Effect of Liberibacter Infection (Huanglongbing Disease) of Citrus on Orange Fruit Physiology and Fruit/Fruit Juice Quality: Chemical and Physical Analyses

ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · JANUARY 2010

Impact Factor: 2.91 · DOI: 10.1021/jf9031958 · Source: PubMed

8 AUTHORS, INCLUDING:

Greg Mccollum
USDA, ARS, Fort Pierce, Florida
74 PUBLICATIONS **1,039** CITATIONS
SEE PROFILE

Michael Irey
Southern Gardens Citrus
62 PUBLICATIONS **571** CITATIONS
SEE PROFILE

Randall Cameron
United States Department of Agriculture
61 PUBLICATIONS **1,069** CITATIONS
SEE PROFILE

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. XXXX, XXX, 000–000 A
DOI:10.1021/jf9031958

Effect of Liberibacter Infection (Huanglongbing Disease) of Citrus on Orange Fruit Physiology and Fruit/Fruit Juice Quality: Chemical and Physical Analyses

Elizabeth Baldwin,*,† Anne Plotto,† John Manthey,† Greg McCollum,‡ Jinhe Bai,† Mike Irey,§ Randall Cameron,† and Gary Luzio†

†USDA-ARS Citrus & Subtropical Products Laboratory, South Atlantic Area, Agricultural Research Service, United States Department of Agriculture, 600 Avenue S N.W., Winter Haven, Florida 33881, ‡Horticultural Research Laboratory, South Atlantic Area, Agricultural Research Service, United States Department of Agriculture, 2001 South Rock Road, Fort Pierce, Florida 34945, and §United States Sugar Corporation, 111 Ponce de Leon Avenue, Clewiston, Florida 33440

More than 90% of oranges in Florida are processed, and since Huanglongbing (HLB) disease has been rumored to affect fruit flavor, chemical and physical analyses were conducted on fruit and juice from healthy (Las −) and diseased (Las +) trees on three juice processing varieties over two seasons, and in some cases several harvests. Fruit, both asymptomatic and symptomatic for the disease, were used, and fresh squeezed and processed/pasteurized juices were evaluated. Fruit and juice characteristics measured included color, size, solids, acids, sugars, aroma volatiles, ascorbic acid, secondary metabolites, pectin, pectin-demethylating enzymes, and juice cloud. Results showed that asymptomatic fruit from symptomatic trees were similar to healthy fruit for many of the quality factors measured, but that juice from asymptomatic and especially symptomatic fruits were often higher in the bitter compounds limonin and nomilin. However, values were generally below reported taste threshold levels, and only symptomatic fruit seemed likely to cause flavor problems. There was variation due to harvest date, which was often greater than that due to disease. It is likely that the detrimental flavor attributes of symptomatic fruit (which often drop off the tree) will be largely diluted in commercial juice blends that include juice from fruit of several varieties, locations, and seasons.

KEYWORDS: Orange juice; Huanglongbing; flavor; limonoids; pectinmethylesterase

INTRODUCTION

Huanglongbing (HLB), yellow shoot, yellow dragon, or citrus greening disease is a serious issue for the citrus industry around the world, as it can kill or debilitate a citrus tree in 2 to 10 years, and as of yet there is no effective treatment (*1*). The suspected causal agent is the Gram-negative bacteria *Candidatus* Liberibacter asiaticus (Las) (*1*), which is vectored by the Asian citrus psyllid, *Diaphorina citri* (*2*, *3*). Fruit from trees infected with Las frequently do not color properly, are small, have an asymmetrical shape, and have been reported to be off-flavored (bitter, metallic, salty) (*2*, *4*−*6*).

HLB symptoms along with the bacterium, Las, were first confirmed in Florida in 2005 and now are present in all Florida citrus-growing counties (*7*). Initially, Las-positive trees were destroyed as quickly as possible in an effort to reduce inoculum; however, this is no longer feasible, as it would greatly impact the Florida citrus industry. Anecdotal reports suggest that fruit from HLB-affected trees have objectionable flavor, and thus, the Florida citrus processors are concerned that diseased fruits entering the juice stream may be affecting orange juice quality. However, reports about off-flavors have not been substantiated by comprehensive scientific chemical and sensory investigations. One report on Brazilian cultivars determined that fruit from trees symptomatic for HLB disease were more acidic and had less juice, total soluble solids per box and per fruit, and a lower solids/acids ratio (*4*). This report, however, did not distinguish between normal looking (asymptomatic) fruit and HLB-symptomatic fruit (small, green, and lopsided), which are likely to have more flavor problems. Another report on late season Florida Valencia juice found the (+)Las juice to have a higher solids/acids ratio and was perceived as sweeter than (−)Las control juice (*8*); however, sampling was limited, since the disease was only just established. Because asymptomatic fruit from Las-infected trees will continue to enter the orange juice processing stream, since unfortunately the disease is now widespread, there is a need to determine qualitative and quantitative impacts of Las or suspected HLB disease on citrus fruit and juice quality. Thus, a chemical study was undertaken, and in a companion paper sensory attributes were also assessed from the same juice samples (*9*).

*To whom correspondence should be addressed. E-mail: Liz.Baldwin@ars.usda.gov. Fax: 863-299-8678.

MATERIALS AND METHODS

Fruit Sampling. In 2007, fruit were sampled from the three principal processing orange varieties (Hamlin, Midsweet, and Valencia). Fruits (30)

Table 1. Sampling of Symptomatic or Asymptomatic Orange Fruit from Diseased (+Las) Trees within the Same Grove for Each of the 2007 and 2008 Seasons[a]

harvest date	variety	no. of trees per treatment	fruit from Las(+) trees	pasteurization
		2006−2007 season		
February 2007	Hamlin	5 × 1 trees	asymptomatic	light
February 2007	Midsweet	5 × 1 trees	asymptomatic	light
March 2007	Valencia	5 × 1 trees[b]	asymptomatic	light
April 2007	Valencia	5 × 1 trees	asymptomatic	light
May 2007	Valencia	5 × 1 trees	asymptomatic	light
June 2007	Valencia	5 × 1 trees	asymptomatic	light
		2007−2008 season		
December 2007	Hamlin	3 × 3 trees	asymptomatic	commercial
February 2008	Hamlin	3 × 3 trees	asymptomatic	commercial
			asymptomatic	not pasteurized
		1 × 3 trees	symptomatic	commercial
			symptomatic	not pasteurized
April 2008	Valencia	3 × 3 trees	asymptomatic	commercial
			asymptomatic	not pasteurized
		1 × 3 trees	symptomatic	commercial
			symptomatic	not pasteurized
June 2008	Valencia	3 × 3 trees	asymptomatic	commercial

[a] For each Las(+) tree, an equal number of healthy trees were harvested in the vicinity. The resulting juice was either lightly pasteurized (71 °C for 15 s), commercially pasteurized (83-90 °C for 8-10 s), or not pasteurized. The 2007 Valencia harvests were from the same five trees (each tree was a replicate sample). The 2008 February Hamlin and April Valencia harvests had three composite replications of three trees (3 × 3 trees), the juice from which was split for commercial pasteurization or was not pasteurized for both diseased and healthy fruit samples. There were also symptomatic fruit samples for the February Hamlin and April Valencia harvests from one tree each, for which the juice was split, half was pasteurized, and half was not. [b] Same trees for March−June 2007 Valencia harvests.

from five replicate trees symptomatic for HLB disease and five replicate healthy trees (±Las) from 1 to 4 harvests were washed, sanitized with 200 ppm NaOCl for 30 s, hand juiced, lightly pasteurized (for minimal flavor impact, 71 °C for 15 s in a water bath), and frozen at −20 °C until analyzed. The juice from all 30 fruits of one tree was pooled as one composite replicate. Trees, symptomatic for HLB disease, were later confirmed to be diseased by polymerase chain reaction (PCR) used to amplify the DNA of the associated Las bacteria (*1*, *3*, *10*), while control trees were confirmed to be PCR negative. The fruit from diseased trees were generally asymptomatic, that is, normal looking, although some were slightly greener and/or smaller. There was one harvest each of Hamlin and Midsweet varieties (February) and four Valencia harvests (March, April, May, and June, same trees) with five tree replicates per variety per harvest date per disease state. The "2008 season" actually included a Hamlin harvest in late (December) 2007. Fruit (200−400) from two harvests each of Hamlin (December 2007 and February 2008, different grove and trees) and Valencia (April and June 2008, different grove and trees) were harvested from three ±Las trees, replicated three times (total of nine infected and nine healthy trees). All replicates for February Hamlin and each of the Valencia 2008 harvests were later combined for pooled analyses. Unfortunately, due to diseased tree removal, it was not possible to use the same trees in 2008. In addition, there was an extra harvest for Hamlin in February 2008 and for Valencia in April 2008 of symptomatic fruit (small, green, and asymmetrical) along with healthy controls from ±Las trees in the same vicinity. All the "2008 season" fruit were extracted using a commercial JBT single head extractor (Lakeland, FL) and pasteurized under simulated commercial conditions (1.2 L m^{-1}, 8−10 s hold time, 83−90 °C using a pilot pasteurizer, UHT/HTST Lab 25EHV Hybrid, Microthermics, Inc.; Raleigh, NC) or not pasteurized, and all samples were stored frozen at −20 °C until analysis of sensory (*9*) or chemical characteristics. A summary of fruit sampling and treatment is presented in **Table 1**. Unless indicated otherwise, Las(+) juice refers to largely asymptomatic fruit taken from Las symptomatic trees and Las(−) refers to fruit taken from Las negative (healthy) trees.

