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Suppression of Chlorophyll Breakdown and Quality Changes by Hot Water Treatment of 'Pichit 1' Lime (*Citrus aurantifolia* Swingle) Fruit

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Hot water treatment (HWT) was applied to a new Thai lime cultivar fruit, 'Pichit 1' (*Citrus aurantifolia* Swingle) to investigate its effect on chlorophyll (Chl) degradation and postharvest quality during storage. Fruit were treated with HWT with temperatures of 45, 48 or 50°C for 3, 5, and 10 min and then kept in the dark at 25°C and 90 ± 5% RH. Lime peels retained a greener color after HWT at 48°C for 5 min compared to the fruit given any other treatment. HWT at 48°C for 5 min efficiently delayed the decrease in the hue angle value acquired during storage. Chlorophyllide *a*, pheophorbide *a*, pyropheophorbide *a*, 13^2-hydroxychlorophyll *a*, pheophytin *a*, and an unknown catabolite, which may be a chlorophyllide *a* derivative, were detected as Chl derivatives in the fresh limes. The levels of chlorophyllide *a*, pheophytin *a*, and 13^2-hydroxychlorophyll *a* gradually decreased during the progression of peel degreening. Those derivative levels were higher in the fruit treated with HWT than the control. Moreover, the organic acid content was maintained at higher levels in the fruit treated with HWT than the control during storage at 25°C. During storage, the sugar content was seen to decrease with or without HWT; however, sugar reduction in the control was more rapid. It appeared that HWT reduced the degradation of Chl by controlling its catabolites. Therefore, HWT affected the fruit quality of green 'Pichit 1' limes in storage.

Key Words: chlorophyll catabolite, chlorophyll degradation, heat treatment, lime, postharvest quality.

Introduction

'Pichit 1', a new lime (*Citrus aurantifolia* Swingle) cultivar that originated in northern Thailand, has been distributed to the southern part of the country as a commercial fruit crop. This cultivar is popular among consumers because of its disease resistance, its thinner peel and a high juice content. However, degradation of Chl causing a loss of its green color is a major problem in limes during transportation and storage (Kaewsuksaeng et al., 2011; Srilaong et al., 2011). The internal quality of limes depends on factors including organic acid and sugar contents (Kaewsuksaeng et al., 2011). In the 'Pichit 1' lime, the commercial value and price strongly depend on its postharvest quality attributes. For quality maintenance, it is important to retain the green color in the peel of the fruit as long as possible.

In green leafy vegetable crops such as broccoli, as well as in limes, initial destruction of Chl *a* has been theorized to result in chlorophyllide formation by chlorophyllase. Then, pheophorbide (Pheide) *a*, which causes a loss of green color, is formed by Mg-dechelating substances. Finally, Pheide *a* is decomposed to fluorescent Chl catabolites, which are primarily colorless, via a red Chl catabolite by both Pheide *a* oxygenase and red Chl catabolite reductase (Matile et al., 1999). Chl-degrading peroxidase (Yamauchi, 2015) has also been suggested to be involved in Chl degradation as the first step enzyme involved in oxidizing Chl *a* to form 13^2-hydroxychlorophyll (OHChl) *a*. It has also been reported that pheophytinase plays a role in the dephytylation of pheophytin (Phein) *a*, resulting in production of Pheide *a* (Schelbert et al., 2009). In addition, pheophorbidase is involved in the Chl degradation pathway and it converts Pheide *a* into pyropheophorbide (Pyropheide) *a* (Shioi et al., 1996).

