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The effect of high pressure processing on clingstone and freestone peach cell integrity and enzymatic browning reactions

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ABSTRACT

HPP-treated fruits and vegetables may undergo undesirable enzymatic browning reactions due to loss of membrane permeability and sub-cellular compartmentalization. Clingstone and freestone peaches were treated from 100 to 500 MPa for 10 min and evaluated for polyphenol oxidase (PPO) activity, color, total phenols, and for cell integrity using light microscopy and ^{1}H NMR. Significant changes in membrane integrity following HPP above 200 MPa were determined by T_2 shifts in the vacuolar compartment from initial levels of 0.79 (clingstone) or 0.88 (freestone) to approximately 0.60–0.68. Clingstone peaches treated at 300, 400 and 500 MPa showed significant decreases (5, 12 and 7%) in % water of the vacuolar compartment and simultaneous increases in the cytoplasmic compartment (4, 8 and 5%). Additionally, there was a reduction in the number of viable cells from an initial 57–58% to 0 and 14% in clingstone and freestone peaches, respectively. These results correlated with the development of increased browning.

Industrial relevance: Clingstone peaches are firm-textured and therefore are preserved primarily through canning, which desirably softens the texture. In this study we evaluated the use of high pressure processing – at a range of MPa levels – for preservation, and found that enzymatic browning took place after 2 weeks in refrigerated storage if processing occurred above 200 MPa. Analytical tools were developed to follow the onset of the browning, and in future work preventative measures will be studied to minimize this reaction.

1. Introduction

High pressure processing (HPP) is a novel food preservation method that is currently receiving the most attention from the food sector, because it is successful in creating products of high nutritional value and sensorial quality. The advantage of this preservation method is that it is not only a microbiologically safe means of providing longer shelf life but also retains the fresh characteristics of products.

In general, pressures in the range of 100–600 MPa are used for food preservation (Palou et al., 2000), but these pressures may also result in loss of cell and therefore tissue integrity. In plant-based materials, cell integrity plays an important role in final product characteristics, in particular color and texture. Loss of cell integrity involves increased membrane permeability, resulting in opening of subcellular organelles and movement of water and metabolites within the cell. High pressure treatment was previously reported to affect the peach enzymes involved in changes in color, polyphenol oxidase activity (Rao et al., 2014) and activity of the enzyme involved in textural changes, pectin methylesterase (Boulekou, Katsaros, & Taoukis, 2010). Undesirable color changes in HPP-processed fruit are a result of enzymatic browning reactions induced by the loss in cell integrity. This loss allows the interaction between the enzyme, initially located in the plastid of the intact fruit, and its substrates, which are initially located in the vacuole. Color is a primary quality attribute in fruits, therefore enzymatic browning reactions have been a crucial problem in HPP treated fruits e.g. mango puree (Guerrero-Beltrán, Barbosa-Cánovas, & Swanson, 2005), banana puree (Palou, López-Malo, Barbosa-Cánovas, Welti-Chanes, & Swanson, 1999), tomato puree (Sánchez-Moreno, Plaza, De Ancos, & Cano, 2006) and navel orange juice (Polydera, Stoforos, & Taoukis, 2005). The complexity of the browning reaction and its substantial impact on food quality has motivated food scientists to explore it at the cellular level. Numerous authors have considered that PPO (1,2-benzenediol; oxygen oxidoreductase, EC 1.10.3.1) and phenolic compounds are the major factors involved in the enzymatic browning reaction (Lee, Kagan, Jaworski, & Brown, 1990; Cheng & Crisosto, 1995; Coseteng & Lee, 1987). However, Cantos, Tudela, Gil, and Espín (2002) observed different results in potatoes, where there was no significant correlation between the degree of browning and any biochemical attribute they tested, e.g. PPO, peroxidase or initial phenolic content. These observations led us to the idea that cell integrity and ability of the enzyme and its substrate to interact may be an important factor controlling browning reactions.

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A combination of NMR-relaxometry and light microscopy were used in this study to characterize the integrity of membranes and cells. These techniques were successfully employed for quantification of onion cell integrity following thermal and HPP processing (Gonzalez, Barrett et al., 2010) and for determining the effect of HPP on strawberry parenchyma tissue (Marigheto, Vial, Wright, & Hills, 2004). ^{1}H NMR relaxometry is widely applied in plant research for probing sub-cellular changes. The proton spin–spin (T_2) relaxation time is related to water content, the properties of water in different cellular locations, and the interaction of water with macromolecules (Snaar & Van As, 1992). Therefore the change in permeability of a membrane, in particular the tonoplast, which encloses the large percentage of cellular water located in the vacuole, should be evident by a change in the T_2 spectrum. The observation of cell integrity using light microscopy is accomplished using a cell viability staining method. In this study, neutral red (NR) is the viability stain used for discriminating intact vacuoles (Admon, Jacoby, & Goldschmidt, 1980). The main property of this initially yellow dye is to diffuse across the tonoplast membrane into the acidic environment of the vacuole, where it undergoes a color change to an intense red. Once damage occurs in plant cells, the tonoplast loses its integrity, releasing the neutral red dye throughout the cell and resulting in a quantifiably less-intense red color.

In general, peaches are classified into two major types, clingstone and freestone. The clingstone type has a stone (endocarp) that tightly clings to the flesh (mesocarp) and usually has a firm texture, which is desired for commercial canning. Freestone peaches are those in which the flesh is easily freed from the stone, and are consumed fresh due to their soft texture. Because of genetically controlled differences between these two types, e.g. integrity of the mesocarp as well as level of PPO activity and concentration of phenolic compounds, browning scenarios in clingstone and freestone peaches following HPP treatment hypothesized to differ. The objectives of this study are to determine the effect of pressure levels in the range of 100–500 MPa on cell integrity in clingstone and freestone peaches, using NMR and microscopic studies, and to correlate that to the development of brown color, PPO activity and total phenols content.

2. Materials and methods

2.1. Raw materials

The clingstone type peach cultivar, Carson, and the freestone type, Summerset, were harvested by hand from Foundation Plant Services, at the University of California, Davis, CA. Peaches were sorted and only fruit at the mid-ripe stage, which corresponded to a firmness of 35–40 N using a TA-XT2 Texture Analyzer (Stable Micro Systems Ltd., Surrey, UK), were stored at 4 °C for approximately two days until processing. Three fruit at the same range of firmness were hand peeled, sliced into approximately 3 cm thick slices and placed into polyethylene bags (4 mil vacuum pouches, Ultrasource, Missouri, USA). Each bag contained 3 peach slices of the same peach type, 1 from each of the 3 different fruits, and separate bags were created for each analysis method. Approximately 2 mL of peach extracts were vacuum packed in polyethylene bags. All of the samples were kept at ambient temperature (22 ± 2 °C) for 30 min after packaging prior to HPP treatment. The same fruit was analyzed for all parameters, e.g. difference in lightness, the paramagnetic study using NMR, PPO activity, total phenols and determination of viable cells using light microscopy. On each replicate day of processing, six packages per each of the peach types were processed at each of the five pressures (100–500 MPa). The control treatment was an unprocessed, sliced peach sample in a vacuum package (approximately 0 MPa).

