

Volatile Emissions of Navel Oranges as Predictors of Freeze Damage

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Volatile emissions of navel orange (*Citrus sinensis* L. Osbeck cv. Washington) fruit were evaluated as a means for predicting and gauging freeze damage. The fruits were subjected to -5 or -7 °C treatments in a laboratory freezer for various time periods of 2–9.5 h and stored at 23 °C for 1, 2, or 7 days, after which time the emission of volatiles from the fruit was measured. Following the final day of volatile measurements the fruits were stored at 5 °C for an additional 2–3 weeks and then evaluated for fruit quality characteristics. Peel injury in the form of brown lesions, drying of the juice vesicles, a decline in acidity, and a loss of flavor were observed to occur as a result of freezing. Corresponding to the loss in fruit quality were large increases in the emissions of ethanol, ethyl butanoate, methyl hexanoate, and ethyl octanoate. With the exception of methyl hexanoate, for which volatile emissions decreased during storage for 7 days at 23 °C, all of the other volatiles were relatively unchanged in amount by storage. Treatment at -7 °C caused greater injury, quality loss, and more volatile emanation than did freezing at -5 °C. The measurement of volatile emissions appears to be a useful approach to identify freeze-damaged navel oranges.

KEYWORDS: Ethanol; esters; freeze injury; citrus

INTRODUCTION

Unpredictable freeze injury to navel orange (*Citrus sinensis* L. Osbeck) fruit caused by prolonged low temperatures in the field continues to pose a menacing problem for the citrus industry. Early symptoms of freeze injury include a water-soaked appearance in regions of the interior fruit segments and the presence of hesperidin crystals in the membranous areas between the segments (1). An off-odor may also be detected from fruit when sliced open soon after a freeze (Mary Lu Arpaia, personal communication). Subsequently, drying of the internal flesh and the development of open spaces between the segments occurs several days to weeks after the freeze event depending on the severity and duration of the freeze. If the freeze was severe, peel damage in the form of brown staining and pitting is seen. It is critical for the grower and the packing house to be able to decide if the freeze event has damaged the crop sufficiently to warrant the fruit not being picked and packed for the fresh market. Making such a decision is problematic as the initial freeze symptoms are in the interior of the fruit and require inspectors to sample and cut numerous lots of the freeze-exposed fruit for examination and evaluation. Such a method of evaluating freeze-damaged oranges is labor intensive and unreliable as the symptoms are often not entirely apparent at

the time of inspection, which can lead to shipment of inferior quality fruit with the accompanying development of more severe symptoms in the marketplace. Flotation tanks have been used to separate injured from noninjured citrus fruit (2), taking advantage of the lesser density of freeze-injured fruit, but this method requires that potentially injured fruit be stored for ≥ 2 weeks to allow interior drying to occur. If an efficient and more accurate means to rapidly identify freeze-damaged oranges following a freeze event can be developed, it would be of great benefit to the industry.

The emanation of volatile compounds from plant tissues has been shown to be influenced by extreme temperatures (3–6), chemical fumigation (4), atmospheric composition (4, 7, 8), and water deficit (4). Freezing also has been found to have a pronounced effect on volatile emanation. For example, volatile hydrocarbons, including both acetaldehyde and ethanol, increased following freezing of the leaves of a number of plant species (4). Forney et al. (9) showed an increase of ethanol and ethyl acetate in two apple cultivars with freezing and suggested that these two volatiles could be useful as indicators of freeze damage in apples. In navel oranges, off-odor has been noted as an early symptom of freeze injury (Mary Lu Arpaia, personal communication), indicating that volatile chemical compounds are emitted following freezing and may serve as indicators of damage. Shaw (10) found that a moderate to heavy freeze caused a decrease in the concentration of hydrogen sulfide in the juice of Valencia oranges. Although hydrogen sulfide was released

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only during extraction of the juice, which precluded its use in the evaluation of whole fruit, he suggested that measurement of this volatile might be useful to indicate freeze damage. However, the study did not test navel oranges, nor did it perform measurements for volatiles other than hydrogen sulfide or relate the decline in hydrogen sulfide to changes of fruit quality. The objective of our study, therefore, was to ascertain whether volatile chemical compounds emitted from navel orange fruit could serve as indicators of freeze-induced quality loss.

