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Original article

The effect of calcium chloride and calcium lactate pretreatment concentration on peach cell integrity after high-pressure processing

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Summary The objective of this study was to determine the effect of calcium chloride and calcium lactate pretreatments at different concentrations (1, 1.5 and 2% w/v) on enzymatic browning of clingstone and freestone peaches following high-pressure processing (HPP) at 200 MPa for 10 min. Proton nuclear magnetic resonance (^{1}H-NMR) relaxometry indicated that following calcium pretreatment and HPP, both peach types had lower percentages of water in the vacuole compartment and a simultaneous increase in the percentage of water in the cytoplasm compartment. Calcium pretreatment of freestone peaches did not affect the development of browning, regardless of the form of calcium or the concentration, whereas calcium lactate pretreatment undesirably enhanced clingstone peach discoloration. Calcium pretreatments have the potential to reduce the loss of cell integrity that results from HPP; however, in this study, the effect was not sufficient to inhibit the interaction between the polyphenol oxidase enzyme that catalyses browning and its substrates.

Keywords Browning, peaches, physical preservation methods, polyphenol oxidase.

Introduction

Peaches (*Prunus persica*) have become a popular fruit consumed worldwide. As they are only available for a short season, in addition to fresh consumption, many peaches are preserved in the form of canned or frozen products. Although there is an advantage to long-term preservation using these methods, fruit characteristics such as texture and flavour as well as vitamin content may dramatically decrease during the preservation process. High-pressure processing (HPP) yields a higher quality product with better retention of texture, flavour, nutrients and health attributes (Barba *et al.*, 2012, 2015); therefore, it has a strong potential to be used as an alternative choice for peach preservation. However, during pressurisation and pressure release, loss of membrane permeability and subcellular compartmentalisation may occur, resulting in textural changes and triggering the enzymatic browning reaction. Discoloration of fruits and vegetables is therefore a major concern after HPP. Colour changes during storage of HPP-processed fruits have been reported, for instance in banana puree (Palou *et al.*, 1999), mango (Guerrero-Beltrán *et al.*, 2005) and navel orange juice (Polydera *et al.*, 2005). This limitation has motivated food scientists to explore enzymatic browning at the cellular level in an attempt to limit these browning reactions.

Calcium chloride and calcium lactate have been employed as firming agents in a wide range of fruits. These compounds function by binding to the negatively charged carboxylic acid groups of galacturonic acid residues in the pectin chain and forming an egg-box gel model resulting in the strengthening of the cell wall (Grant *et al.*, 1973). There is also a possibility that positively charged calcium ions can bind the negatively charged head groups in the lipids that make up cellular membranes (Pedersen *et al.*, 2006). Calcium pretreatment is common not only because of its ability to retain firmness in plant-based materials, but also because there were several reports indicating its use in reducing browning (Drake & Spayd, 1983; Hopfinger *et al.*, 1984; Bolin & Huxsoll, 1989). A primary objective of the pretreatment with calcium chloride or calcium lactate in this study is the desire to have Ca^{2+}

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penetrate into the plasma membrane, which surrounds each plant cell and the tonoplast, which is the membrane surrounding the large water-filled vacuole, and stabilise these membranes prior to HPP.

Nuclear magnetic resonance (NMR), which is a nondestructive method, has been used to detect physiological and biochemical changes in the various water compartments in plant tissues. The application of NMR has been used recently for the determination of the effect of pectin methyl esterase (PME) and $CaCl_2$ infusion on cell integrity in fresh-cut and frozen-thawed mangoes (Kirtil *et al.*, 2014). In this study, NMR relaxometry is used for evaluation of the permeability of each water compartment in samples pretreated with calcium, following HPP.

Observation of peach cells using light microscopy is also included in this study as another tool for determination of the tonoplast integrity. The technique developed by Admon *et al.* (1980) uses neutral red (NR), a lipophilic phenazine dye which diffuses into and ionises in the acidic environment of the vacuole. The intact vacuole appears as an intense red colour, but in damaged vacuoles, the dye spreads throughout the cell and the colour is therefore less intense. The number and colour intensity of intact vacuoles correlate with the ability of the membranes to resist damage from HPP. Thus, the evaluation of micrographs from samples treated with different pretreatments can be correlated with the degree of de-compartmentalisation.

