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Determination and Distribution of 6-Methoxymellein in Fresh and Processed Carrot Puree by a Rapid Spectrophotometric Assay

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Isocoumarin or 6-methoxymellein (6-MM) was extracted from carrot tissue using alkali saponification to solubilize the lactone portion of its structure into an aqueous phase. Acidification and subsequent organic solvent extraction allowed isolates to be quantified and verified as 6-MM by spectrophotometric determination. 6-Methoxymellein was analyzed in carrot cross sections, as a function of depth and before and after thermal processing. A natural propensity for 6-MM accumulation was observed in root tip sections exposed to ethylene, and levels increased as a result of wounding. Consecutive layer peeling demonstrated that small-diameter roots accumulated greater amounts of 6-MM in periderm tissue compared to large roots. Processing carrots into a puree resulted in 10−25% greater extraction of 6-MM than grinding fresh carrot samples, whereas steam-cooked and thermally processed purees had 15% greater extraction than unheated purees. This analytical technique will allow carrot processors to accurately estimate raw and processed products for the bitter compound 6-MM.

Keywords: *Carrots; isocoumarin distribution; phytoalexin; saponification; stress*

INTRODUCTION

Much emphasis has been placed on the contribution of phytoalexins to fruit and vegetable flavor. Carrots (*Daucus carota* L.) can synthesize numerous compounds that negatively impact the flavor of fresh and processed products when exposed to abiotic stress during harvesting, transportation, and storage. The presence of 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (6-methoxymellein, 6-MM) is associated with bitterness in carrots (Sondheimer, 1957a; Sarker and Phan, 1979; Lafuente et al., 1989; Talcott and Howard, 1999). Exposure to ethylene gas stimulates the formation of 6-MM in carrots when it was previously absent (Sarker and Phan, 1975), and accumulation of 6-MM is directly related to elevated storage temperature and ethylene concentration (Lafuente et al., 1989). 6-MM is tasteless in powdered form but extremely bitter in solution, with characteristic absorbances at 267 and 302 nm in ethanol and at 264 and 300 nm in cyclohexane (Sondheimer, 1957a,b). 6-MM has been implicated as the major contributor to bitter and sour flavors in processed carrot puree in association with phenolic acids (Talcott and Howard, 1999). Numerous extraction methods have been established that utilize a variety of solvents such as *n*-hexane (Sondheimer et al., 1955; Chalutz et al., 1969; Lafuente et al., 1996), acetone (Kurosaki and Nishi, 1983), ethyl acetate (Hoffman et al., 1988; Marinelli et al., 1994), and dichloromethane (Mercier and Arul, 1993). However, many of these methods are not suitable as a quality control method for food processors due to lengthy extraction or analysis time and use of expensive instrumentation.

Regulation and monitoring of 6-MM in carrot puree is important to food processors due to increased bitter flavor and subsequent loss of sweet flavors associated with the compound. From sensory analysis of strained carrots we noted that the just noticeable difference (JND) of 6-MM was between 48 and 71 mg/kg (Talcott and Howard, 1999). It was assumed that this JND might be much lower if other bitter compounds such as phenolic acids were simultaneously synthesized with 6-MM. The numerous food matrices that contain carrots may also influence JND concentrations. Therefore, the need to rapidly detect levels of 6-MM below 50 mg/kg was apparent. The objective of this study was to develop a rapid spectrophotometric assay to identify and quantify 6-MM in raw and processed carrot products for use in quality control applications.

MATERIALS AND METHODS

Analytical Procedures. A rapid method for 6-MM quantification was developed and utilized; it involved extraction of both fresh carrots and processed carrot puree with a sodium hydroxide (alkali) solution (Sondheimer, 1957a; Kurosaki and Nishi, 1983), which enhances solubility and facilitates the removal of nonpolar, interfering compounds extracted with traditional solvent systems. Experimentally, 15 mL of 0.5 N sodium hydroxide was volumetrically added to 8 g of carrot tissue (raw or pureed) in a screw-cap vial and vortexed every 5 min for a total extraction time of 20 min. Samples were then filtered through Miracloth (Calbiochem, San Diego, CA), and an aliquot (5 mL) of filtrate was volumetrically added to a solvent-resistant screw-cap vial and acidified with an equal volume of 2 N HCl. Spectrophotometric grade *n*-hexane (10 mL) was volumetrically added, and tubes were mixed by end-over-end agitation for 1 min. The hexane layer was removed, and its absorbance was measured at 238, 262, and 302 nm for 6-MM quantification and verification, using a Hewlett-Packard 8452A diode array spectrophotometer.

