

# Chemical Characterization of Orange Juice from Trees Infected with Citrus Greening (Huanglongbing)

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**ABSTRACT:** The effects due to *Candidatus Liberibacter* infection, commonly called citrus greening or Huanglongbing (HLB), on volatile and nonvolatile components of orange juices, OJ, were examined using GC-MS and high-performance liquid chromatography (HPLC). HLB symptomatic, asymptomatic, and control "Hamlin" and "Valencia" oranges were harvested from December to May during the 2007 to 2008 harvest season. Brix/acid levels in control and asymptomatic juices were similar but symptomatic juices were as much as 62% lower than control juices. No bitter flavanone neohesperidosides were detected and polymethoxyflavone concentrations were well below bitter taste thresholds. Limonin concentrations were significantly higher (91% to 425%) in symptomatic juice compared to control but still below juice bitterness taste thresholds. Juice terpenes, such as  $\gamma$ -terpinene and  $\alpha$ -terpinolene, were as much as 1320% and 62% higher in symptomatic juice than control. Average ethyl butanoate concentrations were 45% lower and average linalool was 356% higher in symptomatic Valencia OJ compared to control. Symptomatic Valencia OJ had on average only 40% the total esters, 48% the total aldehydes, and 33% as much total sesquiterpenes as control juice. Total volatiles between control and symptomatic juices were similar due to elevated levels of alcohols and terpenes in symptomatic juice. There were no consistent differences between asymptomatic and control juices. The chemical composition of juice from HLB/greening symptomatic fruit appears to mimic that of juice from less mature fruit. The reported off-flavor associated with symptomatic juices probably stem from lower concentrations of sugars, higher concentrations of acid as all known citrus bitter compounds were either below taste thresholds or absent.

**Keywords:** bitter off-flavor, GC-MS, HLB, limonin

## Introduction

Citrus greening or Huanglongbing (HLB) is a disease that produces multiple tree and fruit symptoms that is threatening citrus industries throughout the world. It is caused by the bacteria *Candidatus Liberibacter* spp. and is vectored by the Asian citrus psyllid (*Diaphorina citri* Kuwayama). Although harmless to humans, there is no known cure for citrus trees once infected. Prior to twig dieback and decreased productivity due to this phloem limiting bacterium, there are noticeable symptoms in root systems, leaves, and fruit. In an infected tree, the root system starts to decay due to root starvation and new growth is restrained (da Graca 1991). Therefore, the remaining roots do not seem to adequately support the tree (McClellan and Oberholzer 1965). A yellow blotchy mottle, or asymmetrical leaf chlorosis, the result of chloroplast disruption caused by the accumulation of starch granules, is one of the main characteristics of the disease (da Graca 1991). HLB also affects the fruit, and consequently, the juice. Symptomatic fruit are smaller, misshapen, and often contain aborted seeds compared to

fruit from noninfected trees. Fruit from a HLB infected tree tend to fall prematurely and those that remain on the tree fail to mature correctly and retain their green color, hence the name greening for this disease.

Early studies on HLB symptomatic fruit in South Africa have reported that the juice was of poor quality and tasted bitter (McClellan and Oberholzer 1965; McClellan and Schwarz 1970). A study of HLB infected and noninfected Kinnow mandarins determined that there was a lower soluble solids content and higher acidity in HLB fruit than in control fruit (Kapur and others 1978). More recently, it has been reported (Plotto and others 2008) that consumer panels could not distinguish between the taste and smell of juice from asymptomatic fruit of infected trees and that of fruit from uninfected trees from a single late season harvest date. However, an experienced panel perceived juice from HLB fruit as being sweeter and possessing lower acidity than control fruit juice. The conflicting reports of relative sugar acid levels in juices from HLB fruit compared to uninfected (control) juices prompted this study. Therefore, Brix, acid, and other flavor components in juices from control and infected fruit harvested over the entire season from the 2 major orange cultivars grown in Florida were evaluated. The goal of this study was to chemically characterize the flavor components in juices from control (uninfected) fruit with that from both symptomatic and non-symptomatic HLB fruit. One immediate goal was to investigate the off-flavor reportedly found in HLB fruit juice with the ultimate goal of determining the possible impact this disease might have on orange juice flavor.

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## Materials and Methods

“Hamlin” oranges (*Citrus sinensis* (L.) Osbeck) were obtained from commercial groves on December 12, 2007 (Sebring, Fla., U.S.A.), December 18, 2007 (Ft. Pierce, Fla., U.S.A.), and January 30, 2008 (Ft. Pierce, Fla., U.S.A.); the later 2 harvests being from the same grove. “Valencia” oranges were also harvested from commercial groves on April 4, 2008 (Dover, Fla., U.S.A.), April 18, 2008 (Clewiston, Fla., U.S.A.), and May 23, 2008 (Lake Placid, Fla., U.S.A.). The HLB causal bacterium is unevenly distributed within a tree and diagnosis by polymerase chain reaction (PCR) is not always accurate, particularly for asymptomatic tissue (Folimonova and others 2009); therefore, the trees used in this study were selected based on visual symptoms alone to be representative of how most growers and processors are currently diagnosing the disease. Within each grove, fruit were harvested from visually HLB symptomatic trees (displaying blotchy mottle leaves, twig die back, and small fruit) and visually healthy trees (no visible HLB symptoms). From symptomatic trees, fruit showing physical symptoms of HLB infection (that is, small, misshapen) and healthy appearing fruit were harvested and were designated symptomatic and asymptomatic, respectively. Fruit from visually healthy trees were designated control. For each fruit type, approximately 0.2 m<sup>3</sup> (5 1-bushel sacks) were harvested from between 3 and 5 trees at each harvest location. The number of trees sampled depended on the level of HLB severity and how many symptomatic trees needed to be harvested to collect the desired sample size. Healthy fruit samples were collected from the same number of trees as the symptomatic samples. All fruit were randomly harvested from around the trees; however, HLB symptoms are often sectorized within a tree, thereby limiting the extent to which symptomatic fruit could be randomly harvested. Following harvest, fruit were transported to the laboratory and stored overnight at 5 °C and processed the next day. Overnight storage at 4 to 5 °C is the current practice at the Citrus Research and Education Center, UF to eliminate any Asian citrus psyllids that might have been on the surface of the fruit.

All samples were juiced by hand using a reamer juicer model 8R from Sunkist Growers Inc. (Ontario, Calif., U.S.A.) until approximately 18 L were obtained. Care was taken to minimize albedo (and therefore variable extraction of albedo-rich components). The juices were portioned into smaller containers and then stored at -18 °C until analysis.

### Juice quality analyses

Procedures to determine juice quality, especially total soluble solids (°Brix) and total titratable acidity, were done according to FMC FoodTech's Procedures for Analysis of Citrus Products (Cheng 2002). Brix values were determined using a digital Abbe refractometer (Leica Mark II Plus) in triplicate. Acidity was determined by a titration of 25 mL of juice with 0.3123N sodium hydroxide (NaOH) to a phenolphthalein endpoint.

### Solid-phase micro extraction (SPME) volatile collection

Extraction of volatiles using headspace solid-phase micro extraction (SPME) was done using a method described by Bazemore and others (1999) and modified by Mahattanatawee and others (2005). The extraction was accomplished using a 2 cm 50/30 μm DVB/Carboxen<sup>TM</sup>/PDMS StableFlex<sup>TM</sup> fiber (Supelco, Bellefonte, Pa., U.S.A.). An aliquot (10 mL) of whole juice was placed in a 40 mL glass vial with a silicone/PTFE septa screw cap. An internal standard was added (50 μL of 2000 μg/mL benzyl alcohol [Aldrich, St. Louis, Mo., U.S.A.] in methanol) and thoroughly mixed before the vial headspace was purged with nitrogen. The sample was gently

stirred by a stirring bar and allowed to equilibrate in a 40 °C water bath for 30 min. After the equilibration period, the SPME fiber was inserted into the vial headspace and exposed at 40 °C for 45 min.