^{1}H-NMR relaxometry and the observation of peach cells under light microscopy were used to determine whether pretreatment with calcium at different concentrations results in an improvement in cell integrity in peaches following HPP. Parameters involved in the browning reaction, which include degree of browning, polyphenol oxidase (PPO) activity and total phenol content, were also quantified.

Materials and methods

Raw materials

The clingstone peach cultivar Extra Late 1 and the freestone type cultivar Summerset were harvested by hand from Foundation Plant Services on the University of California Davis campus. Peaches were nondestructively sorted with a TA-XT2 Texture Analyzer (Stable Micro Systems Ltd., Surrey, UK) using a compression test for a 3 mm distance and a 5.0-cm-diameter probe, and those with a firmness of 35–40 N were stored at 4 °C for approximately 2 days until processing. The fruit were hand-peeled, and each of the fruit was segmented into different sample sizes based on their further analysis. A flat circular disc of 1 cm thickness was used for colour determination, a cylinder with a 15 mm diameter and 15 mm height was cut using a cork borer for NMR analysis, and a 1.5 × 1.5 cm cube was used for analysis of PPO activity, total phenols and microscopic evaluation of cell viability. There were two control treatments, represented by (i) untreated peaches at approximately 0 MPa packaged in a vacuum package, a polyethylene bag (4 mil vacuum pouch; Ultrasource, North Kansas City, MO, USA) with an oxygen transmission of 1.2 cc sq m^{-1} per day, and (ii) untreated peaches which were vacuum-packaged in the same pouch and then treated with HPP at 200 MPa to compare the effect of pressure without pretreatment. The same fruit was analysed for all parameters, for example difference in lightness, NMR relaxation, PPO activity, total phenols and determination of viable cells using light microscopy.

Calcium pretreatment

The samples were soaked in either calcium chloride or calcium lactate at a concentration of 1, 1.5 and 2% w/v for 5 min before vacuum packaging in polyethylene bags (4 mil vacuum pouches; Ultrasource, Kansas City, MI, USA). Each bag contained peach samples receiving the same pretreatment, one from each of the three replicate fruit, and separate bags were created for each analysis method. All of the samples were kept at ambient temperature (22 ± 2 °C) for 30 min after packaging prior to HPP treatment.

High-pressure processing (HPP)

Following pretreatment, the packaged samples were processed at 200 MPa for 10 min in a HPP unit (Avure Technologies Inc, Kent, WA, USA). The pressure level used in this experiment, at 200 MPa, was justified on the basis of previous studies (Techakanon, 2015), which found that rupture of the plant cellular membranes occurred at this pressure level. The initial high-pressure unit temperature (T_i) was approximately 23 °C. The maximum temperature in the high-pressure chamber was dependent on the set pressure, which for 200 MPa was 28 °C. The high-pressure unit had a 2.0-L vessel and maximum pressure of 600 MPa. The pressurising medium was water.

Nuclear magnetic resonance (NMR) relaxometry

Following high-pressure processing, the samples were removed from the plastic bag and blotted dry before being placed into a covered NMR tube. NMR relaxometry measurements were performed using an NMR spectrometer (Aspect AI; Industrial Area Havel Modi'in, Shoham, Israel) equipped with 1.02 T magnetic field, 43.5 MHz frequency and 42.6 rad s^{-1} T^{-1}gyromagnetic ratio. T_2 was measured using the Carr–Purcell–Meiboom–Gill (CPMG) sequence 15 000 echoes with an

echo time of 0.5 ms. T_2 spectrum inversion was performed on the raw data using a non-negative least square algorithm using Prospa (Magritek, Wellington, New Zealand).

Light Microscopy study

Section preparation

After the HPP process, the cube destined for microscopy evaluation was further cut into small rectangular shapes approximately 1.0 × 0.5 × 0.3 cm^3 before being placed in a sample holder. Then, a Vibratome 1000 Plus (The Vibratome Co., St. Louis, Mo, USA) was used for sectioning specimens with approximately 200 μm thickness. A stain was prepared using 0.5% neutral red in acetone and filtered twice with Whatman paper # 1 before being diluted to 0.04% in 0.55 M mannitol–0.01 M *N*-(2-hydroxyethyl) piperazine-N'-2-ethane-sulphonic acid (HEPES) buffer adjusted to pH 7.8. The specimens were submerged in the solution for 2 h and then rinsed for 0.5 h in the 0.55 M mannitol−0.01 M HEPES buffer solution. An observation under light microscope (Olympus System Microscope, Model BHS, Shinjuku-Ku, Tokyo, Japan) was done at 40× magnification, and colour photomicrographs were captured using a digital colour camera (Olympus MicroFire; Olympus, Tokyo, Japan).