To determine the efficiency of the alkali extraction, it was necessary to extract the same carrot samples using a solvent-based extraction system and to quantify 6-MM by HPLC. Comparison isolates were obtained by extracting carrot tissue (5 g) with 10 mL of methanol for 20 min in a manner similar

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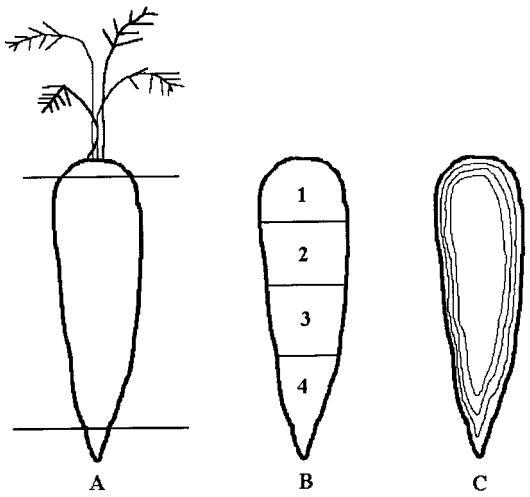

Figure 1. Carrot diagrams of (A) crown and tip removal (1 cm cut) prior to ethylene exposure, (B) cross sections analyzed (1−4), and (C) layered peeling sections analyzed for 6-MM concentration.

to that used for the saponified isolates. Samples were then filtered through Miracloth and a 0.45 μm filter for HPLC analysis according to the method of Talcott and Howard (1999). Total solids were measured by drying samples in a forced-air oven (2 h at 135 °C) and used to convert all values to a dry weight (DW) basis.

Experimental observations concluded that hexane isolates from carrots containing 10−25 mg/kg would generally overestimate actual concentrations of 6-MM by 50−80% due to the presence of interfering compounds. Direct quantification of hexane isolates >25 mg/kg could be made; however, quantification at lower levels required further purification of the hexane fraction by extraction with an equal volume of 75% ethanol/water in a separate solvent-resistant vial. This step selectively partitioned 6-MM into the lower ethanol phase upon mixing and resulted in the removal of interfering compounds as reported by Lafuente et al. (1996). The ethanol was prepared with water to facilitate partitioning from hexane, and absorbance values were measured at 267, 302, and 238 nm for subsequent quantification and verification.

Materials and Processing. Preliminary studies for determining a rapid method for 6-MM extraction and quantification involved spiking commercially processed strained carrots with 6-MM concentrations ranging from 10 to 75 mg/kg and analyzing unprocessed carrots that contained 6-MM. A 20-min saponification time at room temperature was chosen on the basis of time course studies as being the most suitable for a variety of carrot products. However, when carrot purees were analyzed, equivalent extraction was accomplished within 2 min.

Once the methodology was optimized, fresh carrots were obtained from a local market and used for method verification and in determining 6-MM distribution within the root. The carrots were randomly divided into four groups and wounded as follows to simulate common agronomic and handling conditions: removal of tissue 1 cm below the crown; removal of the last 1 cm of root tip; simultaneous crown and tip removal; and whole, unwounded roots (Figure 1A). Additional whole roots were objectively divided by size into small and large roots and stored together for stress treatment to determine the depth of 6-MM synthesis into the root. Average crown diameter for small roots was 22.4 mm and 34 mm for large roots.

