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Polyphenol Oxidase (PPO) and Pectin Methylesterase (PME) inactivation by High Pressure Carbon Dioxide (HPCD) and its applicability to liquid and solid natural products

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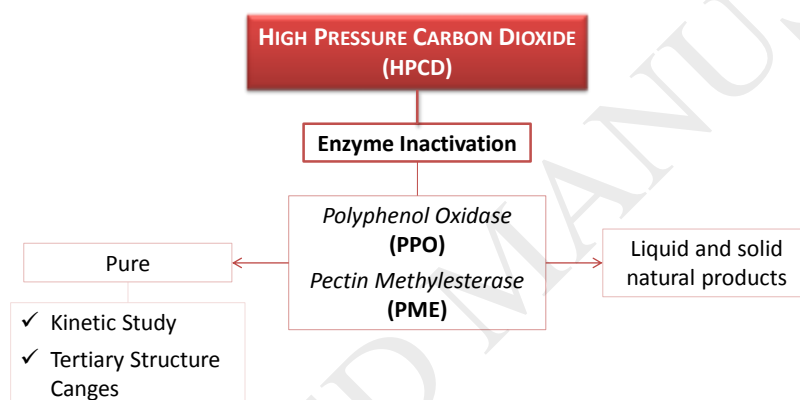
Highlights

- High Pressure Carbon Dioxide (HPCD) was used to inactivate enzymes
- PPO and PME were the selected enzymes
- Inactivation kinetics was studied using pure enzymes
- HPCD technology was applied in fruit juices an solid food
- Enzymes in juices tend to be more resistant than pure enzymes

ABSTRACT

In the present work, High Pressure Carbon Dioxide (HPCD) has been used to inactivate the enzymes Polyphenol Oxidase (PPO) and Pectin Methylesterase (PME). The work has been done in two stages: in the first one, fundamental knowledge about the inactivation mechanism induced by HPCD was obtained using pure commercial enzymes. The influence of the main process parameters was studied: pressure, temperature, time and ratio CO_2/volume of enzymatic solution. The changes in the tertiary structure of the enzymes after the HPCD treatment were analyzed by fluorescence spectroscopy running two different tests: intrinsic fluorescence measurement and ANS binding. In the second stage, the acquired knowledge was used to study the inactivation pattern of these two enzymes in liquid and solid natural products, including fruit and vegetable juices or shrimps. The results revealed differences in the inactivation pattern of the enzymes in these substrates compared to the pure commercial enzymes, but the applicability of HPCD technology for the inactivation of enzymes in real substrates at industrial scale was also revealed.

Key-words. PPO, PME, juice, Supercritical Carbon Dioxide, green process, food technology

1. INTRODUCTION

Enzymes are proteins that accelerate chemical and biological reactions [1]. The use of these biocatalysts is steadily growing since they are active in organic solvents [2] and catalysis takes place at mild conditions. Therefore, enzymes have been used in many applications in the food industry such as reaction or extraction. However, in some cases, enzymes are also responsible of deleterious effects on foods. More specifically, Polyphenol Oxidase (PPO) and Pectin Methylesterase (PME) are two of the main enzymes responsible for the quality loss in freshly squeezed juices. PPO causes the browning of the juice due to the oxidation of polyphenols to quinones [3] and PME causes juice clarification [4], being both effects unaccepted by the consumers. In order to prevent this undesirable effect, it is necessary to inactivate those enzymes. Conventional processes are based on the exposure at high temperatures for short periods of time. This procedure results in an alteration of the organoleptic properties of the juice [5]. For this reason, new technologies to inactivate those enzymes are required in order to preserve the properties of the juices. One of the most promising alternative treatments is High Pressure Carbon Dioxide (HPCD). This technique uses CO_2 in conditions of pressure and temperature usually above the critical point (7.4 MPa and 30.9 °C) that is put in contact with the solution/juice containing the enzyme in a batch, semicontinuous or continuous mode [6]. This technology can be also applied to treat solid foods [7], including shrimps or other sea food [8]. Typical operating values are pressures up to 30 MPa and temperatures in the range 20 to 50 °C [9]. These changes in pressure and temperature tune the physical properties of CO_2 and the way it interacts with the enzyme.

In general, CO_2 induces several effects on the enzyme that can lead to the enzyme inactivation. According to Balaban et al. [6], the main factors that can explain the enzymatic inactivation by HPCD are pH lowering, conformational changes of the secondary and tertiary structure of the enzyme [10], and inhibitory effects of molecular CO_2 on enzyme activity. CO_2 dissolved in aqueous media forms carbonic acid, which drops the pH. In the literature, it is hard to find papers that present the pH of media exposed to HPCD; however the solubility of CO_2 in different media under supercritical conditions has been studied [11,12]. The results published by those authors could be used to calculate the pH drop induced by the CO_2. It has also been reported that the way CO_2 interacts with the core of the protein, usually hydrophobic, is key to explain the inactivation process [13]. The protein tends to be completely folded in

order to protect the hydrophobic core and its functionality from the aqueous media around it, but the presence of a very non polar molecule such as CO_2 can alter the equilibrium.

Although it is possible to find in the literature many papers dealing with the inactivation of enzymes from different foods, the mechanism underlying the inactivation mechanism is yet not clear. In this sense, the enzyme obtained and purified at different extents from foods is not pure enough to avoid the interactions of CO_2 with some other molecules present. Therefore, in order to obtain new and useful insights about the inactivation mechanism, it is necessary to use pure enzymes, given that PPO and PME possess a significantly different structure. Whereas PPO has a fairly complex structure formed by three or four subunits [14] yielding a total molecular weight around 130 kDa, PME is significantly simpler and is formed by only one subunit with a MW in the range 30-35 kDa [15]. These structural differences together with a different spatial arrangement of the residues are responsible for the different inactivation mechanisms and it is necessary to look into them.

Analyzing the works dealing with the inactivation of enzymes in juices already published in the literature, one of the most important drawbacks is the difficulty to compare the results among authors. On one hand because the enzymes are obtained from different sources and extracted following different procedures and on the other hand because different experimental set-ups are used to perform the HPCD treatment. Therefore, it is important to have inactivation data obtained using the same experimental set-up in order to be able to draw conclusions, using first pure enzymes (a work that allows to understand the inactivation mechanism and the conformational changes induced) and then juices and other solid substrates.

In this work, a full approach to the HPCD treatment to inactivate enzymes is presented, in an attempt to overcome the above mentioned limitations. Two pure commercial enzymes (PPO and PME) were exposed to HPCD at different combinations of pressure, temperature, time and CO_2/volume of enzyme ratios in order to study the inactivation kinetics. In a second stage, the HPCD treatment was used to inactivate those two enzymes in juices and shrimps in order to compare the results and validate the results obtained. The aim of this work is to shed light on this aspect based on our broad expertise in the field of the HPCD technology applied to enzymatic inactivation, summarizing and comparing the results obtained using pure enzymes and real substrates.

2. MATERIALS AND METHODS

2.1. Enzymes and Chemicals

Mushroom tyrosinase (polyphenol oxidase -PPO- EC 1.14.18.1) (reference T3824, 25 KU) was purchased from Sigma Aldrich (St. Louis, MO) and dissolved in a phosphate buffer solution (50 mM, pH 6.5) in order to have a 25 U/mL concentration. Pectin methylesterase (Biopectinasa PME Ultra, origin *Aspergillus niger,* EC 3.1.1.11) was purchased from Biocon Española, S.A. (Barcelona, Spain) and diluted in distilled water to have a theoretical activity of 60 U/mL.