Image processing and analysis

Fifteen micrographs were randomly selected from the three replicates of control sample (0 MPa), Control HPP (untreated with calcium, only 200 MPa HPP applied) and each calcium pretreatment for image analysis using software ImageJ (NIH, Bethesda, MD, USA). Cell counter, a plugin software developed by De Vos (2008), was used for semiquantification of the number of viable cells. The cells with a smooth red to pink stain were distinguished as viable cells, whereas inviable cells had a visually rough membrane with no red dye retained.

Degree of browning

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 same fruit samples after 2 weeks storage at 4 °C. Colour was determined using a Minolta CR-410 colorimeter (Minolta camera Co, Ltd., Ramsey, NJ, USA). A white tile was used for calibration (L^* = 96.88, a^* = 0.02, b^* = 2.05). The values were expressed by the CIE $L^*a^*b^*$ system.

Polyphenol oxidase (PPO) assay

The enzyme was assayed using the spectrophotometric method described by Espin *et al.* (1995) with some modifications. Assays were performed at room temperature in 1.0 mL of a medium containing 5.0 mM dihydroxyhydrocinnamic acid (Sigma Aldrich, St. Louis, MO, USA), 0.2 m M 3-methyl-2-benzothiazolinone (MBTH) (Sigma Aldrich), 0.1% (w/v), and 100 m M acetate buffer (pH 5.5). Incubations were started 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, MD, USA). PPO activity was calculated in units using the equation below, where Abs(0) is the initial absorbance and Abs(1) is absorbance at the end of linearity.

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

Analysis of total phenols

Analysis of total phenols was performed by means of the Folin–Ciocalteu Analysis of total phenols was performed by means of the Folin–Ciocalteu method as described by Waterhouse (2002). Peach samples (20 g) were homogenised with 30 mL deionised water, and then 6.4 g of the blended samples was homogenised with 27.6 mL of 76% (v/v) aqueous acetone for 2 min. After 10-min shaking, the cell wall particles were separated using room temperature centrifugation (Centra CL2 tabletop centrifuge, IEC; Needham, MA, USA) at 578.6 × *g* for 10 min. A 0.36-mL aliquot of 2N 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 mixed well before adding 2.64 mL of deionised water. The solution was vortexed again and incubated at 50°C for 5 min. Afterwards, the solution was cooled to room temperature for 1 h, and then, the absorbance was read at 760 nm. Gallic acid (Arcos Organics, Geel, Belgium) concentrations of 0–500 mg L^{-1} were used for calibration of the standard curve. The results were expressed as gallic acid equivalents (GAE)/fresh weight of peaches (g).

Statistical analysis

This experiment was carried out in three replicate processing runs on three separate days. Samples were obtained from three separate peaches, which formed one replicate measurement of each parameter. Analysis of variance was used for determining the effects of each calcium pretreatment on the T_2 relaxation time, % relative area, % viable cells, difference in lightness, PPO activity and total phenols. Tukey's test was used to compare means of each treatment at $P < 0.05$ (SAS version 9.4, Cary, NC, USA).

Results and discussion

T_2 relaxation and relative percentage of water in different compartments of clingstone and freestone peach cells

Following HPP application in untreated control peaches, there are three distinct compartments, each generated from a different proton environment (Fig. 1). Snaar & Van As (1992) pioneered the application of ^{1}H-NMR in plant cell studies and assigned each of these three peaks to the separate cellular water compartment, for example the vacuole, cytoplasm and cell wall/extracellular space. In the present study, the highest peak with the longest T_2 relaxation time and a mean value of 790 ms (peak 1) is assigned to the vacuole, a compartment that contains as much as 50–80% of the total cellular water (Kramer & Boyer, 1995). Plant cell walls are very rigid, being comprised of a cellulose and hemicellulose matrix, which are cemented together with pectin. With its restricted area, the cell wall facilitates proton exchange between water and neighbouring molecules. As a result, the T_2 signal of the water in the cell wall compartment is the shortest, appearing as peak 3 with a mean value of 37 ms. The cytoplasm has the second highest amount of water after the vacuole and has previously been reported with an intermediate T_2 relaxation time (Zhang & McCarthy, 2013; Kirtil *et al.*, 2014). Hence, the cytoplasm water peak is displayed as peak 2 with a mean T_2 value of 143 ms.