Stress was induced by sealing carrot treatments into airtight connecting chambers (27.3 × 16.5 cm) for 3 days at 10 °C. Ethylene gas (1%) was flushed (~200 mL/min for 20 s) into the chambers every 8 h and sealed for 10 min to induce 6-MM synthesis. A continuous feed of 100% oxygen (~30 mL/min) between ethylene treatments was applied to the containers to maintain aerobic respiration.

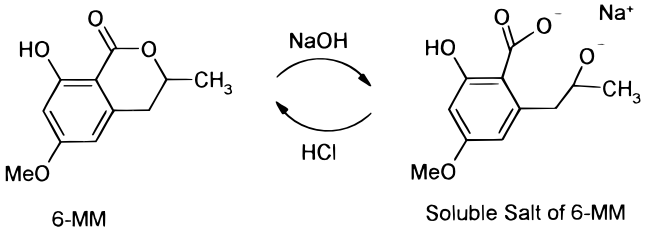

Figure 2. Saponification scheme used to solubilize 6-MM into an aqueous phase.

Samples were obtained for analysis by dividing carrots from each wounding treatment into three groups with five individual roots in each group. Samples from the individual roots were combined within each treatment group and ground into a composite to the smallest attainable particle size in a West Bend High Performance food processor (West Bend, WI). Cross-sectional samples were obtained by cutting each root into four equal length sections (1−4); section 1 contained the crown end, and section 4 contained the root tip (Figure 1B). All sections were analyzed with periderm intact. To determine the effects of processing on 6-MM extraction and recovery, additional whole roots were blended with 50% water (carrot weight), and samples were evaluated unheated, cooked (15-min steam blanch), and after thermal processing (121 °C for 45 min).

Five individual roots were randomly selected from the size sorting of large and small roots and manually peeled to yield consecutive layers of tissue from the periderm to the vascular system (Figure 1C). Each root diameter was measured at three locations (top, middle, and bottom) to determine the depth of each peeled layer, and the corresponding layers from each size designation were blended for composite analysis.

Statistical Analysis. Chemical data represent the mean of three repeated measurements collected at random from three subsamples taken from each stress treatment. Purees were analyzed in triplicate. Simple regression and analysis of variance were conducted on chemical data (SAS Institute, Inc., 1996), and mean separation was conducted by Duncan's multiple-range test ($P < 0.05$).

RESULTS AND DISCUSSION

Experimental Theory. Alkali saponification of 6-MM from carrots was necessary to obtain an optically clear hexane fraction for quantification. Direct extraction of carrots with hexane or methanol, especially in carrot purees, will extract a variety of nonpolar, interfering compounds that either form emulsions with the sample or lack the optical clarity for positive identification and quantification directly from a spectrophotometric measurement. Alkali extraction will saponify the ester bond of the lactone ring of 6-MM near pH 12.3 to form a soluble, sodium salt (Figure 2), with a characteristic absorbance at 330 nm (Figure 3). Partial opening of the lactone ring was observed in the pH range of 11.5−11.8, as noted by a shift in absorbance from the characteristic 302 nm peak, but complete solubility was not spectrally obtained until pH >12.3. Once 6-MM was extracted from the carrot tissue, an acidification step converted 6-MM to its nonpolar state for subsequent partitioning into hexane. Consequently, 6-MM could not be detected in alkali extracts via spectrophotometric analysis. To ensure complete partitioning into hexane upon acidification, the isolate was adjusted to pH <4.0, a step that may require adjustment of acid concentration to compensate for the amount of sample utilized. The saponification/acidification step did not appear to permanently alter the lactone structure as spectrally noted from repeated addition of acid and alkali, and quantification