MATERIALS AND METHODS

Freeze Treatments. Navel oranges (*C. sinensis* L. Osbeck cv. Washington) were obtained from a packing house in the vicinity of Fresno, CA, and placed into storage at 5 °C prior to treatment. Freeze treatments were conducted using a residential chest freezer (Sears) equipped with a temperature controller (Omega, Stamford, CT) and a 100 W light bulb that acted as a resistance heater to provide precise temperatures (± 0.3 °C) in a range that simulated field conditions. The freezer was equipped with an enclosed rack capable of holding 20 fruits at a time and contained three axial fans to circulate cold air across the fruit. Fruit and air temperatures were recorded during the treatments using a Daqbook/DBK 52 temperature logger (Iotech, Cleveland, OH) equipped with type T thermocouples. Thermocouples were taped to the surface of each fruit and used to record the initial time point of heat release (latent heat) that occurs due to the freezing of liquid within the fruit. In addition, one thermocouple was placed in the freezer to monitor freezer temperature during treatment. For experiment 1 fruits were treated at -7 °C for 2, 4, and 6 h, with the control (0 h) fruits held at 5 °C. All treatments were replicated four times by running separate freeze experiments. Following the completion of the treatments the fruits were stored at 23 °C for either 24 h, 48 h, or 7 days to investigate the effect of storage time on volatile emission. Fruits were then placed at 5 °C to await quality evaluations. For experiment 2 fruits were treated at either -5 or -7 °C and stored for 24 h prior to measurement. Treatment times were 4, 6, and 8 h (-7 °C) and 5.5, 7.5, and 9.5 h (-5 °C), the treatment times differing to equalize the amount of time beyond the initial point of intracellular ice formation (fruit took 1.5 h longer to freeze at -5 °C than at -7 °C). All treatments were replicated four times by running separate freeze experiments.

Fruit Quality. Fruit quality evaluations were performed following 3 weeks of storage at 5 °C. Both peel damage and internal drying were evaluated on 20 fruits per treatment. Peel damage in the form of brownish lesions and sunken areas was visually rated using a scale ranging from 0 to 4, where 0 = none, 1 = slight, 2 = moderate, 3 = severe, and 4 = very severe. Ratings of 0 and 1 were taken to be fruit that would still be marketable. Internal drying was assessed by making longitudinal cuts 2 cm from both stem (proximal) and blossom (distal) ends and through the middle and examining the cut sections for evidence of drying and separation between the orange segments. Ratings for individual fruit were 0 (no drying), 1 (slight drying, $\leq 25\%$ of the fruit affected), 2 (moderate drying, 25–75% of the fruit affected), and 3 (severe drying, $> 75\%$ of the fruit affected). A juice sample for each treatment was provided by juicing five fruits using a commercial citrus juicer and straining the juice through a screen to remove pulp. Part of the juice was used to measure the percentage of soluble solids by using a hand-held digital refractometer (Atago, Tokyo, Japan). A 5-mL sample of the juice was titrated with 0.15 M NaOH to estimate titratable acidity using 1% phenolphthalein to indicate the titration endpoint. A portion of the juice was frozen and stored at -20 °C for use in a taste panel. Preliminary studies had shown that although freezing and storage of the juice does alter the flavor, the flavor change induced by freezing of the whole fruit is still very evident in frozen juice. When all samples were available, the juice samples were thawed and presented to an informal taste panel consisting of eight scientists from the Parlier facility. Initial tastings done by the primary researchers indicated that the major effect of freezing was a loss of flavor intensity, so panelists were asked to rate the juice based upon this attribute using a seven-category hedonic scale. Ratings ranged from 1 (imperceptible flat) to 7 (very pronounced).