T_2 distributions and relative area of each water compartment in all samples evaluated in this study are summarised in Table 1. According to these results, HPP treatment at 200 MPa alone (Control HPP) did not affect T_2 relaxation time or % relative area of the main vacuolar compartment (peak 1) compared to the untreated control (Control) in either clingstone or freestone peaches. However, there was a significant decrease in the T_2 time of both the cytoplasm compartment (peak 2) and the cell wall compartment (peak 3) in clingstone peaches. This may have resulted from a high-pressure-induced increase in membrane permeability; however, it is surprising that the vacuolar compartment (peak 1) was not affected. Peak 3 (cell wall) showed a relatively small but significant change, as the cell wall contains only a small amount of water and its structure is more resistant to damage. There was no significant high-pressure effect in freestone peaches, possibly because the middle lamella of this peach type tends to undergo a greater degree of degradation during ripening, which in fact makes the cells more flexible and therefore resistant to HPP (Techakanon, 2015). This finding is also supported by the greater number of viable cells (43% vs. 30%) in freestone samples following HPP alone (Table 2).

Following pretreatment with calcium chloride and calcium lactate at different concentrations and then HPP treatment, peach samples still had three compartments. The T_2 relaxation time of the vacuolar compartment (peak 1) was unaffected by calcium pretreatment in most cases, which implies that Ca^{2+} may not have reached this compartment. The study by Kirtil *et al.* (2014) observed a dramatic change in the T_2 of vacuolar compartment of mango samples (from 930 to 736 ms) following infusion of 0.001 U mL^{-1} PME + 1% (w/w) $CaCl_2$ at 50 kPa. An impregnation technique such as this is therefore recommended, rather than the dipping and passive diffusion that were used in this study, to deliver Ca^{2+} to the target area.

There were some significant changes in the T_2 relaxation times and % relative area of water in peaks 2 and 3 in clingstone peaches. A significant increase in the T_2 of the cytoplasm compartment (peak 2) after pretreatment indicates less interaction of water protons; therefore, calcium treatment may have reached the cytoplasmic (plasma) membrane, improving membrane integrity and decreasing permeability of water in the cytoplasm compartment. Kirtil *et al.* (2014) reported a similar observation of an increased T_2 relaxation time in the cytoplasm compartment of fresh-cut mango pretreated with 1% $CaCl_2$. There was no significant difference among the various

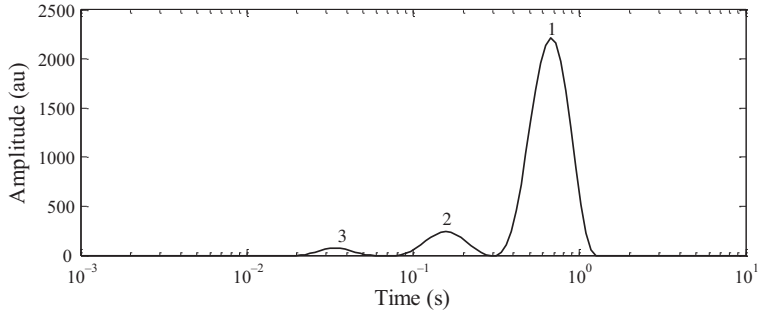

Figure 1 A representative T_2 relaxation spectrum of a control (untreated) peach sample following HPP at 200 MPa.