Physical postharvest treatments including elevated temperature treatment have been examined in regard to

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the suppression of senescence in stored horticultural crops (Lurie, 1998; Sivakumar and Fallik, 2013). Heat treatment after harvest was traditionally used to control decay-causing pathogens and pests in citrus, mango, and papaya, and it has been utilized in a quarantine context (Lurie and Mitcham, 2007). Heat treatment of horticultural fruit has been reported to limit Chl destruction by reducing the gene expression and action of Chl-degrading enzymes (Büchert et al., 2010; Funamoto et al., 2002; Kaewsuksaeng et al., 2007). In green Nagato-yuzukichi fruit (*Citrus nagato-yuzukichi* hort. ex. Y. Tanaka), native to Yamaguchi Prefecture, Japan, and green yuzu fruit (*Citrus junos* Siebold ex. Tanaka), HWT at 40 and 45°C for 5 min efficiently suppressed the decline of hue angle values during storage at 25°C (Ogo et al., 2011). Moreover, Yamauchi et al. (2003) also reported that heat treatment at 50°C for 3 min with a solution of 2% sucrose laurate ester delayed the degreening in Nagato-yuzukichi fruit compared with only heat treatment or 2% sucrose laurate ester without heat treatment. In limes, postharvest stress treatment such as UV-B irradiation was found to suppress Chl degradation during storage at 25°C (Kaewsuksaeng et al., 2011; Srilaong et al., 2011). Thus, postharvest stress treatment such as heat or UV-B treatment could efficiently retard Chl degradation during storage, resulting in quality maintenance during transportation and storage.

We previously investigated the influence of postharvest heat treatment using hot water at 50°C for 5 min on Chl degradation and Chl-degrading enzymes in Thai limes (*Citrus aurantifolia* Swingle 'Paan') during storage (Kaewsuksaeng et al., 2015). In this paper, using a new lime cultivar, 'Pichit 1', we examined the effects of hot water treatments on Chl catabolites and chemical component changes to elucidate the Chl degradation mechanism and internal quality changes during storage.

Materials and Methods

Plant materials and hot water treatment

Samples of mature green limes ('Pichit 1') were collected from a commercial grove in a province in southern Thailand (Nakorn Sri Thammarat). The fruit were transported in an uncontrolled temperature vehicle to a laboratory where they were selected for experimentation based on a number of criteria including: absence of defects, color of peel, shape, size, and uniformity of maturity. Hot water was used to treat the fruit for 3, 5, and 10 min at 45, 48, and 50°C. The control consisted of treatment of the fruit at ambient temperature. The fruit were then dried at ambient temperature for 1 h. After hot water treatment, post HWT, twenty fruits were enclosed in 0.3 mm-thick PE bags with three holes and kept at 25°C, 90 ± 5% RH, in darkness. Every 5 d, 20 fruits were pooled to constitute one replicate. Each treatment was performed with five replicates for postharvest quality analysis.

Surface color and chlorophyll assays

A colorimeter (NF 777; Nippon Denshoku Industries, Japan) was used to measure the hue angle to determine the surface coloration at three positions including the top, middle and bottom of the limes. *N,N*-dimethylformamide was used to assay the Chl *a* and *b* content following the methods of Moran (1982).

Analyses of chlorophyll and resulting derivatives

An acetone-HEPES buffer (AHB) solution containing 20 mL cold acetone and 2.5 mL 0.1 M HEPES buffer (pH 7.5) was prepared. A mortar and pestle were used to grind 2.5 g of fruit flavedo in 22.5 mL of AHB. The sample was held in darkness on ice for 5 min then passed through Whatman # 2 filter paper (GE Healthcare Bio-Sciences, USA). Thereafter, sample aliquots used in HPLC analysis were passed through a DISMIC filter (0.45 μm; AVANTEC, Japan). Chl and the resulting derivatives were analyzed by HPLC with a Hitachi L-700 pump + automatic gradient controller followed by analysis using either a Hitachi L-2450 diode array detector or a Hitachi L-7240 UV–visible spectrophotometer (Hitachi High-Technologies Corporation, Japan). Pigment absorption at 665 nm was monitored. Separation of pigments was done with a 4 × 250 mm LiChropher C18 column (Merck, Germany) with two solvents: (A) 80% methanol (80:20, CH_3OH:H_2O, v/v), and (B) a gradient of 100% ethyl acetate. A linear rate of solvent B was added to solvent A until a 50:50 ratio was reached after 20 min. This mixture was used for an additional 20 min isocratically following the procedures of Eskin and Harris (1981). A flow rate of 1.0 mL·min^{-1} and injection volume of 100 μL were used. Retention time and visible absorption spectra were used to identify Chl and the resulting derivatives. Standards of Chl derivatives such as Pyropheide *a* and Pheide *a* were obtained from Tama Biochemical (Japan) and Wako Pure Chemical Industries (Japan), respectively. The standard of Phein *a* was made by adding three drops of 0.1 M HCl acid to a Chl *a* solution following the methods of Holm-Hansen et al. (1965). OHChl *a* was prepared by adding peroxidase (horseradish, Sigma–Aldrich, USA) into a Chl *a* solution in the presence of hydrogen peroxide and *p*-coumaric acid, as described by Kaewsuksaeng et al. (2007).