2.2. High pressure processing (HPP)

The packaged samples were processed at 100, 200, 300, 400, and 500 MPa for 10 min in a high pressure processing unit (Avure Technologies Inc., Kent, WA). The initial high pressure unit temperature (Ti) was around 23 °C. The maximum temperature in the high pressure chamber was dependent on the set pressure, which for 100–500 MPa was 25, 28, 30, 33, and 35 °C, respectively. The high pressure unit had a 2.0 L vessel and 600 MPa maximum pressure level. The pressurizing medium was water. In each operation, there will be a come-up stage during which pressure is built up to the target pressure, a constant pressure stage for 10 min and a decompression stage. At the end of the holding period, pressure is released to atmospheric pressure within a few seconds. Three replicates were performed on three separate days.

2.3. Nuclear magnetic resonance (NMR) relaxometry

Following high pressure processing, one cylindrical piece was obtained from each of three slices using a cork borer with a 15 mm diameter and 15 mm height. The samples were blotted dry before being placed into a covered NMR tube, which was placed in a plastic sample holder. NMR relaxometry measurements were performed after samples reached room temperature using an NMR spectrometer (Aspect AI, Industrial Area Havel Modi'in, Shoham, Israel) with a magnetic field of 1.02 T and frequency of 43.5 MHz. T_2 was measured using the Carr-Purcell-Meiboom-Gill sequence with an echo time of 0.5 ms and 15,000 echoes. T_2 spectrum inversion using Laplace transformation was performed on the raw data to determine the change in each plant cell compartment. Raw data were then processed by a non-negative least square algorithm using Prospa (Magritek, Wellington, New Zealand).

2.4. Light microscopy

2.4.1. Section preparation

Following the HPP process, the sliced samples were further cut into small rectangular cuboids approximately 1.0 × 0.5 × 0.3 cm and placed in a sample holder. Sections approximately 200 μm in thickness were obtained using a Vibratome1000 Plus (The Vibratome Co., St. Louis, Missouri, U.S.A.).

2.4.2. Neutral red staining

Stain was prepared using 0.5% neutral red in acetone stock solution, which was filtered twice with Whatman paper # 1, and diluted to 0.04% in 0.55 M mannitol–0.01 M HEPES (*N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethane-sulfonic acid]) buffer, pH 7.8. Peach sections were soaked in the staining solution for a period of 2 h, after which they were rinsed for 0.5 h in the 0.55 M mannitol–0.01 M HEPES buffer solution.

2.4.3. Toluidine blue O staining

This technique allows for observation of cell walls following HP treatment and determination of cell size (O'Brien, Feder, & McCully, 1964). Peach sections of 200 μm thickness were immersed in a solution of 0.025% TBO in water for 10 min before being transferred to a microscopic slide.

A drop of de-ionized water was added to the section and covered with a cover slip. Sections were observed with a light microscope (Olympus System Microscope, Model BHS, Shinjuku-Ku, Tokyo, Japan) at 40× and 100× objective magnification. A digital color camera (Olympus MicroFire, Olympus, Tokyo, Japan) was attached to the microscope to capture images (Olympus software, Olympus America, Melville, N.Y., U.S.A.) providing color photomicrographs (800 × 600 pixel resolution).

2.5. Image processing and analysis

Image processing software, Image J (NIH, U.S.A.), was used for micrograph processing and analyzing. Fifteen micrographs were randomly

selected from 3 replicates for the control and each treatment at 100, 200, 300, 400 and 500 MPa for both clingstone and freestone peaches. Micrographs were semi-quantified in percent of viable cells and percent area of viable cells.

2.5.1. Cell count method

A cell counter plug-in developed by de Vos (2008) was used to quantify the number of cells. In the micrographs, viable cells are distinguished by smooth red to pink stained cells, whereas inviable cells have a visually rough membrane without dye retention.

2.5.2. Area of viable cells

Images of micrographs were converted to RBG stacks and then the blue component of the RGB was selected for quantification as it gave a clear discrimination of viable cells from inviable cells. The blue component of each image was then manually adjusted to a threshold level to present black regions as viable cells on a white background. The black areas were then calculated as a percent of stained cells over the total area.

2.6. Degree of browning

Color was determined using a Minolta CR-400 colorimeter (Minolta camera Co, Ltd., Osaka, Japan). The beam diameter was 11 mm with a viewing angle of 0°. The values were expressed by the CIE $L^*a^*b^*$ system. A white calibration plate was used for calibration ($L^* = 96.88$, $a^* = 0.02$, $b^* = 2.05$). The browning of peach samples was reported as the difference in lightness (DL^*), which is the difference between initial lightness and the lightness of the intact fruit sample after 2 weeks storage at 4 °C.

2.7. Partial purification of peach polyphenol oxidase and PPO assay

The enzyme extract was obtained following partial protein purification using Triton X-114 as described by Espin, Morales, Varon, Tudela, and Garcia-Canovas (1995) with some modifications. A 200 g sample was homogenized with 100 mL of cold 0.1 M sodium phosphate dibasic anhydrous (Fisher, Fair lawn, N.J., USA) (pH 7.3), 20 mM EDTA (Fisher, Fair lawn, N.J., USA), and 6% (w/v) Triton X-114 (Sigma Aldrich, St. Louis, M.O., USA) for 2 min. The homogenate was refrigerated at 4 °C for 60 min before centrifugation at 28,373.6 × *g* with a Sorvall-RC5 (E. I. DuPont Co, Wilmington, Del) at 4 °C for 45 min. The supernatant was collected and 8% (w/v) of the surfactant Triton X-114 was added. A 40 °C water bath was used to incubate the mixture for 15 min. A change in color from clear yellow to opaque yellowish was observed at this step because the increased temperature raises the micellar mass and cause the onset of turbidity. The solution was centrifuged at 578.6 × *g* for 10 min at 25 °C. The detergent-rich opaque phase was discarded, and the supernatant was subjected to a second phase partitioning step with 8% (w/v) Triton X-114, and then incubated again in a 40 °C water bath for 15 min. The supernatant was collected after it was centrifuged at 578.6 × *g* for 10 min at 25 °C and stored at −10 °C until usage.

2.8. Polyphenol oxidase (PPO) assay

The enzyme was assayed using a modified spectrophotometric method described by Espin et al. (1995). The principle of this method is to use 3-methyl-2-benzothiazolinone (MBTH) as a chromogenic coupling agent. The adduct formed from this reaction is reddish in color and its generation is followed at 500 nm. Assays were performed at room temperature in 1.0 mL of a medium containing 0.2 mL of 25.0 mM dihydroxyhydrocinnamic acid (Sigma Aldrich, St. Louis, Missouri, USA), 0.2 mL of 2.5 mM MBTH (Sigma Aldrich, St. Louis, Missouri, USA), 0.1% (w/v), and 0.6 mL of 100 mM acetate buffer (pH 5.5). Incubations began with the addition of 10 μL of peach extract. An increase in absorbance at 500 nm was monitored for up to 120 s using UV spectrophotometry (UV2101PC, Shimadzu Scientific Instruments Inc., Columbia, Maryland, USA).

$$\text{PPO activity (units/mL)} = \text{Abs}(1) - \text{Abs}(0) / \text{ min} \cdot \text{mL of juice}$$

The PPO activity was calculated from the above equation, where Abs(0) is the initial absorbance and Abs(1) is absorbance at the end of linearity.