Sugar and Acid Analysis. For titratable acidity (TA) and soluble solids content (SSC), TA was determined by titrating to pH 8.2 with 0.1 N NaOH using an autotitrator (Metler Toledo DL50, Columbus, OH) and SSC using a refractometer (Atago RX-5000cx, Tokyo, Japan). Individual sugar analysis was performed via HPLC. Juice samples were centrifuged (Eppendorf microfuge, Westbury, NY) at top speed up to 15 min; aliquots of the clarified supernatants were diluted 20× with water. Dilute juice was passed through a SepPak (C18) column (Waters/Millipore, Milford, MA) and then filtered through 0.2 μm nylon filter. Aliquots of 1 mL of the extracts were then transferred to 1.5 mL autosampler vials for analysis. Sugars were analyzed using a Shimadzu LC-20 AD Prominence Solvent Delivery system (DGU-20AS Online Degasser, SIL-20A Autosampler, CMB-20A System Controller, Columbia, MD) equipped with an ELSD-LTII detector (Sedex model 85 Low Temperature Evaporative Light Scattering Detector). The column used was the 700 CH Carbohydrate (300 mm × 6.5 mm) (Alltech, Nicholasville, KY) operated at 90 °C in a column heater (Timberline Instruments model 105, Boulder, CO). The mobile phase was water with a flow rate of 0.5 mL min^{-1}. Samples of 10 μL were analyzed. Quantification of sugars was based on the external standard method (EZStart Chromatography software, Justice Laboratory Software, Denville, NJ) using standards for fructose (Amresco, Solon, OH), glucose, and sucrose (Sigma-Aldrich). All results are expressed as g 100 mL^{-1} juice.

For analysis of individual acids, approximately 40 g of juice was extracted using 70 mL of 80% ethanol/deionized water solution. The mixture was boiled for 15 min, cooled, and filtered (Whatman #4 filter paper, Batavia, IL). The filtered solution was brought to 100 mL with 80% ethanol. A total of 10 mL of the filtered solution was then filtered through a C-18 Sep-Pak (Waters/Millipore), followed by a 0.45 μm Millipore (Siemens-Millipore, Shrewbury, MA) filter (*11*). Organic acids, including ascorbic acid, were analyzed using an Altech OA 1000 Prevail organic acid column with a flow rate of 0.2 mL min^{-1} at 35 °C and a mobile phase of 0.01 N H_2SO_4. The injection volume was 20 μL using a Perkin-Elmer Series 200 autosampler (Waltham, MA), a Spectra System P4000 pump, and a Spectra System UV 6000 LP detector (Thermo Fisher Scientific, Waltham, MA).

Volatile Analysis. Two milliliters of the headspace of 10 mL crimped-capped vials with 3 mL of thawed juice was equilibrated at 40 °C for 15 min in a shaker before injection onto an Agilent 6890 (Agilent technologies, Santa Clara, CA) GC apparatus using a Gerstel multipurpose autosampler (Agilent technologies) equipped with Stabilwax column (0.53 mm × 30 m and 1.0 μm film thickness, Restek Corp., Bellefonte, PA) and HP-5 low bleed columns (0.53 mm × 30 m with 1.5 μm film thickness, Agilent Technologies). The flow rate was split equally to the two columns at 17 mL min^{-1} at 40 °C with an increase in temperature at 6 °C/min^{-1} up to 180 °C, where the temperature was held constant for an additional 5.8 min. The GC peaks for all aroma volatile compounds were quantified using standard curves as determined by enrichment of deodorized orange juice

(pumpout of juice from an evaporator) with five concentrations of known authentic volatile compound standards (*12*). Standard aroma compounds were purchased from Sigma-Aldrich, Fluka Chemical Corporation (Buchs, Switzerland), Bedoukian Research, Inc. (Danbury, CT), Roth Chemical Co. (Karsruhe, Germany), K&K Laboratories (Jamaica, NY), and ICN Pharmaceuticals (Cleveland, OH). Volatile peak identities were confirmed by GC-MS. Since headspace volatile concentrations were not detectable by MS, extraction of aroma volatiles using SPME for identity confirmation on MS was performed using a MPS-2 autosampler (Gerstel). The vials were incubated at 40 °C for 30 min, and then a 2 cm SPME fiber (50/30 μm DVB/CAR/PDMS) was inserted into the headspace of the sample vial and exposed for 60 min. The fiber was thermally desorbed in the GC injector (splitless mode) port for 3 min at 250 °C. The separation of volatile compounds was accomplished using an Agilent 6890 GC (Agilent Technologies) instrument equipped with DB-5 (60 m length, 0.25 mm i.d., 1.00 μm film thickness; J&W Scientific, Folsom, CA) and DB-Wax (60 m length, 0.25 mm i.d., 0.50 μm film thickness; Agilent Technologies) columns, coupled with a 5973N MS detector (Agilent Technologies). The column oven was programmed to increase at 4 °C min^{-1} from the initial 40 to 230 °C and then ramped at 100 °C min^{-1} to 260 °C and held for 11.70 min for a total run time of 60 min. Helium was used as carrier gas at flow rate of 1.5 mL min^{-1}. Inlet, ionizing source, and transfer line were kept at 250, 230, and 280 °C, respectively. Mass units were monitored from 40 to 250 m/z and ionized at 70 eV. Data were collected using the ChemStation G1701 AA data system (Hewlett-Packard, Palo Alto, CA). Samples were run in triplicate on the DB-5 column, with a blank run between each sample to ensure fiber cleanness between samples. A mixture of C-5 to C-15 *n*-alkanes was run at the end of each day to calculate retention indices (RIs). Samples were also analyzed (one run per sample) on a DB-Wax column to identify potential coeluting compounds on the DB-5 column.

Volatile compounds were identified by the comparison of their RIs and mass spectra with library entries (NIST/EPA/NIH Mass Spectral Library, version 2.0d; National Institute of Standards and Technology, Gaithersburg, MA). Chemical authentic standards, when available, were run on both columns, and their RIs and spectra confirmed compound identity.

Sample Preparation for Analysis of Secondary Metabolites. Juice extracts were prepared to minimize free sugar (sucrose, glucose, and fructose) content, yet be inclusive of the remaining polar and nonpolar secondary metabolites. Shaken and thawed orange juice (2 mL) was added to 13 mL of methanol, shaken, and then passed through a 0.45 μm PTFE filter. The filter was washed with an additional 0.5 mL of methanol, and the total volume was adjusted to 14 mL. To 12.0 mL of the methanolic juice extract, 1 mL of butanol was added, and the sample was taken to dryness using a Savant centrifugal evaporator. Methanol (2 mL) was added, and each sample was vortexed for 2 min. Samples were centrifuged for 5 min, and then the recovered clear supernatants were quantitatively removed and adjusted to 4.0 mL prior to analysis by HPLC-MS.

An alternative extraction was conducted to obtain higher concentrations of limonin and nomilin aglycones. Juice samples (150 mL) were centrifuged at 10 000*g* for 15 min. Clarified juice sera (100 mL) were extracted three times with equal volumes of methylene chloride. Methylene chloride extracts were rotovapped to dryness, and the residues redissolved in 12 mL of acetone. The solutions were clarified by passage through a 0.45 μm PTFE filter (Siemens, Shrewbury, MA) and then taken to dryness with a Savant centrifugal evaporator (Ontario, Canada). The residues were redissolved in acetone (1.0 mL) containing 4.35 μg of hesperetin (Sigma-Aldrich) internal standard prior to analysis by HPLC-MS.

Quantitative HPLC-MS Analysis for Secondary Metabolites. The secondary metabolites of the orange juice samples were analyzed by HPLC-MS, using a Waters 2695 Alliance HPLC (Waters, Medford, MA) instrument connected in parallel with a Waters 996 photodiode array (PDA) detector and a Waters/Micromass ZQ single quadrupole mass spectrometer equipped with an electrospray ionization source. Compound separations were achieved with a Waters XBridge C8 column (4.6 × 150 mm). Elution conditions included three solvent gradients composed initially of water/acetonitrile/0.5% formic acid (85/10/5, v/v/v) and increased with linear gradients to 75/20/5 (v/v/v) over 10 min, then to 70/25/5 (v/v/v) by 15 min, and then to 55/40/5 and 25/70/5 (v/v/v) by 23 and 40 min, respectively, at a flow rate of 0.75 mL min^{-1}. Data handling was done with MassLynx software version 3.5 (Micromass, Division of Waters Corp., Beverly MA). Postcolumn split to the PDA and mass ZQ detector was 10:1. MS parameters were as follows: ionization mode, ES^+; capillary voltage 3.0 kV; extractor voltage 5 V; source temperature 100 °C; desolvation temperature 225 °C; desolvation N_2 flow 465 L h^{-1}, cone N_2 flow 70 L h^{-1}; scan range m/z 150−1600; scan rate 1 scan s^{-1}; cone voltages 20 and 40 eV. Quantifications of the secondary metabolites were made using either ZQ calculated mass extracted total ion chromatograms (TIC) obtained in scanning mode or single ion response mode. To normalize the mass spectrometer instrument response during sequential runs, an internal standard, hesperetin, was additionally measured at 303 m/z. Endogenous hesperetin accounted for less than 1% of the total level after addition of hesperetin (4.35 μg) as an internal standard. Standards were isolated and identified for feruloyl putrescine as described (*13*). The flavonoids narirutin, narirutin-4′-glucoside, and 6,8-di-*C*-glucosylapigenin were isolated from orange peel and tentatively identified by correlations of their ultraviolet and mass spectra and chromatographic properties with previously published values (*14*−*16*).

