Table 6. Changes in orange juice colour.

Time	L	a	b	ΔE	Chroma
Fresh orange juice	31.62 ± 0.08^{a}	4.26 ± 0.07^{a}	19.9 ± 0.3^{a}		20.4 ± 0.9^{a}
After treatment	28.1 ± 0.2^{b}	4.1 ± 0.1^{a}	16.2 ± 0.2^{b}	5.1 ± 0.5	16.7 ± 0.9^{b}
5 days	28.09 ± 0.06^{b}	4.21 ± 0.04^{a}	16.45 ± 0.05^{b}	4.9 ± 0.3	17.0 ± 0.2^{b}
12 days	28.10 ± 0.07^{b}	3.54 ± 0.08^{b}	15.2 ± 0.1^{c}	5.9 ± 0.3	15.6 ± 0.5^{b}

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.

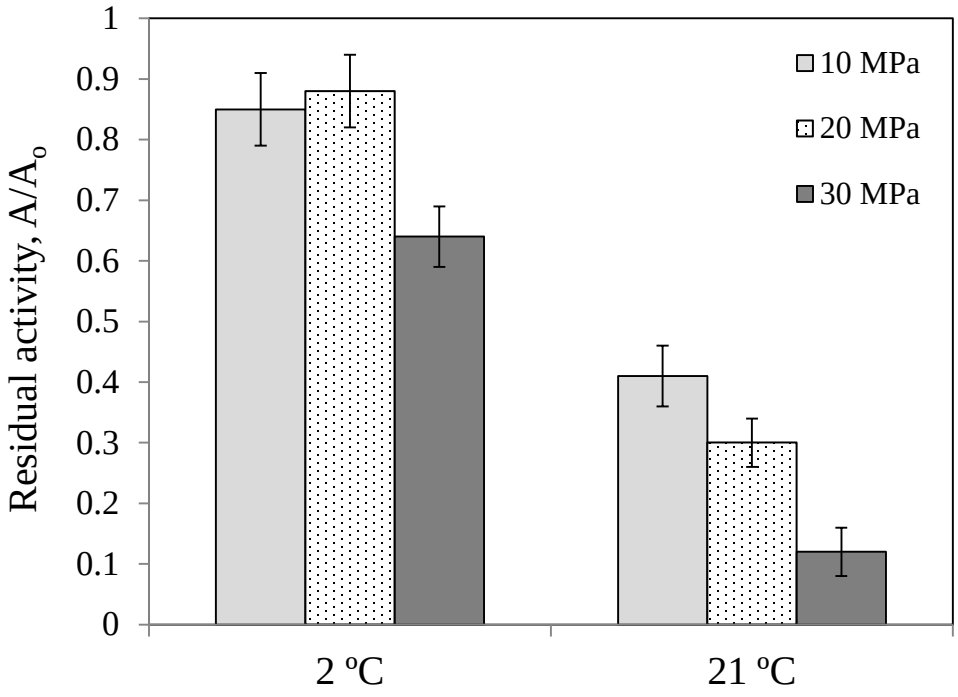

Figure 1. Effect of pressure on orange juice PME inactivation (T = 2 and 21ºC, t = 20 min). Data: mean ± SD (n=3).