2.9. Analysis of total phenols

Peach samples obtained following application of different pressure treatments, 0 (control), 100, 200, 300, 400 and 500 MPa were preserved at −80 °C until analysis. Analysis of total phenols was performed by means of the Folin-Ciocalteu method as described by Waterhouse (2002) with some modifications. Peach samples (20 g) were homogenized with 30 mL deionized water, and then 6.4 g of the blended samples were homogenized with 27.6 mL of 76% (v/v) aqueous acetone for 2 min. After 10 min of shaking, the cell wall particles were separated using room-temperature centrifugation (Centra CL2 tabletop centrifuge, IEC, Needham, Massachusetts, USA) at 578.6 × *g* for 10 min. A 0.36 mL aliquot of 2 N Folin reagent (Sigma Aldrich, Buchs, Switzerland) was added to 1 mL of supernatant. The solution was then vortexed and allowed to stand for 5 min, after which 6 mL of sodium carbonate was added and the solution was vortexed before 2.64 mL of deionized water was then added. The solution was vortexed again and incubated at 50 °C for 5 min. Afterward the solution was cooled to room temperature for 1 h, then the absorbance was determined at 760 nm. Gallic acid (Arcos Organics, Geel, Belgium) at concentrations of 0–500 mg/L was used for calibration of the standard curve. The results were expressed as gallic acid equivalents (GAE)/fresh weight of peaches (g).

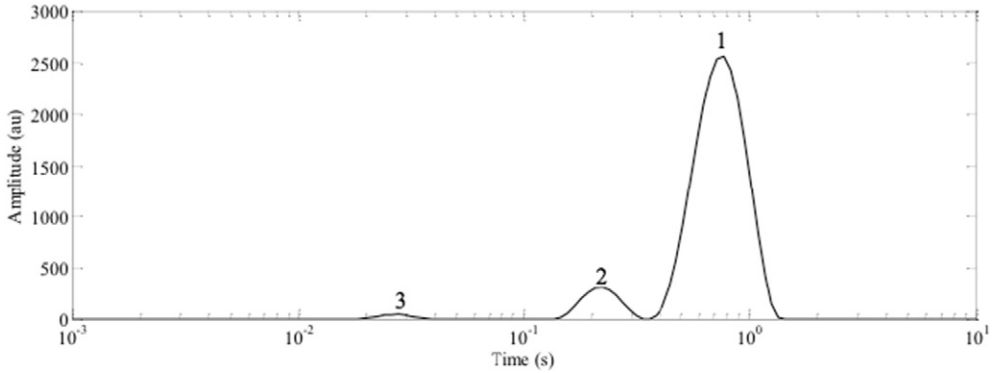

Fig. 1. A representative T_2 relaxation spectrum of unprocessed (control) clingstone peach sample with three water compartments corresponding to the vacuole (1), cytoplasm (2) and cell wall (3).

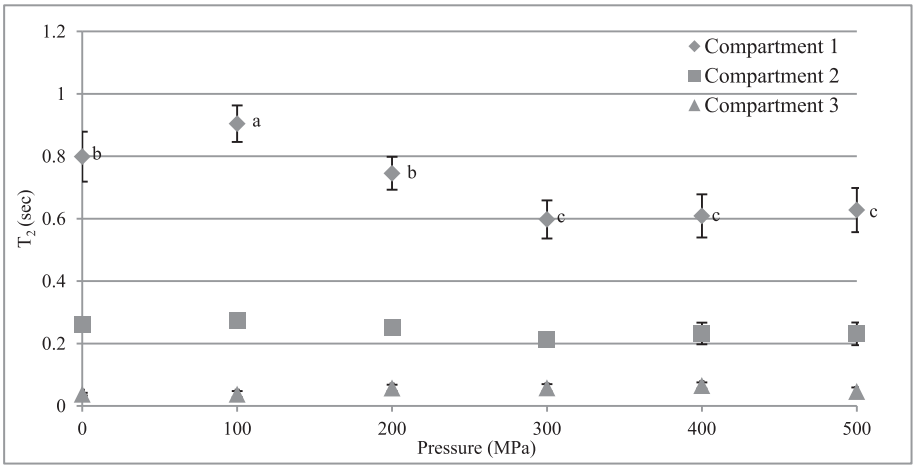

Fig. 2. T_2 relaxation times of compartment 1 (vacuole), compartment 2 (cytoplasm) and compartment 3 (cell wall), of clingstone peaches following HPP treatment at different pressure levels.

2.10. Statistical analysis

This experiment was carried out in three replicate processing runs on three separate days. Analysis of variance was used for determining the effects of pressure level on the T_2 relaxation time, % water, % viable cells, difference in lightness, PPO activity of the intact fruit, PPO activity of the extract and total phenols. Fishers least significant difference (LSD) test was used to compare means of each pressure level at $p < 0.05$ (SAS version 9.4, Cary, North Carolina, U.S.A.). Plots present the mean with its standard deviation for each determination.

3. Results and discussion

3.1. Effect of HPP on cell integrity measured using NMR relaxometry

Commercial HP processes currently use up to 500–600 MPa for fruit preservation, therefore this range was of interest for the current study. Preliminary experiments indicated that treatment of 400 MPa or higher was required for microbial destruction. Previous experiments on onion and mango systems found that membrane integrity was lost at pressures of 200 MPa and above. For these reasons, we determined that evaluation of peach cell integrity followed pressure treatments between 100 MPa (prior to loss of cell integrity) and 500 MPa (which is the limit of our equipment) was relevant.

Water proton relaxometry from NMR has been used to determine the physiological changes in plant tissues after application of a food process. In 1992, Snaar and Van As proposed the use of spin-spin or T_2 relaxation time to correlate with properties of water, the interaction of water with macromolecules, and the permeability of the plant subcellular compartments. These authors used the ^1H NMR technique to probe the water compartments in apple parenchyma tissue and assigned the major compartments to the vacuole (first compartment), cytoplasm (second compartment) and cell wall (third compartment). In the present study, the T_2 distribution of peaches also had 3 distinct compartments; each generated from different proton environments (Fig. 1).

As illustrated in Figs. 2 and 3, which show the T_2 distributions in clingstone and freestone peaches following HPP treatment, it is evident that the HPP-induced change in T_2 relaxation times occurred mostly in the first compartment (vacuole). The T_2 of the control samples (0 MPa) had an initial mean value of 0.79 s and 0.88 s for clingstone and freestone peaches, respectively. Following HPP treatment at 100 MPa, both peach types exhibited the same trend of a small increase in T_2 relaxation time of the vacuolar compartment, but this was only significant in the case of clingstone peaches.

Previously in our group, Gonzalez, Barrett et al. (2010) determined a similar result of increased T_2 in onion samples HPP processed at 100 MPa. Our hypothesis is that during low pressure treatment, the enzyme pectin methylesterase (PME) was liberated from its association with the cell wall, and came into contact with its pectin substrate.