Quantitative limonoid analyses were conducted using modified methods of ref *17*. Limonoid glucosides (*18*) were monitored with positive electrospray ionization (+20 V) measured with the main fragment ions corresponding to the protonated aglycone mass ions (471 m/z for limonin and 515 m/z for both nomilin and nomilinic acid). The TICs for limonin and nomilin were monitored at +40 V. Identifications of the limonoid glucosides and aglycones were made based on the detection of the fragment ions coeluting with authentic standards. Similar techniques were used to detect and measure the levels of selected phenolic secondary metabolites in the orange juice, including feruloyl putrescine (265 m/z), narirutin-4′-*O*-glucoside and narirutin (273 m/z), and 6,8-di-*C*-glucosyl apigenin (393 m/z). Standards for limonin, limonin glucoside, nomilin, and nomilin glucoside for identification were obtained from Hasagawa and coworkers (*18*, *19*).

Cloud Loss Analysis. Commercially processed and pasteurized Valencia juice samples (June 2008 harvest) were brought to 0.02% lithium azide and 4.35 g L^{-1} potassium metabisulfite, placed in glass bottles, and incubated at 30 °C. At selected times, duplicate samples (10 mL per replicate) from each of three replicates for the Las(+) and Las(−) treatments were pipetted into 15 mL graduated, conical centrifuge tubes from each treatment after inverting the glass bottle three times. The samples were centrifuged for 10 min at 360*g*, supernatant (1 mL of each sample) was transferred to a cuvette, and absorbance at 660 nm was recorded (*20*). Reported values are the means and standard errors for each treatment.

Enzyme Activity Assays. Total and thermally tolerant pectinmethylesterase (PME and TT-PME, respectively) activity in raw (unpasteurized) juice was determined titrimetrically on 0.5% Sigma citrus pectin (94% DE) with a Radiometer PHM290 pH-stat controller (assayed at pH 7.5, 200 mM NaCl, 30 °C, using 10 mM LiOH as the titrant). Raw juice pH was adjusted to 7.5 with LiOH prior to titration. TT-PME activity was estimated after the sample had been heated for 20 min in a 70 °C water bath, a treatment that inactivates the thermally labile PMEs. Activity estimates are the means of two or more replicates for each sample. Reported estimates are averages for all commercially processed and pasteurized Valencia Las(+) and Las(−) samples (harvested in June 2008).

Galacturonic Acid Analysis. Pectin content was analyzed in all 2007 and 2008 orange juice samples (*21*). Each juice sample was adjusted to pH 2.4 with concentrated nitric acid. A 7 mL aliquot was removed, placed in a glass reactor tube, heated for 5 min at 110 °C, and immediately cooled to room temperature using a microwave extractor (Discover model 908005, CEM Corp., Mathews, NC) (*22*). All extractions were performed in duplicate and centrifuged for 30 min at 1000*g*. Each supernatant was mixed with 14 mL of cold anhydrous isopropyl alcohol, refrigerated for 60 min at 4 °C, and centrifuged for 1 h at 3000*g*. Supernatant was discarded, and the pellet rewashed once with anhydrous isopropyl alcohol (IPA) and then twice with 70% IPA and centrifuged at 3000*g* for 1 h between each wash while discarding all supernatants. The pellet was dried for 16 h at 50 °C in the centrifuge tube under vacuum. A glass marble and 4 mL of deionized water were added to the dry pellet and then shaken for 24 h. To an 800 μL aliquot of the rehydrated sample, 200 μL of 0.5 M sodium acetate buffer (pH 5.0) and 2 μL of pectinase (Pectinex Ultra SP-L, P-2611, Sigma-Aldrich) were added (*23*). Samples were then incubated at 37 °C for 24 h and then centrifuged for 5 min at 14 000*g*. Determination of

Table 2. Quality Attributes of Valencia Orange Juice from Fruit Harvested from Five Replicate Trees with or without Greening Disease (±Las) Harvested from March to June 2007[a]

	SSC (g 100 mL^{-1})		TA (g 100 mL^{-1})		SSC/TA ratio	
harvest (month)	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
March	10.7ABa[b]	10.3Aa	0.82Aa	0.84Aa	13.2 Da	12.5 Da
April	10.1Ba	9.7Aa	0.68Ba	0.72Ba	15.1Ca	13.6Ca
May	10.6ABa	9.6Ab	0.57Ca	0.54Ca	18.6Ba	18.0Ba
June	11.0Aa	10.1Ab	0.43 Da	0.41 Da	25.8Aa	24.8Aa
ANOVA (*F* values)[c]						
harvest (H)	3.01*		113.24***		128.15***	
disease (D)	15.98***		0.01^{NS}		3.72^{NS}	
H × D	0.67^{NS}		1.08^{NS}		0.16^{NS}	

	total sugars (g 100 mL^{-1})		total sugars/SSC (%)		galacturonic acid (mg g^{-1})	
harvest (month)	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
March	8.7Ba	8.6Aa	80.9Ba	82.9Ba	0.037Bb	0.065Ba
April	9.1ABa	8.0Ab	89.8Aa	81.8Bb	0.095Ba	0.076Ba
May	9.5ABa	8.5Ab	90.0Aa	87.9Aa	0.285Aa	0.214Aa
June	9.7Aa	8.4Ab	87.9Aa	83.7ABa	0.310Aa	0.163ABa
ANOVA (*F* values)						
harvest (H)	1.19^{NS}		5.68**		15.58**	
disease (D)	15.28***		6.54*		4.1^{NS}	
H × D	1.17^{NS}		3.02*		2.12^{NS}	

	sucrose (g 100 mL^{-1})		glucose (g 100 mL^{-1})		fructose (g 100 mL^{-1})	
harvest (month)	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
March	4.9Ba	4.7Aa	1.9Aa	1.9Aa	1.9Aa	1.9Aa
April	5.2ABa	4.4Ab	1.9Aa	1.7Aa	2.0Aa	1.8Aa
May	5.5Aa	4.8Ab	2.0Aa	1.8Ab	2.0Aa	1.9Aa
June	5.6Aa	4.8Ab	2.0Aa	1.8Ab	2.0Aa	1.9Aa
ANOVA (*F* values)						
harvest (H)	2.07^{NS}		0.37^{NS}		0.24^{NS}	
disease (D)	17.71***		11.67**		6.49*	
H × D	1.09^{NS}		1.13 ^{NS}		1.04^{NS}	

[a] All sugars were identified based on reference standards. SSC = soluble solids content; TA = titratable acidity. [b] Values followed by the same capital letter within columns or the same small letter within rows are not significantly different by the Fisher's least significant difference test at $P = 0.05$. [c] ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant.

galacturonic acid was performed on the supernatants using anion exchange chromatography (CarboPac PA1 column, Dionex Corp., Sunnyvale, CA), using 0.0−0.5 M ammonium formate (Fluka Anal. #09735, Sigma-Aldrich) gradient elution with a binary pump (model series 200, Perkin-Elmer) set at a flow rate of 0.6 mL min^{-1} (*24*). Evaporative light scattering was used for detection (Sedex model 85, Sedere Sas, Alfortville, France) set at an evaporator temperature of 99 °C, photomultiplier gain of 9, and nebulizer air pressure of 4.5 atm. Data were collected and analyzed with EZChrome Elite software (version 3.1.6, Agilent Technologies). Galacturonic acid was identified using a standard (Sigma-Aldrich).

Statistical Analysis. SAS version 9.1 (SAS Institute, Gary, NC) was used for analysis of data. For each compound or quality attribute (3−5 replicates), the main and cross effects of harvest time × treatment (disease vs healthy) were analyzed using analysis of variance (PROC ANOVA). One-way ANOVA was used to compare diseased Las(+) and healthy Las(−) samples, while two-way ANOVA was used to determine disease effects across harvest dates and harvest effects across diseased and healthy samples. Within each harvest date or disease states (±Las), treatment means were separated at the 0.001, 0.01, and 0.05 levels of significance using least-squares means (LSD). Statistical comparisons for enzyme activities were performed with GraphPad Prism (version 4.03 for Windows, GraphPad Software, San Diego, CA). Principal components analysis (PCA) was performed with XLSTAT version 2008 (Addinsoft, Paris, France).

RESULTS AND DISCUSSION

For most of the discussion of results, unless otherwise indicated, Las(+) juice refers to asymptomatic fruit juice from Las(+) trees compared to juice from fruit from healthy Las(−) trees. This is true for all the 2007 season data and most of the 2008 season, since the majority of the fruit entering the processed juice stream from Las(+) trees will be of this type. The Las(+) symptomatic fruit (small, green, and lopsided) are less likely to enter the juice stream in large numbers, since they generally abscise (drop off) the tree and may be culled from the processing line. In the 2008 season, there was one harvest each of Hamlin and Valencia symptomatic fruit from Las(+) trees which are indicated as such in a separate subsection.