Volatile Measurements. Ten fruits were sealed in a 8.5-L glass jar for volatile measurements. The use of 10 fruit samples for volatile determination lessened the problem of fruit-to-fruit variability. After the jar had been sealed, a solid phase microextraction (SPME) device [carboxen/poly(dimethylsiloxane) fiber, Supelco, Inc.] was inserted through a septum and the fiber deployed for a period of 1 h at a temperature of 23 °C. This time period was chosen for the initial scans as it maximized recoveries for the widest range of volatiles given the fiber being used. Later, when compounds had been identified that showed a relationship with freeze injury, experiments were conducted indicating that a time of 1 h was necessary for these specific compounds to reach equilibrium. Samples were analyzed using a Hewlett-Packard 6890/5973 gas chromatograph–mass spectrometer (GC-MS). At the end of the trapping period the SPME device was removed from the jar and desorbed at 250 °C in the inlet of the GC-MS for a period of 30 s. The column used was a Hewlett-Packard HP-1MS (30 m \times 0.25 mm) with a helium flow rate of 1 mL/min. After an initial oven temperature of 35 °C, the oven temperature was ramped up at 25 °C/min to 220 °C, at which the temperature was held for 5 min. The MS detector was utilized in scan mode with a mass range set at 15–400. The detector temperature was set at 280 °C. Identification of unknown compounds was performed by comparison with library spectra combined with the use of standard compounds. For quantification, authentic standards were placed onto a piece of filter paper and allowed to volatilize within a sealed 8.5-L glass jar to generate standard curves.

Statistical Analysis. All quality evaluations utilized 20 fruits per treatment, whereas volatile measurements used 10 fruits per evaluation. Treatments were replicated four times by using four separate freeze runs per treatment.

For experiment 1, internal drying, soluble solids, and acidity were analyzed as a linear response to cold exposure time with PROC GLM (SAS Institute Inc., Cary, NC) and 95% confidence intervals used to test the significance of treatment differences. Time was modeled as a discrete factor for the volatiles data. Prior to the analysis the ethanol data were given a reciprocal square root transformation to stabilize the variances. Timepoints 0 and 2 were excluded from the analyses of the ethanol, ethyl butanoate, and methyl hexanoate data as the data were mostly zero. Peel damage displayed a heterogeneity of variance with time and was analyzed by using PROC MIXED (SAS Institute Inc.) to allow incorporation of heterogeneity in the model.

In experiment 2, all quality data were modeled using time as a linear, continuous effect and panelist as a blocking factor with PROC GLM. Time was fit as a discrete effect for the volatile data because of the nonlinearity in the responses. A square root transformation was used for the ethyl octanoate data prior to the analysis to stabilize variances. The control data were deleted from the ethanol, methyl hexanoate, and ethyl butanoate analyses due to a preponderance of zeros. Confidence intervals were used to estimate individual treatment differences for the quality and volatile data.

RESULTS

Experiment 1. Influence of Cold Exposure Time and Storage Duration. Oranges, when transferred from storage at 5 °C to -7 °C, rapidly cooled until 2.5–3 h into the experiment, at which point the fruits exhibited a spike in surface temperature. This increase in temperature was due to latent heat release and indicated the formation of intracellular ice. The mean freeze time averaged over 120 fruits was 161 min (SD = 36). After the initial temperature spike, the fruit surface slowly decreased in temperature for the remainder of the experiment.

Following 3 weeks of storage after freeze treatment, the surface of the peel was found to be uninjured by 2 h of cold treatment but severely injured by the longer 6 h treatment (**Table 1**). Similarly, drying of the internal segments was not evident after 2 h but was apparent in the 4 and 6 h treatments. The soluble solids concentration of the juice was unchanged by freezing, whereas acidity decreased in both the 4 and 6 h treatments.