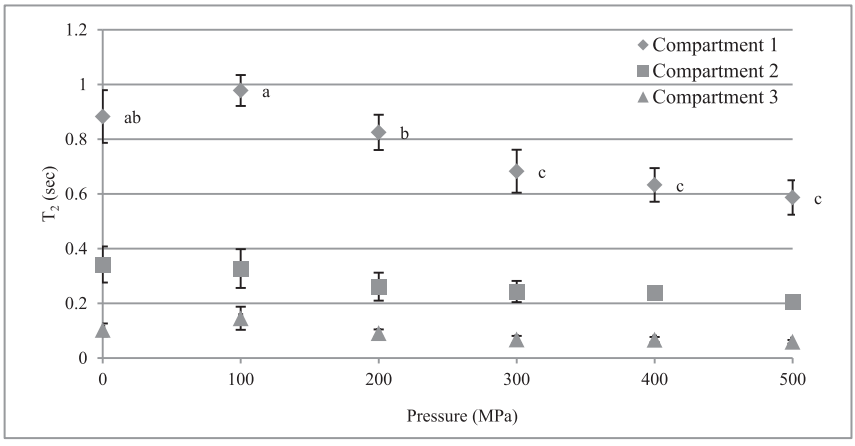

Fig. 3. T_2 relaxation times of compartment 1 (vacuole), compartment 2 (cytoplasm) and compartment 3 (cell wall), of freestone peaches following HPP treatment at different pressure levels.

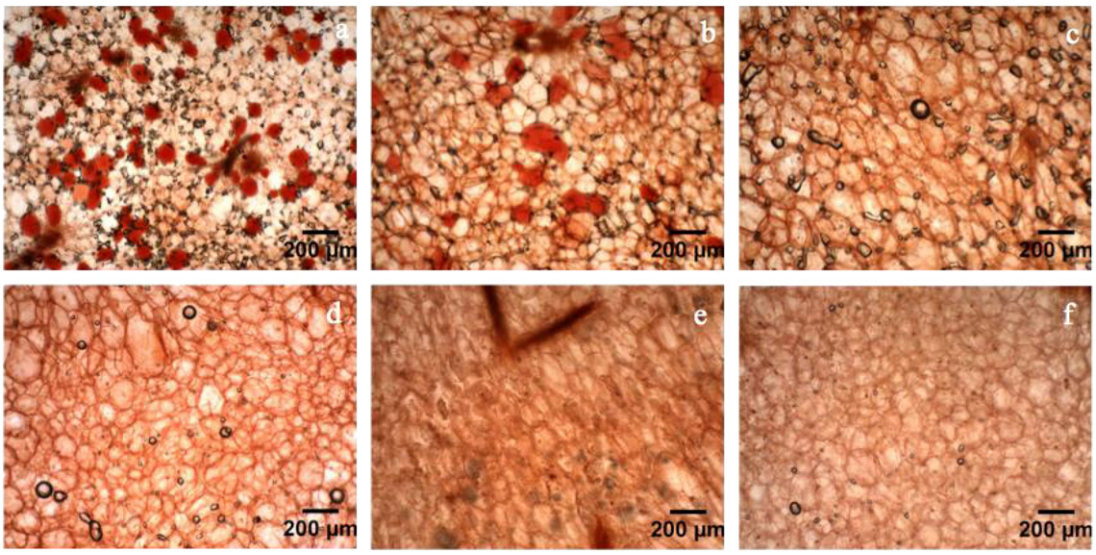

Fig. 4. Micrographs of clingstone peach cells stained with neutral red and observed using a 40× magnification. Samples were given the following treatments: a) control (0 MPa) b) 100 MPa c) 200 MPa d) 300 MPa e) 400 MPa and f) 500 MPa.

PME de-methylated the pectin, creating de-esterified pectin which later formed divalent bonds with either endogenous Ca^{2+} or Mg^{2+} (Basak & Ramaswamy, 1998). This phenomenon is known to result in a more stable, firmer structure (Gonzalez, Jernstedt, Slaughter, and Barrett, 2010), which we hypothesize allowed the cell to withstand mild pressure treatments, as indicated by relatively higher T_2 values.

This hypothesis is supported by the work of Rao et al. (2014), who suggested that low 100 MPa pressure treatments could affect cell organization and separation of the PME enzyme and its substrate. These authors ventured that the PME reaction was accelerated as a consequence of HPP treatment. Boulekou et al. (2010) also found that PME was active in peach pulp, and could only be completely inactivated at 800 MPa and 70 °C for >90 s.

At 200 MPa, a significant decrease in the T_2 was observed, which indicates a change in the proton environment as a result of the loss of cell compartmentalization and rupture of the subcellular membranes (Hills & Remigereau, 1997). The study by Gonzalez, Barrett, et al. (2010) also indicated a similar result of a decrease in T_2 of the vacuolar compartment in 200 MPa treated onions. After processing at 300 MPa, the T_2 decreased significantly and thereafter was stable in the pressure range from 300 to 500 MPa. The complete loss in membrane integrity therefore appeared at 200 or 300 MPa, depending on the peach type, as collaborated by the microscopic study (Figs. 4 and 5).

The T_2 relaxation time of the second and third compartments, which correlated to the properties of water in the cytoplasm and cell wall, respectively, were only slightly changed compared to the first compartment. In general, the T_2 values were in a much shorter after processing (Tables 1 and 2). Clingstone peaches (Table 1) showed a relatively small decrease in T_2 of 11% in compartment 2, while compartment 3 increased by 25% after the 500 MPa treatment. In freestone peaches (Table 2), compartments 2 and 3 showed a decrease in T_2 following 500 MPa, from 0.34 s to 0.21 and from 0.1 s to 0.06 s, respectively. A similar decrease in T_2 of the cytoplasmic compartment was reported in 100 and 300 MPa treated strawberry samples (Marigheto et al., 2004) and 200 MPa processed onions (Gonzalez, Barrett et al., 2010). The change in the cell wall compartment, however, was relatively small compared to the others, since the cell wall contains a relatively lower amount of water and its structure is more resistant to damage.

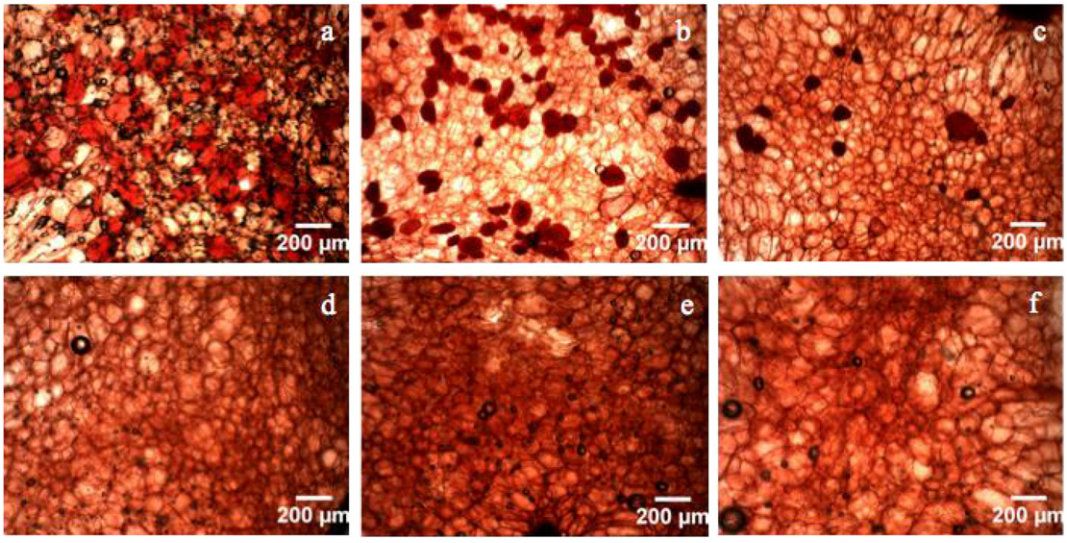

Fig. 5. Micrographs of freestone peach cells stained with neutral red and observed using a 40× magnification. Samples were given the following treatments: a) control (0 MPa) b) 100 MPa c) 200 MPa d) 300 MPa e) 400 MPa and f) 500 MPa.