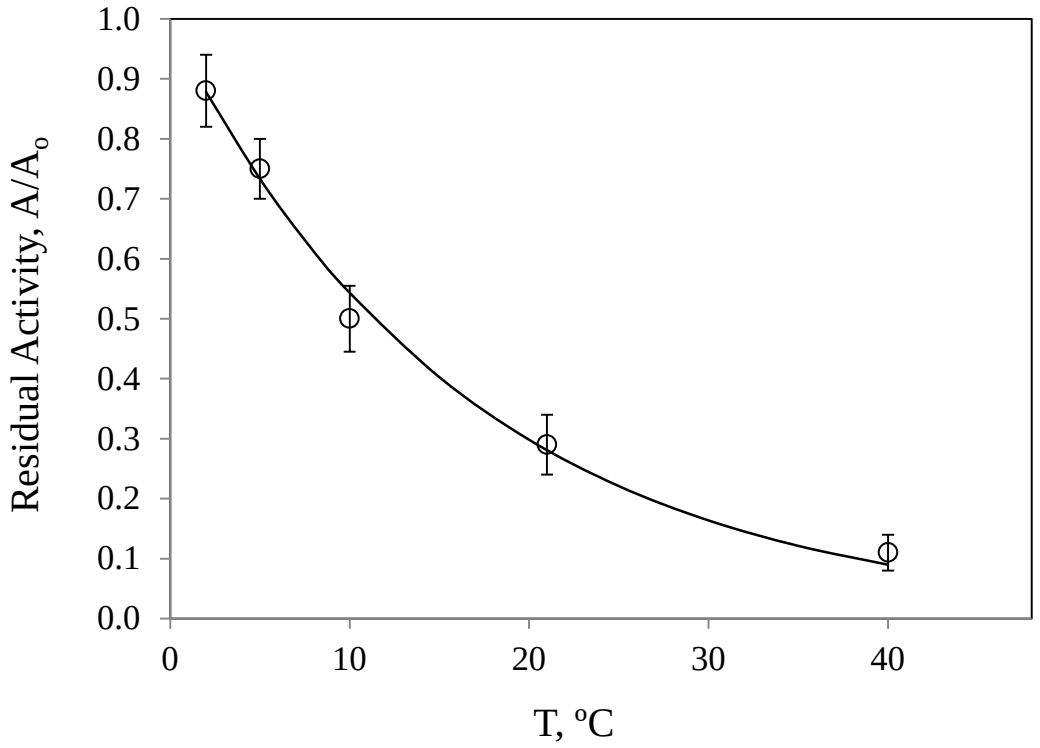

Figure 2. Effect of temperature on orange juice PME inactivation (p = 20 MPa, t = 40 min). Continuous line is an exponential function of residual activity as a function of temperature (Equation 11). Data: mean ± SD (n=3).

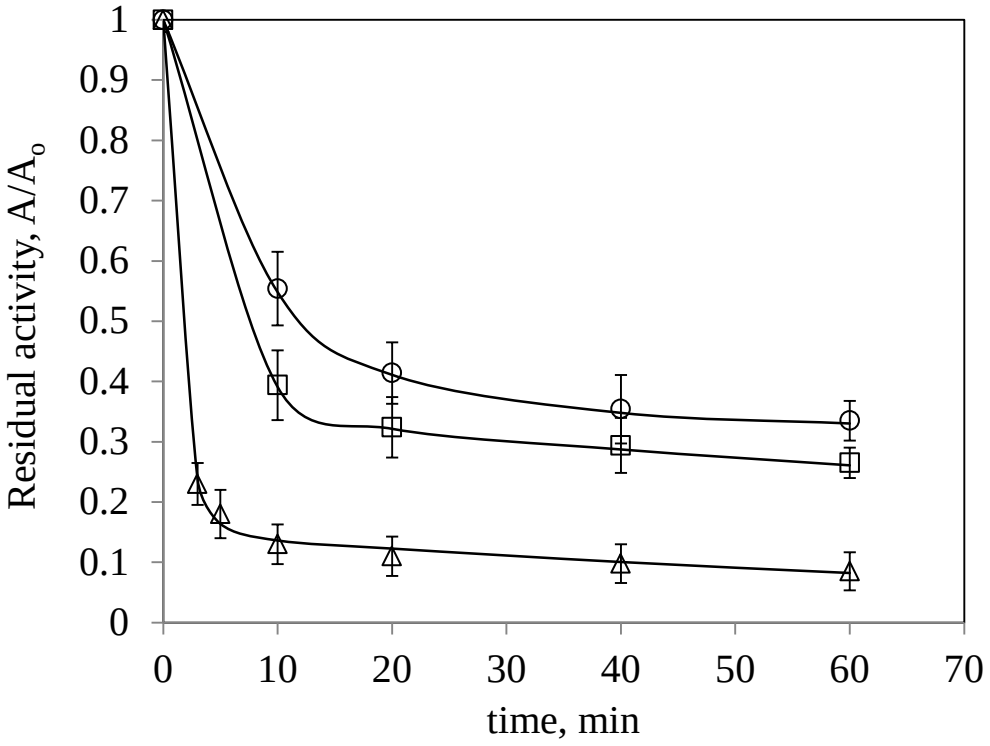

Figure 3. Effect of operating time on PME inactivation at different operating conditions (△ 40ºC and 30 MPa, □ 21ºC, 20 MPa; ○, 21ºC, 10 MPa). Data: mean ± SD (n=3). Continuous lines correspond to the two-fraction model.