Fruits and vegetables used in the present work (apple (cv. Golden Delicious), orange (cv. Valencia), tomato (cv. Canario) and carrot) were purchased in a local market. Shrimps were kindly provided by Gamba Natural (Medina del Campo, Spain) and did not contain sulphites.

Carbon dioxide (99.9%) was supplied by Air Liquide S.A. (Spain). The physical properties of CO_2 were taken from the NIST Database.

2.2. Experimental set-up

All the experiments, either using pure enzymes or juices, were carried out in a stainless steel high pressure batch stirred reactor with an internal volume of approximately 80 mL which was submerged in a thermostatic water bath. A schematic representation of the experimental set up is shown in Figure 1.
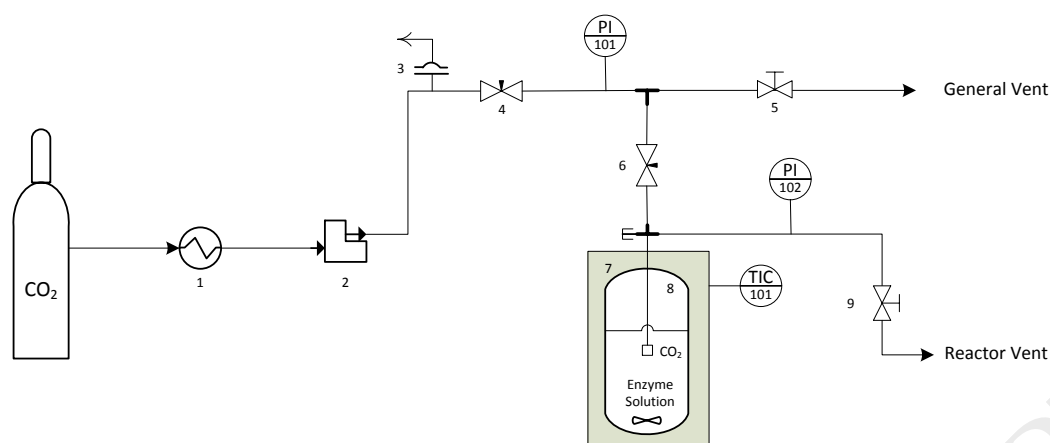
Figure 1. *Experimental set-up for HPCD enzyme treatment. 1: CO_2 cryostat; 2: syringe pump; 3: bursting disk; 4: general process valve; 5: general vent valve; 6: individual inlet valve; 7: water bath; 8: high pressure batch reactor with magnetic stirring; 9: depressurization valve. PI-101: general pressure gauge; PI-102: reactor pressure gauge; TIC-101 temperature controller of water bath.*

In a typical experiment, the enzymatic solution or juice was loaded into the reactor, which was then tightly closed. Subsequently, it was placed in the water bath previously set at the desired temperature. Afterwards, CO_2 was bubbled directly into the enzyme solution (through a sintered stainless steel micro-filter with a pore size of 10μm), in a sufficient amount to reach the desired working pressure, at a pressurization rate of 10 MPa/min. Pressure and temperature were held the desired operating time. Experiments were done in a temperature and pressure range commonly used in non-thermal inactivation HPCD systems: temperature from 40 to 55 °C and pressure from 6 to 18 MPa in the case of the experiments using PME (inactivation times in the range 2 to 75 min) and from 25 to 45 ºC and 6 to 20 MPa in the case of PPO (inactivation times in the range 2 to 15 min). After different inactivation times, the reactor was depressurized (at a depressurization rate of approximately 10 MPa/min) and the enzyme solution was taken out. All the samples were frozen at -20 °C until being analyzed to evaluate the residual activity of the enzyme after treatment. A similar procedure was followed when juices were used.

In the case of the pure commercial enzymes, the single effect of pressure and temperature was studied in order to find out the relevance of the addition of CO_2 in the enzyme inactivation performance. The effect of pressure was evaluated by treating the enzymatic solutions by HPP at Hiperbaric España. The enzymatic solution was placed in sealed polyethylene terephthalate (PET) bottles and introduced into a vessel subjected at pressures up to 600 MPa of isostatic pressure transmitted by water. Pressurization rate was 150 MPa/min and final pressure was held for 15 min. Depressurization occurred in approximately 2 s. The initial operating temperature was 17.2 °C. Physical compression during pressure treatment results in a volume reduction and an increase in temperature and energy, approximately 3 °C per 100 MPa. After the HPP treatment, the enzymatic activity was evaluated. The pure effect of temperature was evaluated by running a set of experiments at atmospheric pressure. In these experiments, the reactor containing 25 mL of the enzymatic solution was placed in a water bath at temperatures in the range 40-60 °C. Samples were taken periodically, up to a total time of 180 min.

2.3. Analysis

2.3.1. Enzyme activity

The PPO enzyme activity was assayed spectrophotometrically (Jasco V-750, Japan) using catechol as substrate and following a slightly modified protocol from Baltacioğlu et al [16]. The PME activity was assayed following the Resolution OIV-OENO 363-2012 for the *"Determination of pectin methylesterase activity in enzymatic preparations (complement to resolution 9-2008)"*. The residual activity (RA) of the enzymes was calculated by dividing the activity measured after the HPCD treatment by the initial activity, and it was expressed as percentage.

2.3.2. Tertiary structure

The tertiary structure of the enzymes was determined by fluorescence spectroscopy using the FLS980 photoluminescence spectrometer (Edinburgh Instruments, Livingstone, UK). The sample was excited at a λ_{em}=280 nm and the emission spectra were recorded in the range from 290 to 450 nm. A 1 cm path length cell was used and the emission and excitation slits were set at 2 and 1 nm respectively when the PPO enzyme was studied. For the PME enzyme, the emission and excitation slits were both set at 2 nm.

2.3.2.1.   ANS binding measurements

ANS (8-Anilino-1-naphthalenesulfonic acid) is an extrinsic fluorescence probe frequently used to study the exposure of the hydrophobic areas on protein surfaces. A stock solution of ANS (1 mM) was prepared in distilled water. In the ANS binding experiments, 0.1 mL of ANS were added to 2.4 mL of the enzyme solution. The excitation wavelength was set at 380 nm and the emission spectra were recorded in the range of 400–650 nm. The excitation and emission slits were set at 2 and 2 nm, respectively. This test was only run for PME.

2.4. Kinetic Models

The first order kinetic model is commonly used to describe the inactivation of enzymes. This model assumes that the logarithm of the residual activity decreases linearly with time. Eq. 1 describes mathematically this model:

$$\ln\left(A/A_0\right) = -kt \qquad (1)$$

A is the activity of the enzyme after treatment at given time t, A_0 the initial activity of the enzyme, k is the inactivation rate constant (kinetic constant) at given pressure and temperature conditions and t is the time the experiment lasts.

The two fraction kinetic model takes into account the existence of several isoenzymes, grouped into two fractions, a labile (L) fraction and a stable (S) one. Both enzymes are considered to be inactivated according to 1st order kinetics, but independently of each other, according to Eq. 2:

$$A = A_L\exp(-k_Lt) + (1 - A_L)\exp(-k_St) \qquad (2)$$

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 the inactivation rate constants of both the labile and stable fractions respectively. From the kinetic constants obtained from both models the pressure and temperature sensitivity parameters (Z_P and Z_T, respectively) were calculated. Conceptually, these parameters are the pressure and temperature increase required to reduce the decimal reduction time (D) by 90%. Decimal reduction time refers to the time needed to reduce the residual activity of the enzyme by 90% [9].