Table 1
T_2 relaxation times and percent water in vacuolar, cytoplasm and cell wall compartments of clingstone peaches following different pressure treatments (0, 100, 200, 300, 400 and 500 MPa). Values with the same letter are not significantly different across treatment variables at a significance level of $p < 0.05$.

Pressure (MPa)	Compartment 1 (vacuole)		Compartment 2 (cytoplasm)		Compartment 3 (cell wall)	
	T_2 (s)	% water	T_2 (s)	% water	T_2 (s)	% water
0	0.79 ± 0.08 b	86.76 ± 0.66 a	0.26 ± 0.02 a	12.02 ± 0.66 c	0.04 ± 0.00 c	1.21 ± 0.22 d
100	0.90 ± 0.06 a	87.11 ± 2.54 a	0.27 ± 0.02 a	10.10 ± 1.86 c	0.04 ± 0.01 c	2.78 ± 0.83 bc
200	0.79 ± 0.05 b	85.66 ± 1.82 a	0.25 ± 0.02 ab	12.72 ± 1.67 c	0.06 ± 0.01 ab	1.62 ± 0.48 cd
300	0.60 ± 0.06 c	81.60 ± 1.69 b	0.21 ± 0.01 c	16.09 ± 1.13 b	0.06 ± 0.01 ab	2.53 ± 1.06 bc
400	0.61 ± 0.07 c	75.16 ± 4.00 c	0.23 ± 0.03 bc	20.36 ± 2.99 a	0.06 ± 0.01 a	4.48 ± 1.38 a
500	0.63 ± 0.07 c	79.65 ± 4.71 b	0.23 ± 0.03 bc	16.98 ± 3.99 b	0.05 ± 0.01 bc	3.36 ± 1.10 ab

In addition to T_2 relaxation time, the percent water in each compartment as indicated by the relative signal intensity (area under the T_2 plot) is used to monitor the change in membrane permeability. The present study observed a redistribution of water in the three compartments following HPP, as shown in Tables 1 and 2. Significant decreases in percent water of the vacuolar compartment of 5, 12 and 7% were observed in clingstone samples treated at 300, 400 and 500 MPa, respectively (Table 1). At the same time, the percent water in the cytoplasmic compartment increased by 4, 8 and 5% in the 300, 400 and 500 MPa treated clingstone peaches. A reduction in signal intensity of compartment 1 simultaneous with an increase in the signal intensity of compartments 2 and 3 indicates the transfer of water from the vacuole to the cytoplasm and cell wall. A similar finding of water redistribution was reported by Zhang and McCarthy (2012). These authors observed a significant decrease of the vacuolar signal from 69.2% to 48.2% in black heart pomegranates with damaged cells, compared to the healthy samples.

3.2. Effect of HPP on cell morphology and number of viable cells

In general, the plant cell is a building block that contains subcellular organelles suspended in a cuboid-shaped cytoplasm that is surrounded by the plasma membrane and a rigid cell wall. According to microscopic observation (Figs. 6 and 7), the size of clingstone peach cells appears to be larger than those of freestone peach cells. Observation of cell walls using Toluidine blue O under light microscopy at 100× objective magnification revealed a change in morphology from the original rectangular cuboid shape of unprocessed (control) cells to a slightly spherical shape in HPP treated cells. Unprocessed samples and mildly treated samples (100 MPa) looked rectangular in section view, while HPP treated samples were more rounded in shape after treatment at pressures of 200 MPa and above in both peach types. Luza, Van Gorsel, Polito, and Kader (1992) observed peach cells using both light microscopy and transmission electron microscopy and also found an angular shape of the cell wall in unprocessed tissues. In agreement with our findings, Marigheto et al. (2004) found that HPP treatment resulted in a rounder shape in strawberry cells; however, this occurred at low pressures of 100 MPa while the rounding in our study occurred after 200 MPa treatments. According to the isostatic principles of HPP processing, the pressure effect is applied instantaneously and uniformly throughout a sample in all directions. During the pressure release stage, we propose that the air in the tissue, which was compressed when pressurizing, expands and ruptures in the cell membranes, causing the loss of turgor pressure and allowing the cell to assume a more spherical shape.

Neutral red staining has been an essential technique for cell viability determination for many years (Repetto, del Peso, & Zurita, 2008). This technique allows for quantification of the intactness of the vacuole and therefore allows for determination of the effect of high pressure on cell integrity. In this study, viable cells were analyzed semi-quantitatively from micrographs using both cell count and area of viable cells calculations. As illustrated in Table 3, unprocessed (control) clingstone samples had an initial mean of 57% viable cells and the percentage of viable cell area was 30%. Freestone peaches had an initial mean of 72% viable cells and this accounted for 58% of the total area. The higher percentage of viable cells in freestone samples is probably due to the more extensive degradation of the middle lamella during ripening, which allows the cells to be more flexible and therefore more resilient during high pressure processing and subsequent sectioning for microscopic study.

After HPP treatment at 100 MPa, the percentage of both viable cell counts and percent stained area generally decreased. There is a strong possibility that compression of the cellular structure occurs without serious disruption of the viability at 100 MPa, while rupture of the cellular membranes of fruits and vegetables occurs at pressures of 200 MPa and above (Basak & Ramaswamy, 1998). Another study by Dörnenburg and Knorr (1993) on *Chenopodium rubrum* (red goosefoot) cells reported a loss of cell viability at 110 MPa. This finding also supports the idea that plant cells are able to sustain up to a 100 MPa pressure treatment. Clingstone peach samples treated at or above 200 MPa showed a complete absence of viable cells, while freestone type peach samples were more resistant, and total loss of viable cells did not occur until 300 MPa. In the present study, a complete rupture of the cellular membranes appears to occur at pressures of 200 MPa or 300 MPa (depending on the peach type), and this is in agreement with the findings of Dörnenburg and Knorr (1993) and Gonzalez, Jernstedt et al. (2010).

Table 2
T_2 relaxation times and percent water in vacuolar, cytoplasm and cell wall compartments of freestone peaches following different pressure treatments (0, 100, 200, 300, 400 and 500 MPa). Values with the same letter are not significantly different across treatment variables at a significance level of $p < 0.05$.