### GC-MS conditions

A Perkin Elmer (Waltham, Mass., U.S.A.) Clarus 500 GC-MS system was used to analyze volatiles. It contained a Stabilwax column (Restek 60 m, 0.25 mmID, 0.5 μm df) with the mass spectrometer scanning 25 to 300 *m/z*. Helium was the carrier gas. The temperature program began at an initial temperature of 40 °C for 2 min, followed by a ramp of 7 °C/min to 240 °C, held for 9.50 min for a 40-min total run time.

### MS peak identification and internal standard quantification

GC-MS chromatograms were analyzed using TurboMass software version 5.4.2 (Perkin Elmer). Peak identifications were confirmed with mass spectra obtained from NIST 2005 (Nat. Inst. of Standards and Technology, Gaithersburg, Md., U.S.A.) mass spectra database libraries and linear retention index (LRI) values calculated using a calibration curve generated from both low and high alkane series (C6-C9 and C8-C25). Since only a single internal standard (benzyl alcohol) was used, the calculated concentrations should be considered semiquantitative.

### Flavanone glycoside sample preparation

Juice flavanone glycosides were extracted using a modified version of methods described by Rouseff and others (1987) and Bronner and Beecher (1995). Rhoifolin (400 μL of 1000 μg/mL) (Indofine, Hillsborough, N.J., U.S.A.), made up in methanol, was added as an internal standard to 5 mL of juice sample and swirled. After centrifuging the sample at 4000 rpm for 15 min, the supernatant was carefully separated from the pellet on the bottom of the centrifuge tube. A C-18 SPE cartridge (Phenomenex Strata C18-E 500 mg/6 mL, 17.5% carbon loading, 461 m<sup>2</sup>/g surface area, 76 Å pore size, 53 μm particle size) was conditioned with 4 mL of methanol followed by 8 mL of deionized water. The supernatant was passed through the C-18 cartridge at approximately 1 drop/s (approximately 3 mL/min). The cartridge was washed with 5 mL of deionized water and the flavanone glycosides were slowly eluted with 3 mL of acetonitrile into a 10 mL volumetric flask. The pellet was sonicated with 3 mL of dimethylformamide and then centrifuged at 4000 rpm for 10 min. The resulting supernatant was added to the 10 mL volumetric flask with the C-18 eluant. Deionized water was used to bring solution to volume before mixing with a small magnetic stirring bar for up to 5 min. About 1.5 mL of the solution was filtered into a HPLC autosampler vial using a 0.45 μm nylon filter (Fisherbrand).

### HPLC conditions, identifications, and quantification

A Thermo (Waltham, Mass., U.S.A.) Finnigan Surveyor HPLC system was used to analyze the flavanone glycosides. Separations were achieved using a C-18 column (Phenomenex Luna 5 μm C18, 250 × 4.60 mm 5μ) and photo diode array, PDA, detector monitoring 240, 280, and 340 nm wavelengths. The mobile phase consisted of a gradient program that began at 18% acetonitrile and 82% aqueous acetic acid (1%) and ended at 60% acetonitrile and 40% aqueous acetic acid (1%) in 30 min. The flow rate was 1 mL/min and the injection volume was 25 μL. Each sample was analyzed in triplicate.

HPLC chromatograms were analyzed using Xcalibur software (Thermo Electron Corp., Waltham, Mass., U.S.A.). Peak identifications were confirmed by retention time by injecting naringin (Acros

Organics, N.J., U.S.A.), hesperidin (Acros Organics), and rhoifolin standards. Sample peak identifications were obtained by comparing both retention times and absorbance spectra from the sample peak with those of standards run under identical conditions. Concentrations were determined using the internal standard method. A flavone glycoside, rhoifolin (20  $\mu\text{g}/\text{mL}$ ) was used as the internal standard.

### Polymethoxylated flavone sample preparation

Extraction of polymethoxylated flavones from orange juice was accomplished using a modified version of a method described by Mouly and others (1999). After centrifuging a 10 mL juice sample at 4000 rpm for 15 min, the supernatant was carefully separated from the pellet at the bottom of the centrifuge tube. Flavone (30  $\mu\text{L}$  of 100  $\mu\text{g}/\text{mL}$ ) (Acros Organics) was added to the supernatant as an internal standard. A C-18 SPE cartridge (Phenomenex Strata C18-E 500 mg/6 mL, 17.5% carbon loading, 461  $\text{m}^2/\text{g}$  surface area, 76  $\text{\AA}$  pore size, 53  $\mu\text{m}$  particle size) was conditioned with 5 mL of methanol followed by 10 mL of deionized water. The supernatant was passed through the C-18 cartridge, then the cartridge was washed with 5 mL of deionized water followed by 3 mL of a solution of 90% water and 10% methanol. The polymethoxylated flavones were then slowly eluted with 2 mL of methanol into a 4 mL amber vial. About 1 mL of the eluant was transferred to a HPLC autosampler vial prior to analysis.

### Polymethoxylated flavone HPLC conditions, peak identification, and quantification

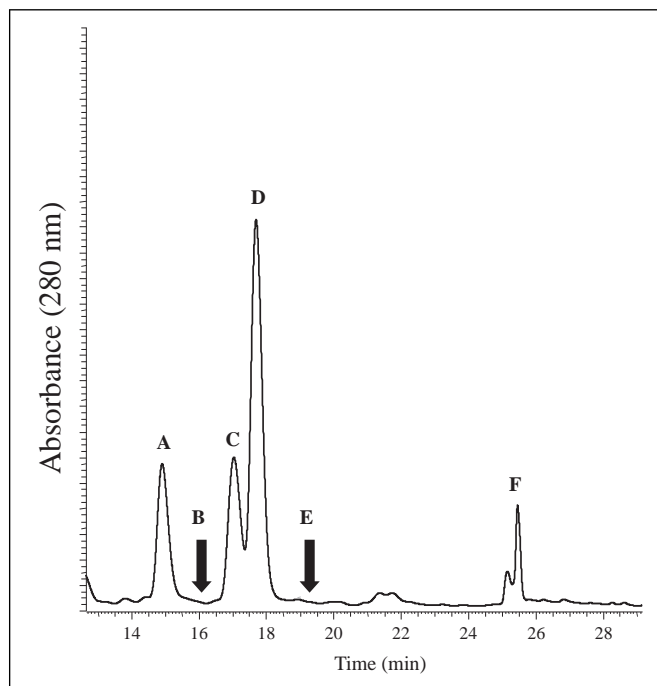
A Thermo (Waltham, Mass., U.S.A.) Finnigan Surveyor HPLC system with a C-18 reversed phase column (Phenomenex Luna 5  $\mu$  C18, 250  $\times$  4.60 mm 5  $\mu$ ) and PDA detector monitoring 240, 280, and 325 nm wavelengths was used to analyze polymethoxylated flavones. Initial mobile phase consisted of 45% acetonitrile, 50% water, and 5% methanol and ended at 45% acetonitrile, 20% water, and 35% methanol using a linear gradient in 20 min. The flow rate was 1 mL/min and injection volume was 25  $\mu\text{L}$ . Each sample was analyzed in triplicate.

HPLC chromatograms were analyzed using Xcalibur software (Thermo Electron Corp.). As with the flavanone glycosides analysis, polymethoxylated flavone, PMF, peak identifications were confirmed matching retention times and UV spectra with those of

standards. Sample concentrations were determined using the internal standard method.

### Limonin sample preparation

Juice samples were prepared according to a modified version of a procedure described by Widmer and Haun (2000). Total of 3 mL of whole juice was placed in a round-bottom centrifuge tube and heated in a 90  $^{\circ}\text{C}$  water bath for 10 min. The sample was diluted with 3 mL of 40% aqueous acetonitrile and thoroughly stirred using a Vortex for 5 s. The solution was filtered using a 0.45  $\mu\text{m}$  nylon filter with a glass microfiber before filling a HPLC autosampler vial.