The Weibull model has also been used to fit the experimental results. This empirical model is presented in Eq. 3 and uses two different parameters: α, the scale parameter (a characteristic time) and β, the shape parameter [17].

$$\log(A/A_0) = -\frac{1}{\ln(10)}\left(\frac{t}{\alpha}\right)^{\beta} \qquad (3)$$

The decimal reduction time can be calculated using Eq. 4, and is named as t_D [18].

$$t_D = \alpha[ln(10^{-1})]^{1/\beta} \qquad (4)$$

The dependence of the scale parameter (α) on temperature can be well modelled using an exponential relationship [17], as shown in Eq. 5:

$$\log(\alpha) = a_1 - b_1T \qquad (5)$$

From Eq. 5, it can be seen that Z_T' equals -1/b_1. Z_T' by definition considers the non-linear part of the curve log(A/A_0) versus time, compared to the classic definition of D, which is calculated from the linear

part of the curve. Conceptually, Z_T' represents the temperature increase necessary to reduce the t_D value by 90%. A similar discussion was done for the dependence of the scale parameter with the pressure, Z_P', representing $\log(\alpha)$ versus pressure. In order to validate these calculations, the pressure and temperature dependence of the shape parameter (β) was evaluated, since there must be no dependence for these Z_P' and Z_T' calculations to be valid [17].

2.5. Statistical analysis

All the statistical analyses were done using the software Statgraphics X64. This software was used to perform the ANOVA test of the screening experiments and the non-linear regression of the experimental data to obtain the kinetic constants of the different models presented in section 2.4. This software was also used to obtain the surface plots of the residual activity obtained for both enzymes, under different experimental conditions.

3. EXPERIMENTAL RESULTS
3.1. Ratio CO_2:enzyme solution

Different volumes of the enzymatic solution were loaded in the reactor, in order to reach the desired ratio CO_2/volume of enzyme dissolution (g/mL), as a function of the pressure and temperature selected. As can be seen in Figure 2, similar trend was observed for both enzymes. It was seen that ratios higher than 3 did not enhance the inactivation kinetics, being a waste of CO_2 from the economic point of view. Extremely high ratios, as can be found in the literature, guarantee the complete inactivation of the enzymes. The importance of these experimental results is linked to the upscaling of the HPCD process, in which the amount of CO_2 used per mL of enzyme is critical in order to assure its economic success.

As can be seen in Figure 2, PME exhibited a higher resistance to the inactivation by HPCD at 45 ºC, presenting a higher residual activity than PPO.
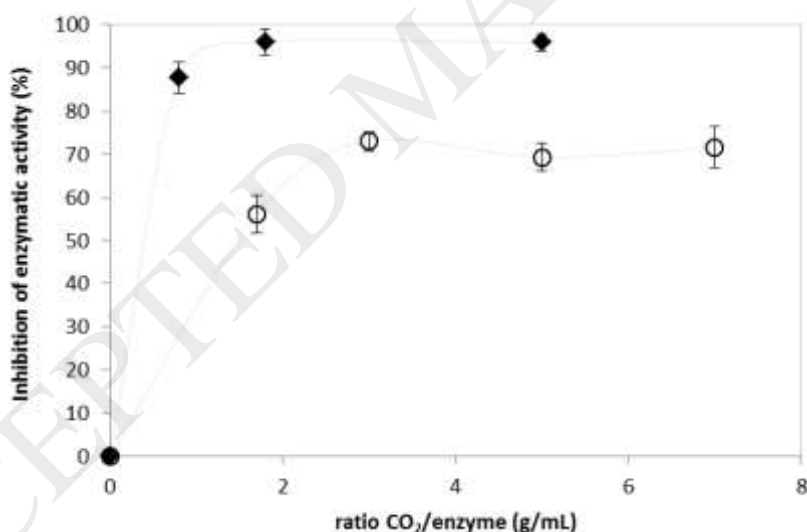
***Figure 2**. Percentage of inhibition measured for pure commercial PME (○) at 48 ºC and PPO (◆) at 45 ºC treated using different ratios CO_2:volume of enzyme solution. All the experiments were carried out at 18 MPa and lasted 15 min.*

3.2. PPO and PME kinetic inactivation study

The experimental results revealed that PPO and PME behaved in a completely different way when treated by HPCD. PPO showed to be very sensitive to both temperature and pressure, whereas PME was almost exclusively affected by temperature. The inactivation pattern was also different: according to the results presented by Benito-Román et al. [19], PPO showed a sharp initial activity decrease followed by a slowed activity decay in all the studied conditions. This pattern proved the presence of two isoenzymes, a labile and a stable fraction, being the latter very insensitive to the presence of CO_2. The two fraction model was

used to explain the experimental data. Regarding the PME, the first order model was used to explain the experimental data observed, according to the results presented by Benito-Román et al. [20]. The main kinetic parameters calculated for each enzyme are presented in Table 1.

Table 1. *Experimental results obtained for both PPO and PME treated by HPCD.*
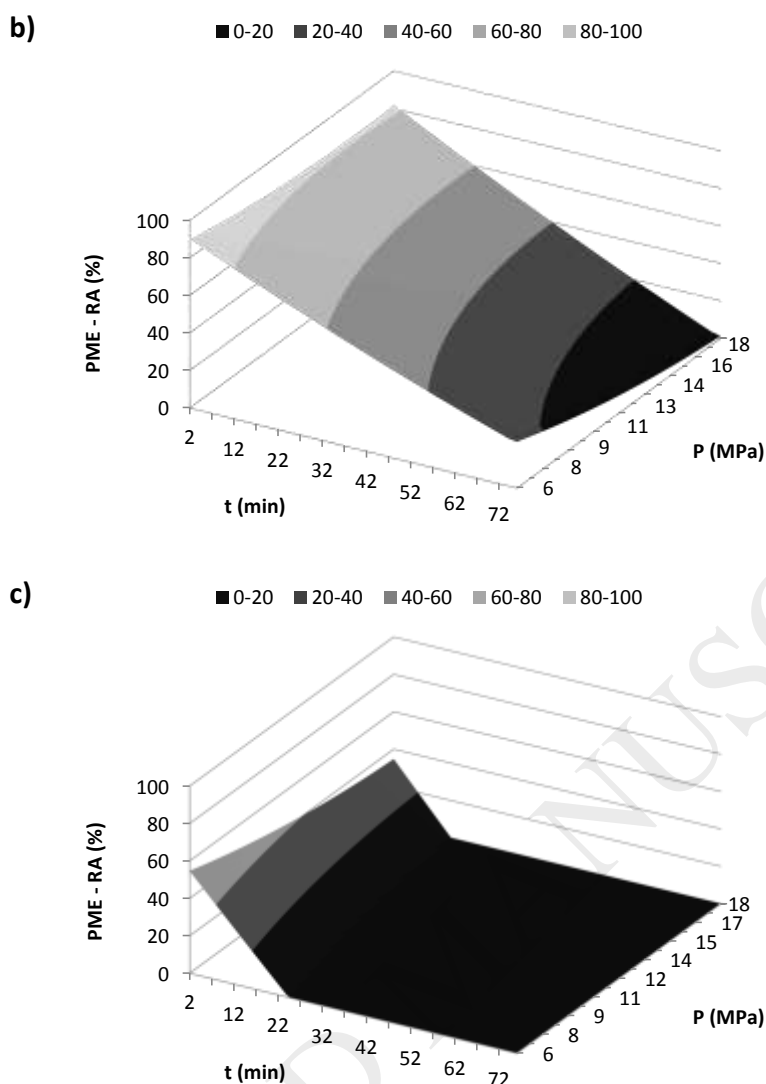
	PPO		PME
Pressure (MPa)	5-20		6-18
Temperature (ºC)	25-45		40-55
Time (min)	Up to 15 min		Up to 75 min
Kinetic Model	Two Fractions	Weibull	First Order
Z_P (MPa)	6.9-7.9	5.0-4.3	27.6-45.0
Z_T (ºC)	27.0-40.7	15.2-7.4	7.9-8.7

In the case of PPO, at constant temperature, higher inactivation rates were observed the higher the pressure. With regards to PME, higher pressures did not lead to faster inactivation rates; only increases in temperature tend to promote the inactivation rate.