Table 1 Average T_2 relaxation times and per cent relative areas (RA) of the three compartments of pretreated clingstone peach cultivar Extra Late 1 and freestone peach cultivar Summerset processed using HPP at 200 MPa for 10 min

		T_2 (ms)			Relative area (%)		
Treatment	**Concentration**	**Peak 1**	**Peak 2**	**Peak 3**	**Peak 1**	**Peak 2**	**Peak 3**
Clingstone peach							
Control		790^{a}	169^{a}	25^{d}	90^{a}	8^{b}	2^{d}
Control HPP		790^{a}	143^{c}	37^{bc}	86^{a}	10^{b}	4^{bc}
Calcium lactate HPP							
	1.0%	791^{a}	166^{ab}	42^{ab}	80^{b}	15^{a}	4^{abc}
	1.5%	771^{ab}	169^{a}	43^{a}	78^{b}	17^{a}	5^{a}
	2.0%	768^{ab}	155^{abc}	40^{abc}	81^{b}	15^{a}	4^{bc}
Calcium chloride HPP							
	1.0%	739^{ab}	165^{ab}	42^{abc}	80^{b}	16^{a}	4^{abc}
	1.5%	745^{ab}	164^{ab}	41^{abc}	79^{b}	17^{a}	5^{ab}
	2.0%	717^{b}	149^{bc}	37^{c}	81^{b}	16^{a}	3^{cd}
Freestone peach							
Control		763^{ab}	159^{ab}	32^{b}	88^{a}	10^{b}	2^{cd}
Control HPP		699^{b}	136^{b}	39^{ab}	86^{a}	12^{b}	2^{d}
Calcium lactate HPP	1.0%	813^{a}	165^{a}	44^{a}	79^{b}	18^{a}	3^{bcd}
	1.5%	770^{ab}	174^{a}	49^{a}	78^{b}	18^{a}	4^{ab}
	2.0%	718^{ab}	171^{a}	48^{a}	79^{b}	17^{a}	4^{a}
Calcium chloride HPP	1.0%	779^{ab}	167^{a}	46^{a}	78^{b}	18^{a}	4^{abc}
	1.5%	772^{ab}	163^{a}	43^{a}	77^{b}	17^{a}	6^{a}
	2.0%	693^{b}	166^{a}	43^{a}	78^{b}	18^{a}	4^{ab}

Values within columns followed by the same letter are not significantly different to each other at $P < 0.05$.

Table 2 Per cent viable cells in clingstone and freestone peaches treated with calcium lactate and calcium chloride at 1, 1.5 and 2% and processed with HPP at 200 MPa for 10 min

	Concentration	**Clingstone (EL1) (%)**	**Freestone (Summerset) (%)**
Control		63 ± 3^{a}	61 ± 7^{a}
Control HPP		30 ± 7^{bc}	43 ± 6^{b}
Calcium lactate	1.0%	28 ± 4^{bc}	23 ± 4^{e}
HPP	1.5%	31 ± 5^{bc}	37 ± 7^{cb}
	2.0%	26 ± 5^{c}	22 ± 6^{e}
Calcium chloride	1.0%	27 ± 2^{c}	24 ± 5^{e}
HPP	1.5%	25 ± 5^{c}	32 ± 5^{cd}
	2.0%	34 ± 3^{b}	29 ± 5^{de}

Values represent the mean with its standard deviation for each determination. Values with the same letter are not significantly different across treatment variables at a significance level of $P < 0.05$.

concentrations of either calcium lactate or calcium chloride used for pretreatment of either peach type; therefore, the lowest concentration (1.0%) of either calcium species was sufficient to impart this change. In contrast to clingstone peaches, calcium treatments did not significantly affect the T_2 relaxation of any of the freestone peach water compartments.

The change in relative area, which refers to a change in the amount of water in each compartment, has been widely used to evaluate the change in cell integrity of fruit and vegetable samples, for instance in strawberry (Marigheto *et al.*, 2004), pear (Hernández-Sánchez *et al.*, 2007), onion (Gonzalez *et al.*, 2010a) and pomegranate (Zhang & McCarthy, 2012). High-pressure treatment alone (Control HPP) resulted in no significant change in relative area in most cases, except in one of the six calcium pretreatments for peak 3 (cell wall) of clingstone peaches, which showed a higher per cent area after HPP. This change, while significant, was quite small in comparison with the amount of water in the whole fruit.