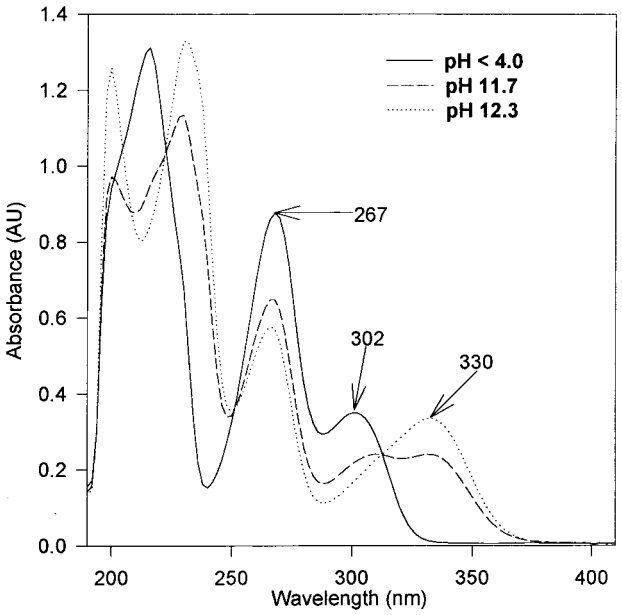

Figure 3. Ultraviolet absorption spectra of 6-MM dissolved in ethanol after alkali saponification of the ester bond in the lactone structure. Solubility into an aqueous phase begins near pH 11.7 and is complete at pH 12.3 as noted by a shift in absorbance from 302 to 330 nm. The peak returns to 302 nm upon acidification.

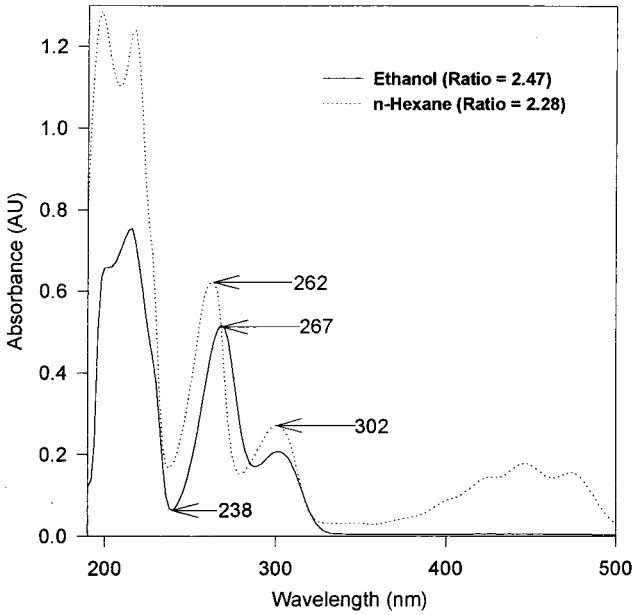

Figure 4. Ultraviolet absorption spectra of identical 6-MM fractions dissolved in ethanol (λ_{max} = 267 nm) and *n*-hexane (λ_{max} = 262 nm). Characteristic peak ratios (λ_{max}/302 nm) are 2.47 for ethanol and 2.28 for *n*-hexane.

was completely unaltered because measurements were based on the spectral characteristics of the benzene ring.

Hexane extraction after acidification of fresh carrots and steam-blanched carrot isolates often resulted in gel formation during vigorous agitation that did not occur in thermally processed carrots. It was concluded that alkali-soluble pectin was extracted simultaneously with 6-MM during saponification and once acidified, would readily precipitate from solution. A filtration step to remove this precipitate was contemplated; however, it was excluded in an attempt to shorten analysis time and prevent possible loss of 6-MM. Processed carrots did not exhibit gel formation, presumably due to pectin depolymerization that occurred via β-elimination during cooking (Buescher and Balmoori, 1981). Therefore, effort should be taken to extract nonthermally processed samples by end-over-end agitation, at the possible expense of extraction recovery, to avoid gel formation in the hexane layer. To maintain method consistency, an end-over-end extraction was conducted for 1 min, which resulted in only minor gel formation.