**Figure 1**—Reverse phase HPLC separation of citrus flavanone glycosides in symptomatic Hamlin orange juice. Flavanone glycosides are: (A) naringin, (D) hesperidin, and (F) didymin. Arrows indicate where the bitter flavanone glycosides (B) naringin and (E) neohesperidin would have eluted. (C) Rhoifolin is the internal standard. Absorbance at 280 nm.

**Table 1**—Brix/acid and oil levels<sup>A</sup> in Hamlin and Valencia orange juices. Significant mean separations are represented by letters a, b, c.

| Date       | Cultivar | Type         | $^{\circ}\text{Brix}/\text{acid}$ ratio | Oil (%) <sup>A</sup> |
|------------|----------|--------------|---|----------------------|
| 12/12/2007 | Hamlin   | Control      | 15.1 $\pm$ 1.8 a                        | 0.003                |
|            | Hamlin   | Asymptomatic | 12.3 $\pm$ 0.54 b                       | 0.002                |
|            | Hamlin   | Symptomatic  | 11.7 $\pm$ 0.18 b                       | 0.002                |
| 12/18/2007 | Hamlin   | Control      | 15.5 $\pm$ 0.40 b                       | 0.003                |
|            | Hamlin   | Asymptomatic | 17.6 $\pm$ 0.61 a                       | 0.007                |
|            | Hamlin   | Symptomatic  | 14.2 $\pm$ 0.17 c                       | 0.008                |
| 1/30/2008  | Hamlin   | Control      | 18.2 $\pm$ 0.70 a                       | 0.003                |
|            | Hamlin   | Asymptomatic | 18.0 $\pm$ 0.40 a                       | 0.009                |
|            | Hamlin   | Symptomatic  | 15.6 $\pm$ 0.22 b                       | 0.004                |
| 4/4/2008   | Valencia | Control      | 13.7 $\pm$ 0.17 a                       | 0.007                |
|            | Valencia | Asymptomatic | 10.8 $\pm$ 0.11 b                       | 0.006                |
|            | Valencia | Symptomatic  | 5.10 $\pm$ 0.04 c                       | 0.005                |
| 4/18/2008  | Valencia | Control      | 14.8 $\pm$ 0.14 a                       | 0.004                |
|            | Valencia | Asymptomatic | 13.0 $\pm$ 0.11 b                       | 0.005                |
|            | Valencia | Symptomatic  | 5.57 $\pm$ 0.04 c                       | 0.008                |
| 5/23/2008  | Valencia | Control      | 18.2 $\pm$ 0.17 b                       | 0.002                |
|            | Valencia | Asymptomatic | 21.5 $\pm$ 0.33 a                       | 0.005                |
|            | Valencia | Symptomatic  | 9.83 $\pm$ 0.06 c                       | 0.004                |

<sup>A</sup>Scott oil test values.



**Figure 2**—Magnified portion of orange segment membrane showing crystalline like structures. Ruler divisions are 1 mm.

### Limonin HPLC conditions, peak identification, and quantification

A Perkin Elmer Series 200 Autosampler connected to 2 Acuflow Series III Pumps and a Perkin Elmer 785A UV/VIS Detector was used to analyze limonin. The system was set up to perform an automated solid-phase extraction using a switching valve as described in Widmer and Haun (2000). One Acuflow pump pumped a solution of 37% acetonitrile in water for limonin analysis, which remained isocratic throughout the analysis. The other pump pumped a solution of 19% acetonitrile in water for naringin analysis. The detector was set at 210 nm for limonin, and the flow rate was 1.10 mL/min. Injection volume was 20  $\mu$ L. Total run time was 20 min, with valve switches at 3 and 15 min. A Zorbax CN column (4.6  $\times$  150 5  $\mu$ L) (Agilent Technologies, Santa Clara, Calif., U.S.A.) was employed.

Chromatograms were analyzed using TotalChrom software (Perkin Elmer). Limonin peak area compared to concentration responses were determined using limonin standards of 20, 10, 5, and 1  $\mu$ g/mL. Sample limonin concentrations ( $\mu$ g/mL) were determined using the external standard method.

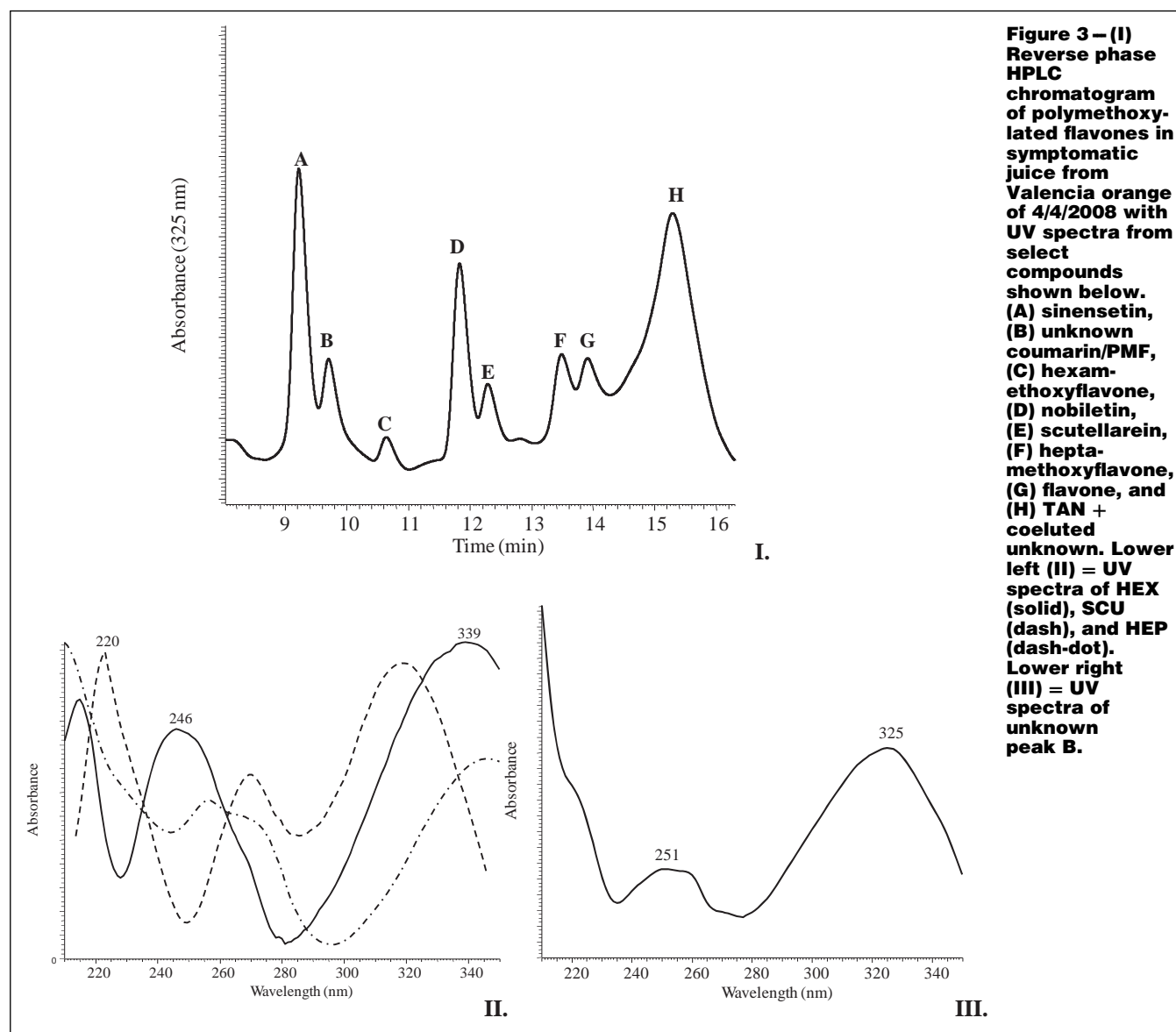
### Statistical analyses

Concentration values of all compounds analyzed were averaged and standard deviations were calculated using Microsoft Excel software. All values were also subjected to one-way analysis of variance (ANOVA) and Tukey's Honestly Significant Differences (HSD) Test to determine if there were significant differences between the 3 juice types (control, symptomatic, and asymptomatic). This was done using Statistica 7.1 from StatSoft (Tulsa, Okla., U.S.A.).