In the present study, both clingstone and freestone peaches demonstrated the same trend of a significant decrease in per cent relative area of the vacuolar compartment (peak 1) simultaneous with an increase in the % RA belonging to the cytoplasmic compartment (peak 2) after pretreatment with either form of calcium and at any concentrations. This scenario demonstrates a migration of water from the vacuole to the cytoplasm. It is possible that the calcium pretreatments act as a hypertonic solution, and plasmolysis takes place with the resulting osmotic differences causing loss of vacuolar membrane integrity and movement of water. Saftner *et al.* (1998) suggested that the immersion of fruits in high calcium concentration salts enhances the risk for fruit injury, which is possibly a result of osmotic effects.

Evaluation of viable cells using light microscopy

Clingstone and freestone peaches both showed a significant decrease in the number of viable cells after high-pressure treatment, from approximately 61–63% to 30–43%, respectively (Table 2). This finding is supported by a previous study of HPP-treated onions, which suggested that pressures above 100 MPa cause membrane damage, which induces a release of PPO as well as its phenolic substrates, and the subsequent enzymatic browning reaction (Butz *et al*., 1994). Basak & Ramaswamy (1998) suggested that the cellular membranes of fruits and vegetables rupture at pressures of 200 MPa and above. A previous study by our group also found a significant decrease in viable cell number in onion samples following HPP at 200 MPa (Gonzalez *et al*., 2010b).

In clingstone peaches, none of the calcium pretreatments used prior to HPP resulted in significant improvements in the maintenance of cell viability. However, in freestone peaches, which had a higher number of viable cells following HPP (43 vs. 30%), there was in fact a significant undesirable decrease in the percentage of viable cells in most of the samples pretreated with either calcium solution at any percentage prior to HPP. Based on our previous experiments, HPP-treated freestone peaches without calcium pretreatment retained their cell integrity better than clingstone types and showed higher percentages of cell viability (Techakanon, 2015). This could be explained by the looser cell matrix of freestone peaches, which undergo more extensive degradation of the middle lamella as the fruit matures. As a consequence, the cells are more flexible and potentially less damaged during HPP and subsequent sectioning for the microscopic study. The calcium treatment, on the other hand, imparts less cell flexibility due to the interaction of calcium with pectin to form bridges. Therefore, following most of the pretreatments and HPP, freestone peaches showed a significant decrease in the percentage of viable cells. This finding is in agreement with the degree of browning results that freestone samples showed a greater difference in lightness after pretreatment and HPP, compared to clingstone peaches.

Degree of browning

Enzymatic browning in fruit is a direct consequence of the interaction between the enzyme polyphenol oxidase, originally located in the plastid and its phenolic substrates, which are initially in the vacuole. After cells lose compartmentalisation, the membrane barrier separating the two components from each other is ruptured. In the present study, peach samples were high-pressure-treated at 200 MPa, and at this level, the cell membranes were ruptured, leading to a release of vacuolar contents to the cytoplasm.

A significant increase in the difference in lightness following HPP treatment alone (Control HPP vs. Control) was observed in both peach types after 2 weeks of storage (Fig. 2). A previous study by our group reported a similar finding in onion cells (Gonzalez *et al*., 2010a,b). These authors found that a loss in membrane integrity was observed using light microscopy at this pressure level. Basak & Ramaswamy (1998) reported significant changes in apple flesh colour after application of pressures of 200 MPa and higher and stated that this was due to the interaction of PPOs and their substrates.

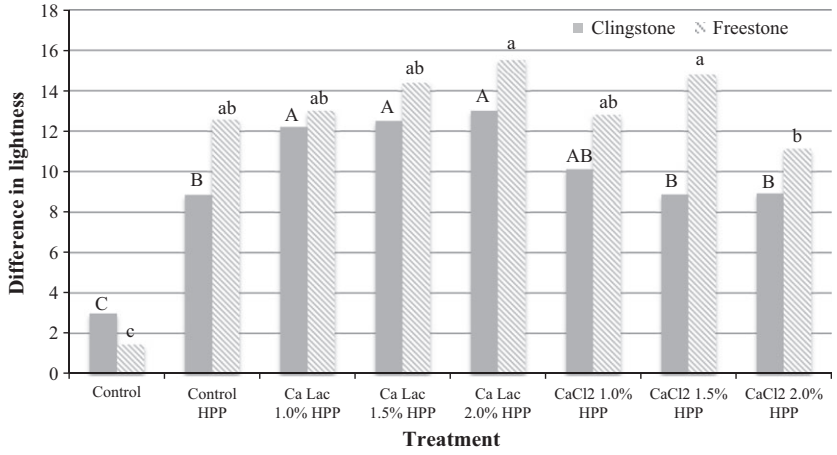

Figure 2 Difference in lightness in clingstone and freestone peaches treated with calcium lactate and calcium chloride at 1.0, 1.5 and 2.0% and then HPP-treated at 200 MPa for 10 min. Values with the same letter are not significantly different across treatment variables at a significance level of $P < 0.05$. Capital letters (A, B, C) refer to clingstone peaches, and small letters (a, b, c) refer to freestone peaches.