Sugars, Acids, and Color for the 2007 Season. For the 2007 season, there was one harvest each for Hamlin and Midsweet fruit and four Valencia harvests and the juice was hand squeezed. There were no significant differences for SSC, TA, SSC/acid ratio, total sugars, individual sugars, or galacturonic acid for Hamlin or Midsweet fruit juice between Las(+) and Las(−) trees (data not shown). Midsweet juice from Las(+) trees showed slightly lower juice color (tristimulus values CIE L^*, a^*, and b^*) (*25*, *26*), although commercially acceptable compared to healthy Las(−) controls (36.8 and 37.9 color values for juice positive or negative for Las, respectively). For Valencia, the SSC for the May and June harvests was lower for juice from Las(+) trees with no differences for TA or the SSC/TA ratio (**Table 2**). There were differences in total sugars for the April, May, and June harvests and in total sugar/SSC ratio for the April harvest (since SSC is made up of soluble solids that include materials that

Table 3. Quality Attributes of Hamlin Orange Juice from Fruit Harvested from Three Composite Replicates of Three Trees Each with or without Greening Disease (±Las) in December 2007 and February 2008[a]

	SSC (g 100 mL^{-1})		TA (g 100 mL^{-1})		SSC/TA ratio	
harvest (month)	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
December 2007	7.8Ba[b]	7.6Ba	0.49Aa	0.50Aa	16.0Ba	15.3Ba
February 2008	11.6Aa	10.4Ab	0.59Aa	0.50Aa	19.8Ab	22.0Aa
ANOVA (*F* values)[c]						
harvest (H)	110.75***		1.52^{NS}		6.14^{NS}	
disease (D)	4.52^{NS}		0.79^{NS}		0.12^{NS}	
H × D	2.89^{NS}		1.74^{NS}		0.45^{NS}	

	sucrose (g 100 mL^{-1})		glucose (g 100 mL^{-1})		fructose (g 100 mL^{-1})	
harvest (month)	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
December 2007	3.9Ba	3.2Bb	1.5Ba	1.3Bb	1.5Ba	1.4Ba
February 2008	5.4Aa	4.0Ab	2.2Aa	1.8Ab	2.2Aa	1.8Ab
ANOVA (*F* values)						
harvest (H)	65.87***		172.37***		122.67***	
disease (D)	51.04***		38.41***		17.07***	
H × D	5.51*		10.35**		7.19*	

	total sugars (g 100 mL^{-1})		juice color (color number)		galacturonic acid (mg g^{-1})	
harvest (month)	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
December 2007	7.0Ba	6.0Bb	34.7Ba	34.5Ba	0.579Ba	0.640Ba
February 2008	9.8Aa	7.6Ab	36.5Aa	36.2Aa	1.329Aa	1.433Aa
ANOVA (*F* values)						
harvest (H)	107.27***		26.01***		104.41***	
disease (D)	46.12***		0.33^{NS}		1.37^{NS}	
H × D	7.73*		0.04^{NS}		0.08^{NS}	

[a] All sugars were identified based on reference standards. SSC = soluble solids content; TA = trtratable acidity. [b] Values followed by the same capital letter within columns or the same small letter within rows are not significantly different by the Fisher's least significant difference test at $P = 0.05$. [c] ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant.

are not sweet, such as cell wall sugars; this ratio measures the components in SSC that are sweet sugars, which affect taste). For individual sugars, there were differences for the same harvests (April and/or May and June) for sucrose and glucose but not fructose. Where there were differences for sugars, values were lower for juice from Las(+) trees compared to juice from Las(−) controls (**Table 2**). For galacturonic acid, representing pectin, levels were higher for the March harvest in Las(+) fruit juice, otherwise there were no differences due to disease status. For individual acids, there were no significant differences for citric or ascorbic acids for Hamlin, Midsweet, or Valencia juices in 2007 (data not shown). Malic acid was significantly higher for juice from Las(−) controls compared to juice from Las(+) trees only for Hamlin (5.1 and 2.7 g L^{-1} for Las(+) and Las(−), respectively), which was also found for Valencia oranges in the 2006 study (*8*). For Valencia, harvest date was significant for peel color, juice ratio, juice color, SSC, TA, SSC/TA, the total sugar/SSC ratio, and galacturonic acid. There was harvest date × disease interaction for the total sugar/SSC ratio (**Table 2**).

Sugars, Acids, Color, and Size for the 2008 Season. For the 2008 season, there were two harvests each of Hamlin and Valencia fruit, and the juice was commercially processed and pasteurized. SSC was higher for juice from Hamlin Las(−) trees for the February harvest, while the SSC/TA ratio was higher in juice from Las(+) trees for that harvest, similar to results from 2006 (*8*); however, there were no differences for TA, galacturonic acid, citric, malic, or ascorbic acids (data not shown for citric, malic, and ascorbic acids). Hamlin juice from Las(−) healthy trees exhibited slight but significantly higher levels of all individual sugars and total sugar compared to juice from diseased Las(+) trees for all but fructose in December. Harvest date was significant for juice color, SSC, sucrose, glucose, fructose, total sugar, and galacturonic acid, which is typical for fruit maturing on the tree. There were harvest date × disease interactions for sucrose, glucose fructose, and total sugar (**Table 3**).

Table 4. Secondary Metabolites (μg mL^{-1}, Excluding Marked Compounds) in Hamlin Orange Juice from Fruit Harvested from Five Trees with or without Greening Disease (±Las) in February 2007

	content (μg mL^{-1})		
compound	(−)Las	(+)Las	*t* test
hydroxycinnamic acid at 6.3 min[a]	5.58	6.33	0.05
hydroxycinnamic acid at 7.2 min[a]	6.32	5.87	0.05
feruloyl putrescine[b]	52.0	56.1	NS
alkaloid[a]	3.69	4.00	NS
narirutin 4′-glucoside[c]	45.2	41.7	NS
limonin glucoside[b]	93.5	127	0.05
narirutin[c]	75.7	70.4	NS
nomilin glucoside[b]	75.2	127	0.001
nomilinic acid glucoside[b]	107	144	0.01
limonin[b]	2.56	3.63	0.01
nomilin[b]	1.16	1.90	0.01

[a] Relative peak area. [b] Identified using reference standards. [c] Tentatively identified.

For Valencia fruit in 2008, there were no differences in juice color, SSC, TA, SSC/TA ratio, individual sugars, or individual acids for juice from Las (±) trees (data not shown). There were significant harvest date effects for sugars (except sucrose), TA, and SSC/TA ratio. However, in the 2008 season, the harvest date differences may also be due to grove location, cultural

conditions, and tree age, since the trees were not the same or from the same grove (unlike the Valencia harvests in 2007 which were from the same trees). In most cases, infected trees are removed so it is difficult to resample from the same trees.

Table 5. Secondary Metabolites (μg mL^{-1}, Excluding Marked Compounds) in Midsweet Orange Juice from Fruit Harvested from Five Trees with or without Greening Disease (±Las) Harvested in February 2007

	content (μg mL^{-1})		
compound	(−Las)	(+Las)	*t* test
hydroxycinnamic acid at 6.3 min[a]	6.10	5.00	NS
hydroxycinnamic acid at 7.2 min[a]	5.82	6.52	0.05
feruloyl putrescine[b]	52.9	59.0	NS
alkaloid[a]	3.54	4.19	0.05
narirutin 4′-glucoside[c]	36.4	43.2	0.05
limonin glucoside[c]	76.4	94.5	0.05
narirutin[c]	61.9	72.5	0.05
nomilin glucoside[b]	86.4	118	0.001
nomilinic acid glucoside[b]	108	132	0.001
limonin[b]	1.43	1.50	NS
nomilin[b]	0.38	0.48	0.05

[a] Relative peak area. [b] Identified using reference standards. [c] Tentatively identified.

Secondary Metabolite Analysis for the 2007 Season. Changes in the levels of certain classes of secondary metabolites frequently reflect stress conditions in plants, and consistent with this many of the secondary metabolites analyzed in this study were higher in juice from Las(+) trees compared to juice from Las(−) trees in fresh squeezed juice. Some of these compounds, like the unidentified alkaloid, limonin and nomilin, can affect flavor (*27*). Levels and/or ratios of some of these compounds may also be useful as chemical indicators for the disease. As might be expected for stressed plants, most secondary metabolites were higher in juice from Las(+) trees compared to juice from Las(−) trees in fresh squeezed juice. For the 2007 season, this included a number of phenolic hydroxycinnamic acids (HCAs) and sesquiterpenoid limonoids including limonin glucoside (LG), nomilin glucoside (NG) and nomilinic acid glucoside (NAG), and limonin (L) and nomilin (N) aglycones in Hamlin juice. Increased levels similarly occurred in Midsweet juice for a number of the HCAs and limoniods, as well as for an unidentified alkaloid and a flavonoid, narirutin (NR) (**Tables 4** and **5**). For the four Valencia harvests, when differences occurred, certain secondary metabolites were higher in juice from Las(+) compared to Las(−) juice (**Table 6**) including L and N for all harvest dates except June for N (although levels were generally low). The only exception was for juice from the March Valencia harvest where HCA (elution

Table 6. Secondary Metabolites (μg mL^{-1}, Excluding Marked Compounds) in Valencia Orange Juice from Fruit Harvested from Five Replicate Trees with or without Greening Disease (±)Las, Harvested from March to June 2007

	hydroxycinnamic acid at 6.3 min[a]		hydroxycinnamic acid at 7.2 min[a]		alkaloid[a]		6,8-di-*C*-glucosyl apigenin[d]	
harvest (month)	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
March	11.7Aa[b]	9.3Bb	1.3Aa	1.3Aa	2.8Ca	2.8Ca	42.6Aa	34.5Ab
April	8.6Ba	9.2Ba	1.4Aa	1.2Aa	2.8Cb	3.5Ca	28.8Ba	29.7Ba
May	7.3Ba	7.1Ca	0.9Bb	1.0Ba	4.0Bb	4.8Ba	33.5Ba	-
June	8.6Bb	10.9Aa	1.1ABa	1.0Ba	5.4Ab	0.9Aa	31.2Ba	32.3ABa
				ANOVA (*F* values)[c]				
harvest (H)	21.61***		7.73***		51.18***		9.29***	
disease (D)	0.01[NS]		0.09[NS]		9.61**		1.76[NS]	
H × D	7.38***		1.08[NS]		1.99[NS]		4.39*	