Postharvest quality parameters

Extracts (15 mL) of the fruit juice of organic acids and sugars were made by treatment with a final concentration of 70% hot ethanol for 15 min. Aliquots (1 mL) of the ethanol extracts were dried by vacuum-evaporation and redissolved in 1 mL of Milli-Q water (Merck). HPLC using a Hitachi L-7420 UV-Visible spectrophotometer was used to determine the citric and malic acid contents. The samples were separated using a 4 × 250 Mightysil RP-18 column (Kanto Chemical

Co., Inc., Japan) and a solvent of water: methanol: 50 mM phosphoric acid (69:1:30). The citric and malic acid spectra were monitored at 210 nm. A flow rate of 1 mL·min^{-1} and an injection volume of 100 µL were used. The ethanol extract was passed through a Sep-pak C18 (Waters, USA) to remove pigments, and the sugar was analyzed by HPLC (Hitachi L-7940 RI detector; Hitachi High-Technologies Corporation) using a 4 × 250 mm NH_2 column (Kanto Chemical Co., Inc., Japan) with an acetonitrile: water solvent (80:20). A flow rate of 1 mL·min^{-1} and injection volume of 100 µL were used. The retention time using the standards was used to identify the organic acids and sugars. The methods of Roe et al. (1948) were used to determine the ascorbic acid content.

The experiments were conducted using a completely randomized design. The analysis of variance (ANOVA) of the data was performed using SAS version 9.0 (SAS Institute Inc., USA). The differences between the means were compared by least significant differences at $P < 0.05$.

Results

Optimization of the hot water treatment

As shown in Table 1, the levels of the hue angle (surface color) and L values in fruits with or without HWT changed due to peel degreening after 20 d of storage at 25°C. Limes treated with HWT at 48°C for 5 min displayed a greener color, had a delayed reduction in hue angle and enhanced L values compared to the fruit with any other treatment during storage at 25°C. This treatment was the best at delaying peel discoloration. Therefore, the HWT at 48°C for 5 min was selected as the optimal method and applied in the next experiment for further investigation. Lime peels started degreening and turned 100% yellow after 20 d storage. However, the limes treated with HWT at 48°C for 5 min remained green up to 20 d after treatment (Fig. 1). During storage there was a significant decline in the hue angle of the control, which increased the degreening of the fruit peel. On the other hand, the HWT fruit showed almost no color change during storage (Fig. 2A).

Table 1. Effect of hot water treatment (HWT) at 45, 48 or 50°C for 3, 5 or 10 min on the hue angle and L value in limes during storage at 25°C. The average values were calculated by the F-test one-way ANOVA with SE (n = 5). Different superscript letters (a–c) within the same column indicate significant differences between treatments. The asterisk (**) indicates that the value is significantly different from the corresponding control ($P < 0.01$).

Treatment	Hue angle	L value
Day 0		
Control	124.41 ± 1.77	45.21 ± 0.70
Day 20		
Control	102.77 ± 1.69c	70.87 ± 1.15c
45°C 3 min	110.22 ± 1.13b	57.85 ± 0.989a
45°C 5 min	114.94 ± 1.18b	68.82 ± 0.30c
45°C 10 min	108.23 ± 1.25c	57.3 ± 0.77a
48°C 3 min	112.93 ± 1.37b	48.74 ± 0.91b
48°C 5 min	119.08 ± 1.94a	44.13 ± 0.67b
48°C 10 min	99.93 ± 1.10c	71.6 ± 1.63c
50°C 3 min	108.22 ± 1.47b	54.18 ± 0.09a
50°C 5 min	110.89 ± 1.64b	61.41 ± 1.13c
50°C 10 min	109.84 ± 1.33b	57.44 ± 1.52a
F-test	**	**
C.V. (%)	0.12	0.15

