VOLATILE AND SELECTED NON-VOLATILE ANALYSIS OF JUICES FROM HUANGLONGBING AFFECTED HAMLIN AND VALENCIA ORANGES

By

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To my family and friends

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

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Huanglongbing (HLB), or citrus greening, is a disease that produces multiple tree and fruit symptoms. Previous studies have reported that juice from HLB symptomatic fruit was of poor quality and bitter. The effects of HLB infection on volatile and non-volatile flavor compounds in Hamlin and Valencia orange juices were studied. Compounds which might produce bitterness, especially non-volatiles such as flavanone glycosides, polymethoxylated flavones, and limonin, were analyzed. HLB symptomatic, asymptomatic, and control Florida Hamlin and Valencia oranges at various maturity stages were harvested and juiced during the 2007-2008 season. Flavanone glycosides (FGs) and polymethoxylated flavones (PMFs) were analyzed using reversed phase HPLC. No bitter FGs (i.e. naringin) were detected. PMF concentrations were were all far below taste threshold levels reported in literature. An unknown compound with a PMF-like UV spectra was only found in HLB symptomatic juice. Limonin was analyzed using HPLC, and concentrations were 91-425% higher in symptomatic juice compared to control. However, concentrations were also below the reported average human detection threshold. Brix/acid ratios were 8-63% lower in symptomatic juice compared to control. Juice volatiles were identified by GC-MS using SPME headspace extractions. Terpenes, such as γ-terpinene and α-terpinolene, were 1,320% and 62% higher in symptomatic juice than control. Esters, such as

ethyl butanoate and ethyl hexanoate, important aroma volatiles, were 87% and 98% lower compared to control. The chemical composition of asymptomatic juices was very similar to control. The compound(s) responsible for producing flavor differences in juice from HLB infected and control fruit appear to be primarily associated with immaturity. The reported off-flavor associated with HLB symptomatic juices apparently stem from a lower concentration of sugars, a higher concentration of acid, and a different volatile profile.

CHAPTER 1
INTRODUCTION

Florida is the leading producer of sweet oranges in the United States. The majority of the oranges produced are used for juice processing. Huanglongbing (HLB), or citrus greening, is a fatal citrus disease that is threatening the world wide orange juice industry and has become a major problem in Florida. Apart from causing multiple vegetative symptoms, HLB also affects the fruit, and consequently, the juice. The main characteristics of symptomatic fruit are its size and color, for it is smaller and misshapened compared to fruit from non-infected trees, and it retains a green color. Early studies on HLB symptomatic fruit in other parts of the world have reported that the juice was of poor quality and tasted bitter (*1, 2*). Another study noted the change in acidity and soluble solids between juices from symptomatic and control fruits (*3*). However, there are no studies which determine how (and if) the infection affects important flavor compounds.

Generally, flavor can be defined as the interaction of volatile and non-volatile compounds through smell and taste. A combination of odor active volatiles define fresh orange aroma. They typically include certain esters, aldehydes, alcohols, and terpenes. Non-volatile compounds, such as limonin and neohesperidosides, are typically responsible for bitterness in citrus juices when found at levels exceeding their taste threshold. Therefore, the objective of this research is to characterize the effects of HLB infection on flavor impact compounds of orange juice, specifically looking at volatile profile differences and non-volatile compounds which might produce bitterness, including flavanone glycosides, polymethoxylated flavones, and limonin.

These flavor impact compounds can be analyzed by employing various sample preparation techniques (i.e. solid-phase extractions) and analytical instruments. Gas chromatography is typically used to study volatile compounds, while high performance liquid chromatography is

used to analyze non-volatile compounds. These results will lay the foundation for more detailed studies (i.e. involving biochemistry, plant physiology and genetics, etc.) and ultimately assist the orange juice industry in preserving a high quality product.

CHAPTER 2
LITERATURE REVIEW

Overview of Huanglongbing

The United States and Brazil are the major sweet orange (*Citrus sinensis*) juice producers in the world. During the 2007-08 season, Florida was responsible for 70% of orange production in the U.S. (*4*). The majority of the oranges produced in Florida are used for juice processing, with Hamlin and Valencia being the predominant cultivars. Hamlins are available from October to January, while Valencias are available from February to June (*5*). Orange production in both countries has been threatened by the rapid spread of Huanglongbing (HLB), a devastating citrus disease. Citrus producing countries in Asia and Africa have been plagued by this disease since the early 1920s, but it has only recently appeared in the Americas. HLB was found in São Paulo, Brazil's largest orange producing state, in March 2004 and in Florida in August 2005 (*6*). In June 2008, the disease was identified in Louisiana (*7*). As of February 16, 2009, there had been a positive confirmation of HLB in thirty-three counties in Florida (*8*).

There are various names associated with this disease, depending on the region. In the Philippines, it is called "mottle leaf," but in India and Indonesia, it is called "dieback" and "vein phloem degeneration," respectively. In South Africa, the disease is known as "greening," a term that has been frequently used until 1995, since one main symptom is fruit that retains a green color. The Chinese term, "huanglongbing," meaning "yellow dragon disease" or "yellow shoot disease," is now the official term of this citrus disease (*6*).

Casual Agents and Insect Vectors

The causal agent of HLB is the bacteria *Candidatus* Liberibacter. There are three strains of this bacteria: '*Candidatus* Liberibacter africanus', '*Candidatus* Liberibacter asiaticus', and '*Candidatus* Liberibacter americanus'(*6*). Liberibacter are Gram-negative, sieve tube-restricted

bacteria. Koch's Postulates, which are criteria to confirm a causal relationship between a microbe and a disease, has yet to be fulfilled for these HLB liberibacters, since they have not been successfully cultured outside of citrus leaves (*9*).

The Asian Citrus Psyllid (ACP), *Diaphorina citri*, is responsible for transmitting HLB. Psyllids obtain the bacteria when they feed off leaves from infected trees, inoculating other sections of the same tree or uninfected trees within a grove when they move around to feed. Younger trees are more susceptible to infection since they produce multiple flushes, or growth of new leaves, throughout the year, as psyllids prefer feeding and breeding on younger leaves (*10*). In Asia and the Americas, the ACP species is prevalent and infect trees with Liberibacter asiaticus or Liberibacter americanus. *Trioza erytreae*, however, has only been found in Africa and is known only to transmit Liberibacter africanus (*11*).

Symptoms and Economic Impact

HLB affects the entire tree, eventually inducing death. Presently, there is no cure for HLB infected trees, but multiple research efforts are underway to try to control and minimize the spread of the disease, especially the psyllid vector. From an economic standpoint, the citrus industry has already seen an appreciable rise in production costs. These additional costs include increased scouting for symptomatic trees and increased insecticide spraying to control the psyllid population (*12*). Since psyllids are more attracted to younger trees, because they have more flushes than older trees, insecticides must be applied more frequently. Tree removal and/or replanting are also options for HLB management that also contribute to increased costs (*10*).

Prior to twig dieback and decreased productivity, there are noticeable symptoms in the root system, leaves, and fruit. In an infected tree, the root system starts to decay due to root starvation, and new growth is restrained (*13*). Therefore, the amount of fibrous roots that are present do not seem to adequately support the tree (*1*). A blotchy mottle, or asymmetrical

chlorosis, in which the inefficient production of chlorophyll causes yellowing or whitening of a leaf, is one of the main characteristics of the disease (*14*). However, these chlorotic patterns may resemble symptoms of other citrus diseases (i.e. citrus tristeza virus) or mineral deficiencies (i.e. iron or zinc). HLB also induces vein yellowing and the growth of small upright leaves (*13*).

Fruit from a HLB infected tree tend to fall prematurely, and those that remain on the tree fail to mature correctly. The fruit is also small and misshapen (i.e. lopsided) with a curved central core, often containing aborted seeds. Another distinctive characteristic of the disease is the failure of the fruit to color properly, remaining green. The flesh and juice from an infected fruit is said to be of poor quality and have a bitter and unpleasant taste (*1, 2*). A study of HLB infected and non-infected Kinnow mandarins determined that there was a lower soluble solids content and higher acidity in HLB fruit than in control fruit (*3*).

Volatile and Non-Volatile Aspects of Juice Flavor

A food's flavor is primarily due to the interaction of aroma and taste substances, which are generally volatile and non-volatile compounds, respectively. Volatile compounds interact with the olfactory epithelium in the nasal cavity orthonasally (through the nose) or retronasally (from the back of the throat via chewing, etc.). Flavor volatiles are considered secondary metabolites that are derived from primary metabolites, such as carbohydrates, lipids, and proteins (*15*). Non-volatile compounds in contrast, interact with taste receptors on the tongue and palate generating perceptions of salty, sour, sweet, bitter, or umami tastes (*16*).

Juice Volatiles and Aroma Active Compounds

The number of volatile compounds a food product contains depends on a number of factors, including the nature of the product and if it was subjected to any processing methods. In the case of fruits, for example, the composition of volatiles can vary with maturity (development) and/or cultivar (i.e. Navel orange vs. Valencia orange). Foods produced via thermal processing

or in conjunction with a fermentation process, such as coffee or tea, can differ greatly in the number of volatiles than their raw/original counterparts. Additional chemical reactions due to heat, enzymatic, or bacterial interactions can produce more than 700 volatile compounds in these products (*16*). These processes, however, may not have favorable effects in other food products. The volatile profile of processed orange juice, for example, differs from that of freshly-squeezed orange juice. The formation of new compounds, loss of desirable compounds, and/or changes in the concentration ratio of certain compounds can produce off-flavors, or aroma not normally present in the food (*17*).

Although foods can contain as many as 700 volatile compounds, only a small fraction is considered odor-active and actually contributes to a food's aroma (*16*). A volatile compound is odor-active only if its concentration in the food matrix exceeds its odor threshold (the lowest concentration in which the odor is recognized). Character impact compounds, or key odorants, are odor-active compounds that provide the characteristic aroma of a food. Methyl anthranilate, for instance, is the character impact compound for a Concord grape, while diacetyl is the key odorant for butter and citral for lemon (*18*). Early studies have identified more than 200 volatiles in freshly squeezed orange juice, but relatively few contribute to its aroma. Moreover, not one is considered an orange flavor character impact compound, so the aroma of fresh orange juice is due to a specific combination of odor-active volatiles (*17*).

The aroma volatiles that primarily contribute to fresh orange juice aroma are aldehydes and esters (*19*). The majority of these compounds are products from the oxidative degradation of fatty acids, such as linoleic and linolenic acids (*18*). Aldehydes (i.e. acetaldehyde, hexanal, octanal, (Z)-hex-3-enal, (E,E)-2,4-nonadienal, etc.) provide fresh, citrus-like, green, grassy, and fatty odor notes to fresh orange aroma. Esters (i.e. ethyl acetate, ethyl butanoate, ethyl hexanoate,

etc.) provide the fruity character of orange juice. Ethyl butanoate, especially, is one of the most potent aroma compounds and the most important ester. Its concentration in orange juice has been reported to increase with increasing fruit maturity (*17*).

Other volatiles, including some alcohols and terpenoid hydrocarbons and their derivatives, contribute to fresh orange juice aroma. Aliphatic alcohols, such as 1-hexanol and (Z)-3-hexen-1-ol, are responsible for woody, green, and grassy notes. However, terpene alcohols, which include linalool and geraniol, contribute floral and fruity notes. The terpene hydrocarbons constitute the majority of orange juice volatiles, and many are found in orange peel oil. Only a few, though, are odor-active, and they include the pinenes (α and β) and β-myrcene, which provide piney and musty odors to fresh orange juice (*17*).

Juice Non-Volatiles and Bitterness

Bitterness in citrus fruits can be caused by limonoids, flavanone glycosides and possibly polymethoxylated flavones. Limonoids and flavonoids are secondary plant metabolites that affect defense mechanisms and molecular signaling in plants, as well as provide health benefits for humans and animals via anticancer and antioxidant activity (*20, 21*).

Flavanone glycosides

There are several classes of flavonoids, but in fresh fruit and their juices, flavanones and flavones predominate. Flavanones are uncommon in plants and citrus is an unusually rich source for these compounds. The flavonoid molecular skeleton consists of two aromatic rings (designated as A and B) connected by a dihydropyrone ring, for flavanones, or a pyrone ring, for flavones (designated as C) (*20*).

These compounds can influence the quality of juice primarily in terms of bitter taste but can in certain cases affect appearance. In *Citrus*, flavanones are usually found with a disaccharide sugar attached at position 7, and are called flavanone glycosides. Not all citrus

flavanone glycosides are bitter. Bitter flavanone glycosides have a neohesperidose sugar (2-*O*-α-L-rhamnosyl-β-D-glucose) whereas non-bitter flavanone glycosides have a rutinose sugar (6-*O*-α-L-rhamnosyl-β-D-glucose) at position 7. Non sugar substitutions like hydroxyl and methoxyl groups at the 3' and 4' position on the aromatic B ring differentiate them. The non-bitter rutionsides, such as hesperidin and narirutin, predominate in sweet orange (*C. sinensis*) varieties (i.e. Valencia, Hamlin, blood, navel) and tangerine, or mandarin, (*C. reticulata*) varieties (i.e. Clementine, Satsuma). In sour oranges (*C. aurantium*) and grapefruit (*C. paradis*), neohesperidosides, such as naringin, are the dominate flavanone glycosides. Other flavanone glycosides that can be found in *Citrus* juices include: didymin and eriocitrin, which are both rutinosides, and neoeriocitrin and poncirin, which are both neohesperidosides (*22*).

Polymethoxylated flavones

Polymethoxylated flavones (PMFs) are another class of compounds that have been suggested to impart bitterness in juice (*23, 24*). Unlike the flavanone glycosides, which have a sugar constituent attached to the A ring, PMFs are found as aglycones (without a sugar moiety) with varying degrees of methoxylation on the aromatic A and B rings. High concentrations can be found in sweet orange and tangerine cultivars, with lower levels found in other citrus (i.e. grapefruit) (*25*). They are present in every part of the fruit, including the peel (flavedo with albedo), membranes, and juice, but the peel contains much higher concentrations compared to the juice (*26*).

Studies conducted by Swift and others (*23, 24*) suggested that orange peel juice (liquid expressed from peel) was bitter. When extracted with benzene, it was found that the neutral fraction, among the acidic and lactone fractions, was the most bitter, and PMFs were the predominant compounds found. They were later identified as sinensetin (SIN), nobiletin (NOB), tangeretin (TAN), 3,5,6,7,8,3',4'-heptamethoxyflavone (HEP), and tetra-O-methylscutellarein

(SCU). The taste thresholds for these PMFs were determined in a synthetic medium that mimicked orange juice. Results from sensory evaluation indicated that SCU had the lowest taste threshold (15 ppm), followed by HEP (28 ppm), SIN (30 ppm), and TAN (33 ppm), with NOB being the least potent (46 ppm) (*27*). For most PMFs, their average concentration in peel juice exceeded their taste thresholds, indicating that they contributed to the bitterness of peel juice (*23*).

The presence of orange peel components in juice depends on the mode of extraction. For instance, hand squeezed oranges would result in little contact of the peel with the extracted juice. Thus, PMF concentration of the juice would remain unchanged. However, during extraction with commercial juice machinery (i.e. Brown or FMC juice extractors), the peel and its components can come in contact with the juice. The amount of orange oil that ends up in the juice depends on the desired juice quality, and it is controlled by setting certain parameters on the machine, such as peel clearance and extractor pressure. Higher extractor pressure, for example, would result in higher juice yield but also with elevated peel oil components in the juice. Lower extractor pressure, however, results in higher quality juice due to a lower concentration of peel components, but at the expense of lower juice yield (*28*).

In the same study in which taste threshold for individual PMFs were established, commercial orange juice concentrates obtained from commercial extractors (Brown and FMC) were also compared. The difference between the two brands is the extraction style, for Brown is a reamer type and FMC is a peel macerating type (*5*). The results were expressed as part per million (ppm) of PMFs of juice reconstituted to 12° Brix, or single-strength. In both cases, the levels of individual PMFs found in each sample were considerably less than their taste threshold, ranging from 0.20 to 2.05 ppm.

Limonin

While the presence of certain flavanone glycosides and polymethoxylated flavones can cause bitterness immediate in orange juice, there is a group of compounds that can cause "delayed bitterness." Limonoids are oxygenated triterpenoid compounds that are present in the seeds and flesh of the fruit. Limonin, the major limonoid found in orange juice, is extremely bitter. Its tasteless precursor, limonoate A-ring lactone (LARL), is present in the flesh of the intact fruit (*21*). When the fruit is physically damaged by, for example, extractors for juice processing, the precursor interacts with the acidic environment and is transformed into limonin. This biochemical transformation is catalyzed by the enzyme limonin D-ring lactone hydrolase and occurs at a pH of 6.5 or lower (*29*).

However, multiple studies have shown that delayed bitterness by the conversion of LARL to limonin decreased throughout fruit growth and maturity, which partly attributed to why juice from mature oranges were less bitter than immature oranges. A study by Hasegawa and others (*30*) discovered that the concentration of LARL decreased as fruit matured, correlating with decreased bitterness in a process termed "natural debittering." The mechanism of this process was not understood until the discovery of limonoid glucosides, which were tasteless compared to their bitter aglycones (*31*). As fruit matured, it was shown that the concentration of limonin 17-β-D-glucopyranoside (LG) increased as the concentration of LARL decreased. It was concluded that LARL metabolized into LG instead of limonin at the late stages of maturity (*30*).

Volatile and Non-Volatile Sample Preparation and Analysis

The extraction of non-volatiles and volatiles from orange juice has traditionally been accomplished using liquid-liquid extraction (LLE), which utilizes different solvents and the interactions between the solvents and the compound of interest to isolate it. Solid-phase extraction (SPE) is a more rapid method that did not require the use of expensive glassware or

large quantities of organic solvents (*32*). The main principle behind this extraction is that the compound of interest is absorbed onto a modified solid support from the sample, which is then desorbed, or eluted, by solvent or thermal means (*33*). The analysis of flavonoids that are found in orange juice, for example, may involve the use of C18-bonded silica, a common sorbent used for reversed-phased SPE (*34*). In reversed-phase SPE, the sample matrix is usually polar, but the compounds of interest are mid- to nonpolar. Therefore, a modified nonpolar sorbent is used. As the sample is passed through the nonpolar stationary phase, the nonpolar compounds are retained while the impurities are washed away. They are then eluted using a strong organic solvent, such as methanol or acetonitrile (*32*).

Unlike traditional solid-phase extraction, solid-phase micro extraction (SPME) is a rapid and solvent-less technique that is commonly used for the analysis of volatiles. The volatiles can be directly extracted and concentrated from the sample headspace onto a fiber coated with a modified sorbent (i.e. polydimethylsiloxane, etc.). After a certain amount of exposure time, the fiber can go straight to the injection port of a gas chromatography system where the analytes are thermally desorbed (*33*, *35*).