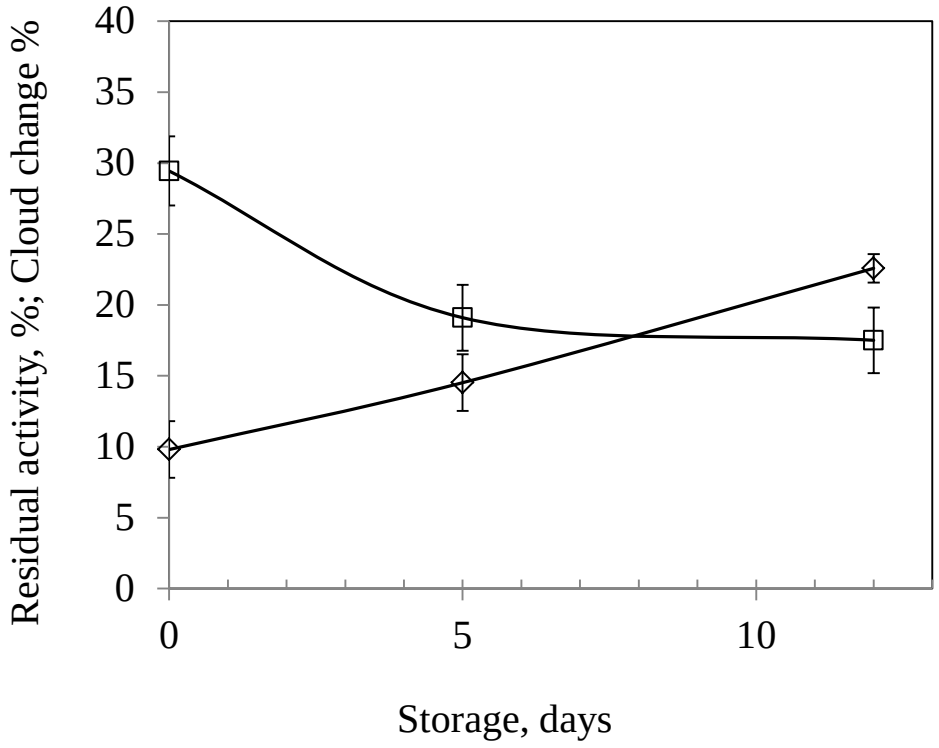

Figure 4. Evolution of PME residual activity and cloud percentage (Eq. 5) in HPDC treated (30 MPa, 40 ºC and 40 min) orange juice during storage at 4ºC. (◇ PME residual activity, □ cloud percentage). Data: mean ± SD (n=3).

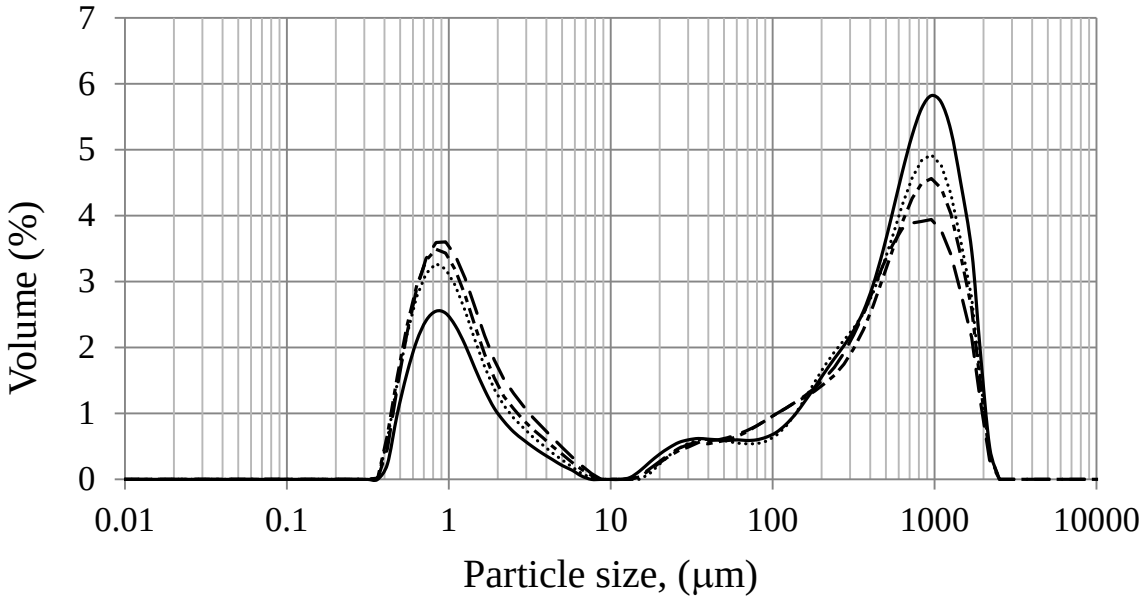

Figure 5. Particle Size Distribution (PSD) of orange juice freshly squeezed (─), immediately after treatment by HPCD at 30 MPa, 40ºC for 40 min (⋯); after 5 days storage at 4ºC (-·-·) ; after 12 days storage at 4ºC (− − −).