Effect of HWT on chlorophyll degradation and chlorophyll derivatives in limes

As shown in Figure 2B, C, HWT also delayed the reduction in Chls *a* and *b* contents in the lime peel, whereas that in the control sharply decreased during the first 5 d of storage and then showed a continued decline during further storage. The HPLC chromatogram of Chl derivatives extracted from fresh limes demonstrated a sequential elution of Chlide *a*, Pheide *a*, Pyropheide *a*, OHChl *a*, Phein *a*, and an unknown peak. As shown in Figure 3, the unknown catabolite was detected after Pyropheide *a* on the chromatogram and the spectral peaks were 432 and 665 nm. Figure 4 shows the changes in Chl derivative levels in limes with or without HWT. Chlide *a* levels decreased in the limes without HWT, while that in the fruit with HWT decreased gradually after a temporary increase at 5 d of storage at 25°C (Fig. 4A). Pheide *a* and Pyropheide *a* levels were mostly found in stored limes with or without HWT on d 5 and their levels in the control were higher than those in the hot water-treated limes during storage (Fig. 4B, C). Phein *a* levels in the control decreased markedly, whereas the level in the hot water-treated fruit increased dramatically during the first 5 d of storage and afterwards showed a marked decline (Fig. 4D). Moreover, the OHChl *a* level in limes treated with HWT also showed a slightly higher level compared to without HWT, as shown in Figure 4E. The unknown catabolite

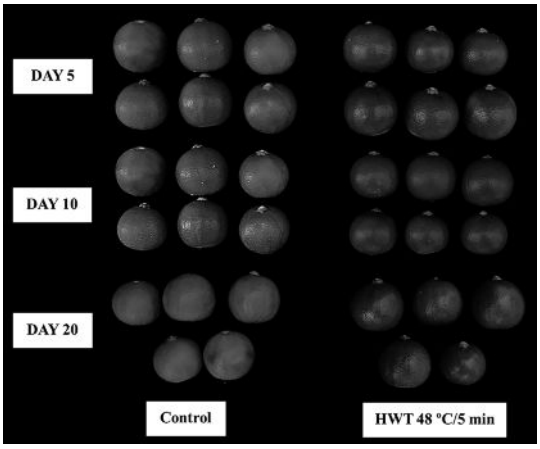

Fig. 1. The peel color change in limes treated with or without hot water treatment (HWT) at 48°C for 5 min during storage at 25°C and then stored for 5, 10, and 20 d.

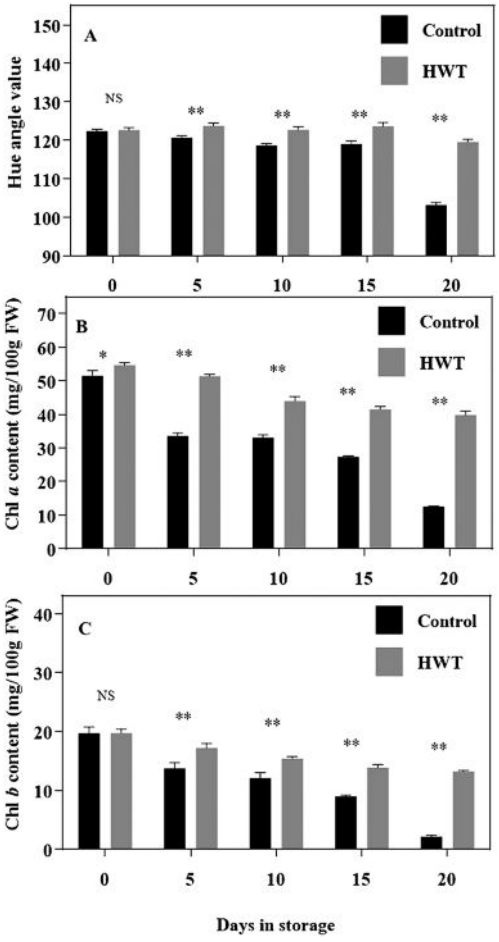

Fig. 2. Changes in the hue angle value (A) chlorophyll (Chl) *a* (B) and *b* (C) contents in limes treated with or without hot water treatment (HWT) at 48°C for 5 min followed by storage at 25°C. Vertical bars represent the average values with SE (n = 5). Significant differences are shown between HWT and the control (**, $P < 0.01$; *, $P < 0.05$ by *t*-test). Chl: chlorophyll.