After the compounds of interest are extracted from the sample matrix, they are separated, identified, and quantified by analytical instruments. Volatile compounds are usually analyzed using gas chromatography, while non-volatiles are analyzed using high performance liquid chromatography.

CHAPTER 3
MATERIALS AND METHODS

The main objective of the study was to determine how HLB affects flavor impact compounds in orange juice, so several sample preparation techniques and analytical instrumentation were used to study volatile and selected non-volatile compounds. A secondary objective was to determine whether changes (if any) from the disease differed during fruit development. Therefore, samples were harvested at different stages of maturity. However, since tree removal is a common practice when HLB is found in groves, it was difficult to sample from the same area at different harvesting dates. Because of this, the majority of the samples were obtained from different areas.

Samples

The Florida Hamlin oranges were obtained from commercial groves on December 12, 2007 (Sebring, FL), December 18, 2007 (Ft. Pierce, FL), and January 30, 2008 (Ft. Pierce, FL). The last two harvesting dates occurred from the same grove. The Valencia oranges were also obtained from commercial groves on April 4, 2008 (Dover, FL), April 18, 2008 (Clewiston, FL), and May 23, 2008 (Lake Placid, FL). Three types of fruit were harvested from the same grove at each date. Fruit that showed physical symptoms of HLB that came from a HLB infected tree was designated as symptomatic (+ +). Fruit that did not show physical symptoms of HLB but came from a HLB infected tree was designated as asymptomatic (+ -). Fruit that was harvested from a non-infected tree served as control (- -). Sampling for each harvesting date was done from 3-5 trees. The number of trees sampled was determined by the severity of symptoms. For example, if a tree was heavily symptomatic, fewer trees were sampled. However, if symptoms were not very severe, more trees were sampled to obtain the needed sample size. The number of trees sampled for asymptomatic and control fruit was determined by the number of trees sampled for

symptomatic fruit. The fruits were picked randomly from around the tree. However, because of the nature of the disease, HLB would be sectored within the tree and a small section of the canopy or a branch would be symptomatic, so symptomatic fruit would be obtained from a limited area.

During the harvesting period, the casual agent of HLB in Florida, *Candidatus* Liberibacter asiaticus, was under the Plant Protection and Quarantine (PPQ) Select Agents and Toxins list of the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA). Therefore, the oranges were juiced under a controlled observation room All samples were juiced by hand using a Sunkist® juice extractor until approximately 4.5 gallons were obtained. The juices were portioned into smaller containers and then stored at -18°C until analysis. All samples were thawed before any analysis.

Analysis of Volatiles

Solid-Phase Micro Extraction (SPME) Procedure

Extraction of volatiles using headspace solid-phase micro extraction (SPME) was done using a method described by Bazemore and others (*36*) and modified by Mahattanatawee and others (*37*). The extraction was accomplished using a 2 cm 50/30 µm DVB/Carboxen™/PDMS StableFlex™ fiber (Supelco, Bellefonte, PA). 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) 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 minutes. After the equilibration period, the SPME fiber was inserted into the vial headspace and exposed at 40°C for 45 minutes.

Gas Chromatography – Mass Spectrometry

A Perkin Elmer (Waltham, MA) Clarus 500 GC-MS system was used to analyze the 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 minutes, followed by a ramp of 7°C/min to 240°C, held for 9.50 minutes for a 40-minute total run time.

Peak Identification and Semi-Quantification

GC-MS chromatograms were analyzed using TurboMass software (Perkin Elmer, Waltham, MA). Peak identifications were confirmed with mass spectra obtained from libraries and linear retention index (LRI) values calculated by a standard curve generated by injecting low and high alkane series (C6-C9 and C8-C25). Since only one internal standard was used, the concentration of each volatile was only semi-quantified. The ratio of the internal standard's concentration to its peak area response is proportional to the ratio of a compound's concentration to its peak area response. Since a known concentration of the internal standard was added to the juice, the concentration of each compound can be calculated. If 50 μL (0.050 mL) of a 2000 μg/mL stock solution of benzyl alcohol is added to 10 mL of orange juice sample, there would be 10 μg/mL of benzyl alcohol in the sample. The concentration of a compound in the sample, [C] = PAC (Peak Area of Compound) / PAIS (Peak Area of Internal Standard) * 10 μg/mL.

Analysis of Flavanone Glycosides

Sample Preparation

Extraction of flavanone glycosides was done using a modified version of methods described by Rouseff and others (*38*) and Bronner and Beecher (*39*). Rhoifolin (400 μL of 1000 μg/mL) (Indofine, Hillsborough, NJ), 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 minutes, 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^2/g surface area, 76Å 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 no faster than 1 drop/second. Afterwards, the cartridge was washed with 5 mL of deionized water. The flavanone glycosides were then 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 minutes. 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 minutes. About 1.5 mL of the solution was filtered into a HPLC autosampler vial using a 0.45 μ nylon filter (Fisherbrand).

High Performance Liquid Chromatography

A Thermo (Waltham, MA) Finnigan Surveyor HPLC system was used to analyze the flavanone glycosides. It contained a reversed phase C-18 column (Phenomenex Luna 5μ C18, 250 x 4.60 mm 5μ) and 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 minutes. The flow rate was set at 1 mL/min and injection volume was 25 μL. Injections were done in triplicate.

Peak Identification and Quantification

HPLC chromatograms were analyzed using Xcalibur software (Thermo Electron Corporation, Waltham, MA). Peak identifications were confirmed by retention time by injecting naringin (Acros Organics NJ), hesperidin (Acros Organics, NJ), and rhoifolin standards. Confirmation was also done through maximum absorbance of certain wavelengths of the UV

spectra as provided by the standards. These patterns were compared with those found in literature. Concentration of compounds (μg/mL) was calculated according to the peak area response of the internal standard, as described above with the GC-MS analysis. The concentration of rhoifolin in the final sample volume was 20 μg/mL. Due to dilution during sample preparation, the concentration of a compound in the sample, [C], = PAC (Peak Area of Compound) / PAIS (Peak Area of Internal Standard) * 20 μg/mL * 2.

Analysis of Polymethoxylated Flavones

Sample Preparation

Extraction of polymethoxylated flavones was done using a modified version of a method described by Mouly, Gaydou, and Arzouyan (*40*). After centrifuging a 10 mL juice sample at 4000 rpm for 15 minutes, the supernatant was carefully separated from the pellet at the bottom of the centrifuge tube. Flavone (30 μL of 100 μg/mL) (Acros Organics, NJ) 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 m^2/g surface area, 76Å pore size, 53 μm 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 at no faster than 1 drop/second. Afterwards, the cartridge was washed with 5 mL of deionized water followed by 3 mL of a purification solution (90% water and 10% methanol). The polymethoxylated flavones were then slowly eluted with 2 mL of methanol into an amber vial. About 1 mL of the eluant was transferred to a HPLC autosampler vial prior to analysis.

High Performance Liquid Chromatography

A Thermo (Waltham, MA) Finnigan Surveyor HPLC system was used to analyze the polymethoxylated flavones. It contained a reversed phase C-18 column (Phenomenex Luna 5μ C18, 250 x 4.60 mm 5μ) and PDA detector monitoring 240, 280, and 325 nm wavelengths. The

mobile phase consisted of a gradient program that began at 45% acetonitrile (kept constant), 50% deionized water, and 5% methanol and ended at 20% deionized water and 35% methanol in 20 minutes. The flow rate was set at 1 ml/min and injection volume was 25 µL. Injections were done in triplicate.

Peak Identification and Quantification

HPLC chromatograms were analyzed using Xcalibur software (Thermo Electron Corporation, Waltham, MA). As with the flavanone glycosides analysis, peak identifications were confirmed by retention time by injecting sinensetin, nobiletin, flavone, and tangeretin standards. Maximum absorbance of certain wavelengths of the UV spectra provided by the standards was also used for confirmation. These patterns were compared with those found in literature. Concentration of compounds (µg/mL) was calculated according to the peak area response of the internal standard, as described by the previous analyses. The concentration of flavone in the final sample volume was 1.5 µg/mL. Due to concentrating during sample preparation, the concentration of a compound in the sample, [C], = PAC (Peak Area of Compound) / PAIS (Peak Area of Internal Standard) * 1.5 µg/mL * 1/5.

Analysis of Limonin

Sample Preparation

Juice samples were prepared according to a modified version of a procedure described by Widmer and Haun (*41*). 3 mL of whole juice was placed in a round-bottom centrifuge tube and heated in a 90°C water bath for 10 minutes in order to convert remaining LARL to limonin and to dissolve any precipitated limonin. The sample was diluted with 3 mL of 40% aqueous acetonitrile and thoroughly stirred using a Vortex for 5 seconds. The solution was filtered using a 0.45 µm nylon filter with a glass microfiber before filling a HPLC autosampler vial.

High Performance Liquid Chromatography

A Perkin Elmer (Waltham, MA) Series 200 Autosampler connected to two 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 µL. Total run time was 20 minutes, with valve switches at 3 and 15 minutes. A Zorbax CN column (4.6 x 150 5 µL) was used for limonin.

Peak Identification and Quantification

Chromatograms were analyzed using TotalChrom software (Perkin Elmer, Waltham, MA). Peak identification was confirmed with injections of limonin standard at different concentrations (20, 10, 5, and 1 µg/mL). Concentration of compounds (µg/mL) was calculated according to a standard curve generated by the limonin standard injections.

°Brix and %Acid Analysis

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 (*42*). °Brix values were determined by placing a couple drops of juice onto a digital abbe refractometer (Leica Mark II Plus) in triplicate. Acidity was determined by a titration method in which 25 mL of juice was brought up to 100 mL with deionized water and mixed thoroughly. A couple drops (4 to 5) of phenolphthalein was added as an indicator. The solution was titrated with 0.3123N sodium hydroxide (NaOH) until the color of the solution was constantly a light pink. °Brix values were corrected using the %acid values obtained from the

titrations, in which $°Brix_c = °Brix + (\%acid*0.2)$. Brix/acid ratios were then calculated by $°Brix_c/\%acid$.

Statistical Analysis

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 three juice types (control, asymptomatic, and asymptomatic). This was done using Statistica 7.1 from StatSoft (Tulsa, OK).

CHAPTER 4
RESULTS AND DISCUSSION

Non-Volatiles

Flavanone Glycosides

The objective of this portion of the study was to determine if bitter flavanone glycosides (FGs) were present in the juice samples and might be responsible for the literature reported bitterness in HLB symptomatic juice. Since bitter FGs, such as naringin and neohesperidin, are present only in sour orange and grapefruit varieties, there was a minimal, if any, chance that these compounds were present in Hamlin and Valencia oranges, which are sweet oranges. Sweet oranges do not contain bitter FGs but are abundant in non-bitter FGs, such as narirutin and hesperidin. It was not known, however, if HLB can cause bitter compounds to develop in symptomatic juice.

Using HPLC, it was determined that all juice samples (control, asymptomatic, and symptomatic) from both Hamlin and Valencia samples did not contain any traces of bitter flavanone glycosides. However, three non-bitter flavanone glycosides, including narirutin, hesperidin, and didymin, were identified. Figure 4-1 shows the elution order of the FGs under reverse phase HPLC conditions, where narirutin is the most polar, followed by hesperidin and didymin. Indications (arrows) also show where naringin and neohesperidin would have eluted if they were present in the juice sample. Although they eluted at different times, the UV spectra of narirutin, hesperidin, and didymin were very similar, having λ_{max} at 330, 280, and 240 nm, as shown in Figure 4-1(A). Rhoifolin, the internal standard used in this analysis, is a flavone glycoside instead of a flavanone glycoside, so its UV spectra differed from the other compounds with λ_{max} at 340, 270, and 240 nm.

Except for narirutin in all samples within the April 4, 2008 harvesting date, there was a significant difference ($\alpha = 0.05$) between control and symptomatic juices. Changes in FG concentration from control ranged as low as 4% and as high as 343%, but there were no consistent trends, as seen in Figure 4-3.

Hesperidin was a difficult compound to examine due to its limited solubility. It is almost insoluble in water, so standard solutions were made up in dimethylforamide (DMF) and acetonitrile. In this study, concentration of hesperidin was reduced after freeze thaw cycles, even though sample preparation and HPLC conditions remained unchanged. During normal sample preparation, the juice was centrifuged and only the supernatant was analyzed while the pellet was discarded. Therefore, only dissolved hesperidin was analyzed. However, hesperidin can readily precipitate out of solution, and work by Gil-Izquierdo and others (*43*) reported that freezing decreased the concentration of dissolved hesperidin, due to precipitation. In order to measure the amount of total hesperidin, extra steps were followed to solubilize precipitated hesperidin. DMF was added to extract and solubilize hesperidin. The resulting solution was combined with that from the supernatant extraction and analyzed with HPLC so that total hesperidin (soluble and insoluble) was determined.

Another symptom of HLB found in symptomatic fruit was found the presence of white crystals, varying in size, within juice segment membranes. Presently, the composition of the crystals is unknown, but given the nature of hesperidin, it has been hypothesized that the crystals are precipitated and crystallized hesperidin.

Polymethoxylated Flavones

Polymethoxylated flavones (PMFs) are also reported to impart bitterness (*24*). Given that these juice samples were hand squeezed instead of mechanically squeezed using commercial extractors, the amount of PMF's and other peel components found in the juice was expected to

be lower than commercial juices. Highest levels of PMF's are found in the peel particularly the flavedo and corresponding peel oil. A subsequent study (*27*) determined PMF concentrations in commercial and hand extracted juices as well as determining bitterness taste threshold levels for five PMFs. In both juice types, the concentrations of all PMF's were far below their respective taste thresholds. However, PMF's in that study were determined using thin-layer chromatography (TLC) and not HPLC. An ensuing HPLC study (*44*) compared PMF values obtained using HPLC with those obtained from the TLC procedure. For almost all PMFs quantified, HPLC values were greatly lower than corresponding TLC values, possibly due to the greater chromatographic resolution of HPLC versus that of TLC.

Six major citrus PMFs have been reported, including sinensetin (SIN), hexamethoxyflavone (quercetogetin) (HEX), nobiletin (NOB), tetramethyl-O-scutellarein (SCU), heptamethoxyflavone (HEP), and tangeretin (TAN). Figure 4-4 shows a chromatogram of the separation between SIN, NOB, flavone (the internal standard), and TAN standards, along with their corresponding UV spectra. TAN was not quantified as it coeluted with another compound of similar spectral properties and approximately equal concentrations.

A unique peak was observed only in chromatograms of symptomatic juice samples from Hamlin dates 12/12/2007 and 1/30/2008 and Valencia dates 4/4/2008 and 4/18/2008. It eluted between SIN and HEX at about 9.7 min and is labeled as peak B in Figure 4-5. The unknown peak's UV spectra and those of HEX, SCU, and HEP are also shown. The unknown peak contains a strong response around 325 nm, which is characteristic of polymethoxylated flavones. However, coumarins, another class of compounds that are derived from the same biochemical pathway as flavonoids, possess similar UV spectra as polymethoxylated flavones (*45*). In plants, coumarins are associated with defense, especially against phytopathogens and stress (*46*). This

suggests that the unknown compound may be a coumarin instead of a polymethoxylated flavone, since it seems to appear only in symptomatic juice.

Concentrations (μg/mL) of the polymethoxylated flavones found in the juice samples are summarized in Table 4-1 for Hamlins and Table 4-2 for Valencias. There were significant differences between control and symptomatic juices for the majority of the PMFs for both Hamlin and Valencia cultivars. However, there were no consistent trends in terms of one type of juice having higher or lower concentrations of PMFs than the other, possibly because of different harvesting locations and cultural practices. PMF values in current study were similar to those reported for hand-squeezed juices and never exceeded reported bitterness thresholds. Therefore, bitterness due to PMFs is highly unlikely. For example, the reported taste threshold for SCU, the most potent of the PMFs, is 15 μg/mL, but its concentration found in the samples ranged from 0.056 – 1.084 μg/mL (about 15x lower than the bitterness threshold) (*27*).

Limonin

Limonin is responsible for delayed bitterness in juices in sweet orange cultivars. When juice is freshly extracted the bitter component 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 (*30, 31, 47*), followed by a study by Fong and others (*48*) 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-β-D-glucopyranoside (LG). Therefore there is less of the LARL to be converted to bitter limonin. This "natural debittering" process might enable limonin to act as an indicator of maturity.

Figure 4-6 is the summary of limonin concentration for all three juice types and all six harvesting dates. Significant differences ($\alpha = 0.05$) were especially seen between control and

symptomatic juices. Limonin concentrations were 91-425% higher in symptomatic juice compared to control. This suggests that the conversion LARL to the non bitter glucoside LG was inhibited or delayed in symptomatic juice. Additional HPLC studies to determine LARL and LG concentrations during normal fruit maturation would have to be done to confirm this hypothesis. Nonetheless, the limonin concentration values suggest that symptomatic fruit appears to be immature. For early season Hamlins (12/12/2007 and 12/18/2007) and Valencias (4/4/2008), the differences between control and asymptomatic juices were not significant. The differences were significant for the later harvesting dates, but were still overshadowed by higher limonin concentrations in symptomatic juice. Overall, it seems that the differences decrease as the fruit matures.

According to Guadagni and others (*49*), the average human detection threshold of limonin in orange juice is approximately 6.5 μg/mL. Although limonin levels were higher in symptomatic juice, and ranged from 2.405 – 5.137 μg/mL, they never exceeded the average threshold. Although some bitter sensitive people might be able to detect bitterness in symptomatic juice, the average person would probably not detect it. Work with a trained descriptive analysis panel, which contained panelists with a wide range of bitterness sensitivities, would have to be done to determine if limonin bitterness would be of concern.

°Brix and Acidity

Other non-volatile compounds that play an important role in flavor, especially pertaining to citrus juice flavor, are sugars (i.e. mono- and di-saccharides) and acids (i.e. citric, malic, etc.). These compounds stimulate taste receptors for sweetness and sourness, respectively. Soluble solids, or °Brix, is mostly made up of sugars, including sucrose, fructose, and glucose. Citric acid is the dominant acid in orange juice, so % acid values generally refers to the percentage of citric acid. In orange juice processing, these two values are very important quality markers since they

are also maturity indicators. The USDA has set minimum and maximum values for these quality markers for different grades of orange juice. Generally, as fruit matures, sugar concentration increases as acid concentration decreases. Oranges used for juice processing must mature long enough to contain a minimum °Brix to % acid ratio in order to be sold as a juice from Florida. (*28*). This ratio denotes the balance between sweetness and acidity (*5*).