These trends can be seen in Figure 3 (a) and (b), where the surface plots for the residual activity versus time and pressure at 45 ºC are shown. For PPO (Figure 3a) after two min the enzyme has significantly lost half of the initial activity at the lowest pressure. Further increases of pressure lead to even faster inactivations at a given time. In general this enzyme only requires 15 min to get a high inactivation degree, whereas at 45ºC, PME required at least 60 min to get similar inactivation degree, as can be seen from Figure 3b. In Figure 3b, the linearity of the inactivation rate with time can be observed as well as the limited effect of pressure, at 45 ºC. However, when temperature is increased up to 55 ºC, PME can be inactivated in a short period of time, as can be seen in Figure 3c. PME requires higher temperatures, in contrast to PPO that can be completely inactivated at 20 MPa after 5 min regardless the temperature.


a)

■0-20 ■20-40 ■40-60 ■60-80 ■80-100

b)

c)
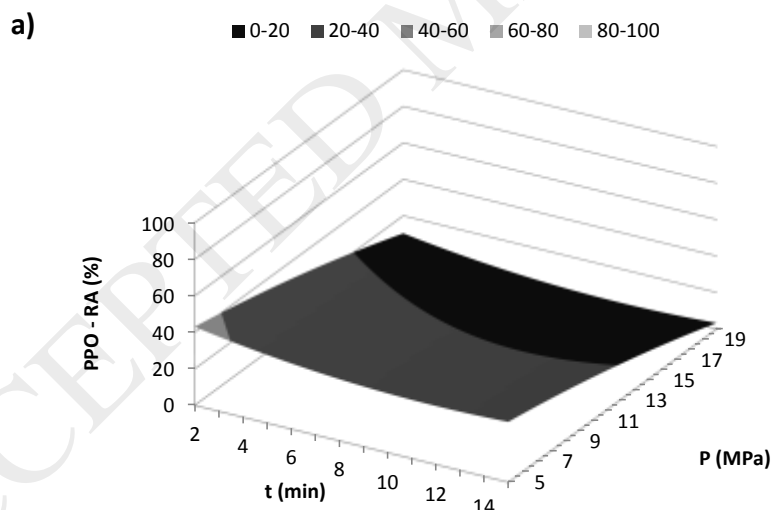
Figure 3. *Surface plots for the residual activity of PPO (a) and PME (b) obtained at 45 ℃ versus pressure and time. In figure (c) the residual activity of PME treated at 55 ℃ versus pressure and time is presented.*

In general, and compared to the PPO, PME has been observed to be more resistant to the HPCD treatment: longer times and higher temperatures are required to achieve significant inactivation (more than 40 °C, preferably above 50 °C). The temperature sensitivity parameter (Z_T) was significantly lower for PME (7.9-8.7 °C) compared to PPO (27.0-40.7 °C). In turn, it was observed that pressure increases did not increase the inactivation rate of the PME (Z_P values for PME were in the range 27.6-45.0 MPa, whereas for PPO were from 6.9 to 7.9 MPa). The value of Z_P proved the experimentally observed trend of an important insensitivity of PME to pressure. The results presented in Table 1, show that PPO is more sensitive to pressure than PME (five or six times more), and in turn, PME is affected by temperature in a greater extent.

a)

b)
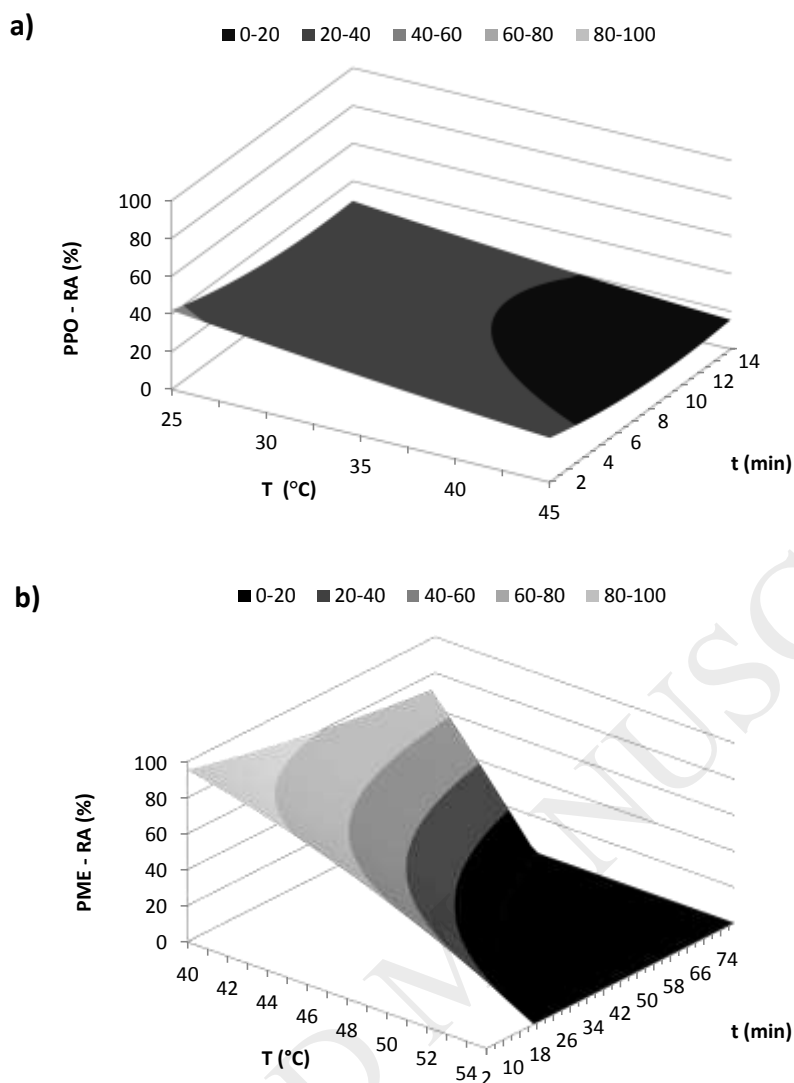
Figure 4. *Surface plots for the residual activity versus temperature and time of PPO (a) and PME (b) obtained at 15 MPa.*

Figure 4, where the residual activity of both enzymes versus temperature and HPCD treatment length has been calculated at 15 MPa, shows that, for PPO, temperature has a limited role, only a slight decrease in residual activity is observed. For PME, a different trend is observed: at low temperatures the inactivation degree is significantly affected by the time the enzyme is exposed to the CO_2, however at the highest temperature studied, it can be seen that, after only 17 min, the complete inactivation of the enzyme is achieved. It can also be seen that, at short times, there is a clear effect of temperature at 15 MPa; similar patterns were observed at other pressures studied.

In general, and compared to PPO, PME has been observed to be more resistant to the HPCD treatment: These different behavior when exposed to HPCD can be attributed to their different structure, which is fairly complex for PPO (3 or 4 subunits with a MW around 130 kDa) whereas PME is formed by only one subunit with a MW around 35 kDa [15]. According to Xu et al. [14] the more complex the structure the more susceptible is to changes in the environment. This also can explain the differences in the inactivation kinetics: PME followed the simplest model, the first order one, while PPO followed a two fraction model, which revealed the presence of two isoenzymes with different inactivation kinetics [19].