level in limes with or without HWT, especially the former, increased a lot in the first 10 d and afterwards showed a decline during storage at 25°C (Fig. 4F).

Effect of HWT on postharvest qualities in lime fruit

The citric and malic acid contents, which are the major organic acids in limes, with or without HWT did not change much during storage (Fig. 5A, B). As shown in Figure 5C, the ascorbic acid content in limes treated with HWT showed an immediate increase and this was subsequently retained at higher levels than that in the control during storage at 25°C.

Figure 6 shows the sugar level changes in the lime fruit treated with or without HWT during storage. The glucose (Fig. 6A) and fructose (Fig. 6B) contents in the control showed a sharp increase during the first 5 d of storage and afterward decreased gradually, whereas the contents in the hot water-treated fruit decreased slightly after a temporary increase on d 5 of storage. On the other hand, the sucrose content (Fig. 6C) with or without HWT showed a slight increase, but showed almost no change during storage.

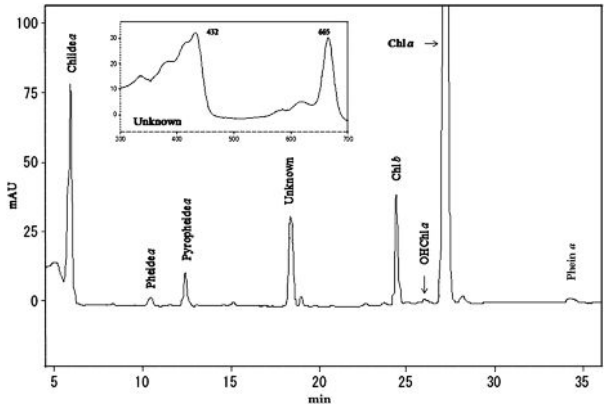

Fig. 3. HPLC chromatograms of Chl and Chl derivatives (Chlide *a*, Chlorophyllide *a*; Pheide *a*, Pheophorbide *a*; Pyropheide *a*, Pyropheophorbide *a*; Unknown; OHChl *a*, 13^2-hydroxychlorophyll *a*, and Phein *a*, Pheophytin *a*) of limes on d 0 during storage at 25°C.

Discussion

A major quality issue in transportation and storage of green horticultural crops is loss of green coloration. The cause of green color loss in limes is the breakdown of Chl (Kaewsuksaeng et al., 2011; Srilaong et al., 2011). The most important factor in maintaining the market value of limes is the maintenance of a green peel color after harvest (Kaewsuksaeng et al., 2011, 2015).

Treatments of a physical nature such as UV irradiation and high/low temperatures or chemical measures such as ethanol vapor have been evaluated to control horticultural crop yellowing during storage (Aiamla-or et al., 2009; Funamoto et al., 2002; Lurie and Mitcham, 2007). One of these stress treatments, heat treatment after harvest, has been traditionally used to manage fruit rot caused by pathogens and pests in citrus, mango and papaya, and has been used in related quarantine issues (Lurie and Mitcham, 2007). Inhibition of yellowing by hot air/water treatment has been evaluated in green citrus fruits (Ogo et al., 2011), broccoli florets (Costa et al., 2006; Funamoto et al., 2002; Terai et al., 1999; Tian et al., 1996), and green leafy vegetables (Gómez et al., 2008).