Figure 4-7 (A) shows that there are significant differences between all three types of juice in °Brix (with acid correction) concentrations for the Hamlins. Also, °Brix values were significantly lower by 13-16% in symptomatic juices compared to control, with the exception of the 12/18/2007 harvesting date. The same general trend was seen with the Valencias in Figure 4-8 (A). °Brix values were significantly lower by 18-24% in symptomatic juices compared to control. Therefore, it can be speculated again that symptomatic juice appears to be from immature fruit even though the juices are from fruit of the same age. In other words, HLB may be delaying maturity.

The Brix/acid ratios are summarized for the Hamlins in Figure 4-7 (B) and the Valencias in Figure 4-8 (B). Since sugar concentration increases as acidity decreases with maturity, an increase in the ratio should be seen. For the Hamlins, the Brix/acid ratio was significantly lower in symptomatic juice compared to control by 8-22%. The same was seen with the Valencias. Because acid titrations for the Valencias could not be repeated because of a lack of sample, statistical analysis on Brix/acid ratios could not be carried out. However, the data seems to suggest that ratios were lower in symptomatic juice compared to control by 45-63%, again suggesting that symptomatic fruit appears to be less mature.

The USDA has specific requirements for grades, or quality standards, of orange juice. For example, Grade A not from concentrate (NFC) orange juice has a minimum Brix/acid ratio of

12.5 (*5*). According to Figure 4-7 (B) and Figure 4-8 (B), almost all symptomatic juice ratios do not meet this minimum requirement.

A Possible Explanation for HLB Apparent Immaturity

A recent study by Kim and others (*50*) on the response of sweet orange (*Citrus sinensis*) leaves to *Candidatus* Liberibacter asiasticus infection, the casual agent of HLB in Florida, indicated that the pathogen affected numerous metabolism categories. 317 genes were down-regulated (decreased gene and corresponding protein expression) while 307 genes were up-regulated (increased gene and corresponding protein expression) in HLB infected leaves. Many of these genes were related to plant defense and stress, nutrient metabolism (i.e. protein, sugar, lipid), metabolite transport, and phytohormones, among others.

Citrus fruit is considered a "sink organ," in which nutrients from mature leaves are transported and stored. Photosynthetic cells are located in the leaves, where light energy is converted to nutrients, especially sugar. Results indicated that genes directly correlated with photosynthesis were not affected by the HLB pathogen. However, more than 4% of the affected genes were related to sugar metabolism, especially for starch synthesis and degradation. Three genes responsible for starch synthesis (AGPase, starch synthase, and granule-bound starch synthase) were up-regulated. There was a higher concentration of sucrose in the HLB leaves compared to the healthy control due to sieve tube blockage, which would also contribute to the accumulation of starch. It was suggested that the blockage would cause nutrient deficiencies in sink organs, which would hinder seed development and fruit maturation. This prior study might help explain why sugar levels were lowest in HLB fruit juice.

Overall, juice from symptomatic fruit may not be bitter, but will probably be more sour and less sweet due to a lower B/A ratio. Also, the claim that symptomatic fruit in South Africa was bitter was from a personal observation and not a trained sensory panel. Furthermore, HLB

disease was already established in the area for more than 30 years by a vector and agent that is different from those found in Florida, where HLB was found in 2005. Therefore, it can be said that the Florida oranges came from newly infected trees.

Candidatus Liberibacter asiaticus, the HLB pathogen, affects orange leaves at a genetic level, resulting in the inability of nutrients to be transported from the leaves to developing fruit. This would cause a delay in maturity and other developmental problems. Elevated limonin and reduced B/A ratios in juices from symptomatic fruit suggest that HLB delays maturity and secondary metabolite formation.

Although limonin causes delayed bitterness, a "natural debittering" process occurs in the fruit as it matures, where limonin's tasteless precursor, limonoate A-ring lactone (LARL), converts to a tasteless limonin glucoside instead of the bitter limonin. Therefore, limonin may be used as a maturity marker. In that sense, significantly higher limonin concentrations in symptomatic juice compared to control suggests that symptomatic fruit appears to be immature.

Volatiles

Volatile Profile

The aroma of a food greatly contributes to its overall flavor, so examining the volatile profile of juices from HLB symptomatic and asymptomatic fruit would determine whether if there were compounds responsible for producing an off-flavor. The polar column (DB-Wax) on the GC-MS separated more than 90 compounds from juice headspace solid phase microextraction (SPME). However, only 50 of these could be identified using the combination of mass spectra and LRI values. Various classes of compounds were found, including alcohols, aldehydes, esters, ketones, and especially terpenes. The compounds that were identified, as well as their concentrations (μg/mL), are summarized in Tables A-1 through A-6. They 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 symptomatic and asymptomatic juice samples compared to control. There were, however, obvious differences in the volatile profile, in terms of concentrations of certain compounds, between control and symptomatic juices, as illustrated in Figure 4-10. Two that stand out are ethyl butanoate, an ester, (B) and valencene, a sesquiterpene, (I). Both happen to be compounds of interest, since they have been associated with fruit maturity. A more detailed discussion on both will follow in the next sections.

Significantly Different Volatiles

Depending on the cultivar, there were a number of compounds out of the 50 identified that were significantly different ($\alpha = 0.05$) between the juice types, Control vs. Symptomatic and Asymptomatic vs. Symptomatic. For Hamlins, the compounds that were consistently significantly different between control and symptomatic juices were ethyl acetate, ethanol, α-pinene, ethyl butanoate, hexanal, β-pinene, myrcene, methyl hexanoate, γ-terpinene, α-terpinolene, hexanol, nonanal, 1-octen-3-ol, linalool, 4-terpineol, ethyl-3-hydroxyhexanoate, α-terpineol, and perillaldehyde. For the comparison of asymptomatic with symptomatic juices, only ethyl octanoate was consistently significantly different. Compounds that were consistent with the comparison of control with symptomatic and asymptomatic with symptomatic included ethyl butanoate, hexanal, β-pinene, linalool, and 4-terpineol.

For the Valencia juices, the compounds that were consistently significantly different between control and symptomatic juices were ethyl hexanoate, γ-terpinene, octanal, ethyl octanoate, 1-octen-3-ol, linalool, 4-terpineol, ethyl-3-hydroxyhexanoate, α-terpineol, and caryophyllene oxide. For the comparison of asymptomatic with symptomatic juices, decyl acetate and nootkatone were consistently significantly different. Compounds that were consistent

with both comparisons included ethyl hexanoate, γ-terpinene, ethyl octanoate, 1-octen-3-ol, linalool, ethyl-3-hydroxyhexanoate, and caryophyllene oxide.

The majority of the data suggests that HLB affects classes of compounds differently. For example, with Hamlin juices, the esters (ethyl acetate, ethyl butanoate, methyl hexanoate, and ethyl-3-hydroxyhexanoate) that were consistently significantly different between control and symptomatic juices were 33 – 87% lower in symptomatic compared to control. However, the terpenes and alcohols derived from terpenes (α-pinene, β-pinene, myrcene, γ-terpinene, α-terpinolene, linalool, 4-terpineol, and α-terpineol) were 79 – 773% higher in symptomatic juices compared to control. No clear trend was seen with the aliphatic alcohols (ethanol, hexanol, and 1-octen-3-ol). Surprisingly, the aldehydes (hexanal, nonanal, and perillaldehyde) were 81 – 337% higher in symptomatic juices compared to control.

Except for the aldehydes, the same trend was seen with the esters and terpenes deemed as consistently significantly different for the comparison between control and symptomatic juices in the Valencias. The esters (ethyl hexanoate, ethyl octanoate, and ethyl-3-hydroxyhexanoate) were 29 – 84% lower in symptomatic compared to control juices. The terpenes and alcohols derived from terpenes (γ-terpinene, linalool, 4-terpineol, and α-terpineol) were 80 – 1,320% higher in symptomatic juices compared to control.

Figure 4-11 illustrates the magnitude of the differences for select compounds that are, according to literature, important odor-active compounds. Esters and aldehydes, for example, contribute the most to orange juice odor. Although most of the compounds detected were terpenes, most have very limited aroma activity. Gas chromatography – olfactometry studies would have to be done to confirm the aroma activity of these compounds from the juice samples. All the esters that were found to be significantly different possess aroma activity according to

literature, but at different degrees, with ethyl butanoate being the most potent. Overall, they provide a fruity aroma to orange juices. Of all the terpenes listed, only α-pinene, β-pinene, myrcene, and α-terpinolene are cited to possess aroma activity, and they offer green, woody, and piney aromas. Linalool, a terpene alcohol, has been cited to be the most potent alcohols in juice, providing a sweet, floral, and fruity smell (*17*).

Biochemical Pathways of Volatiles and Maturity

It has been determined that the casual agent of HLB in Florida has affected multiple genes in the leaves to have an effect on fruit development and maturity. Changes in sugar metabolism and the restriction of nutrient flow from leaves to fruits probably accounts for the lower level of sugars in juice from HLB infected oranges. Sucrose can be broken down into glucose, which is a common precursor to various other biochemical pathways for other metabolites (i.e. lipids, amino acids, etc.) and intermediates (i.e. acetyl-CoA) that are important flavor precursors.

A number of odor-active volatile compounds, especially aliphatic aldehydes, alcohols, and esters, are derived from the oxidation of lipids. In fruit, the oxidation of linoleic (C18:2) and linolenic (C18:3) acids yield these compounds. Pathways for this process include α- and β-oxidation, where removal of two-carbon units, in the form of acetyl-CoA, yield shorter chain acids. These would then react with alcohols to produce esters. For instance, a series of β-oxidations of linoleic acid can produce hexanoic acid (C6), which could then react with an alcohol to yield hexanoate esters. Another pathway involves the catalyzed insertion of oxygen into the fatty acid by the enzyme lipoxygenase to yield hydroperoxides. The hydroperoxides can then be further degraded (with or without enzymes) to produce volatile aldehydes and alcohols. The lipoxygenase catalyzed oxidation of linoleic acid, for example, can produce hexanal, an odor-active compound, which can be converted to hexanol (*18*).

For oranges, one indication of fruit maturation is the color change of the rind, or flavedo, from green to a combination of red, orange, and/or yellow pigments. This occurs when chlorophyll in the flavedo degrades, causing an increase of carotenoid expression that these colors are derived from (*51*). Carotenoids are also precursors to many aroma active compounds, particularly damasecnones, damascones, and ionones, that provide sweet, fruity, and floral notes to ripe fruit (*52*). For example, β-ionone, which has a woody, violet, and/or raspberry-like odor, is one of the main carotenoid-derived aroma compounds found in oranges (*17*). Concentration of carotenoids is fairly constant in mature fruit until the chloroplasts in the cholorophyll begin to disappear and chromoplasts surface due to ripening (*52*). The degradation of chloroplasts also release membrane lipids derived from linoleic and linolenic acids (*53*). A number of enzymes from the degradation process then act on these flavor precursors to yield the characteristic aroma compounds found in mature fruit (*52*).

The flavedo of an orange is also rich in terpenoid hydrocarbons that make up more than 90% of total volatiles (*17*). This diverse class of compounds is generated via the mevalonic acid pathway. The production of terpenes is limited to the action of the HMG-CoA reductase enzyme. Studies on this enzyme report that it is important in the early stages of fruit development, with enzyme activity associating with cell division. However, HMG-CoA reductase does not seem to be active when fruit is matured (*54*). Therefore, it can be speculated that there would typically be more terpenes in an immature fruit than a mature fruit. The GC-MS results, shown in Tables 4-3 to 4-8, indicated that terpene concentrations were significantly higher in symptomatic juices compared to control, which suggests that symptomatic fruit may appear immature. Another indication that symptomatic fruit may appear to be immature is the significantly higher concentration of esters in control juices compared to symptomatic juices. As described above,

the precursors that esters are derived from are released when chloroplasts degrade during fruit maturation, so a ripe and mature orange whose peel colors are orange, red, and yellow, would have a higher concentration of esters than an immature orange whose peel colors are not developed.

Valencene and Maturity

The concentration of valencene is the second highest terpene after limonene. It has been shown to lack aroma activity (*55*). However, unlike the majority of the terpenes, valencene concentrations were higher in control juices compared to symptomatic. In some cases, the difference was significant, as seen in Figure 4-13. For the significant differences, valencene was 50 – 67% lower in symptomatic juice compared to control.

Sharon-Asa and others (*56*) studied the *Cstps1* gene that encoded for the valencene synthase 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 again suggests that symptomatic fruit appears to be less mature.

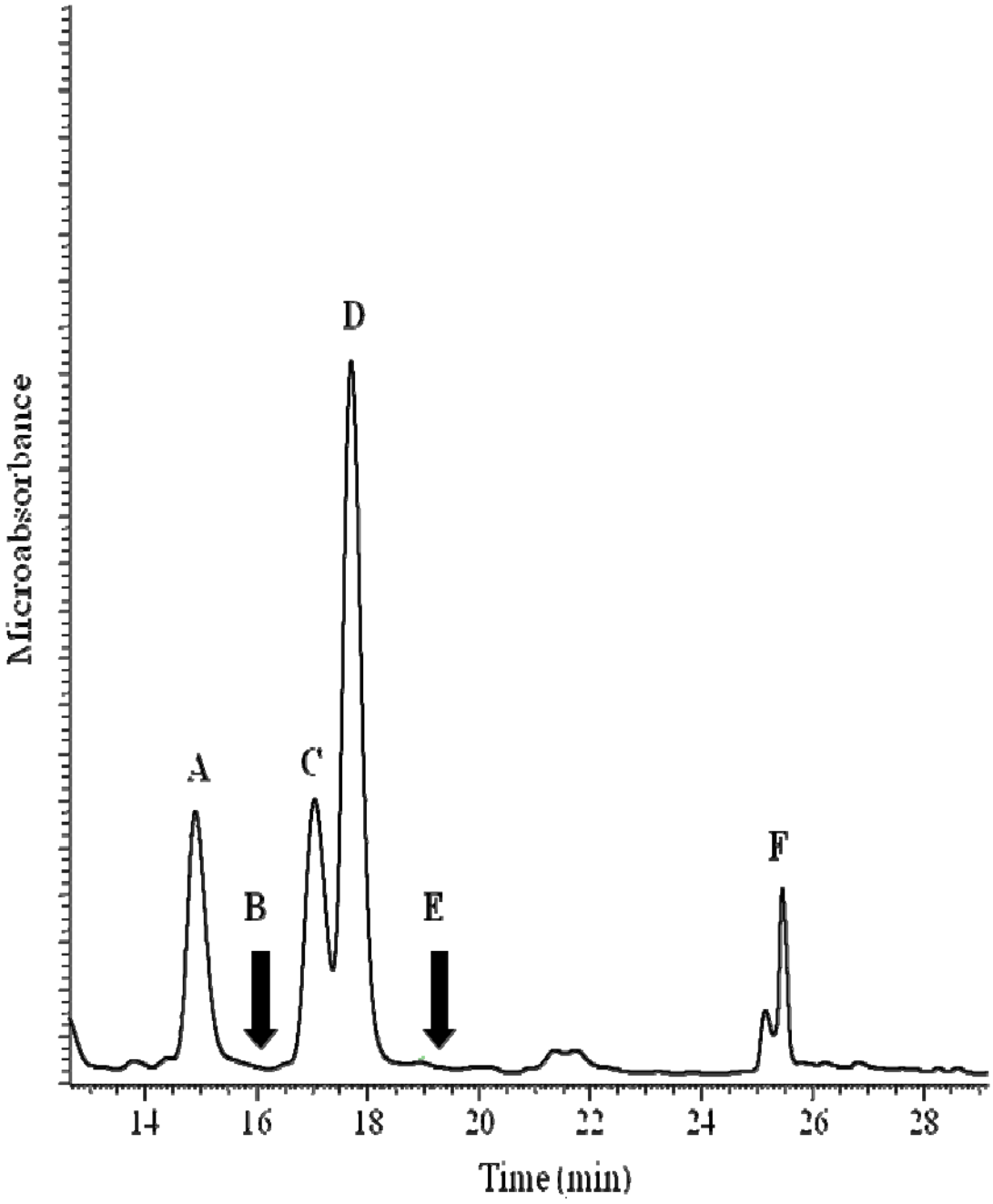


Figure 4-1. Reverse phase HPLC separation of citrus flavanone glycosides of symptomatic juice from 1/30/ 2008. The flavanone glycosides found are labeled: (A) narirutin, (D) hesperidin, and (F) didymin. Arrows indicate where the bitter flavanone glycosides (B) naringin and (E) neohesperidin would have eluted. (C) Rhoifolin is the internal standard used in this analysis.