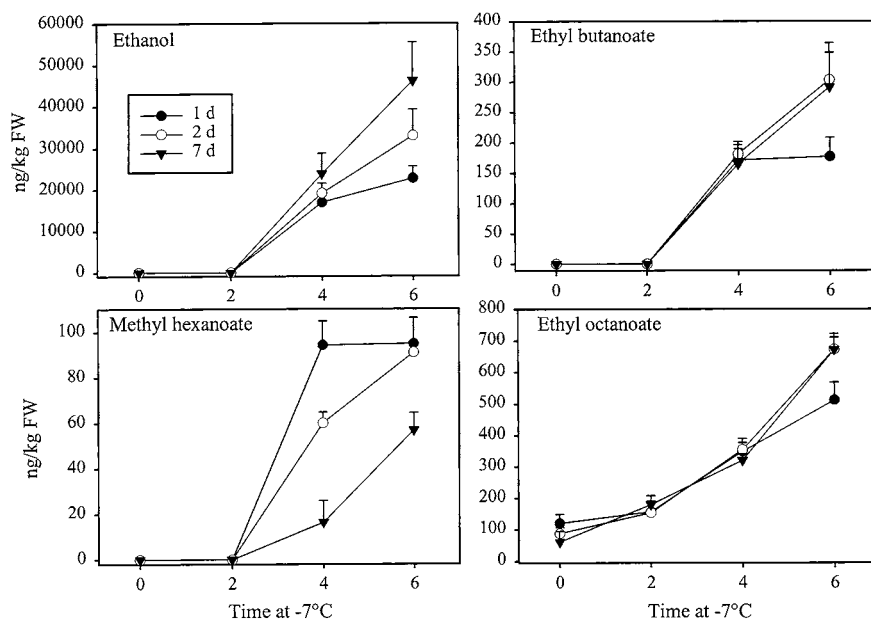


Figure 1. Volatile emanation from navel oranges exposed to -7°C for 0, 2, 4, and 6 h followed by storage at 23°C for 1, 2, or 7 days. Average time at which intracellular ice formation initiated was 140 min. Bars indicate standard errors.

Table 1. Quality Measurements from Navel Oranges Exposed to -7°C for 0, 2, 4, and 6 h and Stored for 3 Weeks at 5°C

time at 7°C (h)	peel damage rating ^a	internal drying rating ^a	soluble solids ^b (%)	acidity ^{b,c} (g/100 mL)
0	0.33 (± 0.10)	0.31 (± 0.05)	13.9 (± 0.21)	0.90 (± 0.03)
2	0.17 (± 0.02)	0.40 (± 0.15)	14.0 (± 0.21)	0.85 (± 0.02)
4	1.87 (± 0.45)	0.72 (± 0.16) [*]	13.9 (± 0.13)	0.80 (± 0.02) [*]
6	3.27 (± 0.14) [*]	1.25 (± 0.12) [*]	13.5 (± 0.04)	0.62 (± 0.03) [*]

^a 0 = none, 1 = slight, 2 = moderate, 3 = severe, 4 = very severe; 20 fruits were evaluated per replication; 4 replications. An asterisk (*) indicates a value significantly different from control (time 0), $P \leq 0.05$. ^b Values based upon the mean of 4 replications. ^c Grams of citric acid per 100 mL.

Volatiles were trapped 1, 2, and 7 days following cold exposure at -7°C , and a listing of chemical compounds present in the trapped headspace was constructed. Of the hundreds of compounds present, ethanol, ethyl butanoate, methyl hexanoate, and ethyl octanoate were selected for further study on the basis of the following criteria: (1) Little or none was present in oranges that had not been frozen. (2) Large and consistent increases occur when the fruits were frozen. (3) The compound was identifiable. Ethanol, ethyl butanoate, and methyl hexanoate were not detectable in the headspace of untreated oranges and those that had received 2 h of cold treatment (**Figure 1**). Ethyl octanoate was present at both of these time points, but the amount following 2 h of freezing treatment was not significantly different from the controls. The increases in volatile emanation following freezing at the 4 and 6 h time points were large and statistically significant from the control and 2--treated fruit for all four volatiles as indicated by non-overlapping confidence intervals (data not shown). Although exposure time was important in determining volatile emanation, storage duration following the cold treatments was a major factor for only ethanol and methyl hexanoate (**Table 2**). In the case of ethanol the response to freezing was enhanced by increasing storage duration, whereas the inverse was true for methyl hexanoate (**Figure 1**).