Pressure (MPa)	Compartment 1 (vacuole)		Compartment 2 (cytoplasm)		Compartment 3 (cell wall)	
	T_2 (s)	% water	T_2 (s)	% water	T_2 (s)	% water
0	0.88 ± 0.09 ab	82.61 ± 2.72 a	0.34 ± 0.07 a	11.62 ± 2.20 c	0.10 ± 0.02 b	3.31 ± 1.30 c
100	0.98 ± 0.06 a	83.60 ± 2.12 a	0.33 ± 0.07 ab	9.12 ± 2.51 c	0.15 ± 0.04 a	4.99 ± 0.55 b
200	0.83 ± 0.06 b	82.72 ± 1.47 a	0.26 ± 0.05 bc	9.60 ± 1.01 c	0.09 ± 0.01 cb	4.95 ± 1.42 b
300	0.68 ± 0.08 c	75.12 ± 3.23 b	0.24 ± 0.04 c	15.27 ± 1.71 b	0.07 ± 0.01 cd	8.43 ± 1.29 a
400	0.63 ± 0.06 c	73.03 ± 2.21 b	0.24 ± 0.03 c	18.33 ± 2.15 a	0.06 ± 0.01 d	7.87 ± 0.48 a
500	0.59 ± 0.06 c	75.01 ± 2.37 b	0.21 ± 0.02 c	16.37 ± 1.37 ab	0.06 ± 0.01 d	8.01 ± 0.99 a

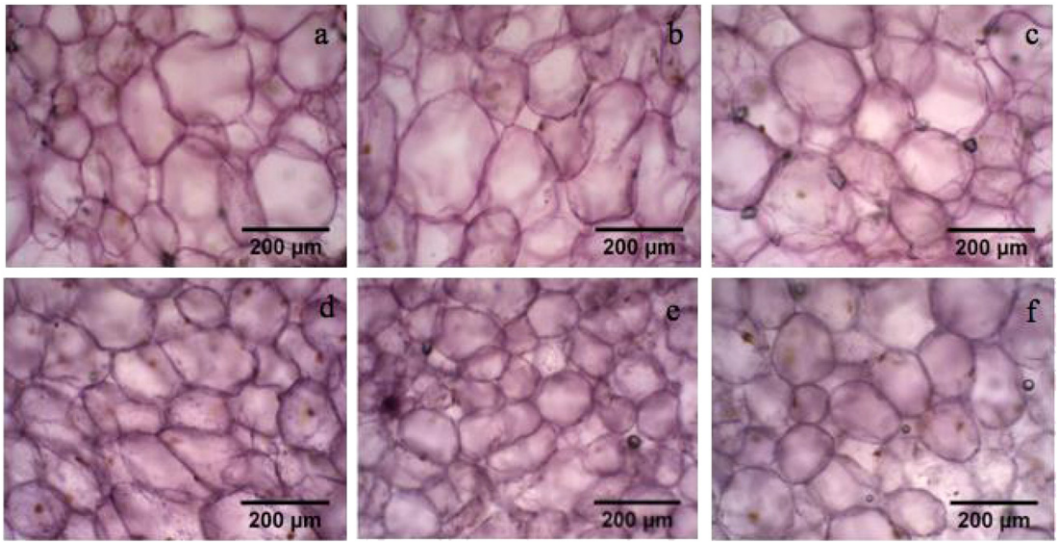

Fig. 6. Clingstone peach tissues stained with 0.025% Toluidine blue O and observed using 100× magnification. Peach samples were processed at a) 0 MPa b) 100 MPa c) 200 MPa d) 300 MPa e) 400 MPa and f) 500 MPa.

3.3. Effect of HPP on enzymatic browning reactions

Following high pressure treatment, vacuum sealed peach samples appeared water-logged or translucent. Prestamo and Arroyo (1998) also found a soaked appearance in cauliflower and spinach after HPP at 400 MPa for 30 min. These authors suggested that the effect of HPP processing on the organization of parenchyma cells was a disappearance of gas in the intercellular spaces and membrane disruption of plant cells, which they observed by cryo-SEM. In the present study, the control samples and the samples processed at a mild pressure levels (100 MPa) were not significantly different from each other in their appearance or difference in lightness (DL) values for either peach type (Fig. 8). Butz, Koller, Tauscher, and Wolf (1994) also reported that pressures up to 100 MPa did not trigger browning reactions in onion samples, since the tonoplast membrane that maintains the phenolic substrates within the vacuole was not disrupted. The difference in color development was much more pronounced after samples were processed at 200 MPa for both peach types, and for freestone peaches, this trend continued up to 500 MPa.

The development of discoloration was observed during the two week refrigerated storage, in accordance with our hypothesis that there is an interaction between polyphenol oxidase (initially in the cytoplasm) and phenolic compounds (initially in the vacuole) after the membranes ruptured. This finding is supported by previous reports of loss in membrane integrity at pressures of 200 MPa and above (Knorr, 1993; Gonzalez & Barrett, 2010). In apples, significant changes in color have also been found to occur at pressures of 200 MPa and higher (Basak & Ramaswamy, 1998). In clingstone peaches, although there was a significant change in DL between the control and 200 MPa treated samples, the difference in lightness in peaches processed at 200, 300, 400 and 500 MPa was not significant. This finding correlates with the stable number of viable cells that were found in samples processed in this range (Table 3). That is, most of the membrane rupture and loss of cellular integrity had already occurred with the application of 200 MPa, therefore higher pressure levels did not increase the damage significantly.

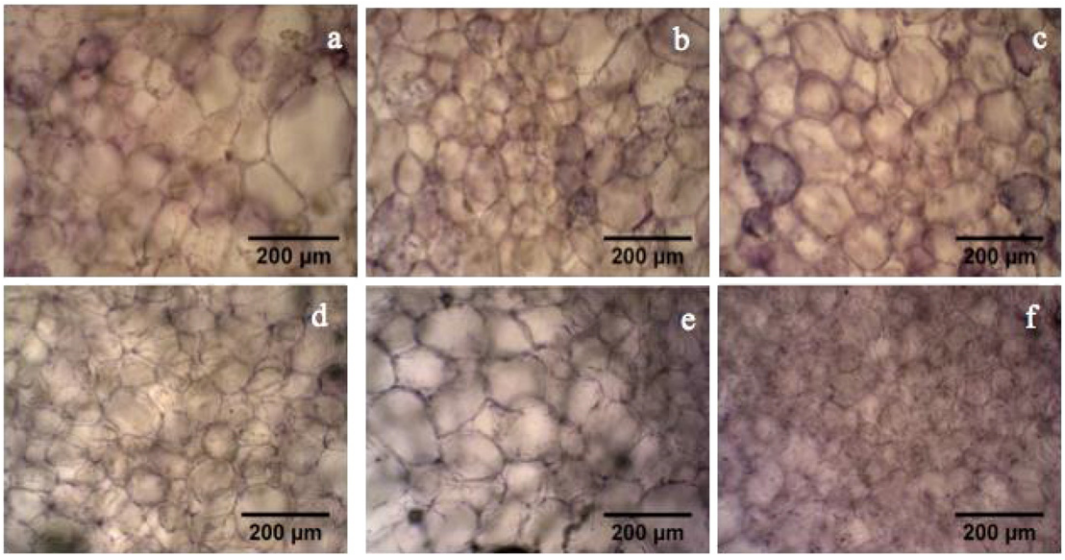

Fig. 7. Freestone peach tissues stained with 0.025% Toluidine blue O and observed using100x magnification. Peach samples were processed at a) 0 MPa b) 100 MPa c) 200 MPa d) 300 MPa e) 400 MPa and f) 500 MPa.

Table 3
Percent viable cells and stained area of clingstone and freestone peaches following different pressure treatments (0, 100, 200, 300, 400 and 500 MPa).