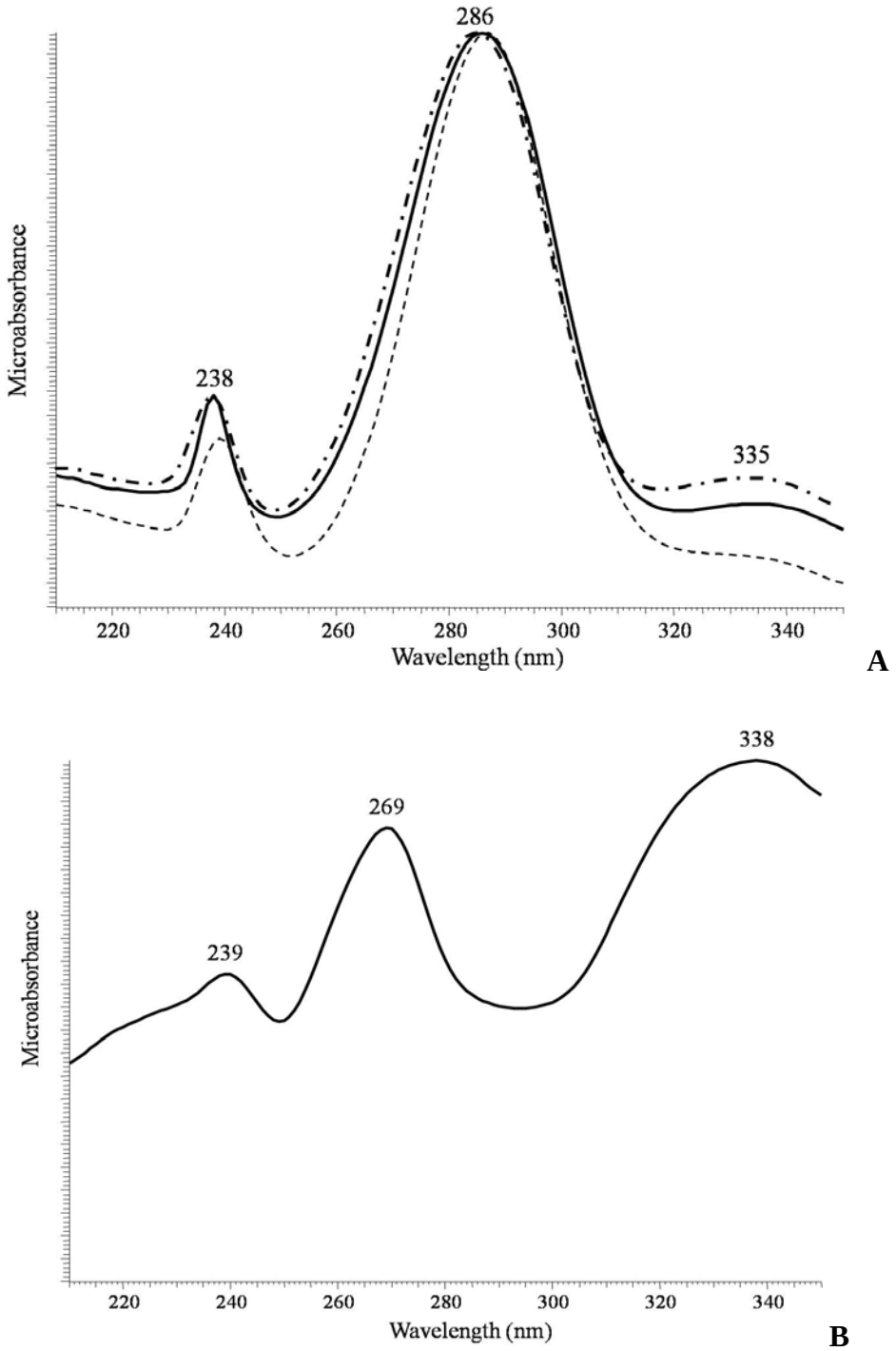
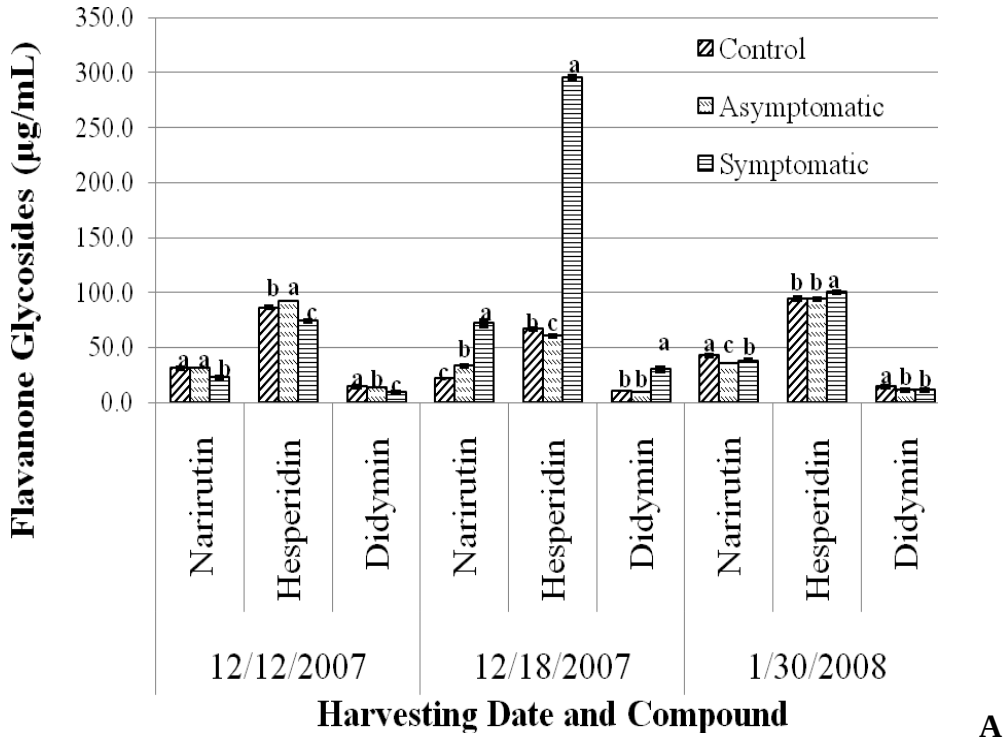
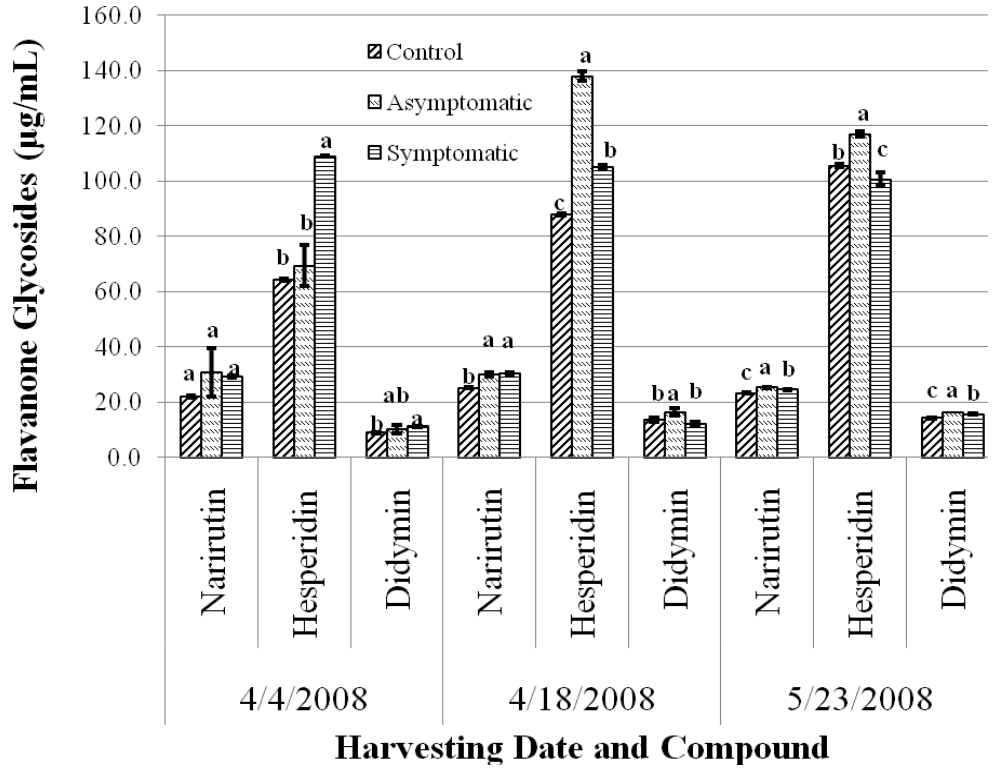


Figure 4-2. UV spectra of eluted chromatographic peaks from Figure 4-1. A) UV spectra of narirutin (solid), hesperidin (dash), and didymin (dash-dot). B) UV spectra of the internal standard, rhoifolin.



A



B

Figure 4-3. Summary of citrus flavanone glycosides. Significant mean separations are represented by letters a, b, c. A) Hamlin samples. B) Valencia samples.

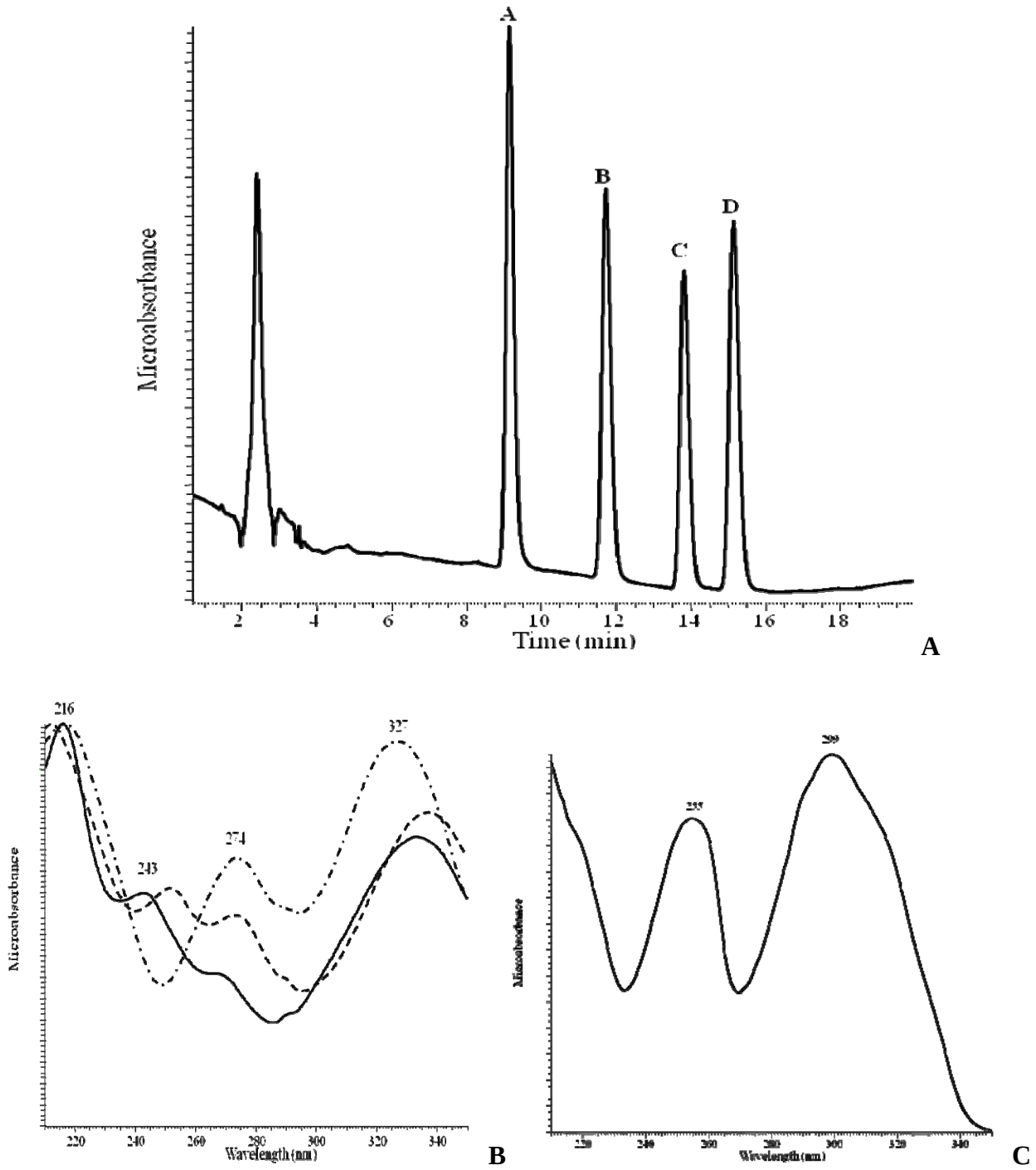


Figure 4-4. HPLC chromatogram and corresponding UV spectra of polymethoxylated flavone standards . A) Reverse phase separation of (A) sinensetin, (B) nobiletin, (C) flavone (internal standard), and (D) tangeretin. B) UV spectra of sinensetin (solid), nobiletin (dash), and tangeretin (dash-dot). C) UV spectra of the internal standard, flavone.

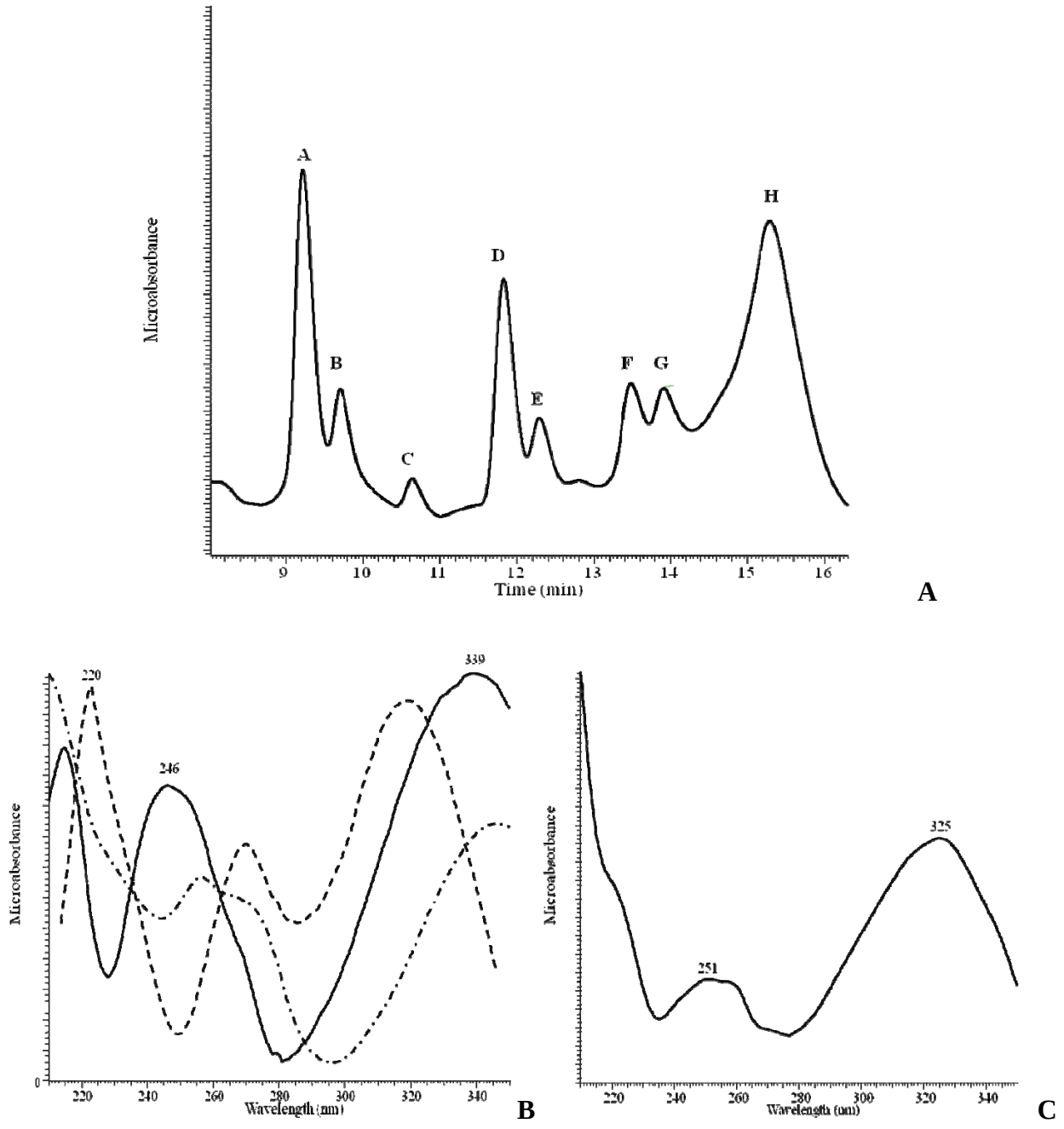


Figure 4-5. HPLC chromatogram of polymethoxylated flavones in symptomatic juice from Valencia 4/4/2008 with UV spectra from select compounds. A) Reverse phase separation of (A) sinensetin, (B) unknown PMF, (C) hexamethoxyflavone, (D) nobiletin, (E) scutellarein, (F) heptamethoxyflavone, (G) flavone, and (H) TAN + coeluted unknown. B) UV spectra of HEX (solid), SCU (dash), and HEP (dash-dot). C) UV spectra of unknown PMF

Table 4-1. Polymethoxylated flavone concentrations (μg/mL) for Hamlin samples at various harvesting dates. Significant mean separations are represented by letters a, b, c.

12/12/2007 Harvesting Date.						
Compound	Control		Asymptomatic		Symptomatic	
Sinensetin	0.289 ± 0.00931	**a**	0.271 ± 0.0153	**a**	0.233 ± 0.0400	**a**
Unknown	N/A		N/A		N/A	
Hexamethoxyflavone	0.135 ± 0.0185	**a**	0.131 ± 0.0135	**a**	0.0366 ± 0.00789	**b**
Nobiletin	0.550 ± 0.0518	**a**	0.510 ± 0.0149	**a**	0.535 ± 0.0268	**a**
Tetramethyl-O-Scutellarein	0.0509 ± 0.0046	**b**	0.118 ± 0.0142	**a**	0.153 ± 0.0247	**a**
Heptamethoxyflavone	0.383 ± 0.00353	**a**	0.241 ± 0.00982	**b**	0.224 ± 0.0244	**b**

12/18/2007 Harvesting Date						
Compound	Control		Asymptomatic		Symptomatic	
Sinensetin	0.135 ± 0.00348	**c**	0.329 ± 0.0457	**b**	1.10 ± 0.0570	**a**
Unknown	N/A		N/A		0.256 ± 0.0164	
Hexamethoxyflavone	N/A		0.0518 ± 0.0140	**b**	0.286 ± 0.0233	**a**
Nobiletin	0.392 ± 0.0118	**b**	0.409 ± 0.100	**b**	1.50 ± 0.0895	**a**
Tetramethyl-O-Scutellarein	0.138 ± 0.00230	**b**	0.113 ± 0.0382	**b**	0.431 ± 0.0682	**a**
Heptamethoxyflavone	0.170 ± 0.00299	**c**	0.314 ± 0.0725	**b**	1.08 ± 0.0635	**a**

1/30/2008 Harvesting Date						
Compound	Control		Asymptomatic		Symptomatic	
Sinensetin	0.301 ± 0.0265	**a**	0.225 ± 0.0193	**b**	0.342 ± 0.0289	**a**
Unknown	N/A		N/A		0.128 ± 0.0250	
Hexamethoxyflavone	0.0996 ± 0.0930	**a**	0.0471 ± 0.00445	**a**	0.0881 ± 0.0179	**a**
Nobiletin	0.200 ± 0.0103	**b**	0.222 ± 0.0291	**b**	0.389 ± 0.0437	**a**
Tetramethyl-O-Scutellarein	0.0183 ± 0.00262	**c**	0.0466 ± 0.00679	**b**	0.128 ± 0.00671	**a**
Heptamethoxyflavone	0.259 ± 0.0285	**a**	0.181 ± 0.0150	**b**	0.286 ± 0.0133	**a**

Table 4-2. Polymethoxylated flavone concentrations (μg/mL) for Valencia samples at various harvesting dates. Significant mean separations are represented by letters a, b, c.