Molar Extinction Coefficients. For quantification of the carrot isolates, an extinction coefficient in *n*-hexane was empirically determined from standards by measuring absorbance values of four hexane isolates of increasing 6-MM concentration (4, 8, 13, and 18 mg/kg) in triplicate at 262 nm. An equal volume of 75% ethanol was thoroughly mixed with the standard, and absorbance of the ethanol layer was recorded at 267 nm. No detectable 6-MM remained in the hexane fraction after extraction with 75% ethanol, indicating that an equal concentration of 6-MM was present in both fractions (Figure 4). When corrected for background interference at 238 nm, the two peaks exhibited similar peak heights and absorbance values. The average molar extinction coefficient for 6-MM in *n*-hexane was then calculated as 16306 ± 126, and the ratio of the 262 nm to the 302 nm peaks was 2.28 based on the extinction coefficient for ethanol (Figure 4). This value was different from Sondheimer's (1957a) molar extinction coefficient for 6-MM in cyclohexane (13638) with an absorption maxima at 264 nm. Either solvent could be utilized in the analysis, but the appropriate extinction coefficient should be utilized for quantification. Sample quantification was conducted according to the following calculations.

Strained Carrots in Hexane

$$\text{mg/kg 6-MM} = \{[(\text{Abs}_{262} \times 208.22 \times 1000)/16306] \times (A + B)D\}/(AC)$$

Ground Carrots in Hexane

$$\text{mg/kg 6-MM} = \{[(\text{Abs}_{262} \times 208.22 \times 1000)/16306](BD)]\}/(AC)$$

Strained Carrots in 75% Ethanol

$$\text{mg/kg 6-MM} = \{[(\text{Abs}_{267} \times 208.22 \times 1000)/14800] \times (A + B)[(DF)/E)]\}/(AC)$$

Ground Carrots in 75% Ethanol

$$\text{mg/kg 6-MM} = \{[(\text{Abs}_{267} \times 208.22 \times 1000)/14800]B[(DF)/E)]\}/(AC)$$

where 208.22 is the molecular weight of 6-MM, A = sample weight (g), B = volume (mL) of NaOH solution added to sample, C = volume (mL) of saponified aliquot, D = volume (mL) of hexane, E = volume (mL) of hexane used for ethanol partitioning, and F = volume (mL) of 75% ethanol. Sample weights and solution volumes can be easily adjusted to determine the level of 6-MM in any given sample.

Recovery and Verification. Methanol consistently extracted more 6-MM than alkali solutions from ground carrot samples. Preliminary studies found that methanol extraction of 6-MM spiked into commercially processed carrot puree resulted in 98−100% recovery by HPLC. Therefore, quantification of the methanol iso-

lates by HPLC was used to calculate extraction recoveries of saponified isolates by the spectrophotometric assay. Similar spiking studies resulted in deviations of ±15% from the HPLC values for saponified purees and ±30% for ground carrots, indicating a direct relationship between particle size and extraction efficiency.

Because commercial standards for 6-MM are not available, a confirmation step was developed for spectrophotometric quantification that is also applicable for positive identification of 6-MM at low concentrations. To confirm the identification of 6-MM in the presence of interfering compounds, a ratio between the characteristic absorption peaks was established. For hexane isolates, the ratio of the 262 to 302 nm peaks was 2.28 ± 0.10, and for ethanol isolates the ratio of 267 to 302 nm peaks was 2.47 ± 0.05 (Sondheimer, 1957b; Figure 4). For all isolates, the ratio of either the 262 or 267 nm peak to the 238 nm peak was <0.60. These ratios made it possible to differentiate background interference from actual 6-MM when sample concentrations were low. Subsequently, 10−15 mg/kg was concluded to be the lowest detection level attainable when 6-MM was extracted from carrot tissue and the ethanol partition step was employed. Without ethanol partitioning, the lowest detection limit for hexane isolates was 25 mg/kg.