Experiment 2. Influence of Temperature. Intracellular ice formation began to occur in oranges exposed to -5°C at a mean time of 220 min (SD = 74) as compared to 128 min (SD

Table 2. Mean Squares for Volatiles from Cold Exposure Time/Storage Duration Experiment (Experiment 1)^a

source	df	ethanol ^b	ethyl butanoate	methyl hexanoate	ethyl octanoate
time	1	0.00001363 [*]	42673 [*]	3456 [*]	677785 [*]
storage	2	0.00000781 [*]	10247	6956 [*]	2452
time \times storage	2	0.00000064	9390	878 [*]	14430 [*]
rep	3	0.00001490 [*]	18201 [*]	1101 [*]	20407 [*]
error	15	0.00000824	4138	120	3873

^a An asterisk (*) indicates statistical significance at $P \leq 0.05$. Analysis was performed with control and 2 h values deleted for ethanol, ethyl butanoate, and methyl hexanoate. ^b Ethanol mean squares are based upon values that have undergone reciprocal square root transformation.

= 35) for fruits exposed to -7°C , the means being based on measurements from 240 fruits per temperature. To more directly compare the effect of temperatures, the treatment times were adjusted for the two temperatures so that the duration of exposure beyond the initial freeze was equivalent for the two temperatures; that is, -5°C treatments were 1.5 h longer than -7°C treatments.

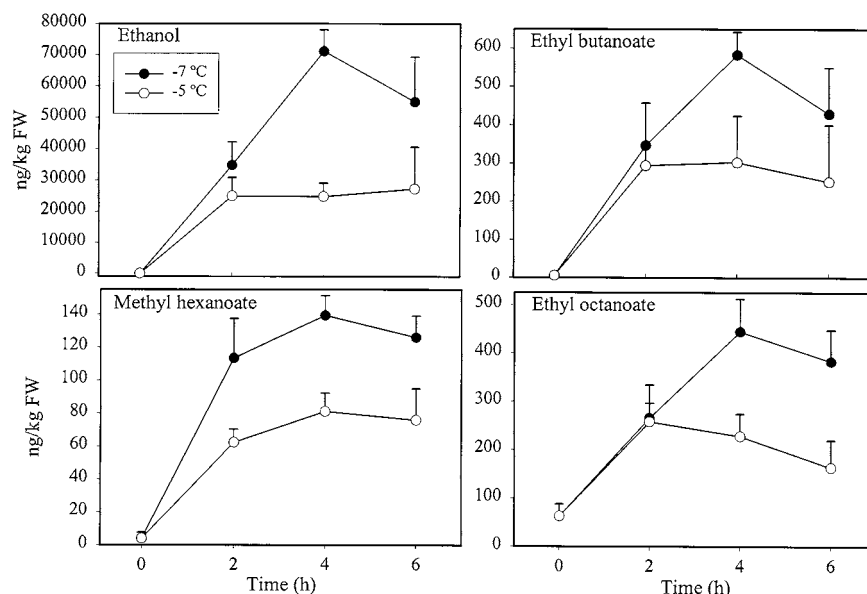
At -5°C peel injury was not significantly increased over the control value, whereas at -7°C the injury became apparent after 4 h of treatment (**Table 3**). Similarly, internal drying was evident only in the -7°C treatments, by which enhanced internal drying occurred at all of the time points. Soluble solids were not affected by cold exposure. Acidity was decreased by 9.5 h of treatment at -5°C and by ≥ 6 h at -7°C . The flavor of juice from treated and control fruits was found to be less intense in treated fruit. The minimum exposure times required to detect the loss of flavor were 7.5 h at -5°C and 6 h at -7°C .

Emissions of ethanol, ethyl butanoate, methyl hexanoate, and ethyl octanoate from oranges exposed to -7°C were greater than from fruits treated at -5°C (**Figure 2**). At 4 h beyond initial intracellular ice formation, the amounts were 1.5–3-fold greater following treatment at -7°C than at -5°C . Although temperature significantly influenced volatile emanation, exposure time was not a significant factor once the initial induction of volatile emission had occurred (**Table 4**).