## Results and Discussion

### $^{\circ}$ Brix and acidity

Sugars (that is, mono- and di-saccharides) and acids (that is, citric, malic, and so on) are major taste components in orange juice. These compounds are responsible for the perception of sweetness and sourness, respectively. Soluble solids, or  $^{\circ}$ Brix, is mostly made up of sugars, primarily sucrose, fructose, and glucose. Citric acid is the dominant acid in orange juice, so % acid values generally refers to the percentage of citric acid. As the fruit



matures sugar level increases and acid levels decrease. Thus the ratio of sugars to acid (Brix/acid ratio) is used as a maturity and quality indicator. The USDA has set minimum and maximum values for these quality markers for different grades of orange juice. For example, USDA minimum and maximum Brix/acid ratios for pasteurized orange juice are 12.5 and 20.5, respectively (USDA 1988).

Brix/acid ratios for the 6 sampling dates are shown in Table 1. This ratio was consistently lower in juice from symptomatic fruit when compared to control juice. These differences were statistically significant ( $P \geq 0.05$ ) in 5 of the 6 harvest dates. °Brix values were as much as 13% to 24% lower and acid levels were as much as 18% higher in symptomatic juices compared to control. These lower sugar and higher acid levels produced ratios that were as much as 62% lower in symptomatic fruit. Symptomatic juice appeared to be from less mature fruit even though the juices are from fruit of the same age. On the other hand, sugar/acid ratios from the juice of asymptomatic fruit were similar to that of control juices. Earlier studies (Plotto and others 2008) had reported that asymptomatic juice always contained higher levels of sugars, lower acidity and higher Brix/acid ratios. However, the Plotto study involved only a single sampling date (July) for a single cultivar (Valencia). In the current study, Brix/acid ratios were higher in 2 of the 6 harvest dates and slightly lower than control juice in 3 of the 6 harvest dates. It is worth noting that 1 of the 2 times where asymptomatic juices contained higher Brix/acid ratios was also the late season Valencia (see Table 1). So on that harvest date and cultivar there is complete agreement with Plotto and others (2008). Therefore, when considering the entire season, the major differences in sugar acid levels were found primarily in symptomatic juices and not asymptomatic juices when compared to control juices.

### Flavanone glycosides

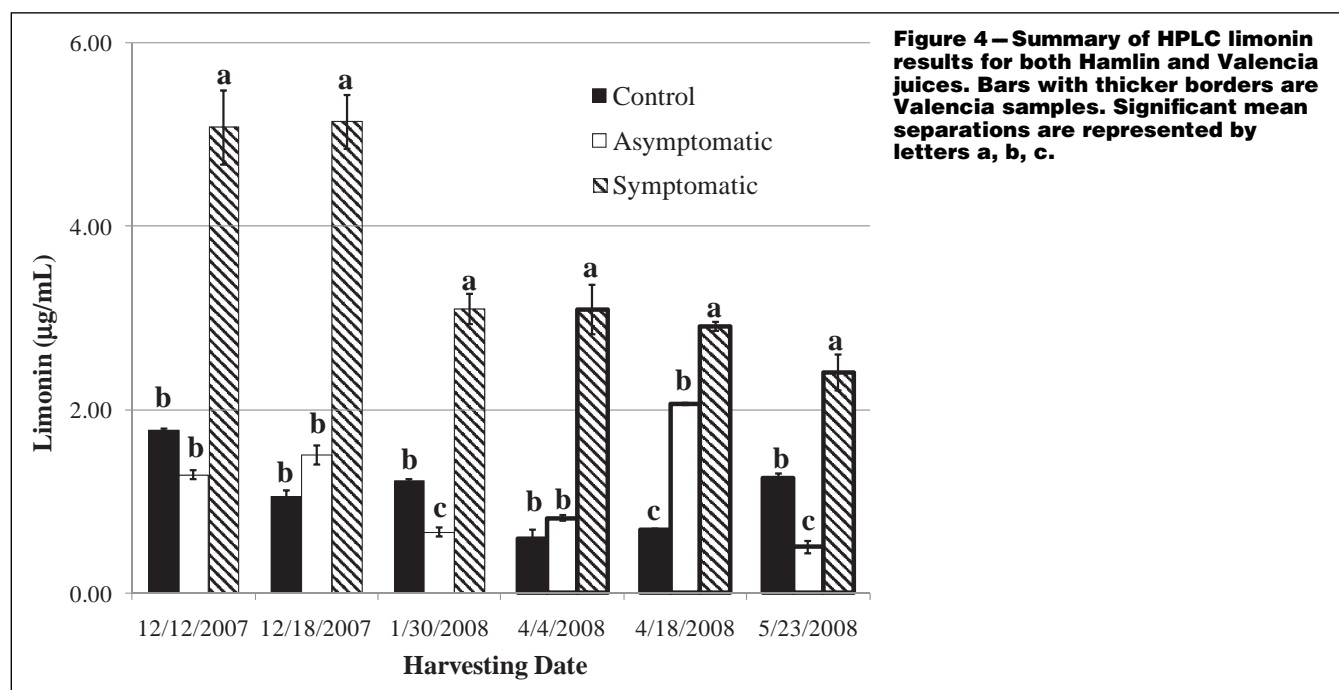
One objective of this study was to determine if bitter flavanone glycosides (FGs) might be responsible for the reported bitterness in HLB symptomatic juice. It was not known if HLB could alter the enzyme mediated chemical pathway to produce bitter flavanones

since both bitter and non bitter flavanones are formed from the same precursor in the Shikimic acid pathway and only differ in the way the 2 sugars (rhamnose and glucose) are linked. Since bitter FGs, such as naringin and neohesperidin, are typically only present in sour orange (*Citrus aurantium*, L.) and grapefruit (*Citrus × paradisi* Macfad.) cultivars (Rouseff and others 1987), they would not be expected to be present in “Hamlin” and “Valencia” sweet orange. Sweet oranges typically do not contain bitter FGs but contain appreciable amounts of nonbitter FGs, such as narirutin and hesperidin.

As shown from the typical HPLC chromatogram in Figure 1, no juice samples (control, asymptomatic, or symptomatic) from both “Hamlin” and “Valencia” cultivars were found to contain bitter flavanone glycosides. As expected, 3 nonbitter flavanone glycosides (narirutin, hesperidin, and didymin) were identified on the basis of retention time and spectral matching with standards. The arrows in this figure are placed where naringin and neohesperidin would have eluted if they were present.

There were differences in observed flavanone glycoside concentration levels between control and symptomatic juices at almost every sampling date. Changes in FG concentration from control ranged as little as -4% and as high as +343%, but there was no consistent trend.

Hesperidin is the major flavanone glycoside in sweet oranges but difficult to measure due to its limited solubility. It is almost insoluble in water and its solubility decreases with decreasing temperature. Hesperidin levels in centrifuged OJ's were observed to diminish after each freeze thaw cycle, even though sample preparation and HPLC conditions remained unchanged. During normal sample preparation, the juice was centrifuged and only the supernatant was analyzed and the pellet was discarded. Gil-Izquierdo and others (2003) reported that freezing decreased concentrations of dissolved hesperidin, due to precipitation. Therefore, dimethylformamide, DMF, was added to the pellet to extract and solubilize precipitated hesperidin and added to the  $C_{18}$  solid phase extraction, SPE, extract so that all the hesperidin in the sample was accounted for.