Method Validation/Sectional Analysis. Wounding treatments to determine the distribution of 6-MM within carrot roots were investigated separately, but comparisons between treatments were made based on percent compositional change. Rates of 6-MM synthesis may have been altered on the basis of location and distance of each storage chamber from the ethylene source and from moisture losses that occurred at the wound sites. Whole roots, having no perceivable wounds, contained higher levels of 6-MM at the root tip (section 4) compared to sections 1−3 after ethylene exposure (Figure 5A). On average, 15% more 6-MM was observed in section 4 for methanol isolates compared to 21% more in saponified isolates with an overall recovery of 70%. The elevated concentration in the root tips was likely a function of reduced root diameter at the carrot tip or a higher proportion of periderm to xylem/phloem in the analyzed sample. Additionally, the root tip is the leading growth tissue into the soil and would be expected to have a higher propensity for phytoalexin production. Analytical testing for 6-MM should concentrate on root tips for obtaining quality control samples representative of the level of stress incurred by the root. Additionally, removal of root-tip sections prior to processing may aid in the reduction of bitter flavors contributed by 6-MM, with minimal loss of production yield.

Root tip removal prior to ethylene exposure accentuated 6-MM synthesis in relation to the remainder of the root (Figure 5B). 6-MM concentration was not significantly different for sections 1−3 but increased by 66−68% at section 4 for both testing methods, with a 65% overall recovery for saponified isolates. Cut or broken root tips are common sources of wounding that may occur during mechanical harvest, transportation, and bulk storage and, subsequently, may have an impact on levels of 6-MM in carrots.

In addition to handling abuse, the majority of carrots destined for storage or transportation are crowned to prevent sprouting. Crown removal prior to ethylene exposure resulted in elevated 6-MM in section 1, in relation to sections 2 and 3, but it was not significantly

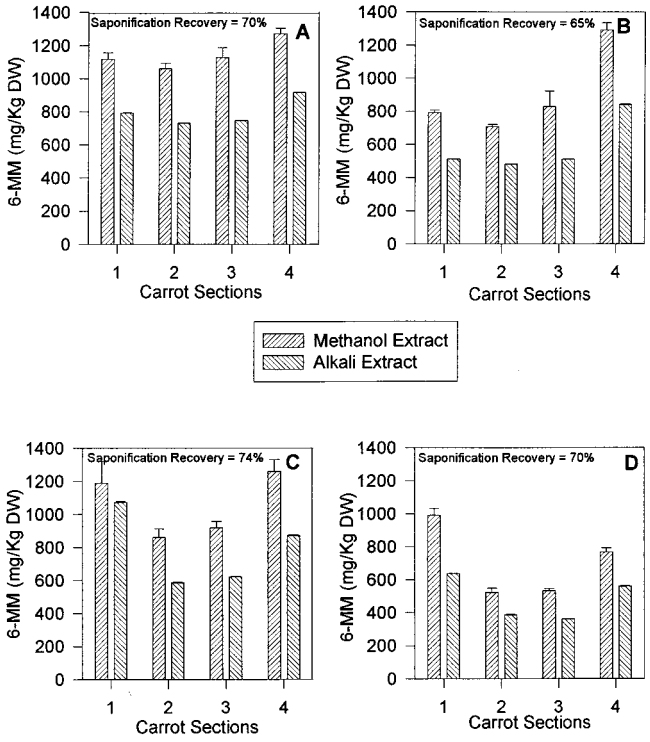

Figure 5. Distribution of 6-MM (milligrams per kilogram of DW) in four equal length carrot sections 1−4 (section 1 contains root crown, whereas section 4 contains root tip) as quantified by methanol and alkali extraction techniques: (A) whole, no wounding; (B) root tip removal; (C) crown removal; (D) crown and tip removal. Roots were stressed with ethylene gas (10 °C for 3 days) after application of wounding treatments. Bars represent standard error of the mean.

different from that in section 4 (Figure 5C). Sections 2 and 3 together were 38 and 61% lower (methanol and saponified isolates, respectively) than sections 1 and 4, resulting in a 74% overall extraction recovery for the saponification method. The impact of crowning was significant due to the large proportion of phloem and xylem tissue present in relation to the periderm. Therefore, high levels of 6-MM at section 1 were attributed to accumulation around the wound site, indicating that crowning steps may adversely impact carrot flavor.