3.3. Pure effect of pressure and temperature on the activity of the enzymes PPO and PME

Results of the experiments done in order to evaluate the pure effect of pressure and temperature revealed that the HPCD technology significantly improved the inactivation rate of PPO and PME. In the case of pressure, the HPP experiments allowed to prove that both enzymes were almost unaffected by high pressures: PME, after having been subjected to 600 MPa, showed a residual activity of 99.5±5.3%. Three replicates were done, and in two of them an increase of the residual activity was observed, which indicates that high pressures might promote the enzymatic activity. PPO exhibited a similar behavior, resulting in a residual activity of 96.3±2.5%. These results proved that just pressure did not inactivate those two enzymes.

Regarding the pure effect of temperature, it was observed that it played an important role in the inactivation kinetics of the enzymes. In the absence of CO_2, it was observed that longer times were required to obtain similar inactivation extents. PPO was subjected to temperatures in the range 25 to 45 ºC for times up to 180 min. The residual activity was 79.0±1.0% and 26.3±2.2% for 25 and 45 ºC, respectively, after 180 min. Longer times did not produced significantly higher decreases in the activity of the PPO. The decimal reduction time obtained for the thermal inactivation experiments done at 45 ºC was 1723±3 min. When HPCD was used, it was seen that it promoted the inactivation of the enzyme: after only 15 min the residual activity was 34.6±2.7% at 5 MPa and even lower at 20 MPa (3.7±0.6%). Under these conditions (20 MPa and 45 ºC), the decimal reduction time was 1.8±0.3 min, compared to the 1723±3 min obtained in the thermal inactivation experiments. Similar results were observed for PME, when subjected to temperatures in the range 45-55 ºC for 180 min. In this case, the decimal reduction times were calculated, being in the range 2424±68 min (at 45 ºC) to 68±2 min (at 55 ºC). The use of HPCD decreased dramatically the decimal reduction time: at 6 MPa it was 1182±60 min (at 45 ºC) and 433±6 min (at 55 ºC), whereas at 18 MPa it was 15.0±0.3 min (at 45 ºC) and 8.1±0.2 min at 55 ºC. Despite the relative long times used in the HPCD treatment, it has been observed that temperature was not the main inactivation effect, being the CO_2 critical in the inactivation process of PME.

The use of HPCD promoted the inactivation of the enzymes PPO and PME, being the effect of the addition of CO_2 significantly higher than the pure effect of pressure and temperature. It is important to remark that the temperature range considered for the HPCD treatments (usually from 20 to 50 ºC [9]) is low enough to be considered as a non-thermal treatment.

### 3.4. Changes in the tertiary structure of pure enzymes

The effect of the HPCD on the tertiary structure of the enzyme was evaluated by fluorescence spectroscopy. Different studies were carried out, which demonstrated that PPO and PME were affected in different ways by the SC-CO_2.

3.4.1. Intrinsic fluorescence experiments

Figures 5 and 6 show the emission spectra of PPO (treated at 45 ºC) and PME (treated at 55 ºC), respectively. Those temperatures led to the highest inactivation rates for each enzyme. In Figures 5 and 6, the HPCD treatment lasted up to 15 min, thus it is possible to see how the length of the exposure of the enzyme to SC-CO_2 is changing the structure of the enzyme, inducing a folding or unfolding effect. In proteins, the fluorescence emission is due to the presence of tryptophan residues. This residue is very sensitive to the polarity of the medium around it [21]: on the one hand, in the presence of a non-polar medium, the maximum intensity is set at 305 nm, but an increasing polarity of the media red-shifts the maximum intensity up to 350 nm. On the other hand, if TRP residues are exposed to a polar solvent, part of the energy absorbed during the excitation period is transferred to that solvent, happening a quenching effect: the emission signal has lower intensity and is red-shifted [22]. All these phenomena indicate that the structure of the enzyme is changing and the residues are being reallocated [23]

Regarding the PPO, the longer the time it is exposed to the CO_2, the higher the emission intensity detected. However the residual activity of the enzyme slightly changed, moving from 7.8% after 2 min to only 3.7% after 15 min of treatment. The longer exposure of the enzyme to the CO_2 did not mean an increase in the inactivation degree. Although there were no significant changes in the residual activity of

the enzyme, a significant increase in the emission intensity was observed. The maximum wave length that provided the maximum intensity also changed: it was clearly red shifted, from 324 nm in the native PPO to 336 nm in the enzyme treated for 2, 5 and 15 min. The time the enzyme was exposed to the CO_2 did not involve a deeper red-shift of the signal. All these results indicate that, compared to the native enzyme, the HPCD treated PPO has suffered important structural changes; on the one hand, the increase in the maximum emission wavelength can be interpreted as a greater exposure of the TRP residues to a more polar environment (what would mean that the enzyme has unfolded), but on the other hand, the increase in the emission intensity would mean that the exposure to water is not involving a quenching effect, which might be contradictory. The complexity of the structure can be behind these results: PPO from mushroom is a type-3 metalloprotein containing a di-nuclear copper active site bound to the protein matrix (four α-helix bundles that surround the active site) by six histidine residues [24]. The exposure to the non-polar CO_2 alters this equilibrium, affecting the stability and activity of the enzyme.
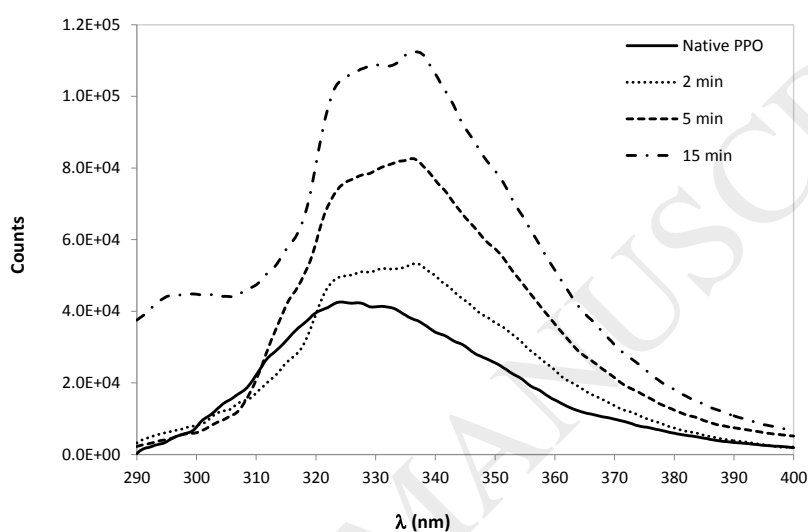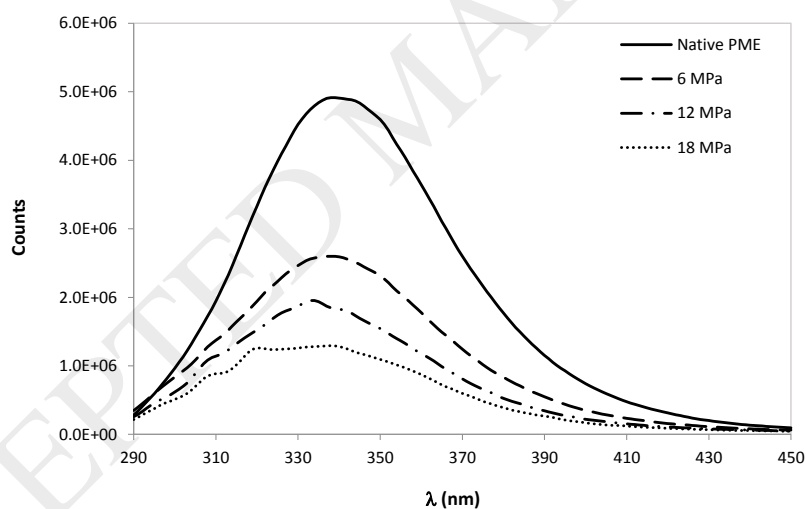