**Figure 4—Summary of HPLC limonin results for both Hamlin and Valencia juices. Bars with thicker borders are Valencia samples. Significant mean separations are represented by letters a, b, c.**

## Crystal formation

Another symptom occasionally seen in HLB symptomatic fruit is shown in Figure 2 was the presence of white crystal-like structures ranging from approximately 0.5 to 3 mm in size found between juice segment membranes. When this material was excised and dissolved in DMF elevated levels of hesperidin were observed compared to segment membranes from the same fruit lacking this feature. Given the solubility of hesperidin, it is likely that the observed crystal-like structures shown in Figure 2 are precipitated and crystallized hesperidin.

## Polymethoxylated flavones

Polymethoxylated flavones (PMFs) have also been reported to impart bitterness (Swift 1965b). Highest PMFs levels are found in the peel, particularly the flavedo and corresponding peel oil. Given that these juice samples were hand squeezed instead of mechan-

ically squeezed using commercial extractors, the amount of PMFs and other peel components typically incorporated into juice was expected to be lower than commercial juices which typically contain higher peel oil levels. Oil levels for all samples are shown in Table 1 and none exceed the maximum USDA level of 0.035% for grade A juice (USDA 1988).

A thin-layer chromatography (TLC) study (Veldhuis and others 1970) determined PMF concentrations in commercial and hand extracted juices as well as determining bitterness sensory thresholds for the 5 major PMFs. In all juices sampled in the current study, PMF concentrations were far below their respective taste thresholds.

As shown in Figure 3, an additional chromatographic peak was observed only in the HPLC chromatograms of symptomatic juice (2 of 3 samples). It eluted between sinensetin (SIN) and hexamethoxyflavone (HEX) at about 9.7 min and is labeled as peak B. The unknown peak's UV spectra and those of HEX,

**Table 2 – Concentrations ( $\mu\text{g/mL}$ ) of Valencia 4/18/2008 orange juice volatiles whose identifications have been confirmed by both MS and LRI values. Significant mean separations are represented by a, b, c.**

| LRI  | Compound                  | Control                | Asymptomatic          | Symptomatic            |
|------|---------------------------|------------------------|-----------------------|------------------------|
| 721  | Acetaldehyde              | 0.55 $\pm$ 0.06(a)     | 0.45 $\pm$ 0.06(a)    | 0.34 $\pm$ 0.12(a)     |
| 901  | Ethyl acetate             | 1.14 $\pm$ 0.11(a)     | 1.10 $\pm$ 0.03(a)    | 0.76 $\pm$ 0.43(a)     |
| 912  | Methanol                  | 14.8 $\pm$ 1.3(a)      | 15.8 $\pm$ 0.3(a)     | 17.8 $\pm$ 2.6(a)      |
| 951  | Ethanol                   | 16.9 $\pm$ 1.9(a)      | 18.1 $\pm$ 1.6(a)     | 17.6 $\pm$ 4.1(a)      |
| 1002 | Methyl butanoate          | 0.54 $\pm$ 0.05(b)     | 0.53 $\pm$ 0.01(b)    | 0.77 $\pm$ 0.11(a)     |
| 1034 | $\alpha$ -Pinene          | 2.02 $\pm$ 0.11(b)     | 4.51 $\pm$ 0.22(a)    | 4.53 $\pm$ 1.02(a)     |
| 1051 | Ethyl butanoate           | 24.2 $\pm$ 2.7(a)      | 14.4 $\pm$ 1.1(b)     | 6.2 $\pm$ 0.6(c)       |
| 1100 | Hexanal                   | 2.65 $\pm$ 0.65(b)     | 6.3 $\pm$ 0.6(a)      | 3.4 $\pm$ 0.6(b)       |
| 1120 | $\beta$ -Pinene           | 0.177 $\pm$ 0.035(b)   | 0.503 $\pm$ 0.014(a)  | 0.448 $\pm$ 0.071(a)   |
| 1162 | $\delta$ -3-Carene        | 0.176 $\pm$ 0.0139(c)  | 0.335 $\pm$ 0.0107(b) | 0.507 $\pm$ 0.0815(a)  |
| 1173 | $\beta$ -Myrcene          | 13.4 $\pm$ 0.761(b)    | 19.7 $\pm$ 0.666(a)   | 22.3 $\pm$ 2.8(a)      |
| 1195 | $\alpha$ -Terpinene       | 0.109 $\pm$ 0.006(b)   | 0.202 $\pm$ 0.013(a)  | 0.225 $\pm$ 0.021(a)   |
| 1207 | Methyl hexanoate          | 0.26 $\pm$ 0.073(a)    | 0.247 $\pm$ 0.075(a)  | 0.712 $\pm$ 0.306(a)   |
| 1228 | Limonene                  | 376 $\pm$ 31(b)        | 741 $\pm$ 32.(a)      | 674 $\pm$ 107(a)       |
| 1248 | Ethyl hexanoate           | 8.69 $\pm$ 0.84(a)     | 5.1 $\pm$ 0.73(b)     | 2.89 $\pm$ 0.30(c)     |
| 1262 | $\gamma$ -Terpinene       | 0.5 $\pm$ 0.03(b)      | 0.12 $\pm$ 0.05(b)    | 7.1 $\pm$ 1.3(a)       |
| 1293 | <i>p</i> -Cymene          | 0.25 $\pm$ 0.02(b)     | 0.543 $\pm$ 0.007(ab) | 0.661 $\pm$ 0.267(a)   |
| 1304 | $\alpha$ -Terpinolene     | 0.336 $\pm$ 0.015(b)   | 1.11 $\pm$ 0.94(a)    | 1.29 $\pm$ 0.33(a)     |
| 1311 | Octanal                   | 0.80 $\pm$ 0.14(c)     | 10.9 $\pm$ 0.9(a)     | 5.4 $\pm$ 0.7(b)       |
| 1366 | 1-Hexanol                 | 1.22 $\pm$ 0.14(a)     | 1.14 $\pm$ 0.26(a)    | 0.869 $\pm$ 0.122(a)   |
| 1402 | ( <i>Z</i> )-3-Hexen-1-ol | 0.856 $\pm$ 0.963(b)   | 0.804 $\pm$ 0.063(b)  | 1.23 $\pm$ 0.18(a)     |
| 1418 | Nonanal                   | 0.697 $\pm$ 0.136(b)   | 2.89 $\pm$ 0.049(a)   | 2.33 $\pm$ 1.19(ab)    |
| 1452 | Ethyl octanoate           | 1.18 $\pm$ 0.03(a)     | 1.3 $\pm$ 0.09(a)     | 0.511 $\pm$ 0.089(b)   |
| 1463 | 1-Octen-3-ol              | 0.165 $\pm$ 0.010(a)   | 0.158 $\pm$ 0.010(a)  | 0.0972 $\pm$ 0.0136(b) |
| 1494 | Octyl acetate             | 0.342 $\pm$ 0.024(c)   | 1.64 $\pm$ 0.01(a)    | 0.976 $\pm$ 0.154(b)   |
| 1525 | Decanal                   | 1.4 $\pm$ 0.3(c)       | 12.6 $\pm$ 0.2(a)     | 5.68 $\pm$ 1.68(b)     |
| 1561 | Linalool                  | 11.6 $\pm$ 1.6(b)      | 19.4 $\pm$ 3.1(b)     | 35.9 $\pm$ 4.4(a)      |
| 1572 | 1-Octanol                 | 5.03 $\pm$ 0.37(b)     | 9.23 $\pm$ 1.13(a)    | 9.14 $\pm$ 0.95(a)     |
| 1621 | $\beta$ -Elemene          | 0.774 $\pm$ 0.062(ab)  | 0.998 $\pm$ 0.13(a)   | 0.678 $\pm$ 0.157(b)   |
| 1635 | 4-Terpineol               | 1.75 $\pm$ 0.23(c)     | 7.27 $\pm$ 1.08(b)    | 11 $\pm$ 1.2(a)        |
| 1639 | $\beta$ -Caryophyllene    | 0.888 $\pm$ 0.091(ab)  | 1.15 $\pm$ 0.28(a)    | 0.608 $\pm$ 0.146(b)   |
| 1673 | 1-Nonanol                 | 0.359 $\pm$ 0.015(b)   | 0.971 $\pm$ 0.099(a)  | 1.17 $\pm$ 0.17(a)     |
| 1681 | Citronellyl acetate       | 0.606 $\pm$ 0.017(b)   | 1.77 $\pm$ 0.07(a)    | 1.63 $\pm$ 0.20(a)     |
| 1690 | $\beta$ -Selinene         | 0.22 $\pm$ 0.02(a)     | 0.205 $\pm$ 0.052(a)  | 0.0848 $\pm$ 0.0097(b) |
| 1697 | Decyl acetate             | 0.404 $\pm$ 0.018(c)   | 0.97 $\pm$ 0.04(a)    | 0.597 $\pm$ 0.083(b)   |
| 1706 | Ethyl-3-hydroxyhexanoate  | 1.37 $\pm$ 0.08(a)     | 1.33 $\pm$ 0.12(a)    | 0.309 $\pm$ 0.0168(b)  |
| 1726 | $\alpha$ -Terpineol       | 0.611 $\pm$ 0.046(c)   | 1.23 $\pm$ 0.07(b)    | 2.2 $\pm$ 0.3(a)       |
| 1766 | Valencene                 | 45 $\pm$ 3.1(a)        | 54.4 $\pm$ 9.2(a)     | 18.7 $\pm$ 3.2(b)      |
| 1770 | $\alpha$ -Selinene        | 4.42 $\pm$ 0.34(a)     | 5.34 $\pm$ 0.91(a)    | 2.62 $\pm$ 0.38(b)     |
| 1779 | $\beta$ -Citronellol      | 1.96 $\pm$ 0.06(a)     | 2.46 $\pm$ 0.38(a)    | 2.66 $\pm$ 0.37(a)     |
| 1785 | Carvone                   | 0.662 $\pm$ 0.059(b)   | 1.51 $\pm$ 0.19(a)    | 0.46 $\pm$ 0.06(b)     |
| 1791 | $\Delta$ -Cadinene        | 0.418 $\pm$ 0.067(a)   | 0.929 $\pm$ 0.302(a)  | 0.93 $\pm$ 0.2(a)      |
| 1820 | Nerol                     | 0.312 $\pm$ 0.017(b)   | 0.535 $\pm$ 0.067(a)  | 0.649 $\pm$ 0.083(a)   |
| 1841 | Perillaldehyde            | 0.609 $\pm$ 0.077(c)   | 1.31 $\pm$ 0.12(b)    | 1.93 $\pm$ 0.15(a)     |
| 1863 | Geraniol                  | 0.839 $\pm$ 0.071(a)   | 0.736 $\pm$ 0.050(a)  | 0.79 $\pm$ 0.10(a)     |
| 1894 | $\alpha$ -Ionone          | 0.294 $\pm$ 0.022(c)   | 0.424 $\pm$ 0.005(b)  | 0.559 $\pm$ 0.069(a)   |
| 1986 | $\beta$ -Ionone           | 0.0971 $\pm$ 0.0104(b) | 0.124 $\pm$ 0.007(a)  | 0.0533 $\pm$ 0.0063(c) |
| 2050 | Caryophyllene oxide       | 0.138 $\pm$ 0.006(c)   | 0.219 $\pm$ 0.009(b)  | 0.249 $\pm$ 0.015(a)   |
| 2272 | $\beta$ -Sinsenal         | 0.161 $\pm$ 0.017(b)   | 0.288 $\pm$ 0.010(a)  | 0.352 $\pm$ 0.082(a)   |
| 2576 | Nootkatone                | 0.995 $\pm$ 0.056(ab)  | 1.35 $\pm$ 0.13(a)    | 0.577 $\pm$ 0.296(b)   |