When the crown and the tip were both removed for ethylene exposure, significantly higher 6-MM in section 1 was observed (13 and 29%) compared to section 4, which was 33 and 45% higher than sections 2 and 3 (methanol and saponified isolates, respectively; Figure 5D). The overall recovery for the saponification method in these samples was 70%. In response to both crown and tip removal, which simulates actual physical abuse during postharvest handling, it was concluded that section 1 had a greater propensity for 6-MM synthesis than section 4. Section 4 may have approached its maximum level of 6-MM synthesis prior to cellular toxicity (Sarker and Phan, 1979). The use of alternative strategies such as application of sprout inhibitors, freezing the roots, or modified atmosphere storage should be investigated on a commercial scale to replace crowning operations. If crowning steps are essential, proper temperature controls (<4 °C) immediately after cutting or use of modified atmosphere should decrease the rate of 6-MM synthesis.

Method Validation/Layered Peeling. On the basis of root diameter, it was concluded that carrot size was a critical factor for the rate and depth of synthesis of

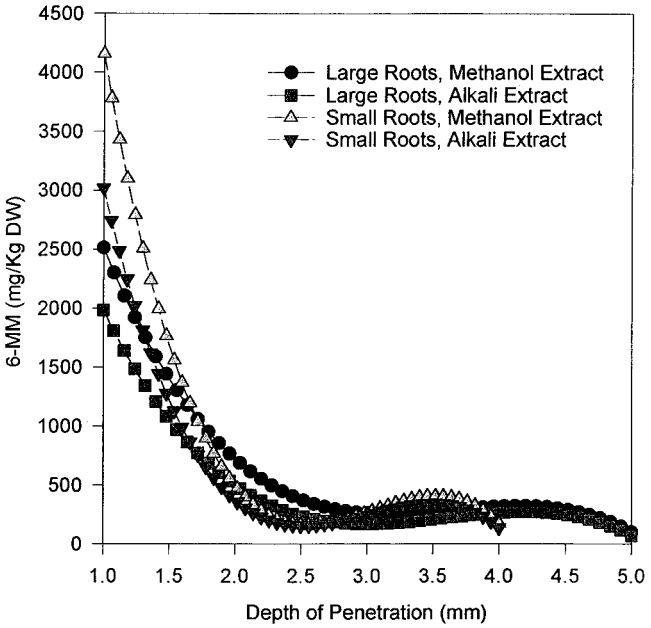

Figure 6. Cubic regression models for the penetration depth (millimeters) of 6-MM (milligrams per kilogram of DW) into small- and large-diameter roots, after exposure to ethylene gas (10 °C for 3 days), as quantified by methanol and alkali extraction techniques.

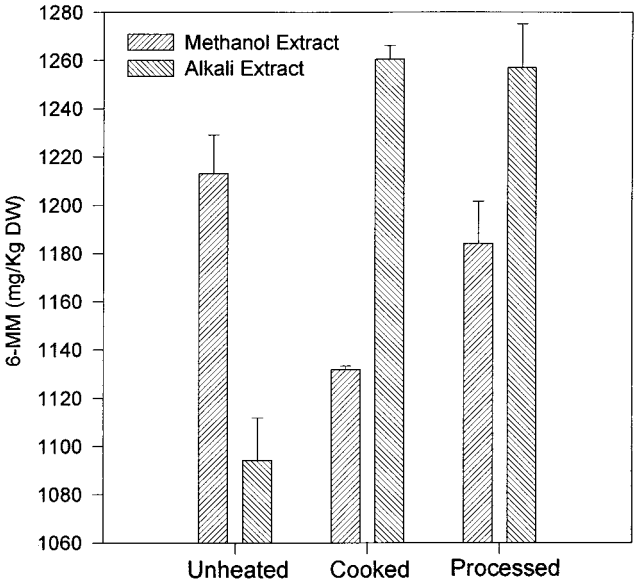

Figure 7. Concentration of 6-MM (milligrams per kilogram of DW) in pureed carrots quantified by methanol and alkali extraction techniques. Saponification recovery was 90% for unheated puree, 111% for steam-cooked puree, and 106% for thermally processed puree. Bars represent standard error of the mean.