4/4/2008 Harvesting Date						
Compound	Control		Asymptomatic		Symptomatic	
Sinensetin	0.594 ± 0.0344	**b**	0.771 ± 0.0126	**b**	1.37 ± 0.134	**a**
Unknown	N/A		N/A		0.489 ± 0.113	
Hexamethoxyflavone	0.0940 ± 0.00873	**a**	0.0982 ± 0.00702	**a**	0.137 ± 0.0328	**a**
Nobiletin	0.399 ± 0.0320	**b**	0.609 ± 0.0406	**ab**	1.12 ± 0.360	**a**
Tetramethyl-O-Scutellarein	0.138 ± 0.0142	**b**	0.183 ± 0.0243	**ab**	0.426 ± 0.179	**a**
Heptamethoxyflavone	0.192 ± 0.0285	**b**	0.204 ± 0.0116	**b**	0.413 ± 0.0469	**a**

4/18/2008 Harvesting Date						
Compound	Control		Asymptomatic		Symptomatic	
Sinensetin	0.161 ± 0.00759	**c**	0.602 ± 0.0362	**a**	0.258 ± 0.0200	**b**
Unknown	N/A		N/A		0.0216 ± 0.000817	
Hexamethoxyflavone	0.116 ± 0.0521	**ab**	0.173 ± 0.0215	**a**	0.0391 ± 0.0201	**b**
Nobiletin	1.06 ± 0.0203	**a**	0.687 ± 0.0574	**b**	0.351 ± 0.0314	**c**
Tetramethyl-O-Scutellarein	0.544 ± 0.00415	**a**	0.302 ± 0.0437	**b**	0.180 ± 0.0140	**c**
Heptamethoxyflavone	0.177 ± 0.0137	**b**	0.334 ± 0.0113	**a**	0.134 ± 0.0162	**c**

5/23/2008 Harvesting Date						
Compound	Control		Asymptomatic		Symptomatic	
Sinensetin	0.159 ± 0.0284	**b**	0.167 ± 0.0193	**b**	0.446 ± 0.0345	**a**
Unknown	N/A		N/A		N/A	
Hexamethoxyflavone	0.0832 ± 0.0297	**ab**	0.0564 ± 0.00876	**b**	0.104 ± 0.00839	**a**
Nobiletin	0.155 ± 0.0283	**c**	0.329 ± 0.0138	**b**	0.452 ± 0.0319	**a**
Tetramethyl-O-Scutellarein	0.0996 ± 0.0145	**c**	0.246 ± 0.00705	**b**	0.332 ± 0.00904	**a**
Heptamethoxyflavone	0.108 ± 0.0174	**b**	0.0564 ± 0.00876	**c**	0.247 ± 0.0255	**a**

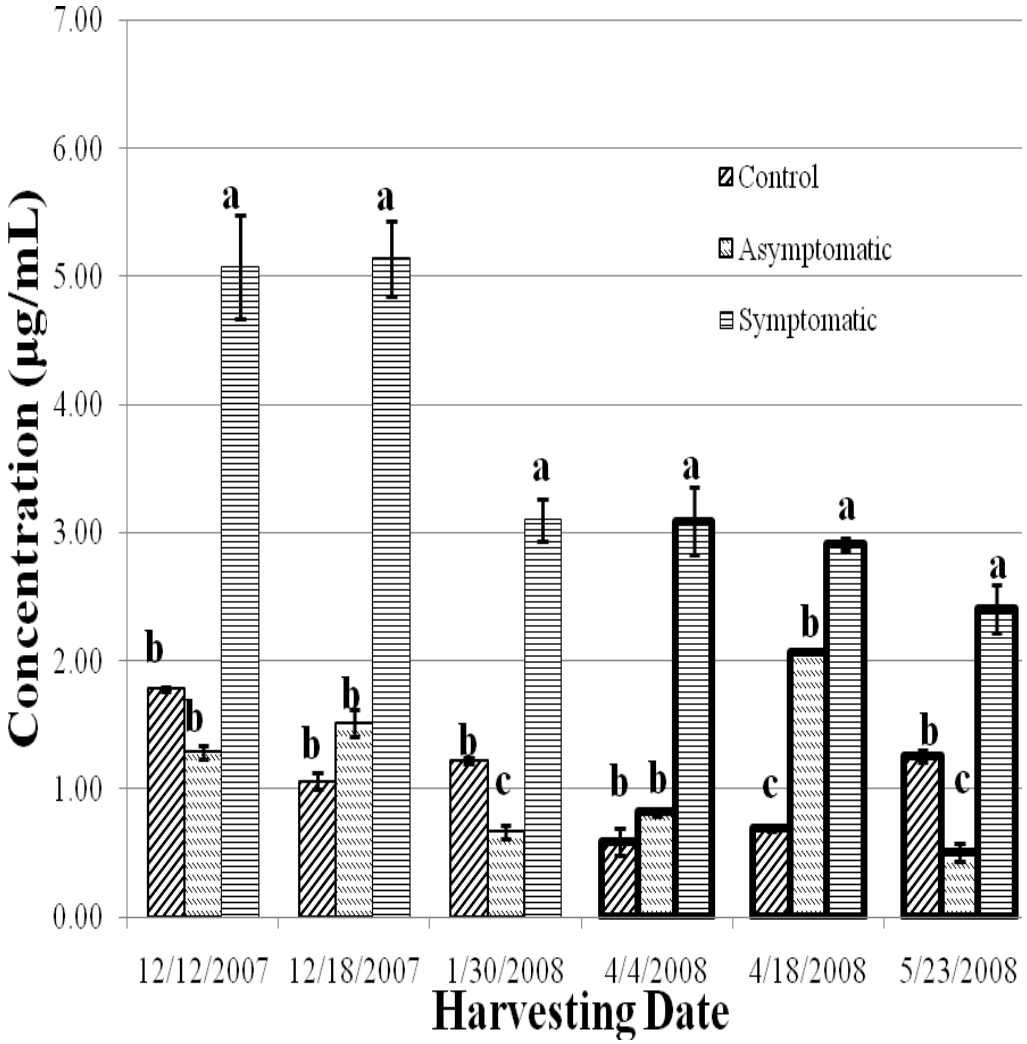
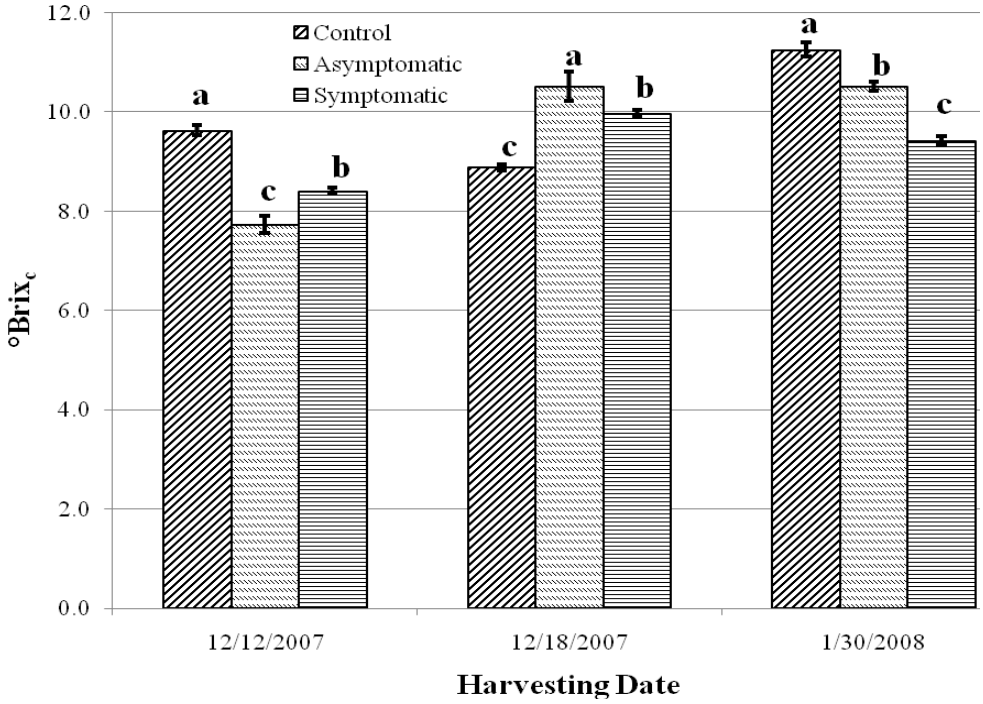
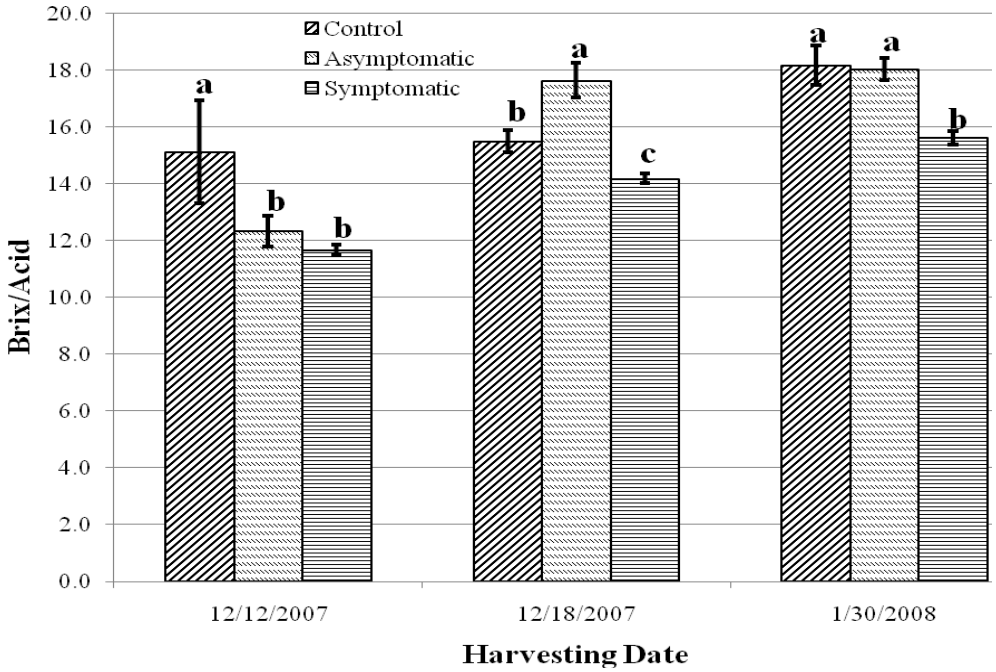


Figure 4-6. Summary of limonin analysis results for both Hamlin and Valencia juice samples. Bars with a weighted outline are Valencia samples. Significant mean separations are represented by letters a, b, c.

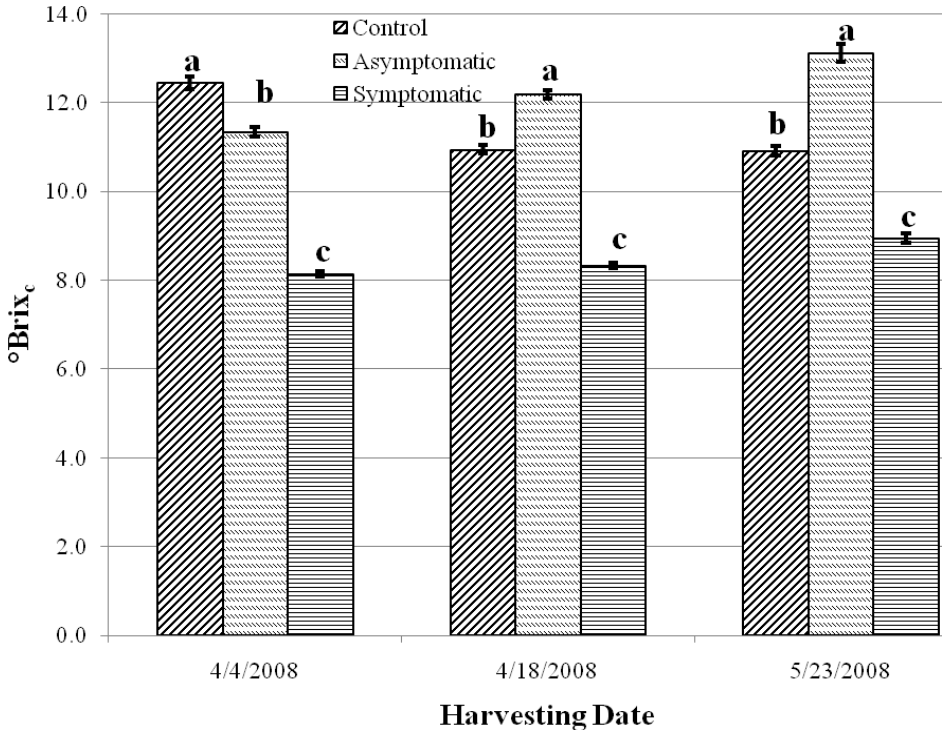


A

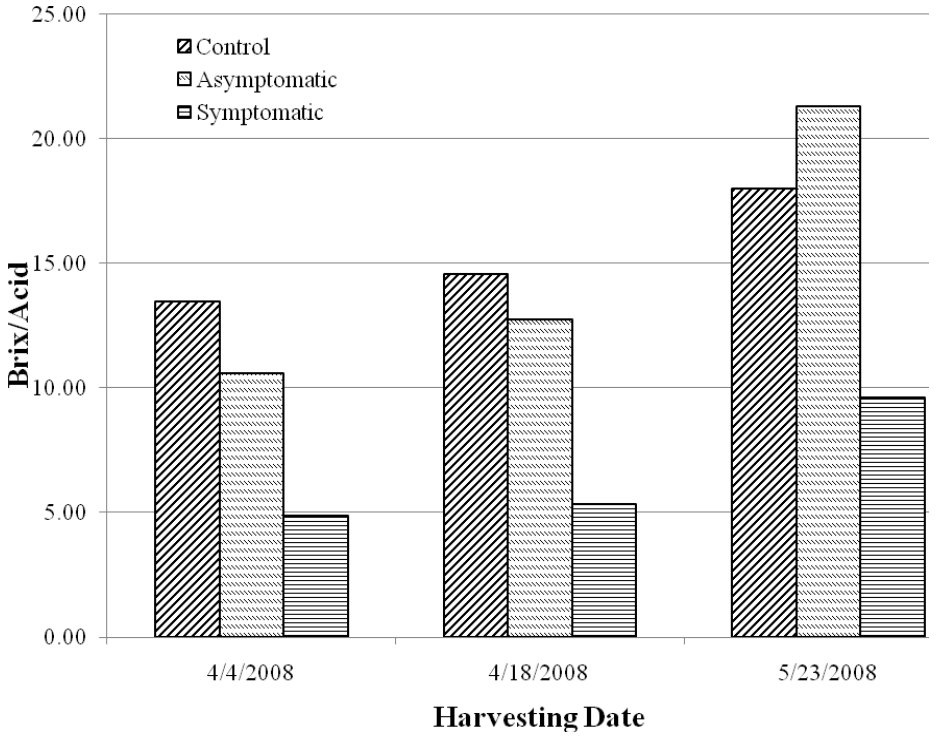


B

Figure 4-7. Results of the sugar and acid analysis performed on the Hamlin samples. Signficiant mean separations are represented by letters a, b, c. A) °Brix results. B) Brix/Acid ratio results.



A



B

Figure 4-8. Results of the sugar and acid analysis performed on the Valencia samples. Signficiant mean separations are represented by letters a, b, c. A) °Brix results. B) Brix/Acid ratio results.

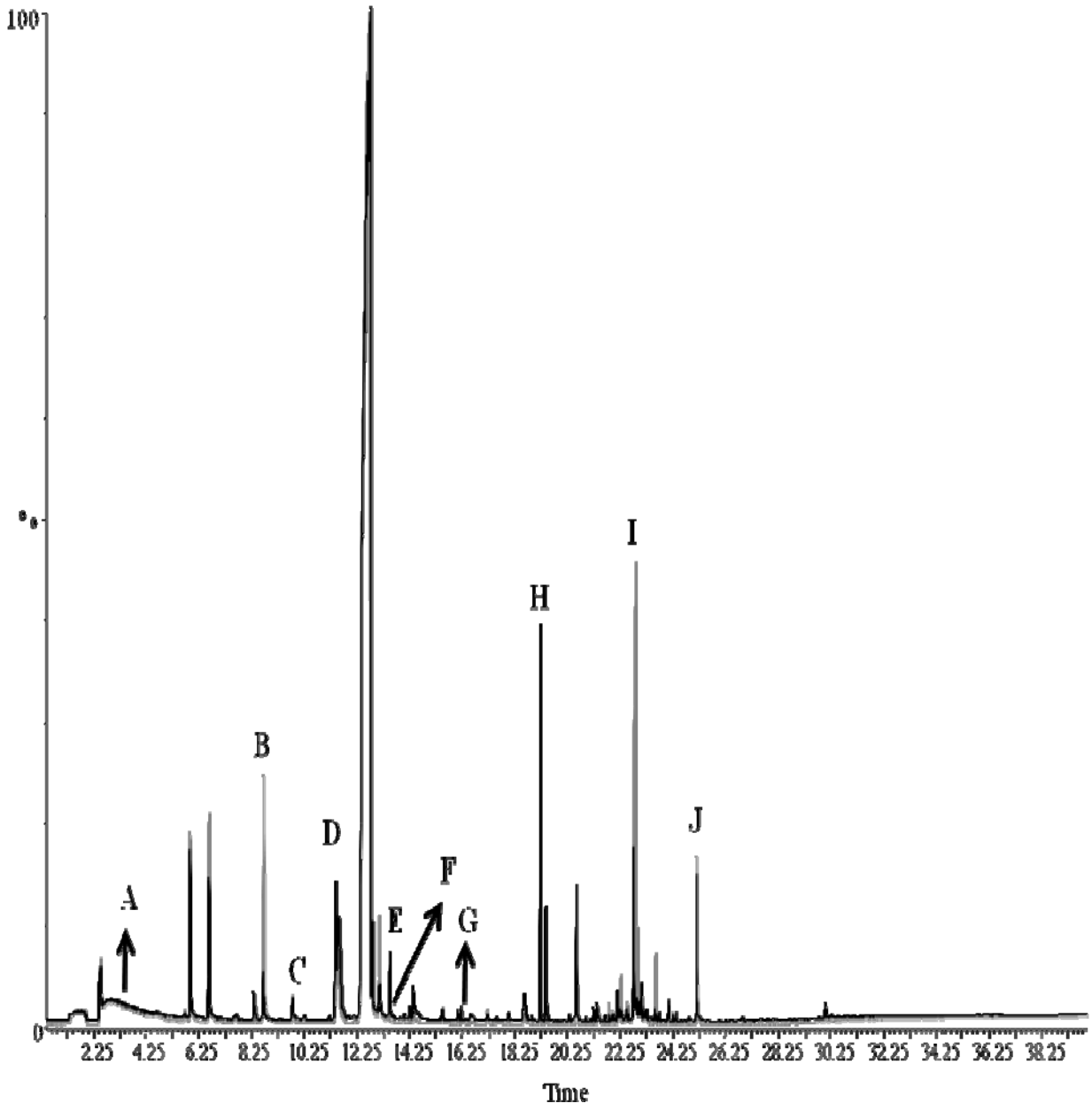


Figure 4-9. GC-MS chromatograms of control and symptomatic juice samples from 4/4/2008. The black outlined chromatogram from the symptomatic sample is superimposed onto the grey outlined chromatogram from the control sample. Select volatiles are labeled: (A) acetaldehyde, (B) ethyl butanoate, (C) hexanal, (D) myrcene, (E) ethyl hexanoate, (F) γ-terpinene, (G) (Z)-3-hexen-1-ol, (H) linalool, (I) valencene, and (J) benzyl alcohol, the internal standard.