tetramethyl-O-scutellarein (SCU), and heptamethoxyflavone (HEP) are also shown below the chromatogram. Tangeretin (TAN) values were not reported as there appeared to be appreciable coelution in the broad peak labeled H in chromatogram shown in Figure 3. The UV spectrum of the unknown peak B (lower left in Figure 3) exhibits a strong  $\lambda_{max}$  at approximately 325 nm, which is characteristic of both polymethoxylated flavones and coumarins (Swift 1965a; Stanley and Jurd 1971). Therefore this peak is probably either an entirely new PMF or a coumarin. In plants, coumarins are associated with defense, especially against phytopathogens and stress (Repcak and others 2001; Abou Zeid 2002).

### Limonin

Limonin is responsible for the delayed bitterness occasionally observed in sweet orange (*Citrus sinensis*) juices. When juice is freshly extracted the bitter limonin exists as a tasteless precursor, limonate-A-ring lactone (LARL). It rapidly converts to the bitter limonin in the presence of acid and heat. Studies conducted by Hasegawa and others (1977, 1989, 1991), followed by a study by Fong and others (1992) determined that the concentration of LARL decreases in the flesh of an orange as it matures. As the fruit matures, the tasteless precursor LARL converts to another tasteless but more stable glucoside, limonin 17- $\beta$ -D-glucopyranoside (LG) leaving less of the LARL to convert to limonin. This strong association of falling limonin levels with maturity has sometimes been employed as a rough estimate of fruit maturity.

Shown in Figure 4 is a comparison of limonin concentrations from all 3 juice types and all 6 harvesting dates. Significant differences ( $\alpha = 0.05$ ) were observed between control and symptomatic juices. Limonin concentrations were 91 to 425% higher in symptomatic juice compared to control. This suggests that the conversion of LARL to the non bitter glucoside LG was inhibited or delayed in symptomatic juice. These elevated limonin concentrations suggest that symptomatic fruit appear to be less mature. Differences between control and asymptomatic juice limonin levels were not significantly different in 3 of the 6 trials. In those sampling dates where significant differences were observed, asymptomatic juices had less limonin than control in 2 of the 3 remaining sampling dates.

According to Guadagni and others (1973), the average human detection threshold of limonin in orange juice is approximately 6.5  $\mu\text{g}/\text{mL}$ . Although limonin levels were higher in symptomatic

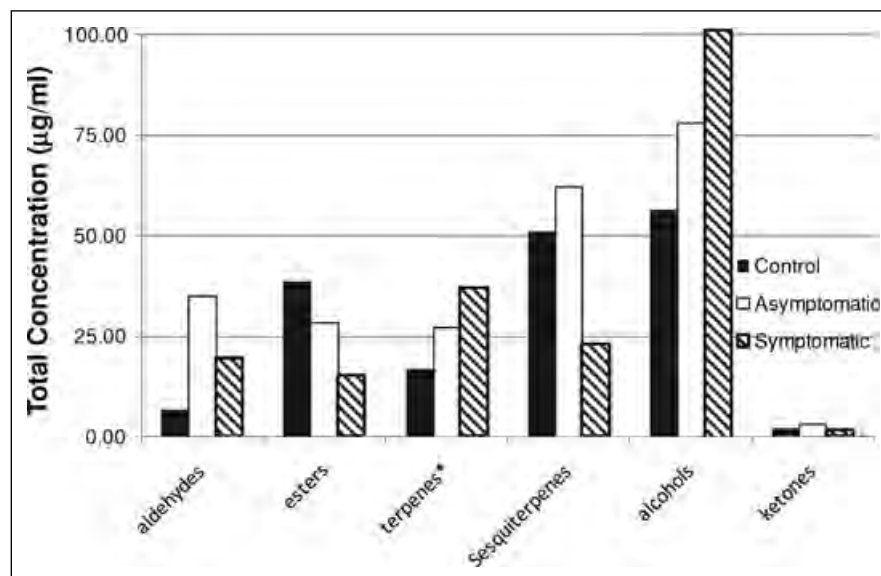
juice, ranging from 2.41 to 5.14  $\mu\text{g}/\text{mL}$ , they never exceeded the average threshold. Even though some bitter sensitive people might be able to detect bitterness in symptomatic juices, the average person would probably not detect bitterness.