Kaewsuksaeng et al. (2015) recently determined that HWT for 5 min at 50°C was effective in delaying Chl deterioration in stored 'Paan' limes. The current study similarly found that HWT for 5 min at 48°C was effective in delaying Chl breakdown in 'Pichit 1' limes during storage at 25°C. From these results, the heat treatments similarly affected these lime cultivars, although their morphology has been found to be different. 'Paan' limes have a thick peel while 'Pichit 1' limes have a thin peel. Therefore, the best temperature for HWT of 'Paan' limes was higher than that of the 'Pichit 1' limes. Moreover, the results suggest that a reduction

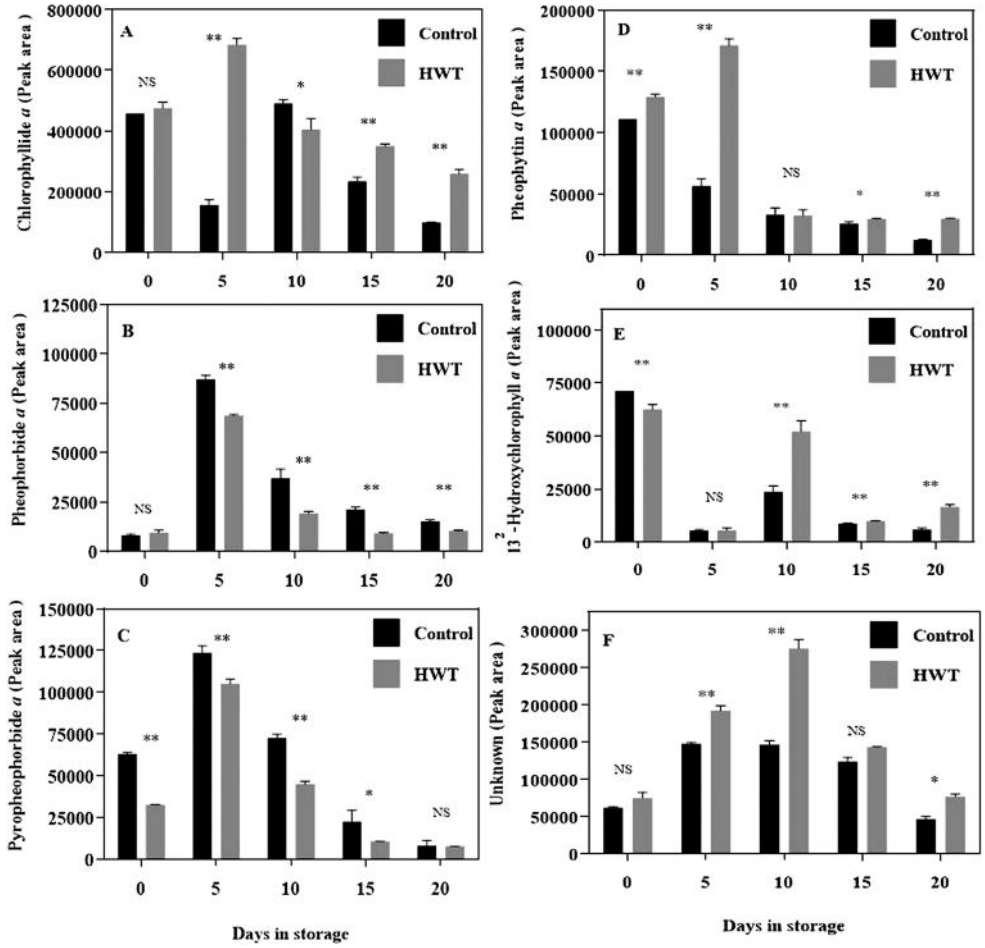

Fig. 4. Changes in Chl derivatives level in limes treated with or without HWT at 48°C for 5 min during storage at 25°C. Chl derivatives were analyzed using an HPLC system. Vertical bars represent the average values with SE (n = 5). Significant differences are shown between HWT and the control (**, $P < 0.01$; *, $P < 0.05$ by *t*-test). Chlorophyllide *a* (A), Pheophorbide *a* (B), Pyropheophorbide *a* (C), Pheophytin *a* (D), 13^2-hydroxychlorophyll *a* (E), and Unknown (F).

in the loss of green coloration may be due to a reduction in Chl destruction by decreasing the activity of enzymes such as pheophytinase, chlorophyllase, Chl-degrading peroxidase, and Mg-dechelation activity (Kaewsuksaeng et al., 2015). Hot air treatment of broccoli florets for 2 h at 50°C effectively reduced storage yellowing, possibly as a result of decreased gene expression and/or activity of the enzymes that degrade Chl (Büchert et al., 2010; Funamoto et al., 2002).