	feruloyl putrescine[e]		narirutin 4′-glucoside[d]		limonin glucoside[e]		narirutin[d]	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
March	84.9Aa	48.4Cb	22.8Ab	25.3Aa	123.2Aa	123.4Ba	49.8Ab	57.2Aa
April	64.6ABa	70.5Ba	22.7Ab	26.3Aa	122.4Ab	137.6Aa	48.4Aa	56.0Aa
May	53.1Bb	74.6Ba	18.6Ba	19.3Ba	134.9Aa	137.7Aa	30.7Ba	31.2Ba
June	78.9Ab	104.7Aa	22.2Aa	22.3Aa	115.4Ab	144.4Aa	33.7Ba	34.8Ba
				ANOVA (*F* values)				
harvest (H)	19.24***		4.49**		1.65[NS]		34.33***	
disease (D)	1.37[NS]		2.29[NS]		8.11**		4.19*	
H × D	18.11***		0.50[NS]		3.49*		0.92[NS]	

	nomilin glucoside[e]		nomilinic acid glucoside[e]		limonin[e]		nomilin[e]	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
March	57.8Aa	54.5Ba	87.7Aa	84.0Ba	0.90Ab	1.37Aa	0.22ABb	0.66Aa
April	56.2Aa	66.1ABa	99.1Aa	93.5Aa	0.78ABb	1.24ABa	0.30Ab	0.54Aa
May	64.1Aa	65.4Ba	78.9Bb	88.1ABa	0.67BCb	1.40Aa	0.12Bb	0.26Ba
June	49.6Ab	71.6Aa	61.6Cb	82.6Ba	0.52Cb	0.93Ba	0.06Ba	0.11Ba
				ANOVA (*F* values)				
harvest (H)	0.90[NS]		7.32***		3.55*		8.45***	
disease (D)	4.00*		1.83[NS]		30.92***		13.29***	
H × D	3.07*		3.13*		0.60[NS]		1.95[NS]	

[a] Relative peak area. [b] Values followed by the same capital letter within columns or the same small letter within rows are not significantly different by the Fisher's least significant difference test at $P = 0.05$. [c] ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant. [d] Tentatively identified. [e] Identified using reference standards.

Table 7. Secondary Metabolites (μg mL^{-1}, Excluding Marked Compounds) in Hamlin Orange Juice from Fruit Harvested from Three Replicates of Three Trees with or without Greening Disease (±Las) in December 2007 and February 2008

	hydroxycinnamic acid at 6.3 min[a]		hydroxycinnamic acid at 7.2 min[a]		alkaloid[a]		6, 8-di-*C*-glucosyl apigenin[d]	
harvest (month)	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
December 2007	2.0Bb[b]	2.7Ba	2.1Ab	2.6Aa	1.6Bb	2.0Ba	47.3Bb	57.1Ba
February 2008	4.0Aa	4.2Aa	2.4Ab	3.0Aa	3.0Aa	2.9Aa	64.0Aa	68.0Aa
				ANOVA (*F* values)[c]				
harvest (H)	21.61***		7.73***		51.18***		9.29***	
disease (D)	0.01^{NS}		0.09^{NS}		9.61**		1.76^{NS}	
H × D	7.38***		1.08^{NS}		1.99^{NS}		4.39*	

	feruloyl putrescine[e]		narirutin 4′-glucoside[d]		limonin glucoside[e]		narirutin[d]	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
December 2007	11.48Ba	13.3Ba	6.9Bb	10.4Ba	72.3Bb	102.0Ba	43.0Bb	54.3Ba
February 2008	21.7Aa	23.3Aa	30.8Ab	34.0Aa	132.1Aa	141.7Aa	75.3Aa	76.2Aa
				ANOVA (*F* values)				
harvest (H)	19.24***		4.49**		1.65^{NS}		34.33***	
disease (D)	1.37^{NS}		2.29^{NS}		8.11**		4.19*	
H × D	18.11***		0.50^{NS}		3.49*		0.92^{NS}	

	nomilin glucoside[e]		nomilinic acid glucoside[e]		limonin[e]		nomilin[e]	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
December 2007	32.3Bb	40.7Ba	35.8Bb	54.1Ba	1.45Ab	3.27Aa	0.43Ab	0.83Aa
February 2008	94.0Aa	95.4Aa	160.3Ab	176.8Aa	0.82Bb	1.54Ba	0.18Bb	0.51Ba
				ANOVA (*F* values)				
harvest (H)	0.90^{NS}		7.32***		3.55*		8.45***	
disease (D)	4.00*		1.83^{NS}		30.92***		13.29***	
H × D	3.07*		3.13*		0.60^{NS}		1.95^{NS}	

[a] Relative peak area. [b] Values followed by the same capital letter within columns or the same small letter within rows are not significantly different by the Fisher's least significant difference test at $P = 0.05$. [c] ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant. [d] Tentatively identified. [e] Indentified using reference standards.

time 6.3 min), 6,8-di-*C*-glucosyl apigenin, and feruloyl putrecine (FP) were higher in Las(−) compared to Las(+) juice. Harvest date was significant for all secondary metabolites measured except for LG and NG. There was harvest date × disease interaction for the 6.3 min HCA, 6,8-di-*C*-glucosyl apigenin, FP and LG, NG, and NAG (**Table 6**).

Secondary Metabolite Analysis for the 2008 Season. For commercially processed Hamlin juice, there were differences between juice from healthy and diseased trees for either one or both harvests for all secondary metabolites measured except FP, although disease effects were not significant for the HCAs, 6,8-di-*C*-glucosyl apigenin, FP, NR-4′-glucoside, or NAG (**Table 7**). In all cases where there were differences, the compounds were higher in juice from Las(+) trees. Harvest date was significant for all compounds except LG and NG. There were harvest date × disease interactions for the 6.3 min HCA, 6,8-di-*C*-glucosyl apigenin, FP, LG, NG, and NAG (**Table 7**).

There were few differences for Valencia orange juice (**Table 8**), and when there were differences, they were all in the juice from the June harvest including FP, L, and LG. Limonin was higher in juice from Las(−) trees compared to juice from Las(+) samples this time, while FP and LG were higher in Las(+) compared to Las(−) controls. Disease effects, however, were significant for all compounds except the HCAs, the alkaloid, FP, and NG, with average values for the two harvest dates being higher for juice from Las(+) trees except for NR and L (data not shown). Harvest date effects were significant for all compounds except the 6.3 min HCA. Harvest date × disease interaction was significant for the alkaloid, NR-4′-glucoside, all the LG, NG, NAG, narirutin, and L (**Table 8**).

Volatile Analysis for the 2007 Season. Volatile compounds, important to orange juice aroma (20−24 compounds) (*12*, *27*−*29*), were analyzed (**Tables 9**−**12**). For fresh squeezed Hamlin juice, only hexanol levels were different, being higher in juice from Las(+) trees than in Las(−) controls (0.76 and 1.86 μg mL^{-1}, respectively). For Midsweet, ethanol was higher in juice from Las(+) trees compared to juice from Las(−) trees, while hexanal, *cis*-3-hexenol, and linalool were higher in Las(−) controls (**Table 9**).

For Valencia juice, the levels of volatiles showed more variation by harvest date than by disease (**Table 10**). Also, as with Hamlin and Midsweet juice, there was no apparent pattern due to disease. For aldehydes, when there was a difference (mostly for May), aldehyde volatiles were higher in the juice from Las(−) controls. For alcohols, levels were higher for the juice from the Las(−) controls for methanol, *trans*-2-hexenol, linalool, and octanol in May, and for Las(+) for methanol and hexanol in April and June, 2-methylpropanol in June, *cis*-3-hexenol in March, May, and June, linalool in June, and α-terpineol in April. For terpenes, limonene and valencene were higher in juice from Las(−) controls in May and March, respectively, while sabinene was higher in juice from Las(+) trees in June. For esters, ethyl acetate, ethyl butanoate, and methyl butanoate were higher in Las(−) control juice for the May harvest while methyl butanoate and ethyl hexanoate were higher in juice from Las(+) trees in April (methyl butanoate), May, and June (ethyl hexanoate). Harvest date was highly significant for all volatiles except α-terpineol, valencene, and methyl butanoate. For all harvest dates combined, disease was only significant for about half the volatiles measured: octanal, decanal, 2-methylpropanol, *cis*-3-hexenol,

Table 8. Secondary Metabolites (μg mL^{-1}, Excluding Marked Compounds) in Valencia Orange Juice from Fruit Harvested from Three Replicates of Three Trees with or without Greening Disease (±Las) in April and June 2008

	hydroxycinnamic acid at 6.3 min[a]		hydroxycinnamic acid at 7.2 min[a]		alkaloid[a]		6, 8-di-C-glucosyl apigenin[d]	
harvest (month)	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
April	8.0Aa[b]	8.0Aa	1.4Ba	1.2Ba	1.1Ba	1.0Ba	48.9Ba	51.19Ba
June	7.0Aa	7.1Aa	6.9Aa	8.1Aa	2.1Aa	2.0Aa	99.5Aa	105.8Aa
				ANOVA (F values)[c]				
harvest (H)	4.21^{NS}		4.60*		189.95***		19.10***	
disease (D)	0.87^{NS}		12.87**		0.91^{NS}		4.33*	
H × D	0.16^{NS}		0.47^{NS}		11.07**		1.44^{NS}	

	feruloyl putrescine[e]		narirutin 4′-glucoside[d]		limonin glucoside[e]		narirutin[d]	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
April	18.4Ba	18.4Ba	21.8Ba	22.1Ba	66.9Ba	54.9Ba	28.2Ba	25.8Ba
June	61.1Ab	76.8Aa	10.0Ba	11.7Ba	107.5Ab	125.9Aa	42.4Aa	34.0Aa
				ANOVA (F values)				
harvest (H)	76.94***		871.84***		88.46***		249.42***	
disease (D)	1.66^{NS}		11.01**		11.86**		9.28**	
H × D	0.76^{NS}		6.59*		5.76*		12.58**	

	nomilin glucoside[e]		nomilinic acid glucoside[e]		limonin[e]		nomilin[e]	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
April	63.6Aa	71.2Aa	113.6Aa	114.7Aa	0.61Ba	0.52Aa	0.50Aa	0.59Aa
June	55.1Ba	69.5Aa	51.6Ba	61.0Ba	0.66Aa	0.43Bb	0.08Ba	0.08Ba
				ANOVA (F values)				
harvest (H)	574.35***		872.24***		94.01***		18.13***	
disease (D)	1.61^{NS}		10.90**		103.48***		29.21***	
H × D	4.40*		6.55*		13.46***		0.00^{NS}	

[a] Relative peak area. [b] Values followed by the same capital letter within columns or the same small letter within rows are not significantly different by the Fisher's least significant difference test at $P = 0.05$. [c] ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant. [d] Tentatively identified. [e] Identified based on reference standards.

trans-2-hexenol, sabinene, valencene, ethyl butanoate, and ethyl hexanoate. There was significant harvest date × disease interactions for octanal, hexanal, decanal, 2-methylpropanol, *trans*-2-hexenol, linalool, octanol, and methyl butanoate (**Table 10**).