### GC-MS volatile differences

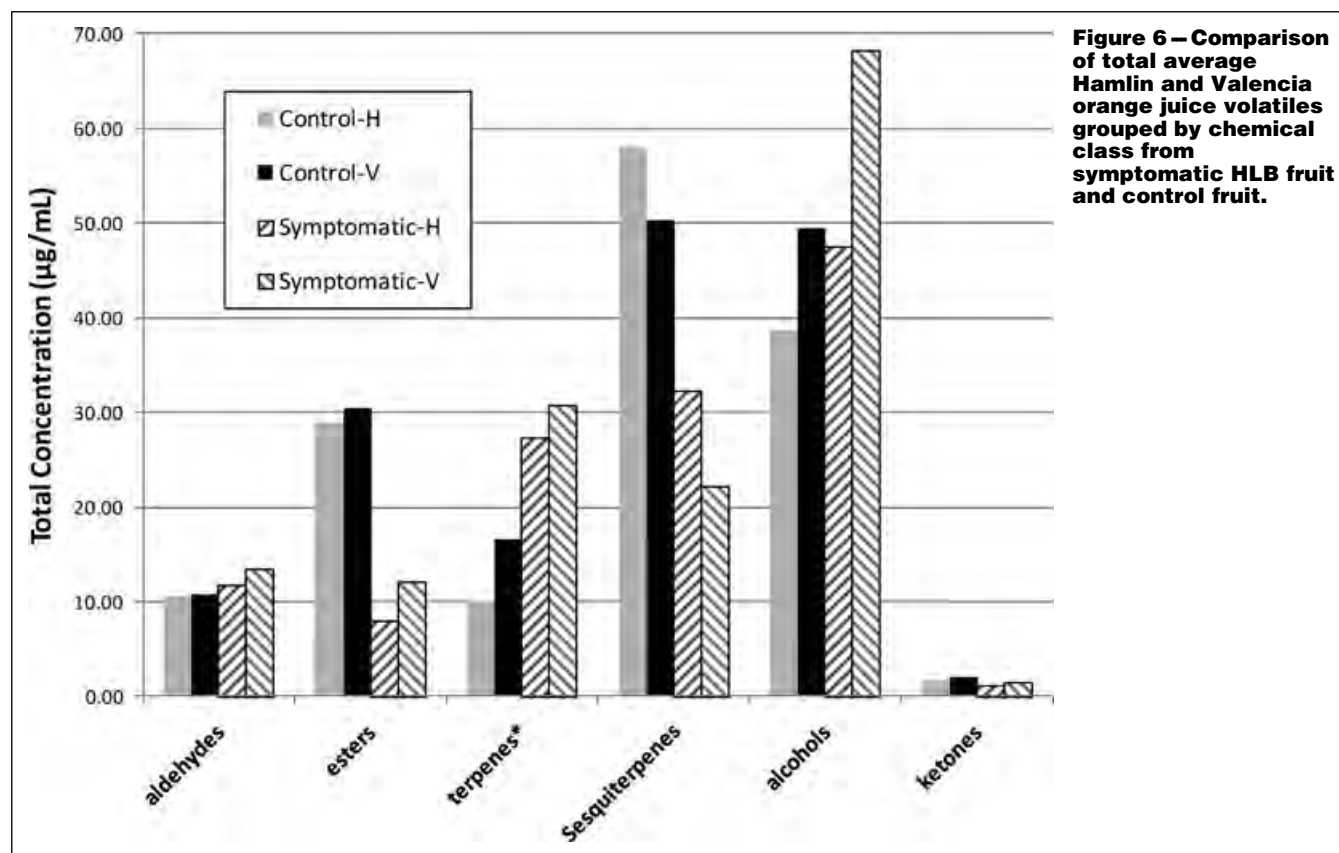
Over 90 volatiles were detected in the GC-MS studies of which 50 were identified (see Table 2) using a combination of both mass spectra and LRI value matching. Identified compounds included 15 terpenes, 13 alcohols (including terpene alcohols), 10 esters, 7 aldehydes, 4 ketones, and 1 oxide. New compounds that might have been responsible for an off-flavor were not detected in either symptomatic or asymptomatic juice samples compared to control. HLB affects classes of volatiles differently. For example, Hamlin juice esters (ethyl acetate, ethyl butanoate, methyl hexanoate, and ethyl-3-hydroxyhexanoate) were 33 to 87% lower in symptomatic compared to control. However, terpenes and alcohols derived from terpenes ( $\alpha$ -pinene,  $\beta$ -pinene, myrcene,  $\gamma$ -terpinene,  $\alpha$ -terpinolene, linalool, 4-terpineol, and  $\alpha$ -terpineol) were 79 to 773% higher in symptomatic juices compared to control. Unexplainably, aldehydes (hexanal, nonanal, and perillaldehyde) were as much as 81% higher in symptomatic juices compared to control.

As shown in Figure 5, symptomatic fruit average values from all 3 Valencia harvests contained higher levels of total alcohols (primarily linalool) and total terpenes, but lower levels of total esters and total sesquiterpenes. Concentration trends for asymptomatic juices were not as clear. Asymptomatic juices contained higher average levels of total aldehydes than either control or symptomatic juice. However, its average volatile profile was most similar to that of the control, especially considering esters, ketones, alcohols and sesquiterpenes. Control juices also generally contained higher levels of esters and sesquiterpenes compared to symptomatic juice.

As expected the volatile profiles of Hamlin and Valencia juices are somewhat different as shown in Figure 6. Comparing control Hamlin and Valencia juice volatiles it can be seen that aldehyde, ester, and ketone totals are similar. The major effect of HLB infection appears to be reflected in higher levels of terpenes and alcohols and lower levels of esters and sesquiterpenes in symptomatic juices compared to control juices. The elevated terpene totals but reduced sesquiterpene totals suggest that the enzymes responsible for the formation of sesquiterpenes from terpenes have been partially inhibited. Similarly, the elevated levels of alcohols and reduced



**Figure 5 – Total averaged values for major volatiles sorted by chemical classes from all 3 Valencia harvest dates. \*Limonene has been excluded from the terpene values.**



**Figure 6 – Comparison of total average Hamlin and Valencia orange juice volatiles grouped by chemical class from symptomatic HLB fruit and control fruit.**

levels of esters might suggest that esterase enzymes are also partially inhibited.

There were also individual volatile differences between Hamlin and Valencia juices. For Hamlin juices, the compounds that were consistently different between control and symptomatic juices were ethyl acetate,  $\alpha$ -pinene, ethyl butanoate, hexanal,  $\beta$ -pinene, myrcene, methyl hexanoate,  $\gamma$ -terpinene,  $\alpha$ -terpinolene, hexanol, nonanal, 1-octen-3-ol, linalool, 4-terpineol, ethyl-3-hydroxyhexanoate,  $\alpha$ -terpineol, and perillaldehyde. For the Valencia juices, the compounds that were consistently significantly different between control and symptomatic juices were ethyl hexanoate,  $\gamma$ -terpinene, octanal, ethyl octanoate, 1-octen-3-ol, linalool, 4-terpineol, ethyl-3-hydroxyhexanoate,  $\alpha$ -terpineol, and caryophyllene oxide. Only 5 volatiles were consistently different in both cultivars. Individual Valencia esters (for example, ethyl hexanoate, ethyl octanoate, and ethyl-3-hydroxyhexanoate) ranged from 29% to 84% lower in symptomatic compared to control juices. Terpenes and alcohols derived from terpenes ( $\gamma$ -terpinene, linalool, 4-terpineol, and  $\alpha$ -terpineol) ranged from 80% to 1320% higher in symptomatic juices compared to control.