6-MM. Average root diameter for large roots was 34 mm at the crown, 26.5 mm at midsection, and 20.2 mm near the root tip, whereas small roots were 22.4 mm at the crown, 17.9 mm at midsection, and 14.6 mm near the tip. Decreasing concentrations of 6-MM from the peel to the vascular tissue were observed. Accumulation of 6-MM has been directly linked to elevated respiration rate induced by ethylene (Lafuente et al., 1996), and epidermal tissue has been reported to respire at a higher rate than internal tissue (Sarker and Phan, 1979). The depth of 6-MM accumulation from the peel to the vascular system was individually plotted using a cubic regression model based on root size and method of quantification (Figure 6). Small roots had 1.5 times more 6-MM in the epidermal layer than large roots and incrementally decreased to an average depth of 3 mm, where the concentrations were nearly equal in both small and large roots. Elevated levels of 6-MM in the first 3 mm of crown tissue represented 26.8% of total tissue from the periderm to the center of the root for small roots and 17.6% of tissue in large roots. Saponification recovery was 82% for small roots and 74% for large roots, which may be attributed to differences in the thickness of the layers resulting from manual peeling. It is uncertain if elevated levels of 6-MM in small roots were linked to a higher respiration rate compared to large roots or if cell size and density affected ethylene transport into the root. Consequently, small (narrow) roots had a deeper penetration of 6-MM relative to large roots, which may reduce overall yield if carrots are peeled to remove the bitter compound.

Method Validation/Pureed Carrots. The extraction efficiency of 6-MM using the saponification method for carrot purees was superior to that for fresh ground carrots due to the reduction of particle size associated with preparing the puree. However, additional time for particle size reduction may not be conducive for rapid quality control evaluation. Uncooked puree resulted in 90% extraction efficiency when compared with methanol extracts and was 10−25% higher in recovery than corresponding fresh ground samples. Cooked and retorted puree (recoveries of 111 and 106%, respectively) had similar concentrations for the saponification method and resulted in 15% better extraction recovery of 6-MM compared to uncooked purees (Figure 7). Mercier and Arul (1993) noted that 6-MM will readily leach into boiling water, indicating that heating in conjunction with reduced particle size may account for the elevated extraction efficiency. However, methanol isolates were not significantly different between uncooked and thermally processed purees, indicating that the saponification method may be more effective for the analysis of carrot purees.

Time of Analysis. Many methods have been utilized for the extraction and quantification of 6-MM in carrot tissue. However, many of these procedures are not suitable for use in quality control applications due to long analysis time, limited sample throughput, and use of expensive instrumentation (HPLC) for quantification. The method of Lafuente et al. (1996) requires an overnight extraction in hexane followed by ethanol partitioning for quantification. The Hoffman and Heale (1987) procedure requires solvent-extracted isolates to be purified by TLC for subsequent ethanol elution and quantification. Finally, the method of Mercier and Arul (1993) requires boiling carrot isolates prior to solvent extraction. With the saponification method described here a large number of samples can be prepared, extracted, analyzed, and confirmed simultaneously for 6-MM covering a wide range of sample matrices. The method is flexible enough to allow for varied extraction times, solvent volume, and sample weights to suit a given analytical need. The analysis time is essentially limited to the rate of sample preparation, and all extracts can be retained from one day to the next, allowing flexibility for routine quality control.

Conclusions. A rapid method for 6-MM was developed, which is suitable for quality control testing of either whole or pureed carrots. This rapid technique will allow processors to gauge the level of stress incurred in

a given lot of carrots. Saponification of carrot samples will decrease analysis time while minimizing the presence of interfering compounds that affect quantification. Understanding the distribution of 6-MM within carrots may benefit processors in selectively determining tissue to be removed prior to processing. Removal of root tips prior to processing may be an effective tool for reducing bitterness, while serving as a critical sampling point for 6-MM quantification. Crowning and root tip damage prior to storage and ethylene exposure was conducive to 6-MM production; therefore, measures to eliminate stress conditions during postharvest handling are critical. Utilization of large-diameter roots for processing is recommended due to the greater percentage of 6-MM present in periderm tissue that can be removed by peeling.

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