Volatile Analysis for the 2008 Season. For commercially processed Hamlin juice, aldehyde volatiles acetaldehyde and octanal were higher in the juice from Las(−) control trees for the February and December harvests, respectively, compared to juice from Las(+) trees (**Table 11**). For alcohols, ethanol and *cis*-3-hexenol were higher in controls for December and February harvests, respectively. For terpenes, myrcene was higher in juice from Las(+) trees for both harvests and sabinene was higher in juice from Las(−) trees for February. For esters, ethyl acetate and ethyl butanoate were higher in Las(−) controls for February, while ethyl hexanoate was higher in juice from Las(+) trees for both harvests. There were highly significant harvest date effects for about half of the volatiles studied: acetaldehyde, octanal, hexanal, methanol, ethanol, hexanol, 2-methylpropanol, sabinene, ethyl butanoate, methyl butanoate, and ethyl hexanoate. There was a significant disease effect for the combined harvest dates only for sabinene and ethyl hexanoate and a significant harvest date × disease interaction for acetaldehyde, octanal, and *cis*-3-hexenol (**Table 11**).

For commercially processed Valencia juice, only ethanol, hexanol, sabinene, and ethyl acetate aroma volatiles showed differences between the juice from Las(+) and Las(−) trees, and only in the June harvest. Hexanol and ethyl acetate were higher in the juice from Las(−) controls, while ethanol and sabinene were higher juice from Las(+) trees (**Table 12**). There were more differences due to harvest date than due to disease with harvest date being significant for octanal, decanal, methanol, hexanol, *cis*-3-hexenol, octanol, α-pinene, myrcene, sabinene, ethyl acetate, ethyl butanoate, and methyl butanoate. There was one significant harvest date × disease interaction for ethanol (**Table 12**).

Table 9. Volatile Content in Midsweet Orange Juice from Fruit Harvested from Five Replicate Trees with or without Greening Disease (±Las) in February 2007[a]

	content (μg mL^{-1})		
compound	(−)Las	(+)Las	t test
acetaldehyde	59.10	56.50	NS
hexanal	0.028	0.017	0.05
decanal	0.158	0.004	NS
methanol	153.5	165.7	NS
ethanol	4241	5355	0.05
hexanol	1.447	1.449	NS
cis-3-hexenol	0.656	0.527	0.05
trans-2-hexenol	0.004	0.004	NS
linalool	1.239	1.121	0.05
α-terpineol	1.329	1.219	NS
octanol	0.035	0.010	NS
α-pinene	0.081	0.076	NS
limonene	46.38	37.90	NS
sabinene	0.003	0.003	NS
valencene	4.373	4.248	NS
ethyl acetate	3.948	4.217	NS
ethylbutanoate	0.112	0.118	NS
methylbutanoate	0.039	0.054	NS
ethyl hexanoate	0.006	0.006	NS

[a] All aroma volatiles identified based on reference standards.

Table 10. Volatile Content (μg mL^{-1}) in Valencia Orange Juice from Fruit Harvested from Five Replicate Trees with or without Greening Disease (±Las) from March to June 2007[a]

	aldehydes							
	acetaldehyde		octanal		hexanal		decanal	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
March	48.7Ba[b]	41.2Cb	0Ba	0Ba	0.039Ba	0.037Ba	0.52BCa	0.52BCa
April	80.5Aa	72.8Ba	4Ba	4Ba	0.018Ca	0.022Ca	0.54Ba	0.49Ca
May	71.6Aa	60.9Ba	0.528Aa	0.337Ab	0.044Ba	0.021Cb	2.56Aa	1.26Ab
June	78.1Aa	85.4Aa	0.046Ba	0.048Ba	0.067Aa	0.067Aa	1.01Ba1	1.08Ba
ANOVA (F value)[c]								
harvest (H)	27.95***		129.10***		45.32***		21.57***	
disease (D)	2.28^{NS}		6.57*		2.80^{NS}		4.92*	
H × D	1.72^{NS}		6.74**		4.34*		5.36*	

	alcohols (1)									
	methanol		ethanol		hexanol		2-methylpropanol		*cis*-3-hexenol	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
March	78.3Aa	77.6Aa	4435Ba	3821Ca	0.30Ca	0.41Ca	0Ba	0Ca	0.82Ab	0.96Aa
April	69.5Ab	81.5Aa	4814Ba	4350BCa	0.70Cb	1.45Ba	0Ba	0Ca	0.40Ba	0.38Ba
May	47.5Ba	26.5Cb	5060Ba	4739Ba	3.24Aa	2.91Aa	0.091Aa	0.127Ba	0.47Bb	0.82Aa
June	41.4Bb	51.6Ba	5655Aa	5912Aa	2.25Bb	3.19Aa	0.063Ab	0.177Aa	0.74Ab	0.95Aa
ANOVA (F value)										
harvest (H)	18.70***		16.32***		38.75***		33.22***		16.60***	
disease (D)	0.00^{NS}		2.71^{NS}		3.03^{NS}		10.62**		9.15**	
H × D	2.53^{NS}		1.21^{NS}		1.95^{NS}		5.41**		1.77^{NS}	

	alcohols (2)							
	trans-2-hexenol		linalool		octanol		α-terpineol	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
March	0.040Ca	0.029Ba	1.66Ca	1.64 Da	0.173Ba	0.135Ca	0.94Aa	0.90Aa
April	0.033Ca	0.008Ba	2.10Ba	2.01Ca	0.188Ba	0.144BCa	0.80Bb	1.50Aa
May	0.283Aa	0.204Ab	3.15Aa	2.61Bb	0.476Aa	0.274Bb	0.76Ba	0.81Aa
June	0.206Ba	0.221Aa	2.90Ab	3.58Aa	0.460Aa	0.560Aa	0.99Aa	0.74Aa
ANOVA (F value)								
harvest (H)	213.53***		80.11***		27.79***		1.04^{NS}	
disease (D)	9.96**		0.01^{NS}		1.97^{NS}		0.53^{NS}	
H × D	6.12**		9.55**		3.57*		1.78^{NS}	

	terpenes									
	α-pinene		myrcene		limonene		sabinene		valencene	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
March	0.104Ba	0.080Ba	0Ba	0Ba	58Ba	49Ca	0.060Ba	0.049Ba	2.71Aa	2.31Ab
April	0.145Ba	0.215Ba	0Ba	0Ba	90Ba	109Ba	0.069Ba	0.097Ba	2.59Aa	2.49Aa
May	0.828Aa	0.755Aa	2.64Aa	2.56Aa	336Aa	274Ab	0.512Aa	0.598Aa	2.46Aa	2.44Aa
June	0.789Aa	0.869Aa	2.53Aa	2.84Aa	302Aa	302Aa	0.572Ab	0.890Aa	2.49Aa	2.50Aa
ANOVA (F value)										
harvest (H)	82.13***		37.01***		100.32***		29.01***		0.35^{NS}	
disease (D)	0.09^{NS}		0.05^{NS}		0.96^{NS}		4.92*		3.53*	
H × D	0.73^{NS}		0.11^{NS}		1.71^{NS}		1.51^{NS}		1.92^{NS}	

	esters							
	ethylacetate		ethyl butanoate		methyl butanoate		ethyl hexanoate	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
March	3.86Aa	3.83Aa	0.0548Ba	0.0408Ba	0.0106Ba	0.0112Ba	0Ba	0.005Ba
April	3.27BCa	3.40Ba	0.0864Aa	0.0694Aa	0.0202ABb	0.0452Aa	0Ba	0.016Ba
May	3.43Ba	3.00Cb	0.0858Aa	0.0574ABb	0.0400Aa	0.0112Bb	0.075Ab	0.159Aa
June	3.02Ca	2.82Ca	0.0814Aa	0.0674Aa	0.0284ABa	0.0194Ba	0.035ABb	0.113Aa
ANOVA (F value)								
harvest (H)	20.92***		5.00**		2.42^{NS}		14.63***	
disease (D)	2.61^{NS}		9.02**		0.27^{NS}		10.31**	
H × D	1.96^{NS}		0.31^{NS}		3.67*		1.99^{NS}	

[a] All aroma volatiles identified based on reference standards. [b] Values followed by the same capital letter within columns or the same small letter within rows are not significantly different by the Fisher's least significant difference test at $P = 0.05$. [c] ***, $P < 0.001$; **, $P < 0.01$;*, $P < 0.05$; NS, not significant.