Figure 5. *Fluorescence emission spectra for PPO treated by HPCD (45 °C, 20 MPa) for different times.*

PME exhibited a different behavior, as can be seen in Figure 6: the longer the enzyme was exposed to the HPCD the lower the emission intensity was, in experiments lasting from 2 to 15 min, at 55 ºC and 18 MPa. Native PME exhibited the maximum intensity at 338 nm, which remained almost unaltered at the different exposure times to CO_2. In this case, it seems that the exposure of the TRP residues to the environment has not changed significantly, as the insignificant changes in the maximum emission wavelength demonstrate; however, the decrease in the intensity seems to indicate that a quenching effect is happening, and that energy absorbed in the excitation step is being transferred somewhere else. In any case, as mentioned above, besides having a significantly simpler structure than PPO, PME has 5 different tryptophan residues [25], two of them exposed to the solvent, another two partially exposed to the solvent and finally one completely buried in the protein structure.
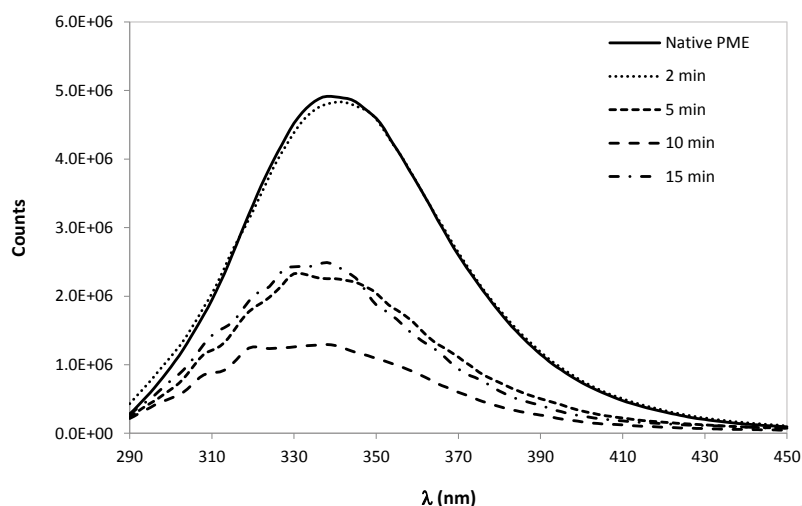
***Figure 6.*** *Fluorescence emission spectra for PME treated by HPCD (55 °C, 18 MPa) for different times.*

In Figure 7, the effect of pressure (6-18 MPa) on the emission spectra of PME treated at 55 ºC for 10 min is shown. It can be seen how the higher the pressure the lower the intensity. It is possible to see how the lower the pressure the smoother the signal is, which proves that changes in the structure of the enzyme are happening. The detected changes in the intensity correlate with the changes detected in the residual activity, which changed from 24.5% at 6 MPa to 5.8% at 18 MPa. This indicates that pressure reduces the activity of the enzyme affecting its tertiary structure.

Figure 7. *Fluorescence emission spectra for PME treated by HPCD at different pressures (at 55 ºC and 10 min).*

3.4.2. ANS binding experiments

In Figure 8, the ANS binding experiments carried out for PME inactivated thermally and by HPCD are shown.

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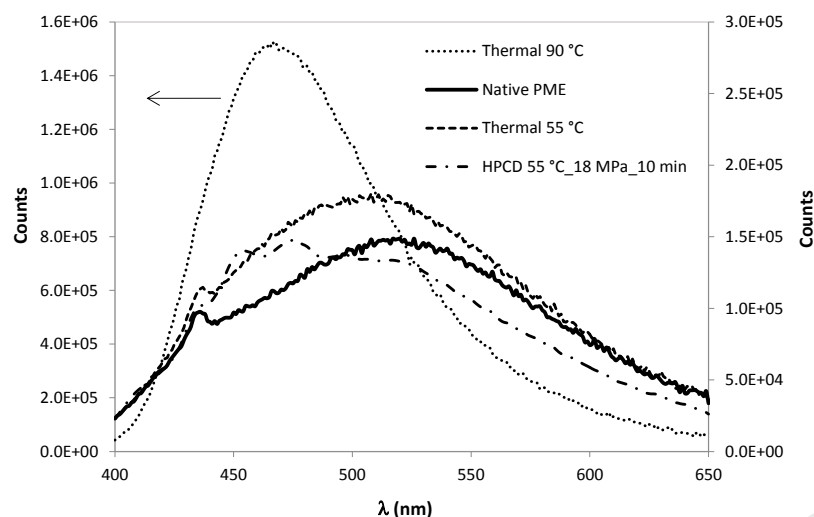
Figure 8. *ANS binding experimental results presented for PME treated thermally and by HPCD (at 55 ºC and 18 MPa. Results for PME thermally treated at 90 ºC are presented in the primary Y axis.*

The emission spectra for the ANS binding experiments carried out for the PME enzyme, revealed that HPCD and thermal inactivation induce significantly different changes in the structure of the enzyme. Thermal inactivation at 90 ºC revealed a completely destroyed structure, as supports the important blue shift of the emission spectra (maximum was detected at 467 nm, whereas native PME had the maximum at 526 nm), together with the dramatic increase in the emission intensity (10 times higher than native PME) detected. These two facts indicate that the hydrophobic core of the enzyme has been completely open. When the enzyme was treated by HPCD, the emission intensity did not change significantly: 38% higher than native PME at 48 ºC and 34% higher at 55 ºC, with a residual activity of 10% and 6%, respectively. At 55 ºC maximum emission was detected at 475 nm and at 490 nm at 48 ºC. These experiments reveal that, although in both cases the enzyme is almost completely inactivated (thermally and HPCD treated), both processes affect in different ways to the structure of the enzyme, and therefore the inactivation mechanism is different in both cases.

3.5. Application of HPCD to the inactivation of enzymes in real substrates

The experimental set-up used to inactivate pure enzymes was also used to treat juices obtained from fruits and vegetables, as well as solid products (shrimps). The most significant results, regarding kinetic parameters, for both pure enzymes and their natural media are presented in Tables 2 (PPO) and 3 (PME).

Table 2. *Kinetic parameters calculated for PPO obtained from different sources*

Source	Model	P (MPa)	T (ºC)	Z_P (MPa)	Z_T (ºC)
PPO [19]	Two Fraction	5-10-20	25-35-45	6.9-7.0-7.9	27.0-33.9-40.7
	Weibull	5-10-20	25-35-45	5.0-5.2-4.3	15.2-11.8-7.4
Apple [9]	1st order	10	35-45	-	29
		12.5-20	45	25	-
	Weibull	10	35-40-45	-	27
		12.5-15-20	45	27	-
Shrimps	Weibull	20	30-40-50	-	24

Table 3. *Kinetic parameters calculated for PME obtained from different sources*

Source	Model	P (MPa)	T (ºC)	Z_P (MPa)	Z_T (ºC)
PME [20]	1st order	6-12-18	40-48-55	27.6-45.0-44.4	7.9-8.7-8.7
Orange [26]	no parameters were calculated				
Tomato [27]	Weibull	20	35-45-55	-	10
		8.5-10-15-20	45	43	-
Carrot	Weibull	10-30	20-40	123	15

3.5.1. Fruit and Vegetable juices

Juices from several fruits and vegetables were treated by HPCD. The main results are presented in the following paragraphs.