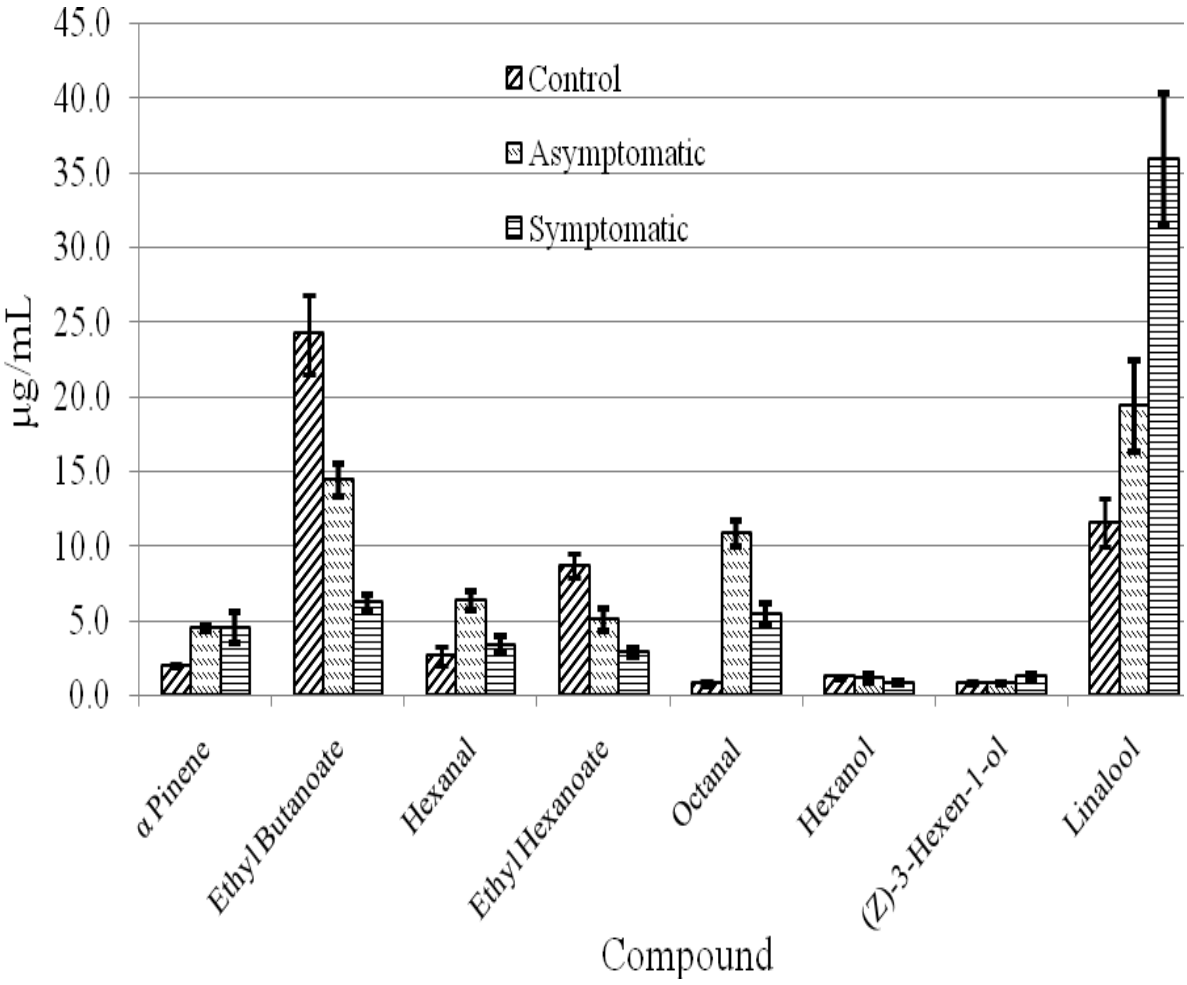
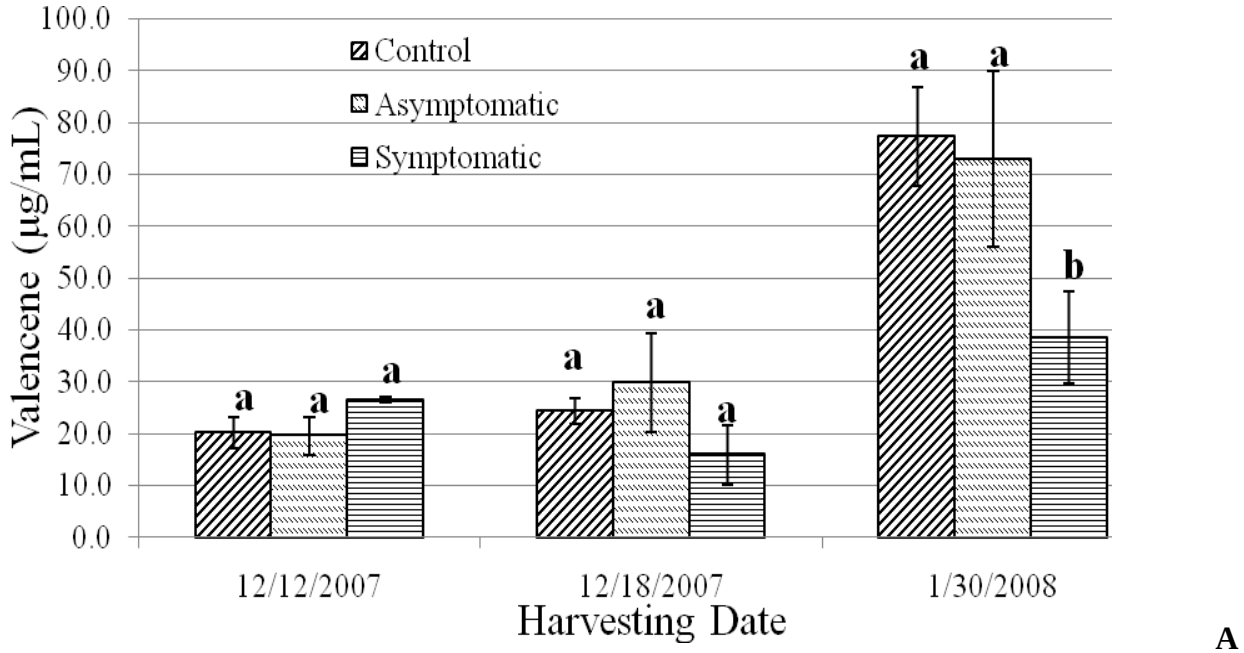
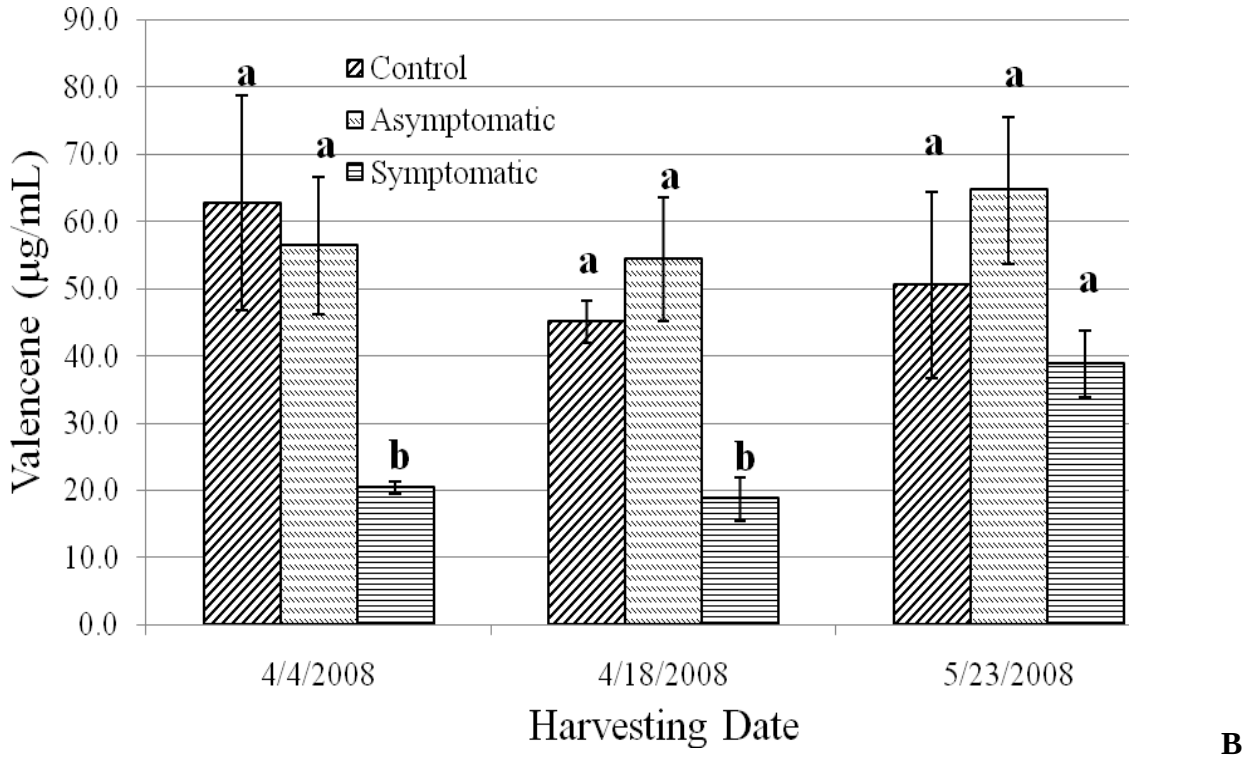


Figure 4-10. Differences of select odor-active volatiles in Valencia 4/18/2008 symptomatic, asymptomatic, and control juices.



A



B

Figure 4-11. Summary of Valencene concentrations (µg/mL). Significant mean separations are represented by letters a, b. A) Hamlins. B) Valencias.

CHAPTER 5
CONCLUSION

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

Overall, there were differences between HLB symptomatic and control juices for sugars, acids, and limonin. However, there was little to no difference in juices from fruit which did not exhibit external HLB symptoms (asymptomatic fruit) to control juices. HLB delays maturity in symptomatic fruit. Sugars (measured as °Brix) were lower and acid levels were higher in symptomatic fruit. Esters, especially ethyl butanoate, were 29-87% lower in symptomatic juices compared to control, while some terpenes (i.e. β-pinene, myrcene, γ-terpinene, linalool) were 79-1,320% higher 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 limonin (91-425% higher than control), no limonin level exceeded the bitterness threshold in orange juice. Therefore, bitterness would not be detected by most people who taste the juice. Flavanone glycosides and polymethoxylated flavones were also eliminated as possible sources of bitterness. Therefore, reported off-flavor associated with HLB symptomatic juices is not bitterness, but apparently stem from a lower concentration of sugars, a higher concentration of acid, and an imbalance of certain volatile compounds.

Results from this study can be used to generate a database on HLB orange juice and flavor. The biochemical pathways that produce the primary (sugars and acids) and secondary metabolites (flavanone glycosides, esters, terpenes, etc.) that were shown to be affected by HLB will have to be closely examined. The idea that symptomatic fruit appears to be immature can be

explored through a comparative study between HLB symptomatic fruit and non-infected immature fruit. Differences in juice peel oil content should be explored as this study only examined hand squeezed juices where oil levels were much lower than commercially extracted juice.

APPENDIX: VOLATILE TABLES

Table A-1. Concentration (μg/mL) of Hamlin 12/12/2007 volatiles confirmed by MS and LRI values. Significant mean separations are represented by a, b, c.

LRI	Compound	Control		Asymptomatic		Symptomatic	
721	Acetaldehyde	0.167 ± 0.0228	**a**	0.232 ± 0.0491	**a**	0.246 ± 0.0638	**a**
901	Ethyl Acetate	0.915 ± 0.171	**a**	0.453 ± 0.0414	**b**	0.556 ± 0.0387	**b**
912	Methanol	13.1 ± 2.28	**a**	13.4 ± 1.74	**a**	16.6 ± 2.24	**a**
951	Ethanol	14.4 ± 1.99	**a**	6.65 ± 0.753	**b**	9.27 ± 1.69	**b**
1002	Methyl Butanoate	0.387 ± 0.0664	**ab**	0.260 ± 0.0314	**b**	0.404 ± 0.0571	**a**
1034	α-Pinene	1.45 ± 0.178	**b**	2.67 ± 0.419	**a**	2.18 ± 0.0470	**a**
1051	Ethyl Butanoate	0.191 ± 0.0339	**c**	7.19 ± 0.888	**b**	14.5 ± 2.06	**a**
1100	Hexanal	0.0884 ± 0.0283	**c**	2.27 ± 0.318	**b**	4.57 ± 0.701	**a**
1120	β-Pinene	0.0681 ± 0.00761	**b**	0.190 ± 0.0489	**a**	0.295 ± 0.0596	**a**
1162	δ-3-Carene	0.115 ± 0.0133	**a**	0.119 ± 0.0124	**a**	0.0770 ± 0.00993	**b**
1173	β-Myrcene	6.39 ± 0.683	**b**	17.6 ± 1.25	**a**	16.3 ± 0.616	**a**
1195	α-Terpinene	0.0747 ± 0.00796	**b**	0.173 ± 0.0236	**a**	0.172 ± 0.0119	**a**
1207	Methyl Hexanoate	0.0933 ± 0.0182	**b**	0.273 ± 0.0217	**ab**	0.562 ± 0.321	**a**
1228	Limonene	166 ± 16.8	**b**	439 ± 45.7	**a**	385 ± 24.5	**a**
1248	Ethyl Hexanoate	0.581 ± 0.0473	**c**	7.77 ± 0.874	**b**	11.1 ± 0.984	**a**
1262	γ-Terpinene	0.426 ± 0.0525	**c**	0.999 ± 0.101	**b**	1.34 ± 0.0324	**a**
1293	*p*-Cymene	0.236 ± 0.200	**b**	0.270 ± 0.00196	**b**	0.548 ± 0.107	**a**
1304	α-Terpinolene	0.182 ± 0.0206	**b**	0.442 ± 0.0198	**a**	0.523 ± 0.0524	**a**
1311	Octanal	0.0829 ± 0.0232	**a**	0.212 ± 0.00736	**a**	1.15 ± 0.744	**a**
1366	1-Hexanol	0.631 ± 0.0573	**b**	0.764 ± 0.0783	**b**	2.48 ± 0.265	**a**
1402	(Z)-3-Hexen-1-ol	2.48 ± 0.235	**a**	1.45 ± 0.157	**b**	3.10 ± 0.353	**a**
1418	Nonanal	0.141 ± 0.0955	**b**	0.615 ± 0.140	**ab**	3.76 ± 2.39	**a**
1452	Ethyl Octanoate	0.0545 ± 0.0356	**c**	1.45 ± 0.134	**a**	1.09 ± 0.134	**b**
1463	1-Octen-3-ol	0.0367 ± 0.00690	**b**	0.0771 ± 0.00791	**b**	0.168 ± 0.0514	**a**
1494	Octyl Acetate	0.115 ± 0.0556	**a**	0.112 ± 0.0122	**a**	0.182 ± 0.0349	**a**
1525	Decanal	0.889 ± 0.431	**a**	1.26 ± 0.220	**a**	3.88 ± 2.29	**a**
1561	Linalool	3.45 ± 0.272	**b**	4.43 ± 0.645	**b**	6.43 ± 0.773	**a**
1572	1-Octanol	0.457 ± 0.164	**b**	2.12 ± 0.194	**a**	2.29 ± 0.167	**a**
1621	β-Elemene	0.162 ± 0.0301	**b**	0.546 ± 0.124	**a**	0.599 ± 0.0259	**a**
1635	4-Terpineol	2.54 ± 0.437	**b**	1.99 ± 0.268	**b**	5.08 ± 0.456	**a**
1639	β-Caryophyllene	0.504 ± 0.0651	**c**	0.840 ± 0.214	**b**	1.21 ± 0.0425	**a**
1673	1-Nonanol	0.188 ± 0.127	**ab**	0.0641 ± 0.00799	**b**	0.371 ± 0.160	**a**
1681	Citronellyl Acetate	0.556 ± 0.0554	**b**	0.735 ± 0.215	**ab**	0.991 ± 0.0413	**a**
1690	β-Selinene	0.188 ± 0.0430	**a**	0.0981 ± 0.0197	**b**	0.1378 ± 0.00146	**ab**
1697	Decyl Acetate	0.152 ± 0.0335	**a**	0.143 ± 0.0230	**a**	0.152 ± 0.0218	**a**
1706	Ethyl-3-Hydroxyhexanoate	0.490 ± 0.0422	**c**	0.669 ± 0.0544	**b**	1.37 ± 0.0658	**a**
1726	α-Terpineol	41.1 ± 2.90	**a**	0.567 ± 0.221	**b**	0.439 ± 0.114	**b**
1766	Valencene	20.2 ± 3.04	**a**	19.6 ± 3.69	**a**	26.5 ± 0.593	**a**

Table A-1. Continued.

LRI	Compound	Control		Asymptomatic		Symptomatic	
1770	α-Selinene	1.73 ± 0.261	**b**	1.91 ± 0.293	**b**	2.55 ± 0.183	**a**
1779	β-Citronellol	0.496 ± 0.0792	**c**	0.942 ± 0.0824	**b**	1.23 ± 0.0660	**a**
1785	Carvone	0.0394 ± 0.0244	**b**	0.400 ± 0.0580	**a**	0.437 ± 0.0222	**a**
1791	Δ-Cadinene	1.02 ± 0.203	**a**	1.25 ± 0.355	**a**	0.839 ± 0.0112	**a**
1820	Nerol	0.0636 ± 0.0108	**b**	0.196 ± 0.0240	**a**	0.207 ± 0.00991	**a**
1841	Perillaldehyde	0.0547 ± 0.0168	**b**	0.261 ± 0.0300	**a**	0.223 ± 0.0181	**a**
1863	Geraniol	0.817 ± 0.0682	**a**	0.534 ± 0.0345	**b**	0.627 ± 0.00937	**b**
1894	α-Ionone	0.141 ± 0.0202	**a**	0.137 ± 0.0394	**a**	0.204 ± 0.0196	**a**
1986	β-Ionone	0.0533 ± 0.0146	**a**	0.0657 ± 0.00562	**a**	0.100 ± 0.0326	**a**
2050	Caryophyllene Oxide	0.0463 ± 0.00542	**b**	0.0702 ± 0.00607	**a**	0.0786 ± 0.00490	**a**
2272	β-Sinensal	0.0972 ± 0.0364	**a**	0.110 ± 0.0368	**a**	0.0911 ± 0.0323	**a**
2576	Nootkatone	0.707 ± 0.0157	**a**	0.580 ± 0.138	**a**	0.769 ± 0.692	**a**

Table A-2. Concentration (μg/mL) of Hamlin 12/18/2007 volatiles confirmed by MS and LRI values. Significant mean separations are represented by a, b, c.