Table 11. Volatile Content (μg mL^{-1}) in Hamlin Orange Juice from Fruit Harvested from Three Replicates of Three Trees Each with or without Greening Disease (±Las) in December 2007 and February 2008[a]

	aldehydes							
	acetaldehyde		octanal		hexanal		decanal	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
December 2007	41.7Ba[b]	44.3Ba	0.55Aa	0.21Bb	0Ba	0Ba	2.03Aa	2.09Aa
February 2008	82.3Aa	67.7Ab	0.63Aa	0.63Aa	0.045Aa	0.024Aa	2.32Aa	2.27Aa
ANOVA (*F* value)[c]								
harvest (H)	104.28***		6.87*		11.74**		0.47^{NS}	
disease (D)	3.58^{NS}		2.54^{NS}		1.09^{NS}		0.04^{NS}	
H × D	7.54*		11.13**		1.09^{NS}		0.06^{NS}	

	alcohols (1)									
	methanol		ethanol		hexanol		2-methylpropanol		*cis*-3-hexenol	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
December 2007	25.3Ba	21.1Ba	2476Ba	2560Ba	0.98Ba	3.16Ba	0.513Aa	0.672Aa	1.35Aa	0.65Bb
February 2008	84.8Aa	71.5Aa	8909Aa	5873Ab	8.02Aa	7.47Aa	0.273Ba	0.375Ba	1.05Aa	1.24Aa
ANOVA (*F* value)										
harvest (H)	62.91***		25.73***		58.48***		13.02**		3.37^{NS}	
disease (D)	1.60^{NS}		2.36^{NS}		1.21^{NS}		3.08^{NS}		2.14^{NS}	
H × D	0.43^{NS}		2.64^{NS}		3.40^{NS}		0.14^{NS}		4.27*	

	alcohols (2)							
	trans-2-hexenol		linalool		octanol		α-terpineol	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
December 2007	0.138Aa	0.141Aa	1.72Aa	1.93Aa	0.554Ba	0.624Ba	2.33Aa	2.4 1Aa
February 2008	0.117Aa	0.163Aa	2.03Aa	1.96Aa	1.084Aa	1.045Aa	2.19Aa	2.27Aa
ANOVA (*F* value)								
harvest (H)	0^{NS}		0.90^{NS}		1.25^{NS}		1.30^{NS}	
disease (D)	0.28^{NS}		0.30^{NS}		0^{NS}		4.19^{NS}	
H × D	0.23^{NS}		0.05^{NS}		0.02^{NS}		1.30^{NS}	

	terpenes									
	α-pinene		myrcene		limonene		sabinene		valencene	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
December 2007	1.90Aa	2.57Aa	8.98Ab	11.14Aa	489Aa	533Aa	0.823Aa	0.989Aa	2.17Aa	2.95Aa
February 2008	1.75Aa	1.99Aa	4.90Bb	8.16Ba	481Aa	512Aa	0.756Aa	0.188Bb	3.59Aa	3.44Aa
ANOVA (*F* value)										
harvest (H)	0.88^{NS}		3.04^{NS}		0.15^{NS}		29.47***		2.35^{NS}	
disease (D)	1.35^{NS}		1.79^{NS}		0.96^{NS}		14.62**		0.25^{NS}	
H × D	0.30^{NS}		0.07^{NS}		0.03^{NS}		2.31^{NS}		0.55^{NS}	

	esters							
	ethyl acetate		ethyl butanoate		methyl butanoate		ethyl hexanoate	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
December 2007	2.45Ba	2.67Aa	0.126Ba	0.121Ba	0.514Aa	0.62Aa	0.278Ab	0.397Aa
February 2008	3.72Aa	1.95Bb	0.546Aa	0.357Ab	0.377Ba	0.30Ba	0.069Bb	0.102Ba
ANOVA (*F* value)								
harvest (H)	3.47^{NS}		45.15***		43.61***		82.83***	
disease (D)	3.91^{NS}		3.96^{NS}		0.63^{NS}		7.53*	
H × D	1.69^{NS}		3.54^{NS}		0.74^{NS}		2.45^{NS}	

[a] All aroma compounds identified based on reference standards. [b] Values followed by the same capital letter within columns or the same small letter within rows are not significantly different by the Fisher's least significant difference test at $P = 0.05$. [c] ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant.

Symptomatic Las(+) Fruit from HLB-Affected Trees and Effect of Pasteurization. All of the above analysis was done with healthy fruit from Las(−) trees or asymptomatic fruit from Las(+) trees. The Hamlin December 2007 and Valencia April 2008 harvests, however, also included sets of clearly symptomatic fruit (small, green, lopsided) from Las(+) trees. Large volumes of symptomatic

Table 12. Volatile Content (μg mL^{-1}) in Valencia Orange Juice from Fruit Harvested from Three Replicates of Three Trees Each with or without Greening Disease (±Las) in April and June 2008[a]

	aldehydes							
	acetaldehyde		octanal		hexanal		decanal	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
April	121.6Aa[b]	112.4Aa	2.99Aa	2.62Aa	0.106Aa	0.081Aa	5.82Aa	6.24Aa
June	105.1Aa	114.2Aa	0.66Ba	0.82Ba	0.114Aa	0.124Aa	3.64Ba	3.53Ba
ANOVA (*F* value)[c]								
harvest (H)	1.62^{NS}		17.89^{**}		2.35^{NS}		5.79^{*}	
disease (D)	0^{NS}		0.04^{NS}		0.21^{NS}		0.3^{NS}	
H × D	2.49^{NS}		0.29^{NS}		1.14^{NS}		0.51^{NS}	

	alcohols (1)									
	methanol		ethanol		hexanol		2-methylpropanol		*cis*-3-hexenol	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
April	99.89Aa	112.9Aa	12116Aa	11901Aa	5.06Ba	4.29Ba	0.299Aa	0.308Aa	1.32Ba	1.19Ba
June	53.59Ba	54.13Ba	8418Bb	13307Aa	9.40Aa	7.10Ab	0.319Aa	0.281Aa	1.52Aa	1.63Aa
ANOVA (*F* value)										
harvest (H)	31.26^{***}		1.42^{NS}		7.72^{*}		0^{NS}		6.47^{*}	
disease (D)	0.52^{NS}		5.93^{*}		1.43^{NS}		0.04^{NS}		1.37^{NS}	
H × D	0.44^{NS}		7.07^{*}		0.35^{NS}		0.13^{NS}		1.46^{NS}	

	alcohols (2)							
	trans-2-hexenol		linalool		octanol		α-terpineol	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
April	0.136Aa	0.121Aa	6.74Aa	5.86Aa	2.51Aa	2.09Aa	2.16Aa	2.00Aa
June	0.082Aa	0.131Aa	4.58Ba	5.30Aa	0.93Ba	1.01Ba	2.16Aa	2.53Aa
ANOVA (*F* value)								
Harvest (H)	0.11^{NS}		4.34^{NS}		29.34^{***}		1.00^{NS}	
Disease (D)	0.07^{NS}		0.02^{NS}		0.48^{NS}		1.00^{NS}	
H x D	0.24^{NS}		1.48^{NS}		1.02^{NS}		1.00^{NS}	

	terpenes									
	α-pinene		myrcene		limonene		sabinene		valencene	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
April	2.29Aa	2.16Aa	9.60Aa	9.29Aa	520Aa	512Aa	0.84Aa	0.87Ba	3.02Aa	2.04Aa
June	1.38Ba	1.35Ba	3.77Ba	4.47Ba	422Aa	378Aa	1.05Ab	1.60Aa	3.23Aa	3.25Aa
ANOVA (*F* value)										
harvest (H)	8.99^{*}		19.68^{**}		3.92^{NS}		11.24^{**}		1.22^{NS}	
disease (D)	0.08^{NS}		0.03^{NS}		0.20^{NS}		3.99^{NS}		0.56^{NS}	
H × D	0.03^{NS}		0.18^{NS}		0.10^{NS}		1.16^{NS}		0.60^{NS}	

	esters							
	ethyl acetate		ethyl butanoate		methyl butanoate		ethyl hexanoate	
	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las	(−)Las	(+)Las
April	1.81Ba	1.80Ba	0.587Aa	0.610Aa	0.514Aa	0.620Aa	0.066Aa	0.109Aa
June	3.40Aa	2.16Ab	0.187Ba	0.180Ba	0.377Aa	0.299Ba	0.072Aa	0.094Aa
ANOVA (*F* value)								
harvest (H)	18.64^{**}		179.98^{***}		6.57^{*}		0.02^{NS}	
disease (D)	5.91^{*}		0.08^{NS}		3.04^{NS}		0.90^{NS}	
H × D	1.32^{NS}		0.24^{NS}		1.11^{NS}		0.09^{NS}	

[a] All aroma volatiles identified based on reference standards. [b] Values followed by the same capital letter within columns or the same small letter within rows are not significantly different by the Fisher's least significant difference test at $P = 0.05$. [c] ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant.

fruit were difficult to find, since most trees are removed before they reach the stage of producing symptomatic fruit; therefore, these harvests could not be replicated, but trees in the same vicinity were found from which Las(−) healthy fruit, Las(+) asymptomatic fruit, and Las(+) symptomatic fruit were collected. Therefore, fruit and juice of three replicates of 15 fruits of Las(−)