3.5.1.1. Apple Juice

Illera et al, 2018 [9] dealt with the inactivation of PPO in juices obtained from apple (*Malus domestica* cv. Golden Delicious). These authors studied the effect of pressure in the range 10-20 MPa and temperature from 35 to 45 ºC, in experiments lasting up to 120 min. It was observed that the inactivation rate of PPO from apple increased the higher the pressure and temperature: at 20 MPa and 45 ºC the residual activity of the enzyme was only 10%. They also observed that the enzyme seemed to be more sensitive to temperature rather than to pressure changes. The experimental data fitted the 1st order model and the Weibull models reasonably well, in contrast with the results obtained when pure PPO had been subjected to the HPCD treatment, where the experimental results were modeled using the two fraction model. The kinetic parameters obtained for apple PPO were the following: Z_T, 29ºC and Z_P, 25 MPa. As can be seen, Z_T obtained for apple PPO is quite similar to that obtained for the pure PPO, whereas Z_P is significantly higher (up to 3 times), which indicates that apple juice requires much higher increases in pressure to reduce the decimal reduction time by 90%. Juice obtained from apple is a complex mixture of molecules, sensitive to temperature but quite resistant to pressure. The interactions that might appear among all these molecules serve as a protective network for the enzyme, hindering the effect to pressure. Marszałek et al [28] also studied the inactivation of PPO from apple juice by HPCD and compared it with the inactivation of a pure commercial enzyme. These authors also concluded that the resistance of the enzyme to the HPCD treatment depends on its source, being more resistant the enzymes found in juices compared to the commercial ones.

Initial pH of cloudy apple juice was 3.89±0.01 and immediately after HPCD treatment it fell down to 3.74±0.01. The particle size distribution, measured by the Mastersizer 2000 (Malvern Panalytical), of untreated cloudy apple showed three maxima at 0.63, 19.96 and 208.93 μm. After HPCD treatment (20 MPa, 45 ºC and 60 min), the particle range shifted to smaller particle sizes with one main maximum at 0.182 μm. The average particle size ($D_{4,3}$) before and after the treatment were 109±3 μm and 2.4±0.2 μm, respectively.

3.5.1.2. Orange Juice

Briongos et al. [26], studied the effect of SC-CO₂ on the enzyme PME present in juice squeezed from oranges (*Citrus sinensis* cv. Valencia). Orange juice was treated by HPCD at temperatures up to 40 ºC, in the range 10-30 MPa and times up to 60 min. Orange PME was significantly affected by temperature, whereas pressure did not produce a significant effect on the inactivation performance of this enzyme. These results match the results presented when pure PME was used. Nevertheless, important differences between the inactivation trend of pure and orange juice PME were observed. The inactivation kinetics of pure PME were explained by the first order model, while the inactivation kinetics of the PME obtained from juice was explained by the two fraction model, presenting a residual activity around 10% (at 40 ºC and 30 MPa for 60 min) or 37% at (21 ºC, either at 10 or 20 MPa for 60 min). Despite the relatively low temperature used, compared to the pure enzyme, the inactivation rate of PME from orange juice was quite high and significantly higher to that obtained for the pure PME (residual activity was

74%, at 40ºC and 18 MPa for 60 min). As it was shown when pure PME was used, high temperatures are required to inactivate this enzyme, since it has proved itself to be resistant to the HPCD treatment.

The pH of the orange juice before and after the HPCD treatment did not change significantly, being 4.17±0.01 and 4.14±0.02, respectively. The particle size distribution of the juice was also measured. Two maxima around 0.8 μm and 850 μm were observed. The size of stable cloud particle has been reported to be in the range of 0.4-5 μm, whereas the larger particle size can be due to the presence of settling pulp [26]. The freshly squeezed juice had an average particle size ($D_{4,3}$) of 523±37 μm, whereas immediately after the HPCD treatment it was 438±26 μm. It could be observed a decrease in the average particle size of the juice, as well as a homogenization of the particle size distribution, probably induced during the depressurization stage.

3.5.1.3. Tomato Juice

With regards to the tomato juice (*Lycopersicon esculentum* cv. Canario), it was loaded in the reactor and treated by HPCD, as described Illera et al., [27]. The pressure range was 8.5 to 20 MPa and temperatures were in the range 35 to 55 ºC. Compared to the orange juice inactivation experiments, higher temperatures were tried, given the low inactivation degrees observed for PME at 40 ºC. In this case, the experimental results fitted the Weibull Model, and the main kinetic parameters were Z_T'=10±2 ºC and Z_P'=43±3 MPa. A nearly complete inactivation of the enzyme was observed when it was treated at 55 ºC and 20 MPa. In general it was observed that, at 20 MPa, increases in temperature increased the inactivation rate. Data obtained at 45 ºC showed an inactivation rate increase with pressure; residual activity ranged from 60% at 8.5 MPa to 45% at 20 MPa, which is in agreement with the results presented for the pure PME.

The pH of the tomato juice was not significantly affected by the HPCD treatment at 20 MPa, only a minimum decrease from 4.09 to 4.05 at 45 ºC, from 4.16 to 4.15 at 35 ºC and from 4.40 to 4.38 at 25 ºC. However, more significant differences were observed in the average particle size distribution (from 356±7 to 72±1 μm), as shown in Figure 9:
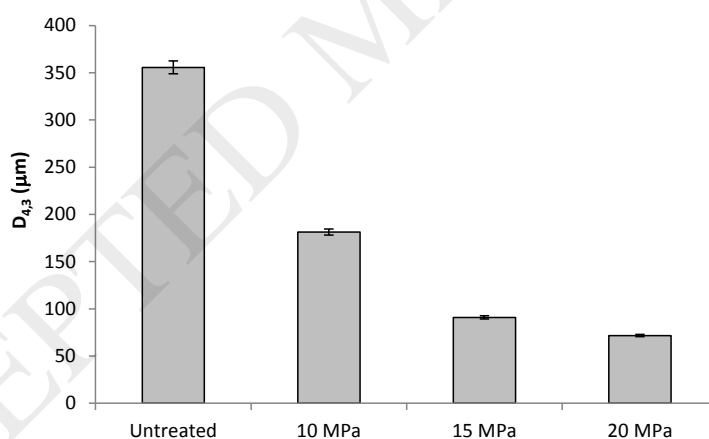
Figure 9. *Average particle size distribution of tomato juices treated by HPCD (45 ºC, 60 min) at different pressures.*

Similarly to the results obtained for orange juice, the HPCD treatment induced the reduction of the average particle size of the tomato juice, leading to a more homogenous distribution. The untreated juice exhibited two maxima around 0.8 and 800 μm. In treated juices the weight of the smallest maximum in the distribution grew, the higher the pressure. The maximum with the largest particle size was centered in 100 μm for the juices treated by HPCD, decreasing the higher the pressure.