Table 3. Quality Measurements of Navel Oranges Exposed to either -5 or -7 °C for Various Amounts of Time^a

temp (°C)	time in freezer (h)	time past initial freeze ^{b,c} (h)	peel damage rating ^d	internal drying rating ^d	soluble solids ^e (%)	acidity ^e (g/100 mL)	taste rating ^f
control	0	0	1.70 (±0.25)	1.08 (±0.13)	13.5 (±0.23)	1.2 (±0.03)	5.8 (±0.25)
-5	5.5	2	1.75 (±0.23)	1.43 (±0.32)	14.5 (±0.03)	1.3 (±0.10)	4.3 (±0.59)
	7.5	4	2.01 (±0.20)	1.74 (±0.24)	13.6 (±0.21)	1.0 (±0.09)	4.1 (±0.35)*
	9.5	6	2.15 (±0.21)	1.73 (±0.27)	13.8 (±0.55)	0.9 (±0.05)*	3.5 (±0.50)*
-7	4	2	2.47 (±0.19)*	1.83 (±0.35)*	13.2 (±0.36)	1.0 (±0.03)	4.3 (±0.53)
	6	4	2.77 (±0.23)*	2.18 (±0.32)*	13.2 (±0.17)	0.9 (±0.03)*	4.0 (±0.57)*
	8	6	3.11 (±0.12)*	2.03 (±0.16)*	13.5 (±0.32)	0.9 (±0.05)*	3.4 (±0.57)*

^a Exposure times were designed to equalize the amount of time at each temperature following the detection of intracellular ice formation (freeze initiation was detected in -7 °C fruit 1.5 h prior to that in -5 °C fruit). ^b Initial freeze as indicated by latent heat release. ^c Within columns, numbers followed by an asterisk are significantly different ($P \leq 0.05$) from the control. ^d 0 = none, 1 = slight, 2 = moderate, 3 = severe, 4 = very severe; 20 fruits were evaluated per replication; 4 replications. ^e Values based upon means of four replications; acidity expressed in grams of citric acid/100 mL. ^f Ratings are mean values from eight panelists based upon the intensity of the flavor. Ratings ranged from 1 (imperceptible flat) to 7 (very pronounced).

**Figure 2.** Volatile emanation from navel oranges exposed to either -5 or -7 °C and stored for 24 h at 23 °C. Timepoints are the length of time beyond the initiation of intracellular ice formation. Bars indicate standard errors.**Table 4.** Mean Squares for Volatiles from Influence of Temperature Experiment (Experiment 2)^{a,b}

source	df	ethanol ^b	ethyl butanoate	methyl hexanoate	ethyl octanoate ^c
temperature	1	4705*	173961*	16939*	116*
time	2	665	34532	1014	11
temperature × time	2	669	26131	38	29
replication	3	668	90633	1978	27
error	15	346	37486	741	9

^a An asterisk (*) indicates statistical significance at $P \leq 0.05$. ^b Actual mean squares for ethanol, ethyl butanoate, and methyl hexanoate = values $\times 10^6$. ^c Ethyl octanoate values are square root transformations. Analysis was performed with control values deleted.

DISCUSSION

Commercially ripe California navel oranges are considered to be in danger of freezing when field temperatures reach -3.3 to -3.9 °C for durations of 3–4 h (11). Due to unpredictable seasonal/climatic irregularity in the occurrence of freezing conditions in California, it was necessary in this study to simulate natural freezing by exposing navel oranges to freezing temperatures in the laboratory. Two temperatures, -5 and -7 °C, were chosen to provide freeze damage of differing severities. Exposure of navel oranges to these temperatures caused damage