In the current study, it was hoped that this problem would be solved using pretreatments with calcium solutions, which theoretically may aid in retention of cell integrity during HPP and prevent loss of membrane integrity. However, results in Fig. 2 illustrate that there was no significant effect of pretreating with calcium chloride on reducing the discoloration of HPP-treated and cold-stored peaches. This is supported by previous findings of strawberry pretreatment with $CaCl_2$ at 1% and 4%, which also found that there was no significant effect of calcium pretreatment on strawberry firmness (Marigheto *et al.*, 2004).

Calcium lactate, in contrast, had a significant negative effect on clingstone peach browning, with a significant increase in the difference in lightness in the samples pretreated with all concentrations prior to HPP processing. There was no significant difference in freestone peaches pretreated with any level of calcium lactate, as compared to the Control HPP sample.

In a previous study by Gorny *et al.* (2002) in fresh-cut pears, these authors also found that treatment with calcium lactate did not prevent cut surface darkening. In fact, the pretreated sample developed a lower hue angle (higher flesh darkening) than the control sample (water dip) during 8 days of storage at 0 °C in continuously flowing (100 mL min^{-1}) humidified air. Another study on calcium treatment of peach fruit (cv. Andross) confirmed the same finding, as significant discoloration compared to the control was found in samples during 4 weeks of cold storage following calcium lactate treatment at high concentration of 187 mM Ca (Manganaris *et al.*, 2007).

Effect of pretreatment on PPO activity

Figure 3 illustrates that high-pressure treatment alone had no effect on PPO activity of freestone peaches, but caused a significant increase in that of clingstone peaches. A similar observation was reported in our previous study, in which clingstone peach cultivars Carson and Ross had a significantly higher PPO level following HPP at 100 MPa for 10 min (Techakanon, 2015). PPO activation induced by HPP has been reported in other fruits and vegetables, for instance, pear extracts following HPP at 400 MPa or higher (Asaka *et al.*, 1994), strawberries treated at 400 MPa (Cano *et al.*, 1997) and HPP onions treated at 100–500 MPa (Butz *et al.*, 1994).

This study further compared the PPO activity after use of different pretreatments in the two peach types. Freestone peaches are generally higher in PPO activity, with a mean value of 31 141 units mL^{-1} in the control group, while the untreated clingstone peaches had a mean PPO activity of 9776 units mL^{-1}. These PPO activity levels are in agreement with our previous study (Techakanon, 2015). There was no significant difference ($P < 0.05$) in PPO activity following pretreatment with calcium lactate or calcium chloride in either clingstone or freestone types. Although other studies have suggested an effect of $CaCl_2$ on PPO inhibition, through the interaction between the chloride ion and copper at the active site of the enzyme (Garcia & Barrett, 2002), this experiment did not observe any significant changes in PPO activity. One possible reason could be the low concentration of calcium chloride used in the current experiment, as chloride is a weak inhibitor. Some authors have suggested that an