It has been reported that Chls *a* and *b* decrease in horticultural crops that are fresh and senescing during storage and a number of Chl *a* derivatives have been detected, including Pheide *a*, Pyropheide *a*, Phein *a*, Chlide *a*, and 13^2-OHChl *a* (Gross, 1991). We found a new Chl derivative in fresh lime fruit and its derivative accumulated during the Chl degradation process. The spectroscopic characteristics of the unknown catabolite appeared at 432 and 665 nm, suggesting that it may be a Chlide *a* derivative since its spectral peak values were similar to those of Chlide *a*. Vergara-Domínguez et al. (2011) also reported that a Chlide *a* derivative was present in olives and the spectral peak values had the same values as Chlide *a*. In this study, high levels of Chlide *a* and Phein *a* were found in fresh limes and they decreased during storage at 25°C. As might be expected, the decrease in those Chl derivatives may be strongly associated with the action of Chl-degrading enzymes, including chlorophyllase, pheophytinase, and Mg-dechelating substances, during storage. On the other hand, these enzyme levels were highly retained in limes treated with HWT compared to the control throughout the storage period, inferring that the activities of Chl-degrading enzymes may be effectively suppressed by HWT treatment (Kaewsuksaeng et al., 2011, 2015).

Pheide *a* and Pyropheide *a* levels in limes treated with HWT slowly accumulated compared to the fruit without HWT. Srilaong et al. (2011) demonstrated that the Pheide *a* level declined in UV-B-treated limes, especially during the degreening. This indicates that the degradation of Chlide *a* to Pheide *a* may be suppressed by UV-B, as was apparent in the HWT fruits in our study. 13^2-OHChl *a* is formed as an intermediate and does not accumulate during storage in horticultural

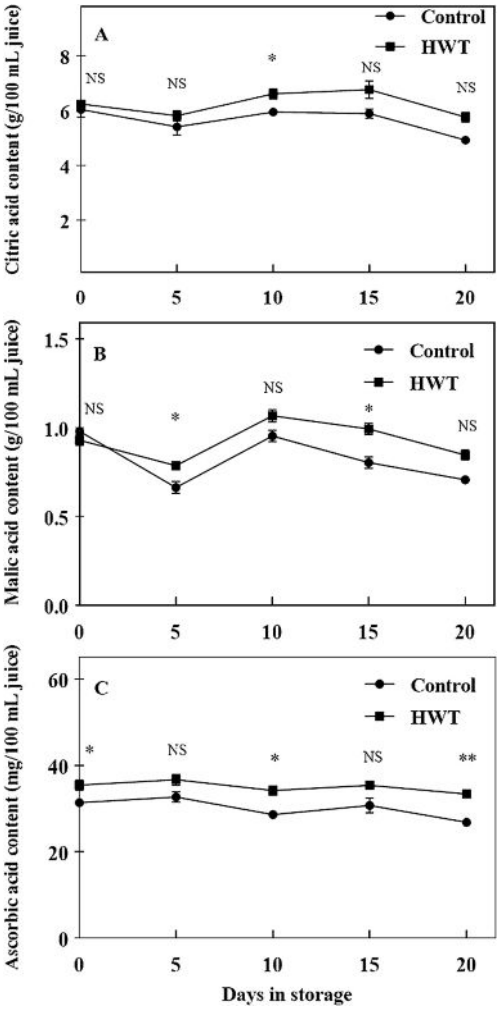

Fig. 5. Changes in citric acid (A), malic acid (B), and ascorbic acid (C) contents in the juice from limes treated with or without HWT at 48°C for 5 min during storage at 25°C. Vertical bars represent the average values with SE (n = 5). Significant differences are shown between HWT and the control (**, $P < 0.01$; *, $P < 0.05$ by *t*-test).