3.5.1.4. Carrot Juice

Juice obtained from carrot (*Daucus carota*) was treated by HPCD, in order to study the inactivation of the PME enzyme. Carrot juice was loaded in the reactor shown in Figure 1, and the effect of pressure (10-30 MPa) and temperature (20-40 ºC) were studied in experiments up to 60 min long. PME from carrot

resulted to be the most resistant to HPCD treatment among all the fruits and vegetables studied: at 20 ºC, only a 20% decrease in the residual activity at 10 and 20 MPa was observed, increasing slightly at 30 MPa. Indeed, at 40 ºC the pressure sensitivity parameter was 123±3 MPa, which provides a clear idea of the insensitivity of this enzyme to pressure, as was seen experimentally. From the experimental work, it was concluded that high temperatures were required to inactivate PME from carrot. The temperature sensitivity parameter obtained at 30 MPa was 15±3 ºC, slightly higher than that obtained for the pure PME. Other pressures studied resulted in similar values for the sensitivity parameter, supporting the idea of the limited effect of pressure on this enzyme. Figure 10 shows the residual activity versus time for the carrot juice inactivation experiments carried out at 20 MPa and different temperatures. All these kinetic parameters were calculating using the Weibull model, which fitted reasonably well the experimental data: a sudden decrease in the activity was observed followed by slowed activity decay. Dashed lines in Figure 10 represent the inactivation curve calculated using the Weibull model.
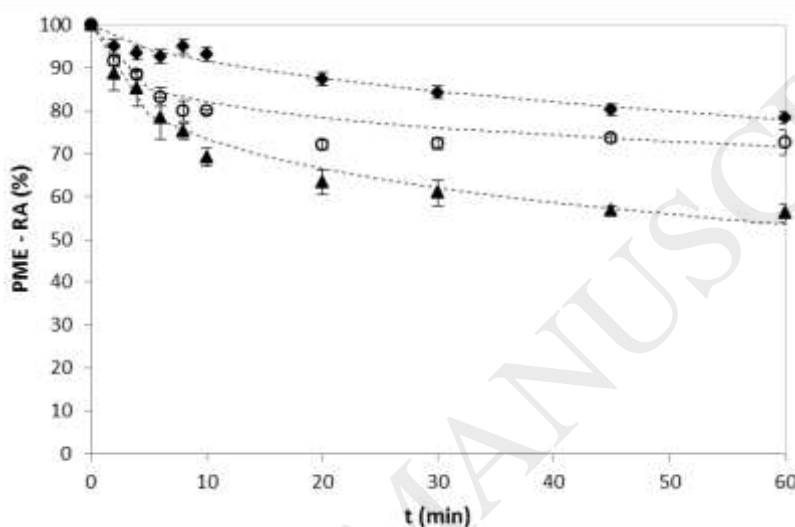
Figure 10. *Residual activity for PME, present in carrot juice, treated by HPCD at 20 MPa: ◆, 20 ºC;○, 30 ºC; ▲, 40 ºC. Dashed lines represent the Weibull model.*

The pH of the carrot juices was significantly affected by the HPCD treatment, decreasing dramatically. The initial pH of the juice was 6.10±0.01. When this juice was treated at 20 MPa for 60 min, the pH dropped to 5.25±0.02 at 30 ºC and to 5.19±0.02 at 40 ºC. Freshly squeezed carrot juice has a higher pH compared to orange or tomato juice. The presence of CO_2 tends to decrease it, and therefore the organoleptic properties of this juice can be significantly more affected that those of more acidic juices.

3.5.2. Shrimps

The HPCD technology was also applied to inactivate PPO extracted from shrimp head. In this case, the PPO was extracted using a phosphate buffer solution at pH 7.2 and 4 ºC. Afterwards, it was treated with supercritical carbon dioxide for 60 min at 20 MPa and at three different temperatures in the range of 30-50 ºC. The experimental results fitted the Weibull model and the temperature sensitivity parameter was calculated at 20 MPa, resulting to be 24±6 ºC. This is significantly higher than the value obtained for the pure PPO using the same model (7.4±1.4 ºC) which indicates that a higher temperature increase in required to reduce the decimal reduction time of shrimps PPO by 90%. This is probably due to a protective effect that other molecules present in the extract hinder the action of the CO_2 on the enzyme. It is also possible that the buffer solution presents a mitigating effect, which is translated in a lower solubility of the CO_2 in the media and hence a lower pH drop.

Finally, shrimps were directly treated by HPCD. They were introduced in the reactor with and without water and treated at 12 MPa and 40 ºC for 30 min. The appearance of the shrimps after the treatment can be seen in Figure 11. After the HPCD treatment the color of the shrimps changed, having the appearance of boiled shrimps. The activity of the PPO was significantly reduced. Indeed, shrimps treated without the presence of aqueous media in the reactor, did not developed a black color in the head until the 5th day;

however the presence of water in the reactor did not favor the PPO inactivation, since two days after the treatment the head of the shrimp was already black.

a)

b)

c)
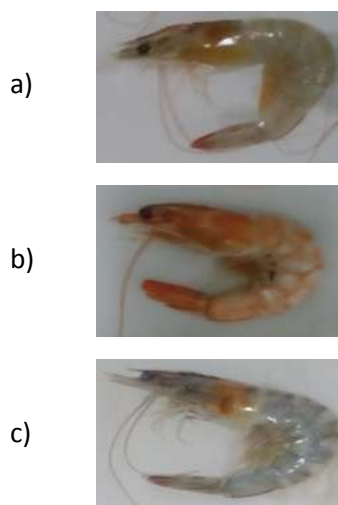
Figure 11. *Appearance of the shrimps: a) shows the native shrimp; b) shows the shrimp after HPCD treatment without water and c) shows the shrimp after HPCD treatment with water. In both cases, HPCD treatment was carried out at 12 MPa, 40 ºC and 30 min.*

3.5.3. General remarks

The experimental results above presented indicate a reasonably good agreement between the inactivation degrees obtained for pure enzymes and those obtained for fruits and vegetables after HPCD treatment. In general, the enzymes PPO and PME in juices and solid foods tend to be more resistant than the pure enzymes to the HPCD treatment, which indicates that more intense treatments will have to be carried out in order to achieve successful industrial inactivation rates. Higher ratios CO_2/volume of enzyme treated might also help to enhance the enzymatic inactivation. The experimental data obtained for fruits and vegetables have been fitted to the Weibull model. The reason behind this fact is related to the nature of this model: it is a statistical model of distribution of inactivation times and assumes that not all the enzymes present have the same probability of being inactivated [17]. On the other hand, the first order kinetic model assumes that all the enzymes have the same probability of being deactivated. In a complex mixture, as a juice or purified enzyme obtained from fruits, vegetables or shrimps, it seems difficult to accept that assumption, since the enzymes might be linked to other molecules and their interaction with the SC-CO_2 might be affected. That is the reason why the pure enzyme experiments were carried out in an attempt to understand the inactivation mechanism and the conformational changes that the enzyme is suffering as a consequence of the exposure to CO_2.

4. CONCLUSIONS

The use of HPCD significantly promoted the inactivation of pure PPO and PME, compared to the inactivation obtained only by high pressures or temperatures. PPO and PME activity is significantly affected by HPCD, but in different ways: on the one hand PPO is affected by both pressure and temperature and the inactivation kinetics is described by the two fraction model; on the other hand, PME is only affected by temperature and the inactivation pattern follows the first order model. The analysis of the kinetic parameters reveals a higher stability of PME to the HPCD treatment. Both enzymes suffer dramatic changes after the HPCD treatment, as the fluorescence spectroscopy analysis revealed. Despite all these differences, both enzymes shared the importance of the ratio CO_2/volume of enzyme loaded in the reactor on the inactivation performance, since ratios higher than 3:1 guaranteed a complete inactivation.

The inactivation of PPO and PME in juices from several species followed similar pattern to the inactivation kinetics presented by the pure enzymes (pressure and temperature sensitivity parameters were in the same order of magnitude), but some differences existed. These differences between pure enzymes

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and enzymes from juices can be explained by the presence of other molecules and the interaction/protective effects that might appear.

The HPCD treatment induced a slight drop of pH in juices as has been presented and also induced changes in the particle size, leading to a homogenization of them. These two facts might affect the organoleptic properties of the juices, and should be considered. All in all, HPCD technology is useful to inactivate enzymes in juices, and the selection of the working parameters will have to be done taking into account the substrate to be treated.

5. ACKNOWLEDGEMENTS

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