Pressure (MPa)	Clingstone		Freestone	
	% viable cells	% stained area	% viable cells	% stained area
0	57 ± 10	30.19 ± 7.15	73 ± 5	58.17 ± 11.43
100	38 ± 3	20.04 ± 6.41	47 ± 10	50.01 ± 8.93
200	0	0	4 ± 3	14.43 ± 5.76
300	0	0	0	0
400	0	0	0	0
500	0	0	0	0

A relatively high initial level of PPO activity in freestone peaches (Fig. 10), as compared to clingstone peaches (Fig. 9), may have led to the approximately 3-fold greater difference in lightness (Fig. 8). In general, clingstone peach tissues have a more organized tissue matrix, with strong linkages between the cells through middle lamella cross-linking. Freestone peach cells, on the other hand, are more independent and less strongly linked by pectic bonds in the middle lamella (Personal communication with Dr. Thomas Gradziel, UC Davis). The concentration of secondary metabolic compounds e.g. flavor, color and phenolic substrates, is also higher in freestone peaches. The high degree of browning that occurred in freestone peaches is consistent with the higher PPO activity level (indicating greater decompartmentalization) and the lesser degree of tissue coherence in this variety (suggesting reduced ability to withstand damage by HPP).

3.4. Effect of HPP on PPO activity in peach fruit and partially purified extracts

This study compared PPO activity in partially purified peach extracts and in peach fruit, in both clingstone and freestone type peaches, following HPP treatment at levels from 100 to 500 MPa. As illustrated in Fig. 9, PPO activity in control clingstone peach fruit had a mean value of 16,000 units/mL. A significant increase in activity (42%) was observed in samples following HPP treatment at 100 MPa, but the trend showed a significant decrease (39%) following pressure application at 200 MPa. PPO activity in clingstone peach fruit was fairly constant after processing at between 200 and 500 MPa. In contrast, clingstone peach extracts had a higher activity overall, with a mean value of 49,000 units/mL in the extracts taken from control samples. In contrast to the results in the peach fruit, PPO activity did not change significantly in the extracts treated at between 100 and 400 MPa, but a sharp decrease (35%) in PPO activity was observed after processing at 500 MPa, when activity declined to 32,000 units/mL.

Freestone peach fruit had almost twice the PPO activity as clingstone peaches in the unprocessed control samples, with a mean value of 30,000 units/mL (Fig. 10). The trend in PPO activity reduction with the application of high pressure was slightly different to that in clingstone fruit. There was no increase in PPO activity following the 100 MPa treatment, rather, the activity was constant. There was a significant decline (12%) in PPO activity after 300 MPa and, similar to the clingstone type, and the activity level was stable from 300 to 500 MPa. However, the percent reduction in activity after the 500 MPa treatment (35%) was less than that in the clingstone peaches (74%).

In the partially purified freestone PPO extracts, the initial activity of 87,000 units/mL was also much higher than that found in the fruit, and it was greater than that in the extracts from clingstone peaches. The PPO activity level was not significantly affected by HPP until the 300 MPa treatment (12% reduction). The reduction in activity was lower and occurred earlier than observed in the clingstone peach extracts, but then the activity did not decline further following 400 or 500 MPa treatments. Rao et al. (2014) made different observations in peach juice, where PPO activity decreased following HPP in a range from 400 to 600 MPa, as described by a first order kinetic model. The presence of phenolic substrates in peach juice may have resulted in the decrease in PPO activity observed by these authors. There is very little information in the literature comparing the differences in browning rate or polyphenol oxidase activity in clingstone and freestone type peaches (Lee et al., 1990).

High pressure treatment has a direct effect on protein structure, which in this case is the conformation of the enzyme. The primary structure is almost unaffected by high pressure, but changes in the secondary structure have been reported to occur at very high pressures

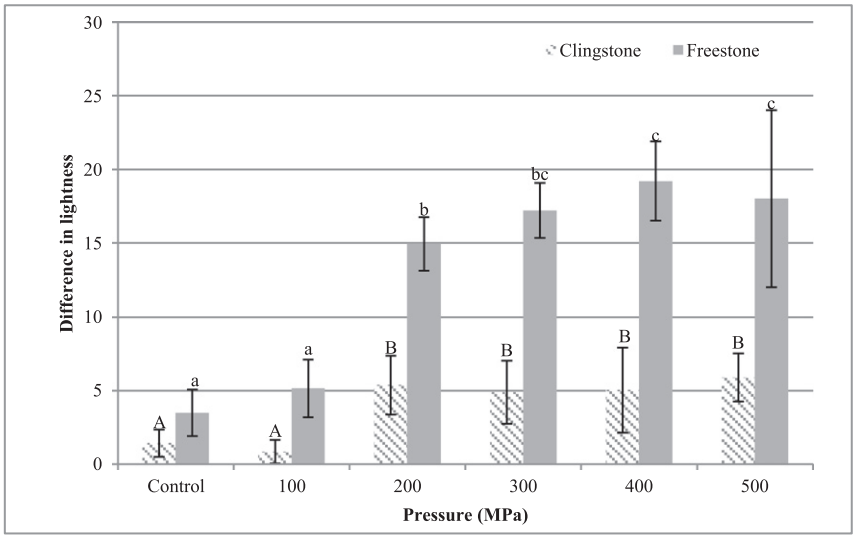

Fig. 8. Difference in lightness of clingstone and freestone peaches following HPP treatment at 0 (control) and 100–500 MPa. Capital letter indicates comparison between clingstone peaches and small case letter compares freestone peaches. Values with the same letter are not significantly different across treatment variables at a significance level of $p < 0.05$.

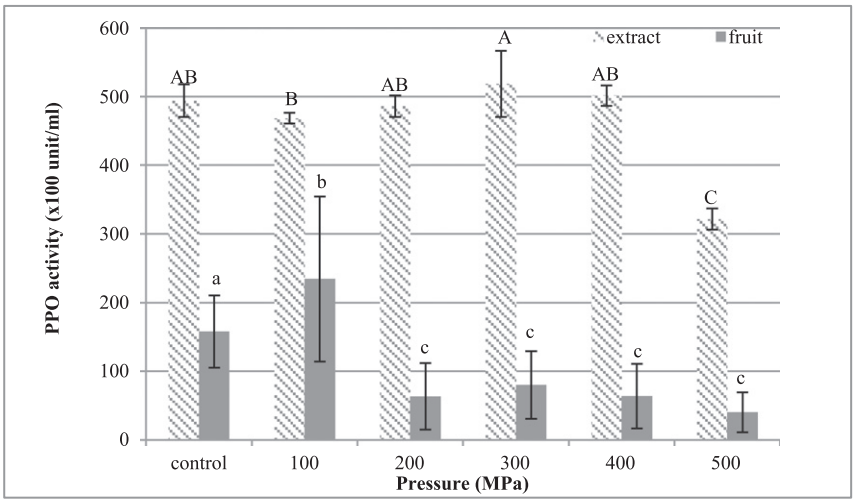

Fig. 9. PPO activity in clingstone peaches, in both the whole fruit and partially purified extracts, following HPP treatment at 0 (control) and 100–500 MPa. Capital letter indicates comparison between PPO from extract and small case letter compares PPO from intact fruit. Values with the same letter are not significantly different across treatment variables at a significance level of $p < 0.05$.