An earlier, single harvest study of late season Valencia juice (Plotto and others 2008) found higher levels of acetaldehyde, methanol, 2-methylpropanol and  $\alpha$ -pinene in juice from HLB trees. In the current study, juices from both Hamlin and Valencia cultivars showed lower levels of ethyl butanoate, a key aroma ester, and elevated levels of linalool a major aroma terpene alcohol in symptomatic fruit compared to control.

### Valencene and maturity

Valencene is the 2nd most abundant terpene after limonene in orange juice. Although it lacks aroma activity (Elston and others 2005) it has been used for many years as a quality/maturity marker. As shown in Table 2, Valencene concentrations were lower

in symptomatic juice compared to control and asymptomatic juices.

Sharon-Asa and others (2003) studied the *Cstps1* gene that encoded for valencene synthase, the enzyme that produces valencene. They reported that gene expression occurred as the fruit matured, causing an accumulation of valencene. A higher concentration of valencene in control juices compared to symptomatic juices suggests that symptomatic fruit has not matured in a normal way.

### Conclusions

The objective of this study was to determine the effects of citrus greening/Huanglongbing on both volatile and selected non-volatile flavor compounds in Florida orange juices. Special attention was directed at compounds that may produce bitterness.

Overall, major differences were observed for sugars, acids, and limonin between HLB symptomatic and control juices. However, there was little to no difference in juices from fruit that did not exhibit external HLB symptoms and that of control juices. HLB appears to arrest or slow maturity as evidenced by lower sugars (measured as °Brix) and elevated acid levels. Esters, especially ethyl butanoate, were significantly lower and alcohols were significantly lower in symptomatic juices compared to control. The concentration of valencene, a commonly accepted maturity marker, was also 50-67% lower in symptomatic juice compared to control.

Although symptomatic juice contained elevated levels of the bitter limonin (91% to 425% higher than control), no limonin level exceeded the average bitterness threshold in orange juice. Therefore, most people who taste the juice would not detect bitterness. Flavanone glycosides and polymethoxylated flavones were not found in sufficient concentrations to contribute significant bitterness. Therefore, reported off-flavor associated with HLB symptomatic juices is likely not bitterness, but apparently the result of the juices



being less sweet (lower sugars), more sour (often confused with bitterness) due to higher acid levels, and an imbalance of certain volatile compounds. The lower levels of valencene support the suggestion that HLB juices possess the juice volatile profiles of less mature fruit.

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### References

- Abou Zeid AHS. 2002. Stress metabolites from *Corchorus olitorius* L. leaves in response to certain stress agents. *Food Chem* 76(2):187–95.
- Bazemore R, Goodner K, Rouseff R. 1999. Volatiles from unpasteurized and excessively heated orange juice analyzed with solid phase microextraction and GC-olfactometry. *J Food Sci* 800–3.
- Bronner WE, Beecher GR. 1995. Extraction and measurement of prominent flavonoids in orange and grapefruit juice concentrates. *J Chromatogr A* 705(2):247–56.
- Cheng G. 2002. Procedures for analysis of citrus products. 4th ed. Lakeland, Fla.: FMC Technologies, Inc. FMC FoodTech, Citrus Systems. 192 p.
- da Graca JV. 1991. Citrus greening disease. *Ann Rev Phytopathol* 29:109–36.
- Elston A, Lin JM, Rouseff R. 2005. Determination of the role of valencene in orange oil as a direct contributor to aroma quality. *Flav Fragr J* 20(4):381–6.
- Folimonova SY, Robertson CJ, Garnsey SM, Gowda S, Dawson WO. 2009. Examination of the responses of different genotypes of citrus to Huanglongbing (citrus greening) under different conditions. *Phytopathology* 99(12):1346–54.
- Fong CH, Hasegawa S, Coggins CW, Atkin DR, Miyake M. 1992. Contents of limonoids and limonin 17-beta-d-glucopyranoside in fruit tissue of Valencia orange during fruit-growth and maturation. *J Agric Food Chem* 40(7):1178–81.
- Gil-Izquierdo A, Gil MI, Tomas-Barberan FA, Ferreres F. 2003. Influence of industrial processing on orange juice flavanone solubility and transformation to chalcones under gastrointestinal conditions. *J Agric Food Chem* 51(10):3024–8.
- Guadagni DG, Maier VP, Turnbaug JG. 1973. Effect of some citrus juice constituents on taste thresholds for limonin and naringin bitterness. *J Sci Food Agric* 24(10):1277–88.
- Hasegawa S, Bennett RD, Herman Z, Fong CH, Ou P. 1989. Limonoid glucosides in citrus. *Phytochemistry* 28(6):1717–20.
- Hasegawa S, Hoagland JE. 1977. Biosynthesis of limonoids in citrus. *Phytochemistry* 16(4):469–71.
- Hasegawa S, Ou P, Fong CH, Herman Z, Coggins CW, Atkin DR. 1991. Changes in the limonoate a-ring lactone and limonin 17-beta-d-glucopyranoside content of navel oranges during fruit-growth and maturation. *J Agric Food Chem* 39(2):262–5.
- Kapur SP, Kapoor SK, Cheema SS, Dhillon RS. 1978. Effect of greening disease on tree and fruit characters of kinnow mandarin. *Punjab Horticult J* 18:176–9.
- Mahattanatawee K, Rouseff R, Valim MF, Naim M. 2005. Identification and aroma impact of norisoprenoids in orange juice. *J Agric Food Chem* 53(2):393–7.
- McClellan APD, Oberholzer PCJ. 1965. Greening disease of the sweet orange: evidence that it is caused by a transmissible virus. *South African J Agric Sci* 8:253–76.
- McClellan APD, Schwarz RE. 1970. Greening or blotchy-mottle disease of citrus. *Phytophylactica* 2:177–94.
- Mouly PP, Gaydou EM, Arzouyan C. 1999. Separation and quantitation of orange juices using liquid chromatography of polymethoxylated flavones. *Analisis* 27(3):284–8.
- Plotto A, Baldwin E, McCullum T, Narciso J, Irely M. 2008. Effect of early detection Huanglongbing on juice flavor and chemistry. *Proc Fla State Hort Soc* 121:261–9.
- Repcak M, Imrich J, Franekova M. 2001. Umbelliferone, a stress metabolite of *Chamomilla Recutita* (L.) *Rauschert*. *J Plant Physiol* 158(8):1085–7.
- Rouseff RL, Martin SF, Youtsey CO. 1987. Quantitative survey of naringin, naringin, hesperidin, and neohesperidin in citrus. *J Agric Food Chem* 35(6):1027–30.
- Sharon-Asa L, Shalit M, Frydman A, Bar E, Holland D, Or E, Lavi U, Lewinsohn E, Eyal Y. 2003. Citrus fruit flavor and aroma biosynthesis: isolation, functional characterization, and developmental regulation of Cstps1, a key gene in the production of the sesquiterpene aroma compound valencene. *Plant J* 36(5):664–74.
- Stanley WL, Jurd L. 1971. Citrus coumarins. *J Agric Food Chem* 19(6):1106–10.
- Swift LJ. 1965a. Flavones of neutral fraction of benzene extractables of an orange peel juice. *J Agric Food Chem* 13(5):431–3.
- Swift LJ. 1965b. Peel juice flavor: proximate analyses of Florida orange peel juice extract for 1962-63 and 1963-64 seasons. *J Agric Food Chem* 13(3):282–4.
- USDA. 1988. United States standards for grades of orange juice. In Code of the Federal Registrar, Vol. 21 CFR 146.140-153.
- Veldhuis MK, Swift LJ, Scott WC. 1970. Fully-methoxylated flavones in Florida orange juices. *J Agric Food Chem* 18(4):590–2.
- Widmer WW, Haun CA. 2000. Analysis of limonin and flavonoids in citrus juices and byproduct extracts by direct injection and in-line sample clean-up. In: Berhow MA, Hasegawa S, Manners GD, editors. Citrus limonoids: functional chemicals in agriculture and food. Washington, D.C.: American Chemical Society. p 60–72.