LRI	Compound	Control		Asymptomatic		Symptomatic	
721	Acetaldehyde	0.312 ± 0.0294	**a**	0.330 ± 0.0102	**a**	0.179 ± 0.109	**a**
901	Ethyl Acetate	0.286 ± 0.0435	**a**	0.283 ± 0.0582	**a**	0.144 ± 0.0286	**b**
912	Methanol	14.7 ± 1.57	**a**	15.7 ± 1.24	**a**	18.4 ± 1.72	**a**
951	Ethanol	5.02 ± 0.534	**a**	5.49 ± 0.408	**a**	1.47 ± 0.231	**b**
1002	Methyl Butanoate	0.566 ± 0.137	**a**	0.483 ± 0.0197	**a**	0.377 ± 0.0624	**a**
1034	α-Pinene	1.58 ± 0.126	**b**	3.44 ± 0.0556	**a**	4.35 ± 0.724	**a**
1051	Ethyl Butanoate	16.8 ± 4.11	**a**	9.48 ± 1.90	**b**	2.10 ± 0.176	**c**
1100	Hexanal	5.82 ± 1.20	**b**	5.98 ± 0.402	**b**	12.4 ± 0.913	**a**
1120	β-Pinene	0.184 ± 0.0420	**b**	0.411 ± 0.0966	**b**	1.32 ± 0.125	**a**
1162	δ-3-Carene	0.0990 ± 0.00223	**b**	0.111 ± 0.0122	**b**	0.134 ± 0.00513	**a**
1173	β-Myrcene	9.18 ± 0.638	**b**	21.1 ± 1.77	**a**	22.8 ± 1.48	**a**
1195	α-Terpinene	0.423 ± 0.516	**a**	0.125 ± 0.0203	**a**	0.201 ± 0.0398	**a**
1207	Methyl Hexanoate	0.955 ± 0.316	**a**	0.548 ± 0.149	**b**	0.380 ± 0.162	**b**
1228	Limonene	237 ± 5.92	**b**	605 ± 32.8	**a**	620 ± 72.4	**a**
1248	Ethyl Hexanoate	7.56 ± 0.897	**a**	8.88 ± 0.843	**a**	1.89 ± 0.122	**b**
1262	γ-Terpinene	0.405 ± 0.0488	**b**	1.01 ± 0.0621	**a**	1.24 ± 0.248	**a**
1293	*p*-Cymene	0.182 ± 0.0218	**a**	0.232 ± 0.0204	**a**	0.238 ± 0.0718	**a**
1304	α-Terpinolene	0.174 ± 0.0178	**b**	0.628 ± 0.0980	**a**	0.586 ± 0.110	**a**
1311	Octanal	0.464 ± 0.0322	**a**	0.296 ± 0.0616	**b**	0.426 ± 0.0782	**ab**
1366	1-Hexanol	1.71 ± 0.179	**b**	2.37 ± 0.232	**b**	3.32 ± 0.359	**a**
1402	(Z)-3-Hexen-1-ol	1.94 ± 0.221	**b**	2.68 ± 0.208	**a**	2.90 ± 0.285	**a**
1418	Nonanal	0.597 ± 0.137	**b**	0.767 ± 0.0781	**ab**	1.02 ± 0.142	**a**
1452	Ethyl Octanoate	0.387 ± 0.0695	**b**	1.05 ± 0.0782	**a**	0.0937 ± 0.0168	**c**
1463	1-Octen-3-ol	0.235 ± 0.0210	**a**	0.133 ± 0.0158	**b**	0.134 ± 0.0197	**b**
1494	Octyl Acetate	0.136 ± 0.0295	**c**	0.524 ± 0.0410	**b**	0.941 ± 0.0888	**a**
1525	Decanal	0.752 ± 0.0704	**b**	0.784 ± 0.353	**ab**	1.45 ± 0.312	**a**
1561	Linalool	4.75 ± 0.776	**a**	2.37 ± 0.268	**b**	4.81 ± 0.629	**a**
1572	1-Octanol	3.62 ± 0.495	**a**	3.22 ± 0.205	**a**	3.49 ± 0.324	**a**
1621	β-Elemene	0.541 ± 0.0418	**a**	0.670 ± 0.302	**a**	0.320 ± 0.162	**a**
1635	4-Terpineol	2.44 ± 0.459	**b**	2.58 ± 0.263	**b**	4.37 ± 0.802	**a**
1639	β-Caryophyllene	0.778 ± 0.0744	**a**	0.757 ± 0.216	**a**	0.589 ± 0.155	**a**
1673	1-Nonanol	0.355 ± 0.533	**a**	0.416 ± 0.0253	**a**	0.460 ± 0.0536	**a**
1681	Citronellyl Acetate	0.797 ± 0.0267	**a**	0.876 ± 0.217	**a**	1.14 ± 0.296	**a**
1690	β-Selinene	0.111 ± 0.0128	**a**	0.1194 ± 0.0368	**a**	0.0744 ± 0.0450	**a**
1697	Decyl Acetate	0.181 ± 0.0329	**a**	0.287 ± 0.0649	**a**	0.284 ± 0.0423	**a**
1706	Ethyl-3-Hydroxyhexanoate	1.22 ± 0.254	**a**	1.07 ± 0.607	**a**	0.161 ± 0.00410	**b**
1726	α-Terpineol	0.408 ± 0.0617	**b**	0.372 ± 0.0273	**b**	0.778 ± 0.0772	**a**
1766	Valencene	24.3 ± 2.42	**a**	29.9 ± 9.53	**a**	15.9 ± 5.74	**a**
1770	α-Selinene	2.18 ± 0.241	**a**	3.06 ± 1.22	**a**	1.57 ± 0.608	**a**
1779	β-Citronellol	0.974 ± 0.149	**a**	1.21 ± 0.175	**a**	0.891 ± 0.118	**a**

Table A-2. Continued.

LRI	Compound	Control		Asymptomatic		Symptomatic	
1785	Carvone	1.02 ± 0.230	**a**	0.860 ± 0.0690	**a**	0.357 0.0445	**b**
1791	Δ-Cadinene	0.651 ± 0.159	**a**	0.786 ± 0.149	**a**	0.940 0.332	**a**
1820	Nerol	0.229 ± 0.0352	**a**	0.234 ± 0.0175	**a**	0.225 ± 0.0198	**a**
1841	Perillaldehyde	0.274 ± 0.0277	**b**	0.339 ± 0.0168	**b**	0.496 ± 0.101	**a**
1863	Geraniol	0.624 ± 0.0767	**a**	0.722 ± 0.0441	**a**	0.671 ± 0.00685	**a**
1894	α-Ionone	0.132 ± 0.0150	**b**	0.143 ± 0.0236	**b**	0.407 ± 0.125	**a**
1986	β-Ionone	0.0734 ± 0.00839	**a**	0.0826 ± 0.0152	**a**	0.0570 ± 0.0227	**a**
2050	Caryophyllene Oxide	0.0788 ± 0.00440	**b**	0.131 ± 0.00882	**a**	0.0751 ± 0.0186	**b**
2272	β-Sinensal	0.119 ± 0.00411	**a**	0.128 ± 0.0448	**a**	0.0911 ± 0.0331	**a**
2576	Nootkatone	0.708 ± 0.117	**ab**	0.908 ± 0.123	**a**	0.474 ± 0.214	**b**

Table A-3. Concentration (µg/mL) of Hamlin 1/30/2008 volatiles confirmed by MS and LRI values. Significant mean separations are represented by a, b, c.

LRI	Compound	Control		Asymptomatic		Symptomatic	
721	Acetaldehyde	0.299 ± 0.0565	**a**	0.415 ± 0.0774	**a**	0.309 ± 0.0773	**a**
901	Ethyl Acetate	0.176 ± 0.0123	**b**	0.397 ± 0.0509	**a**	0.363 ± 0.0780	**a**
912	Methanol	12.2 ± 0.891	**a**	13.7 ± 1.51	**a**	14.8 ± 1.64	**a**
951	Ethanol	3.48 ± 0.340	**c**	10.1 ± 1.30	**a**	6.32 ± 0.952	**b**
1002	Methyl Butanoate	0.409 ± 0.0219	**a**	0.506 ± 0.0889	**a**	0.376 ± 0.0138	**a**
1034	α-Pinene	0.879 ± 0.0668	**c**	2.12 ± 0.461	**b**	3.05 ± 0.200	**a**
1051	Ethyl Butanoate	9.61 ± 0.0849	**b**	16.4 ± 1.94	**a**	4.20 ± 0.611	**c**
1100	Hexanal	8.27 ± 0.155	**a**	3.87 ± 0.429	**b**	2.89 ± 0.400	**c**
1120	β-Pinene	0.400 ± 0.0767	**a**	0.202 ± 0.0310	**b**	0.222 ± 0.0639	**b**
1162	δ-3-Carene	0.271 ± 0.0211	**b**	0.615 ± 0.972	**a**	0.383 ± 0.0128	**b**
1173	β-Myrcene	5.78 ± 1.13	**b**	16.5 ± 1.31	**a**	17.8 ± 0.286	**a**
1195	α-Terpinene	0.0640 ± 0.00591	**b**	0.165 ± 0.0392	**a**	0.154 ± 0.0116	**a**
1207	Methyl Hexanoate	1.67 ± 0.3112	**a**	0.483 ± 0.177	**b**	0.366 ± 0.0831	**b**
1228	Limonene	164 ± 8.15	**c**	399 ± 74.2	**b**	527 ± 44.0	**a**
1248	Ethyl Hexanoate	6.52 ± 0.699	**b**	9.77 ± 1.47	**a**	0.123 ± 0.0215	**c**
1262	γ-Terpinene	0.162 ± 0.0158	**c**	0.782 ± 0.238	**b**	1.41 ± 0.144	**a**
1293	*p*-Cymene	0.209 ± 0.0262	**b**	0.319 ± 0.618	**a**	0.209 ± 0.0214	**b**
1304	α-Terpinolene	0.127 ± 0.0221	**b**	0.455 ± 0.113	**a**	0.611 ± 0.0646	**a**
1311	Octanal	0.807 ± 0.126	**a**	0.987 ± 0.132	**a**	0.441 ± 0.131	**b**
1366	1-Hexanol	3.44 ± 0.134	**a**	2.338 ± 0.252	**b**	2.51 ± 0.568	**b**
1402	(Z)-3-Hexen-1-ol	4.64 ± 0.108	**a**	2.80 ± 0.281	**b**	1.36 ± 0.128	**c**
1418	Nonanal	2.46 ± 0.273	**a**	1.15 ± 0.158	**b**	1.44 ± 0.139	**b**
1452	Ethyl Octanoate	0.260 ± 0.0458	**b**	1.252 ± 0.279	**a**	0.395 ± 0.0160	**b**
1463	1-Octen-3-ol	0.442 ± 0.0346	**a**	0.235 ± 0.0297	**b**	0.127 ± 0.0169	**c**
1494	Octyl Acetate	0.205 ± 0.010	**b**	0.150 ± 0.0476	**b**	0.765 ± 0.0406	**a**
1525	Decanal	0.676 ± 0.205	**a**	1.14 ± 0.158	**a**	1.65 ± 0.759	**a**
1561	Linalool	5.81 ± 0.378	**c**	10.1 ± 1.05	**b**	17.1 ± 2.18	**a**
1572	1-Octanol	2.80 ± 0.233	**b**	4.11 ± 0.385	**a**	3.85 ± 0.455	**a**
1621	β-Elemene	1.486 ± 0.272	**a**	1.71 ± 0.376	**a**	0.987 ± 0.286	**b**
1635	4-Terpineol	1.31 ± 0.235	**b**	2.02 ± 0.221	**b**	3.37 ± 0.461	**a**
1639	β-Caryophyllene	1.32 ± 0.228	**a**	1.51 ± 0.419	**a**	1.34 ± 0.394	**a**
1673	1-Nonanol	0.396 ± 0.0413	**a**	0.365 ± 0.0374	**a**	0.351 ± 0.0236	**a**
1681	Citronellyl Acetate	2.36 ± 0.244	**a**	2.25 ± 0.605	**a**	0.551 ± 0.0310	**b**
1690	β-Selinene	0.405 ± 0.0492	**b**	0.361 ± 0.109	**b**	0.986 ± 0.293	**a**
1697	Decyl Acetate	0.331 ± 0.0171	**b**	0.330 ± 0.0366	**b**	0.812 ± 0.0116	**a**
1706	Ethyl-3-Hydroxyhexanoate	1.07 ± 0.0978	**b**	2.27 ± 0.0793	**a**	0.511 ± 0.0384	**c**
1726	α-Terpineol	0.503 ± 0.0667	**b**	0.626 ± 0.0532	**ab**	0.708 ± 0.0753	**a**
1766	Valencene	77.34 ± 9.56	**a**	72.9 ± 17.0	**a**	38.5 ± 8.86	**b**
1770	α-Selinene	8.12 ± 1.19	**a**	7.73 ± 2.09	**a**	3.70 ± 0.979	**b**
1779	β-Citronellol	3.97 ± 0.625	**a**	3.70 ± 1.50	**a**	1.57 ± 0.318	**a**

Table A-3. Continued.

LRI	Compound	Control		Asymptomatic		Symptomatic	
1785	Carvone	1.28 ± 0.149	**a**	0.890 ± 0.0986	**b**	0.339 ± 0.111	**c**
1791	Δ-Cadinene	0.896 ± 0.222	**a**	0.866 ± 0.241	**a**	0.983 ± 0.195	**a**
1820	Nerol	0.191 ± 0.0282	**b**	0.246 ± 0.0327	**ab**	0.296 ± 0.0299	**a**
1841	Perillaldehyde	0.310 ± 0.0112	**b**	0.285 ± 0.0469	**b**	0.6801 ± 0.0633	**a**
1863	Geraniol	0.974 ± 0.110	**ab**	1.080 ± 0.808	**a**	0.850 ± 0.243	**b**
1894	α-Ionone	0.365 ± 0.0296	**a**	0.449 ± 0.0618	**a**	0.480 ± 0.0549	**a**
1986	β-Ionone	0.0931 ± 0.0140	**a**	0.0873 ± 0.0103	**a**	0.0670 ± 0.0159	**a**
2050	Caryophyllene Oxide	0.238 ± 0.0336	**a**	0.291 ± 0.104	**a**	0.229 ± 0.0182	**a**
2272	β-Sinensal	0.106 ± 0.00442	**a**	0.110 ± 0.0324	**a**	0.131 ± 0.0133	**a**
2576	Nootkatone	0.672 ± 0.175	**a**	0.781 ± 0.305	**a**	1.22 ± 0.335	**a**

Table A-4. Concentration (μg/mL) of Valencia 4/4/2008 volatiles confirmed by MS and LRI values. Significant mean separations are represented by a, b, c.

LRI	Compound	Control		Asymptomatic		Symptomatic	
721	Acetaldehyde	0.329 ± 0.0529	**a**	0.349 ± 0.449	**a**	0.224 ± 0.100	**a**
901	Ethyl Acetate	0.594 ± 0.0387	**a**	0.548 ± 0.0400	**a**	0.210 ± 0.0316	**b**
912	Methanol	13.4 ± 1.10	**a**	14.9 ± 1.27	**a**	14.8 ± 0.846	**a**
951	Ethanol	12.6 ± 0.212	**a**	12.7 ± 0.706	**a**	10.3 ± 0.868	**b**
1002	Methyl Butanoate	0.392 ± 0.340	**a**	0.361 ± 0.0329	**a**	0.393 ± 0.0516	**a**
1034	α-Pinene	2.44 ± 0.428	**a**	3.36 ± 0.859	**a**	2.66 ± 0.0230	**a**
1051	Ethyl Butanoate	13.2 ± 0.328	**a**	12.4 ± 0.779	**a**	5.15 ± 0.454	**b**
1100	Hexanal	4.45 ± 0.278	**a**	3.06 ± 0.4111	**b**	1.35 ± 0.1888	**c**
1120	β-Pinene	0.283 ± 0.0301	**a**	0.278 ± 0.0544	**a**	0.246 ± 0.0185	**a**
1162	δ-3-Carene	0.196 ± 0.0131	**a**	0.110 ± 0.00788	**b**	0.139 ± 0.0180	**b**
1173	β-Myrcene	15.8 ± 1.36	**a**	17.9 ± 1.24	**a**	16.9 ± 0.151	**a**
1195	α-Terpinene	0.119 ± 0.00883	**a**	0.204 ± 0.0321	**a**	0.254 ± 0.165	**a**
1207	Methyl Hexanoate	0.641 ± 0.487	**a**	0.887 ± 0.487	**a**	0.123 ± 0.0354	**a**
1228	Limonene	514 ± 40.7	**b**	621 ± 57.0	**a**	433 ± 8.16	**b**
1248	Ethyl Hexanoate	11.2 ± 0.802	**a**	11.7 ± 0.821	**a**	3.09 ± 0.570	**b**
1262	γ-Terpinene	0.792 ± 0.113	**c**	2.49 ± 0.272	**b**	3.16 ± 0.0706	**a**
1293	*p*-Cymene	0.367 ± 0.0713	**b**	0.581 ± 0.0471	**a**	0.422 ± 0.0399	**b**
1304	α-Terpinolene	0.535 ± 0.0569	**b**	0.814 ± 0.0639	**a**	0.629 ± 0.0189	**b**
1311	Octanal	1.29 ± 0.0580	**a**	0.697 ± 0.0563	**b**	0.281 ± 0.0394	**c**
1366	1-Hexanol	1.33 ± 0.0742	**a**	1.30 ± 0.0229	**ab**	1.13 ± 0.116	**b**
1402	(Z)-3-Hexen-1-ol	1.44 ± 0.103	**a**	1.44 ± 0.0889	**a**	1.48 ± 0.108	**a**
1418	Nonanal	0.624 ± 0.0662	**ab**	0.507 ± 0.0286	**b**	0.782 ± 0.164	**a**
1452	Ethyl Octanoate	2.04 ± 0.165	**b**	2.72 ± 0.189	**a**	0.590 ± 0.0378	**c**
1463	1-Octen-3-ol	0.166 ± 0.00375	**a**	0.129 ± 0.00777	**b**	0.0888 ± 0.0167	**c**
1494	Octyl Acetate	2.31 ± 0.149	**a**	2.52 ± 0.265	**a**	1.12 ± 0.0967	**b**
1525	Decanal	2.10 ± 0.307	**a**	1.71 ± 0.694	**ab**	0.989 ± 0.113	**b**
1561	Linalool	9.15 ± 0.685	**b**	7.37 ± 0.430	**b**	19.2 ± 1.49	**a**
1572	1-Octanol	8.41 ± 0.408	**a**	6.10 ± 0.231	**b**	3.82 ± 0.207	**c**
1621	β-Elemene	1.34 ± 0.428	**a**	1.21 ± 0.396	**a**	0.762 ± 0.0664	**a**
1635	4-Terpineol	1.96 ± 0.465	**c**	4.02 ± 0.261	**b**	6.17 ± 0.544	**a**
1639	β-Caryophyllene	1.26 ± 0.189	**a**	1.08 ± 0.227	**a**	0.616 ± 0.0332	**b**
1673	1-Nonanol	0.871 ± 0.0379	**a**	0.658 ± 0.0452	**b**	0.336 ± 0.0180	**c**
1681	Citronellyl Acetate	1.10 ± 0.131	**b**	3.55 ± 0.624	**a**	1.98 ± 0.136	**b**
1690	β-Selinene	0.312 ± 0.113	**a**	0.256 ± 0.0673	**ab**	0.102 ± 0.0136	**b**
1697	Decyl Acetate	0.864 ± 0.135	**b**	1.845 ± 0.544	**a**	0.754 ± 0.218	**b**
1706	Ethyl-3-Hydroxyhexanoate	1.46 ± 0.080	**a**	1.08 ± 0.0921	**b**	0.222 ± 0.0183	**c**
1726	α-Terpineol	0.571 ± 0.0169	**b**	0.543 ± 0.0643	**b**	1.11 ± 0.0880	**a**
1766	Valencene	62.8 ± 16.0	**a**	56.4 ± 10.2	**a**	20.4 ± 0.900	**b**
1770	α-Selinene	7.02 ± 2.61	**a**	6.09 ± 1.84	**a**	2.68 ± 0.351	**a**
1779	β-Citronellol	3.41 ± 1.39	**a**	2.22 ± 0.358	**a**	1.84 ± 0.868	**a**

Table A-4. Continued.