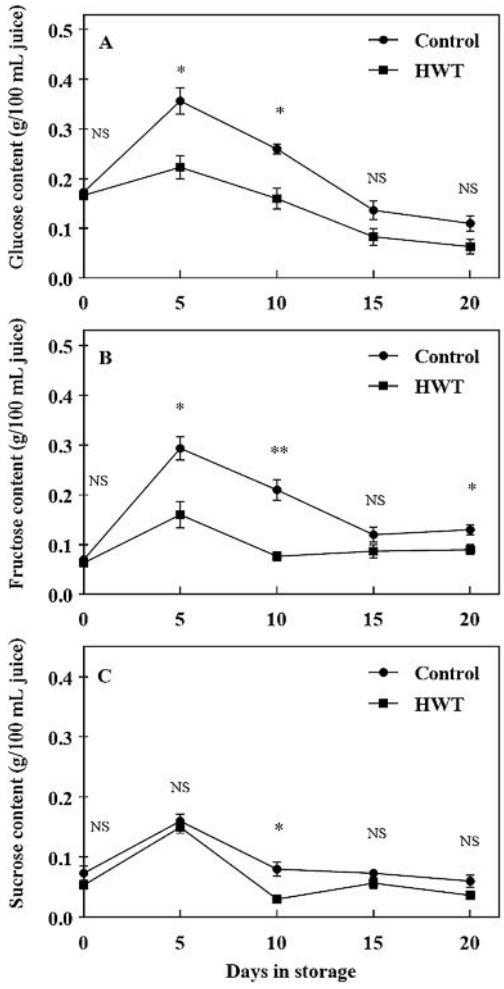

Fig. 6. Changes in glucose (A), fructose (B), and sucrose (C) contents in the juice from limes treated with or without HWT at 48°C for 5 min during storage at 25°C. Vertical bars represent the average values with SE (n = 5). Significant differences are shown between HWT and the control (**, $P < 0.01$; *, $P < 0.05$ by *t*-test).

crops. Therefore, the content of 13^2-OHChl *a* usually shows a decrease with senescence during storage (Yamauchi and Watada, 1991, 1998). In accordance with the Chl derivative changes, as previously reported, the decrease in the 13^2-OHChl *a* level was also delayed by HWT during storage. The findings obtained here indicate that HWT could be involved in the suppression of Chl and its derivative changes through inhibition of the action of Chl-degrading enzymes.

In addition to Chl degradation and Chl derivatives, chemical composition changes in relation to internal quality occur during the storage of limes. The citric acid and malic acid contents in limes showed a slight change during storage. These contents were higher in the fruit treated with HWT than in the control during storage. HWT may suppress the respiration rate, which is necessary to maintain organic acids in limes and is similar to that seen with UV-B irradiation (Kaewsuksaeng et al., 2011, 2015).

As shown in Figure 5C, HWT had a positive effect on antioxidants such as ascorbic acid since their content in the fruit after HWT immediately showed an increase and was subsequently retained at a higher level than that in the control during storage. Kaewsuksaeng et al. (2011) found that the content of ascorbic acid in UV-B treated fruit increased after UV-B treatment. A possible explanation for these findings is that stress treatments such as high temperature and UV-B could induce the activation of an ascorbate-glutathione cycle.

Changes in sucrose, fructose and glucose during storage were also reduced by HWT. However, as senescence advanced in the control, the contents of these sugars increased. Our results are consistent with other research that indicated that heat treatment reduced sugar level changes (Lemoine et al., 2008). Therefore, HWT appears to be an effective technique for preserving the internal fruit quality of limes.

Conclusion

The current research found that hot water treatment of limes for 5 min at 48°C was optimally effective in slowing the destruction of Chl during storage. The same treatment reduced the levels of a number of Chl derivatives, including Chlide *a*, Phein *a*, and 13^2-OHChl *a*. A gradual increase in the malic and citric acid contents and a reduction in fructose, glucose, and sucrose also occurred during storage. In addition, HWT preserved the level of ascorbic acid in storage. We believe that HWT for 5 min at 48°C could be an effective technique for prolonging the postharvest quality of limes in storage.

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