(Hendrickx, Ludikhuyze, Van den Broeck, & Weemaes, 1998; Tauscher, 1995). Previously, authors have also found that at the level of the tertiary structure, which is dominated by hydrophobic and ionic interactions, changes have been observed at pressures above 200 MPa (Hendrickx et al., 1998). The biological activity of enzymes is governed by the active site; therefore, even small changes in the active site can trigger a loss in enzyme activity. PPO is one of the food quality-related enzymes that is most resistant to pressure, although sensitivity varies in different commodities. Several authors have found that treatment at 800 MPa and higher was required to cause PPO inactivation in mushrooms and potatoes (Gomes & Ledward, 1996; Yi et al., 2012). However, other reports indicate that there may be inactivation at lower levels. This was true in the case of guacamole treated at 689 MPa (Palou et al., 2000) and in guava puree following 400 and 600 MPa treatment (Yen & Lin, 1996). The finding proposed by Hendrickx et al. (1998) supports the theory that high pressure could affect the intramolecular arrangement of proteins leading to the unfolding or denaturation of enzyme. Another study claimed the effect of HPP on unfolding of β-lactoglobulin (Belloque, López-Fandiño, & Smith, 2000). The authors reported that β-lactoglobulin started increasing its conformation flexibility at pressure ≥ 200 MPa and had a very flexible conformation or became denatured at 300 and 400 MPa. The decrease in enzyme activity of the peach extract in the present study may have resulted from conformational changes at the active site of the enzyme.

The primary difference between the PPO activity in the partially purified extract and that in the fruit is the presence of phenolic compounds in the fruit sample. The reduction in PPO activity in the whole fruit may have been affected by two factors. The first factor is the effect of high pressure, causing enzyme denaturation through conformational changes as described above. The other cause is due to product inhibition or self-inactivation and turnover of the enzyme. In case of product inhibition, *o*-quinones, which are generated after interaction between PPO and phenolic substrates, have been reported to affect protein structure and function (Loomis & Battaile, 1966; Mason & Peterson, 1965; Pierpoint, 1969; Mathew & Parpia, 1971; Sánchez-Ferrer, Pérez-Gilabert, Núñez, Bru, & García-Carmona, 1994). Moreover, quinones can damage proteins, amino acids or lipids by generating free radicals through redox-recycling (Felton, Donato, Broadway, & Duffey, 1992). For enzyme turnover, PPOs can irreversibly inactivated due to loss of histidine and the release of copper at the active site

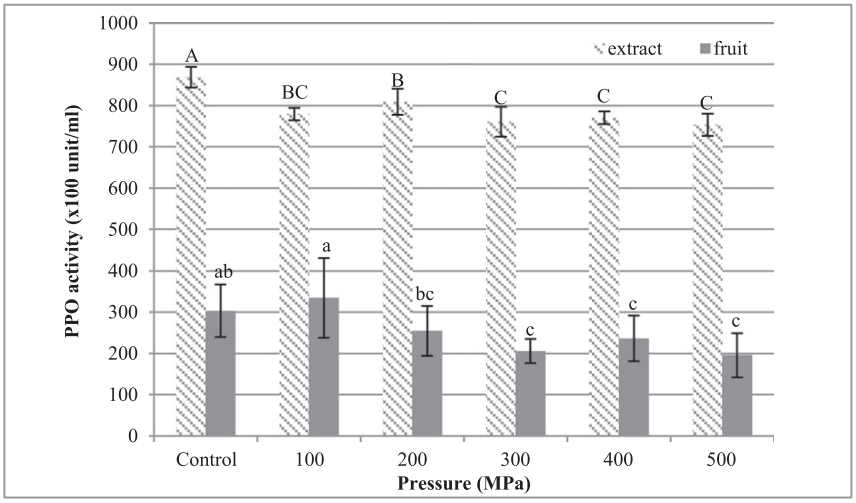

Fig. 10. PPO activity in freestone peaches, in both the whole fruit and partially purified extract, following HPP treatment at 0 (control) and 100–500 MPa. Capital letter shows comparison between PPO from extract and small case letter compares PPO from intact fruit. Values with the same letter are not significantly different across treatment variables at a significance level of $p < 0.05$.

(Golan-Goldhirsh & Whitaker, 1985; Nelson & Dawson, 1944; Dietler & Lerch, 1982). Golan-Goldhirsh and Whitaker (1985) reported inactivation of mushroom PPO at the rate of approximately one in 5000 turnovers of the substrate to product. In the preparation of partially purified PPO extracts, the phenolic compounds were removed by a phase partitioning separation prior to HPP treatment. Therefore, the inactivation of the partially purified enzyme due to interaction with quinones and/or turnover of enzyme were prevented.

3.5. Effect of HPP on concentration of total phenols

The total phenol content of clingstone peaches was stable throughout high pressure levels from 0 to 500 MPa with a mean value in the range of 5.79 ± 0.81 to 7.34 ± 0.79 mg/g sample (data not shown). Freestone peaches are generally rich in flavor, color and phenolic compounds. There was no significant change in total phenol content, with mean value for this peach type in the range of 9.22 ± 0.63 to 10.86 ± 0.96 mg/g sample (data not shown). Therefore, the role of phenolic concentration in the enzymatic browning scenario appears to be negligible. High pressure treatment is therefore beneficial in terms of the ability to retain these nutrients. In similar study, no significant difference in total phenols was reported in strawberry and blackberry purées that were pressure treated at 400 and 500 MPa (Patras, Brunton, Da Pieve, & Butler, 2009). This was also true for Granny Smith apple purée following HPP treatment at 400 MPa (Landl, Abadias, Sárraga, Viñas, & Picouet, 2010).

4. Conclusions

High pressure processing level has a significant influence on the final quality of peach products. This study found different effects on peach membrane integrity following HPP treatments in the range of 0–500 MPa. Changes in membrane integrity of the vacuolar compartment after HPP at 300, 400 or 500 MPa were determined by T_2 shifts from initial levels of 0.79 (clingstone) or 0.88 (freestone) to approximately 0.60–0.68 after any treatment above 200 MPa. Clingstone peaches treated at these higher levels also showed significant decreases (5, 12 and 7%) in the percent water in the vacuolar compartment and a simultaneous increases in the cytoplasmic compartment (4, 8 and 5%). Additionally, there was a reduction in the number of viable cells from an initial 57–58% to 0 and 14% in clingstone and freestone peaches, respectively.

As a consequence of the loss of membrane integrity, the PPO enzyme is allowed to interact with its phenolic substrates, creating brown pigments. In this study, the relationship between the degrees of browning with the loss of cell integrity showed strong agreement. Once cells are damaged by pressure treatments above 200 MPa, both the T_2 relaxation time and the number of viable cells decreased significantly, simultaneous with the development of brown color. On the contrary, PPO activity tended to decrease following HPP, while total the phenol content was stable in the entire pressure range studied.

These results indicate that the degree of browning in peaches following HPP was not significantly correlated with either PPO activity or phenolic content, but rather with loss in cell integrity. Use of a combination of microstructural and ^{1}H NMR tools was an effective means of determining loss of membrane integrity and de-compartmentalization. Future investigations may develop a mathematical model, using T_2, percent water and light microscopy measurements, to predict browning in fruits. In addition, calcium pretreatments have the potential to reduce the loss of cell integrity, since calcium ions bind to the carboxylic acid groups of galacturonic acid residues in the pectin chain, resulting in the strengthening of the cell wall. Therefore, future studies may investigate the ability of calcium treatments to prevent loss of membrane integrity and initiation of enzymatic browning.

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