LRI	Compound	Control		Asymptomatic		Symptomatic	
1785	Carvone	0.994 ± 0.105	**a**	0.878 ± 0.172	**a**	0.808 ± 0.0536	**a**
1791	Δ-Cadinene	0.441 ± 0.262	**a**	0.890 ± 0.639	**a**	0.473 ± 0.0690	**a**
1820	Nerol	0.588 ± 0.0343	**a**	0.444 ± 0.0379	**b**	0.578 ± 0.0413	**a**
1841	Perillaldehyde	0.666 ± 0.0160	**a**	0.704 ± 0.0415	**a**	0.681 ± 0.0672	**a**
1863	Geraniol	1.21 ± 0.0137	**a**	1.17 ± 0.0888	**a**	0.751 ± 0.0376	**b**
1894	α-Ionone	0.443 ± 0.01061	**a**	0.481 ± 0.0566	**a**	0.295 ± 0.126	**a**
1986	β-Ionone	0.114 ± 0.00517	**a**	0.126 ± 0.00993	**a**	0.0549 ± 0.00460	**b**
2050	Caryophyllene Oxide	0.243 ± 0.00969	**a**	0.244 ± 0.0151	**a**	0.0802 ± 0.00127	**b**
2272	β-Sinensal	0.223 ± 0.0249	**a**	0.243 ± 0.0174	**a**	0.298 ± 0.0702	**a**
2576	Nootkatone	1.47 ± 0.0488	**a**	1.69 ± 0.184	**a**	0.639 ± 0.308	**b**

Table A-5. Concentration (μg/mL) of Valencia 4/18/2008 volatiles confirmed by MS and LRI values. Significant mean separations are represented by a, b, c.

LRI	Compound	Control		Asymptomatic		Symptomatic	
721	Acetaldehyde	0.545 ± 0.0610	**a**	0.452 ± 0.056	**a**	0.339 ± 0.123	**a**
901	Ethyl Acetate	1.14 ± 0.110	**a**	1.10 ± 0.0301	**a**	0.758 ± 0.429	**a**
912	Methanol	14.8 ± 1.30	**a**	15.8 ± 0.305	**a**	17.8 ± 2.59	**a**
951	Ethanol	16.9 ± 1.94	**a**	18.1 ± 1.64	**a**	17.6 ± 4.11	**a**
1002	Methyl Butanoate	0.538 ± 0.0498	**b**	0.532 ± 0.0151	**b**	0.770 ± 0.112	**a**
1034	α-Pinene	2.02 ± 0.113	**b**	4.51 ± 0.215	**a**	4.53 ± 1.02	**a**
1051	Ethyl Butanoate	24.2 ± 2.65	**a**	14.4 ± 1.11	**b**	6.19 ± 0.599	**c**
1100	Hexanal	2.65 ± 0.650	**b**	6.30 ± 0.617	**a**	3.40 ± 0.598	**b**
1120	β-Pinene	0.177 ± 0.0351	**b**	0.503 ± 0.0141	**a**	0.448 ± 0.0714	**a**
1162	δ-3-Carene	0.176 ± 0.0139	**c**	0.335 ± 0.0107	**b**	0.507 ± 0.0815	**a**
1173	β-Myrcene	13.4 ± 0.761	**b**	19.7 ± 0.666	**a**	22.3 ± 2.80	**a**
1195	α-Terpinene	0.109 ± 0.00679	**b**	0.202 ± 0.0133	**a**	0.225 ± 0.0209	**a**
1207	Methyl Hexanoate	0.260 ± 0.0736	**a**	0.247 ± 0.0753	**a**	0.712 ± 0.306	**a**
1228	Limonene	376 ± 31.0	**b**	741 ± 32.1	**a**	674 ± 107	**a**
1248	Ethyl Hexanoate	8.69 ± 0.837	**a**	5.10 ± 0.727	**b**	2.89 ± 0.303	**c**
1262	γ-Terpinene	0.500 ± 0.0293	**b**	0.120 ± 0.0454	**b**	7.10 ± 1.29	**a**
1293	*p*-Cymene	0.250 ± 0.0205	**b**	0.543 ± 0.00733	**ab**	0.661 ± 0.267	**a**
1304	α-Terpinolene	0.336 ± 0.0150	**b**	1.11 ± 0.937	**a**	1.29 ± 0.326	**a**
1311	Octanal	0.795 ± 0.140	**c**	10.9 ± 0.874	**a**	5.40 ± 0.737	**b**
1366	1-Hexanol	1.22 ± 0.141	**a**	1.14 ± 0.264	**a**	0.869 ± 0.122	**a**
1402	(Z)-3-Hexen-1-ol	0.856 ± 0.963	**b**	0.804 ± 0.0631	**b**	1.23 ± 0.184	**a**
1418	Nonanal	0.697 ± 0.136	**b**	2.89 ± 0.0486	**a**	2.33 ± 1.19	**ab**
1452	Ethyl Octanoate	1.18 ± 0.0299	**a**	1.30 ± 0.0894	**a**	0.511 ± 0.0885	**b**
1463	1-Octen-3-ol	0.165 ± 0.0103	**a**	0.158 ± 0.0101	**a**	0.0972 ± 0.0136	**b**
1494	Octyl Acetate	0.342 ± 0.0239	**c**	1.64 ± 0.0120	**a**	0.976 ± 0.154	**b**
1525	Decanal	1.40 ± 0.297	**c**	12.6 ± 0.209	**a**	5.68 ± 1.68	**b**
1561	Linalool	11.6 ± 1.59	**b**	19.4 ± 3.07	**b**	35.9 ± 4.39	**a**
1572	1-Octanol	5.03 ± 0.372	**b**	9.23 ± 1.13	**a**	9.14 ± 0.947	**a**
1621	β-Elemene	0.774 ± 0.0626	**ab**	0.998 ± 0.130	**a**	0.678 ± 0.157	**b**
1635	4-Terpineol	1.75 ± 0.232	**c**	7.27 ± 1.08	**b**	11.0 ± 1.24	**a**
1639	β-Caryophyllene	0.888 ± 0.0909	**ab**	1.15 ± 0.277	**a**	0.608 ± 0.146	**b**
1673	1-Nonanol	0.359 ± 0.01530	**b**	0.971 ± 0.0986	**a**	1.17 ± 0.167	**a**
1681	Citronellyl Acetate	0.606 ± 0.0172	**b**	1.77 ± 0.0651	**a**	1.63 ± 0.204	**a**
1690	β-Selinene	0.220 ± 0.0233	**a**	0.205 ± 0.0523	**a**	0.0848 ± 0.00970	**b**
1697	Decyl Acetate	0.404 ± 0.0177	**c**	0.970 ± 0.0397	**a**	0.597 ± 0.0829	**b**
1706	Ethyl-3-Hydroxyhexanoate	1.37 ± 0.0780	**a**	1.33 ± 0.115	**a**	0.309 ± 0.0168	**b**
1726	α-Terpineol	0.611 ± 0.0461	**c**	1.23 ± 0.0692	**b**	2.20 ± 0.279	**a**
1766	Valencene	45.0 ± 3.13	**a**	54.4 ± 9.18	**a**	18.7 ± 3.24	**b**
1770	α-Selinene	4.42 ± 0.339	**a**	5.34 ± 0.913	**a**	2.62 ± 0.384	**b**
1779	β-Citronellol	1.96 ± 0.0604	**a**	2.46 ± 0.379	**a**	2.66 ± 0.371	**a**

Table A-5. Continued.

LRI	Compound	Control		Asymptomatic		Symptomatic	
1785	Carvone	0.662 ± 0.0586	**b**	1.51 ± 0.186	**a**	0.460 ± 0.0567	**b**
1791	Δ-Cadinene	0.418 ± 0.0665	**a**	0.929 ± 0.302	**a**	0.930 ± 0.200	**a**
1820	Nerol	0.312 ± 0.0166	**b**	0.535 ± 0.0666	**a**	0.649 ± 0.0831	**a**
1841	Perillaldehyde	0.609 ± 0.0768	**c**	1.31 ± 0.119	**b**	1.93 ± 0.151	**a**
1863	Geraniol	0.839 ± 0.0710	**a**	0.736 ± 0.0499	**a**	0.790 ± 0.102	**a**
1894	α-Ionone	0.294 ± 0.0220	**c**	0.424 ± 0.00457	**b**	0.559 ± 0.0690	**a**
1986	β-Ionone	0.0971 ± 0.0104	**b**	0.124 ± 0.00672	**a**	0.0533 ± 0.00632	**c**
2050	Caryophyllene Oxide	0.138 ± 0.00553	**c**	0.219 ± 0.00933	**b**	0.249 ± 0.0153	**a**
2272	β-Sinensal	0.161 ± 0.0167	**b**	0.288 ± 0.00990	**a**	0.352 ± 0.0824	**a**
2576	Nootkatone	0.995 ± 0.0563	**ab**	1.35 ± 0.129	**a**	0.577 ± 0.296	**b**

Table A-6. Concentration (μg/mL) of Valencia 5/23/2008 volatiles confirmed by MS and LRI values. Significant mean separations are represented by a, b, c.

LRI	Compound	Control		Asymptomatic		Symptomatic	
721	Acetaldehyde	0.655 ± 0.137	**a**	0.793 ± 0.132	**a**	0.750 ± 0.282	**a**
901	Ethyl Acetate	1.67 ± 0.0774	**a**	1.84 ± 0.0730	**a**	2.44 ± 0.709	**a**
912	Methanol	14.7 ± 0.561	**a**	15.2 ± 1.04	**a**	16.9 ± 3.25	**a**
951	Ethanol	19.0 ± 0.480	**a**	18.7 ± 2.08	**a**	21.8 ± 6.37	**a**
1002	Methyl Butanoate	0.655 ± 0.0179	**a**	0.530 ± 0.00521	**a**	0.713 ± 0.182	**a**
1034	α-Pinene	1.63 ± 0.232	**b**	2.61 ± 0.497	**a**	1.70 ± 0.287	**b**
1051	Ethyl Butanoate	25.1 ± 0.248	**a**	18.8 ± 0.284	**a**	19.9 ± 5.74	**a**
1100	Hexanal	8.68 ± 0.113	**a**	9.14 ± 0.293	**a**	8.31 ± 2.44	**a**
1120	β-Pinene	0.374 ± 0.0100	**a**	0.552 ± 0.173	**a**	0.522 ± 0.294	**a**
1162	δ-3-Carene	0.164 ± 0.0200	**b**	0.239 ± 0.0366	**b**	0.332 ± 0.0487	**a**
1173	β-Myrcene	12.3 ± 0.475	**a**	17.5 ± 2.08	**a**	14.8 ± 3.56	**a**
1195	α-Terpinene	0.159 ± 0.0148	**b**	0.144 ± 0.0508	**b**	0.340 ± 0.0513	**a**
1207	Methyl Hexanoate	1.11 ± 0.0331	**a**	0.824 ± 0.407	**a**	0.839 ± 0.172	**a**
1228	Limonene	305 ± 33.5	**b**	464 ± 51.3	**a**	325 ± 55.5	**b**
1248	Ethyl Hexanoate	11.3 ± 0.951	**a**	11.5 ± 0.600	**a**	7.94 ± 1.76	**b**
1262	γ-Terpinene	0.118 ± 0.00664	**b**	0.232 ± 0.00360	**b**	1.67 ± 0.276	**a**
1293	*p*-Cymene	0.309 ± 0.0571	**a**	0.432 ± 0.0581	**a**	0.531 ± 0.180	**a**
1304	α-Terpinolene	0.300 ± 0.0389	**b**	0.534 ± 0.0570	**a**	0.489 ± 0.126	**ab**
1311	Octanal	2.88 ± 0.230	**a**	0.876 ± 0.0255	**b**	1.12 ± 0.304	**b**
1366	1-Hexanol	1.11 ± 0.0557	**b**	2.32 ± 0.883	**a**	1.74 ± 0.483	**ab**
1402	(Z)-3-Hexen-1-ol	0.679 ± 0.0260	**b**	1.27 ± 0.0246	**a**	1.07 ± 0.279	**ab**
1418	Nonanal	0.927 ± 0.231	**a**	0.645 ± 0.0503	**a**	1.07 ± 0.461	**a**
1452	Ethyl Octanoate	0.823 ± 0.117	**b**	1.12 ± 0.0499	**a**	0.486 ± 0.0670	**c**
1463	1-Octen-3-ol	0.239 ± 0.00864	**a**	0.271 ± 0.00890	**a**	0.154 ± 0.0342	**b**
1494	Octyl Acetate	0.599 ± 0.576	**a**	0.241 ± 0.0368	**b**	0.186 ± 0.0756	**b**
1525	Decanal	2.00 ± 0.267	**a**	1.29 ± 0.468	**a**	1.45 ± 0.331	**a**
1561	Linalool	14.0 ± 0.219	**b**	13.6 ± 0.283	**b**	26.7 ± 6.63	**a**
1572	1-Octanol	6.59 ± 0.105	**a**	4.38 ± 0.0514	**b**	4.96 ± 0.780	**b**
1621	β-Elemene	1.06 ± 0.285	**a**	1.73 ± 0.341	**a**	1.10 ± 0.188	**a**
1635	4-Terpineol	1.48 ± 0.0353	**b**	3.27 ± 0.0222	**b**	5.77 ± 1.36	**a**
1639	β-Caryophyllene	0.977 ± 0.327	**a**	1.39 ± 0.360	**a**	0.784 ± 0.114	**a**
1673	1-Nonanol	0.467 ± 0.329	**a**	0.384 ± 0.0128	**a**	0.398 ± 0.0626	**a**
1681	Citronellyl Acetate	0.317 ± 0.405	**b**	1.13 ± 0.0777	**a**	0.426 ± 0.135	**b**
1690	β-Selinene	0.237 ± 0.0777	**a**	0.304 ± 0.0638	**a**	0.189 ± 0.0233	**a**
1697	Decyl Acetate	0.203 ± 0.0246	**b**	0.443 ± 0.0595	**a**	0.208 ± 0.0317	**b**
1706	Ethyl-3-Hydroxyhexanoate	1.67 ± 0.0229	**b**	1.97 ± 0.0164	**a**	0.929 ± 0.114	**b**
1726	α-Terpineol	0.450 ± 0.0806	**b**	0.730 ± 0.0325	**a**	0.813 ± 0.168	**a**
1766	Valencene	50.5 ± 13.8	**a**	64.6 ± 10.9	**a**	38.8 ± 5.03	**a**
1770	α-Selinene	5.43 ± 1.68	**bc**	7.63 ± 1.65	**ac**	4.03 ± 0.403	**b**
1779	β-Citronellol	1.70 ± 0.490	**b**	3.67 ± 0.959	**a**	2.10 ± 0.297	**ab**

Table A-6. Continued.

LRI	Compound	Control		Asymptomatic		Symptomatic	
1785	Carvone	1.25 ± 0.0931	**a**	1.25 ± 0.0355	**a**	0.669 ± 0.0389	**b**
1791	Δ-Cadinene	0.252 ± 0.154	**a**	0.460 ± 0.131	**a**	0.295 ± 0.0223	**a**
1820	Nerol	0.273 ± 0.00644	**b**	0.451 ± 0.0231	**a**	0.420 ± 0.0486	**a**
1841	Perillaldehyde	0.476 ± 0.0269	**b**	0.513 ± 0.0255	**b**	0.654 ± 0.0742	**a**
1863	Geraniol	0.733 ± 0.0655	**a**	0.164 ± 0.0114	**b**	0.739 ± 0.0944	**a**
1894	α-Ionone	0.244 ± 0.0150	**b**	0.386 ± 0.0412	**a**	0.395 ± 0.0396	**a**
1986	β-Ionone	0.0724 ± 0.00580	**b**	0.124 ± 0.00592	**a**	0.0670 ± 0.00230	**b**
2050	Caryophyllene Oxide	0.224 ± 0.0137	**b**	0.339 ± 0.0158	**a**	0.114 ± 0.0217	**c**
2272	β-Sinensal	0.153 ± 0.0414	**a**	0.186 ± 0.0103	**a**	0.159 ± 0.00672	**a**
2576	Nootkatone	0.819 ± 0.0557	**ab**	1.15 ± 0.0682	**a**	0.606 ± 0.220	**b**

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BIOGRAPHICAL SKETCH

Lilibeth Rubio Dagulo graduated *cum laude* with a Bachelor of Science in food science and human nutrition at the University of Florida in May 2007. She is the first American born child of Filipino immigrants to receive an undergraduate degree from an American institution. Lilibeth wanted to pursue a specialization in flavor chemisty. Therefore, in August 2007, she entered the Master of Science Program in the Food Science and Human Nutrition Department at the University of Florida. Under the supervision of Dr. Russell L. Rouseff, Lilibeth received training in citrus flavor analysis. Lilibeth plans to pursue a career as a flavor chemist and/or flavor analyst at a flavor or beverage company.