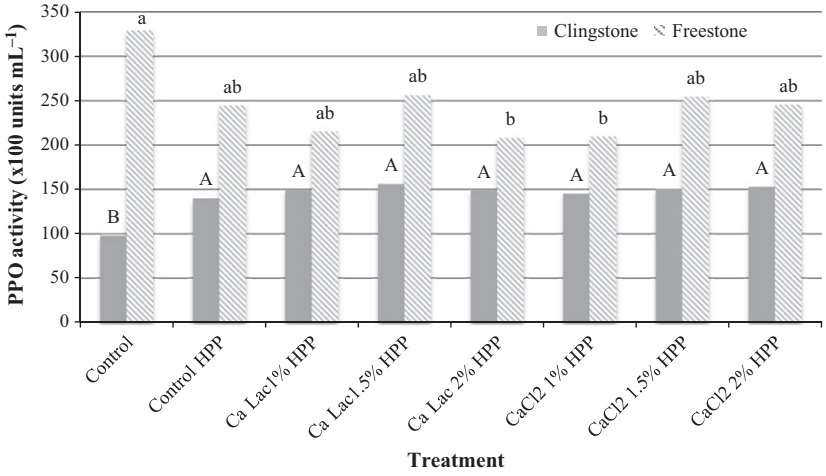

Figure 3 PPO activity in clingstone and freestone peaches treated with calcium lactate and calcium chloride at 1.0, 1.5 and 2.0% followed by HPP treatment at 200 MPa for 10 min. Values with the same letter are not significantly different across treatment variables at a significance level of $P < 0.05$. Capital letters (A, B, C) refer to clingstone peaches, and small letters (a, b, c) refer to freestone peaches.

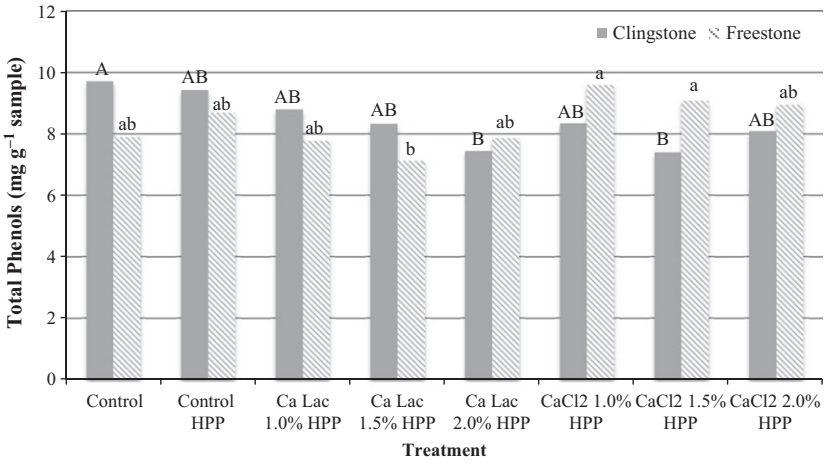

Figure 4 Total phenol content in clingstone and freestone peaches treated with calcium lactate and calcium chloride at 1, 1.5 and 2% following HPP treatment at 200 MPa. Values with the same letter are not significantly different across treatment variables at a significance level of $P < 0.05$. Capital letters (A, B, C) refer to clingstone peaches, and small letters (a, b, c) refer to freestone peaches.

elevated level of chloride is required to cause PPO inhibition (Mayer & Harel, 1991).

Effect on total phenols

The selected cultivars of clingstone and freestone peaches had similar levels of phenolic compounds, for example in the range of 6–12 mg g^{-1} sample (Figure 4). These compounds act as substrates for PPO in enzymatic browning reactions. Both peach types showed a stable level of total phenols following HPP as compared to the control samples. No significant differences in total phenols in other HPP-treated fruits were also reported by other investigators, for example strawberry and blackberry purées (Patras *et al*., 2009) and Granny Smith apple purée (Landl *et al*., 2010). High-pressure treatment is therefore excellent for retaining these nutrients.

Summary and conclusions

Enzymatic browning in fruit following HPP directly involves the loss of cell integrity, which leads to the interaction between the enzyme in the plastid and the substrates stored in the vacuole. Calcium pretreatments may help prevent enzymatic browning reactions during HPP if the penetration of Ca^{2+} reaches the target area, which is the tonoplast or vacuolar membrane of peach cells. However, the results of this study showed no significant changes in T_2 relaxation time of the vacuolar compartment and no improvement in the number of viable cells in most of the pretreated samples, in either clingstone and freestone peaches. These findings indicate a high probability that Ca^{2+} could not penetrate all the way to the tonoplast membrane. This result is also in agreement with the observation of the difference in lightness, in which calcium chloride and calcium lactate pretreatments at 1–2% could not improve peach colour following 2 weeks of refrigerated storage. For future investigations, a higher calcium concentration and/or improved impregnation (potentially using vacuum infusion) is suggested for efficient delivery of Ca^{2+} inward to the tonoplast membrane.

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