to the peel surface and a drying of the internal fruit vesicles (Tables 1 and 3), two quality alterations that an inspector visually grading fruit can potentially detect. Injury to the peel (flavado), however, is not a common symptom associated with natural freezing of oranges (1) and in our study was only significantly enhanced by treatment at -7 °C and not at -5 °C (Table 3), even though intracellular ice formation occurred at -5 °C. Internal drying of the juice vesicles, which is the quality trait commonly utilized for grading of fruit for freeze damage, was found to not increase at -5 °C and was often fairly subtle at -7 °C. We found acidity and taste to be more sensitive to freezing than peel injury or internal drying, with alterations in both of these quality characteristics being apparent at -5 °C (Table 3). These observations highlight the likely possibility that evaluating solely for internal drying, as is currently practiced by the industry, could allow off-flavored fruits to reach the consumer.

The emission of ethanol, ethyl butanoate, methyl hexanoate and ethyl octanoate was found to be strongly enhanced by freezing (Figures 1 and 2) and to correspond to subsequent damage to the fruit following storage. As with fruit injury, there was no enhancement in volatile emission induced by cold treatment unless intracellular ice formation was detected (Figure 1), even following long exposures at higher, nonfreezing temperatures (data not shown). To determine the effect of

storage on volatile emission, oranges were stored for various amounts of time (1, 2, and 7 days) following a freeze treatment prior to the measurement of volatile emission. Storage altered the emission of ethanol and methyl hexanoate, whereas ethyl butanoate and ethyl octanoate were unchanged (**Figure 1**). All of the volatiles, however, were emitted in substantial amounts following all of the storage durations, indicating that these volatiles may remain as viable markers of freeze injury for a considerable time after the freeze event. Even after cold exposure times had been adjusted to equalize the amount of time beyond the point of initial intracellular ice formation, it was clear that there were greater amounts of all of the volatiles being emitted following treatment at $-7\text{ }^{\circ}\text{C}$ as compared to $-5\text{ }^{\circ}\text{C}$ (**Figure 2**). Injury to the fruit also followed this trend (**Table 3**). Although volatile emission was less following treatments of lesser severity, it nonetheless was always predictive of injury in our experiments and appears to be a sensitive indicator of freeze damage.

Enhanced emission of ethanol following the freezing of fruit tissues has been previously reported in apples (9). As the authors noted, it is likely that the ethanol is a product of fermentation resulting from disruption of aerobic respiration by freezing. Ethanol is present as a normal constituent of orange juice (12) and can increase in concentration as a result of waxing (13) and prolonged storage (14). However, we detected no ethanol emanation from whole unwaxed oranges that had not been subjected to freezing temperatures. Acetaldehyde, another common product of fermentation, was not detected by our analysis, and no attempt was made to determine if it was present in the samples. The other volatiles examined in this study are esters that can be formed from reactions between organic acids and alcohols. Knee and Hatfield (15) applied alcohol vapors to apples and found that this treatment increased the emanation of esters from the fruit. Increased levels of ethanol in the frozen oranges may be responsible for the greater emanation of ethyl butanoate and ethyl octanoate from these tissues given that ethanol is precursor for these two esters. Although we were not able to measure methanol using our system, it is possible that methanol was also enhanced as a result of freezing, thus providing additional substrate to increase the formation of methyl hexanoate. Both ethanol and methanol were reported to be strongly enhanced by the storage of oranges in conditions favoring anaerobiosis (16). Ethyl acetate, an ester that has been previously linked to plant stress (9), was also often present in the headspace of frozen oranges but was not chosen as a marker volatile for this study because it was not always detectable in the frozen samples.

In summary, the emission of ethanol, ethyl butanoate, methyl hexanoate, and ethyl octanoate increased rapidly following the freezing of navel oranges and is predictive of damage to the fruit that will be present following a 3 week period of storage. This indicates that one or more of these volatiles could be useful as indicators that a damaging freeze has occurred and that the oranges should not be packed for the fresh market. Ethanol, particularly, is an attractive indicator due to its ease of measurement. It is recognized, however, that the manner in which detached oranges freeze under simulated laboratory conditions may differ from oranges on a tree under field

conditions. Further research needs to ascertain whether these volatiles can be useful in the evaluation of naturally frozen oranges.

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