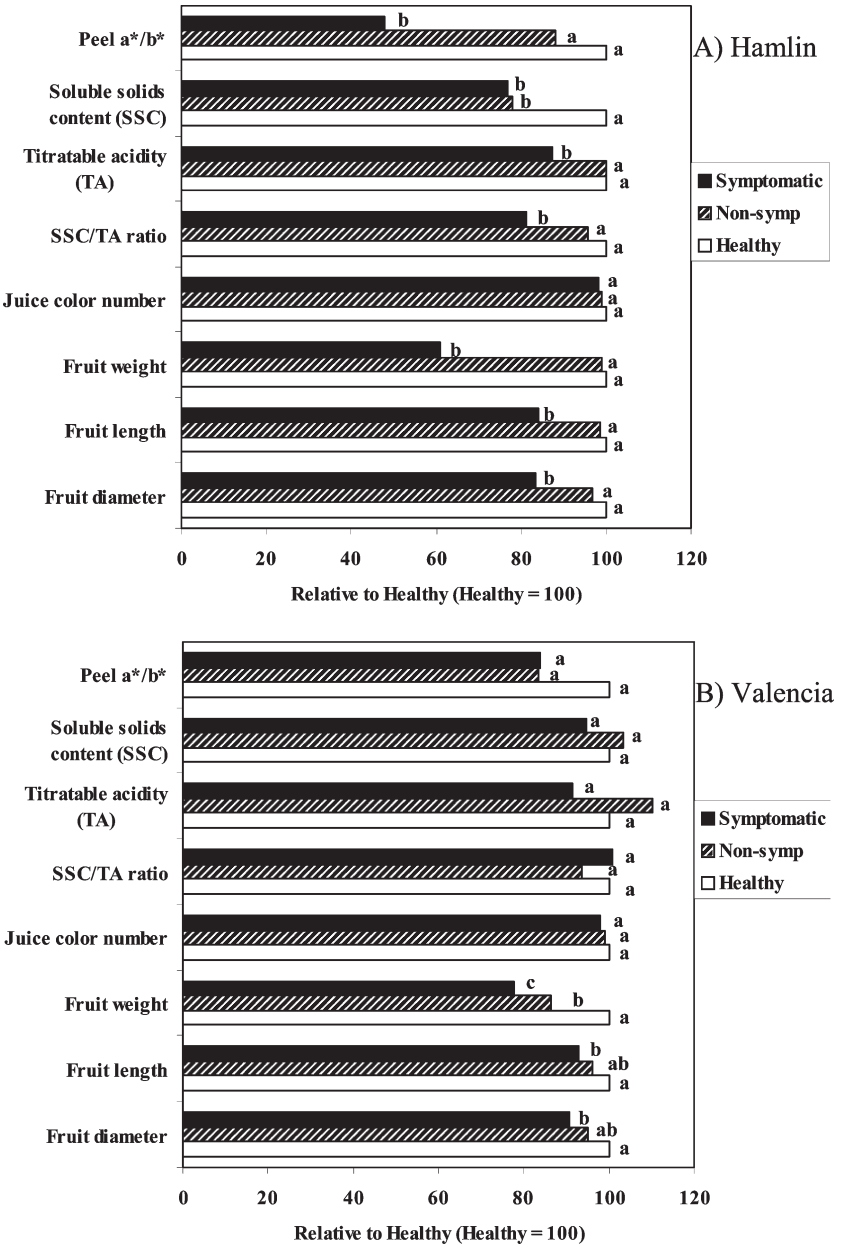

Figure 1. Relative quality attributes of Las(+) symptomatic and nonsymptomatic (nonsymp) fruit and juice for (**A**) Hamlin and (**B**) Valencia as a percentage of healthy Las(−) controls (controls = 100%). Means are calculated from three replicates.

healthy, Las(+) asymptomatic, and Las(+) symptomatic samples were compared (Las+ asymptomatic or "nonsymptomtic" and "symptomatic" fruit values are expressed as a percent of Las− controls) for Hamlin and Valencia. For these samples, the juice of these fruits was prepared with and without pasteurization. Results showed that pasteurization had little effect on many of the measured juice quality attributes (data not shown). For Hamlin quality attributes of fruit and pasteurized juice, peel color was greener (a^*/b^* values lower) for Las(+) symptomatic fruit, SSC was higher for Las(−) controls, TA was lower for Las(+) symptomatic fruit juice, as was the SSC/ acid ratio, fruit weight, length, and diameter compared to Las(+) nonsymptomatic and Las(−) healthy control fruit and juice, with no differences between the latter two groups except for SSC (**Figure 1A**). It is not known how the low fruit size affects fruit yield/tree in Florida, but studies are underway. For Valencia fruit and pasteurized juice, there were much less differences. There were no differences for peel color, SSC, TA, SSC/acid ratio, or juice color; however, Las(−) control fruit were heavier, longer, and wider than symptomatic fruit, and heavier than Las(+) nonsymptomatic fruit, which were in turn heavier than Las(+) symptomatic fruit (**Figure 1B**).

The effect of disease in Hamlin samples resulted in higher levels of N, L, FP, the alkaloid, and the HCAs in Las(+) symptomatic fruit juice (**Figure 2A**) compared to Las(+) nonsymptomatic or Las(−) control juice. Higher levels of N also occurred in Las(+) nonsymptomatic fruit juice compared to Las(−) controls. NG and NR were also higher in Las(+) symptomatic than Las(+) nonsymptomatic fruit juice but not significantly different from controls. For Valencia juice secondary metabolites, N was higher in the Las(+) symptomatic and Las(+) nonsymptomatic juice compared to Las(−) healthy controls, as was NG (**Figure 2B**). L, NAG, 6,8-di-*C*-glucosyl apigenin, and the 6.3 min HCA were higher in Las(+) symptomatic samples than in Las(+) nonsymptomatic or Las(−) control juice. For both Hamlin and Valencia juice, when there were differences due to pasteurization, generally

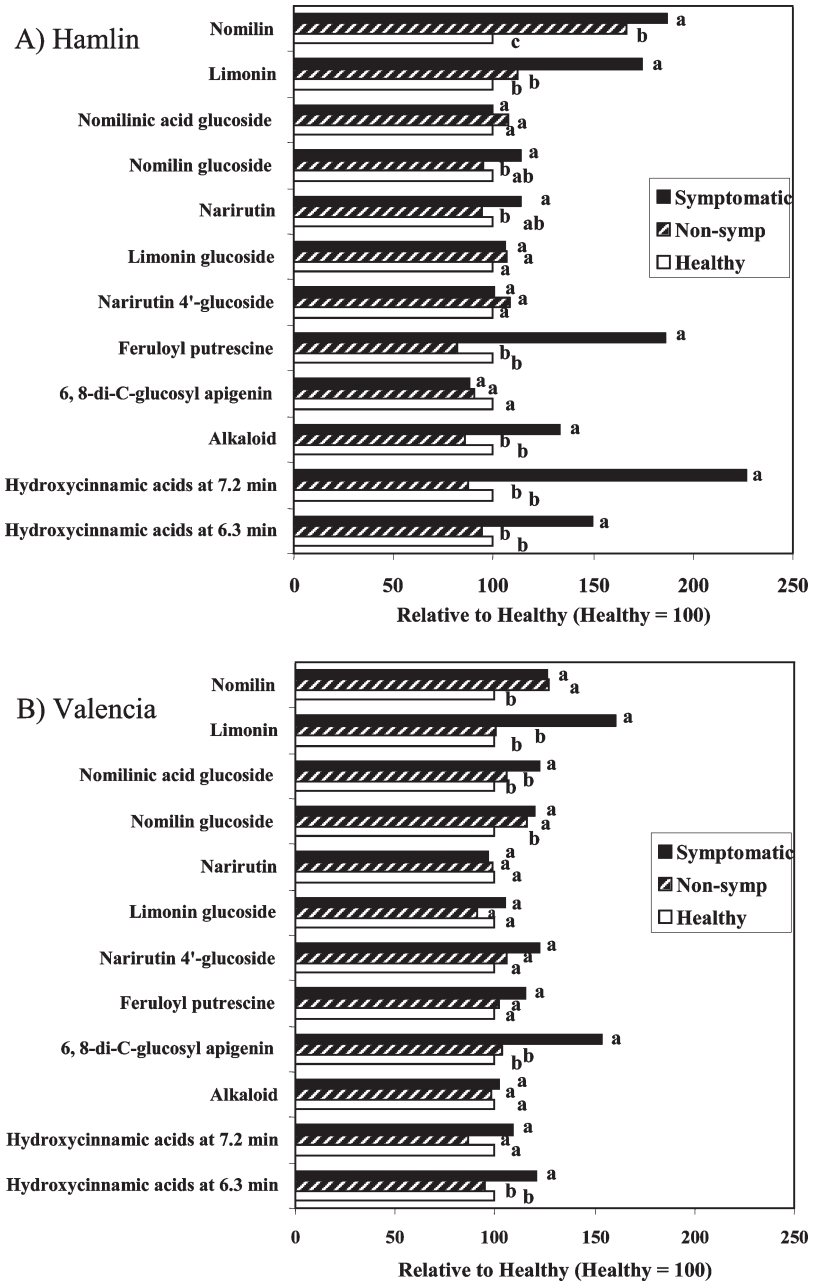

Figure 2. Relative levels of secondary metabolites of Las(+) symptomatic and nonsymptomatic (nonsymp) fruit and juice for (**A**) Hamlin and (**B**) Valencia as a percentage of healthy Las(−) controls (controls = 100%). Means are calculated from three replicates. Nomilin, limonin, and their glucosides as well as nomilinic acid glucoside and feruloyl putrescine were identified using reference standards. The other compounds are tentatively indentified.

levels of secondary metabolites were higher in nonpasteurized versus pasteurized juice (data not shown).

Cloud Loss and PME Activity in Las(+) and Las(−) Juice. The reduced absorbance values in all the unpasteurized samples used in the cloud loss study suggest some juice cloud destabilization had occurred prior to being frozen (**Figure 3**). Fresh and pasteurized juice from Valencia fruit typically have cloud absorbance values ~2.0 AU (absorbance at 660 nm). Juice cloud became less stable after 24 h for the Las(+) samples. By 72 h, juice cloud had fallen below 1.0 AU in the Las(+) juice, indicative of a destabilized cloud. Absorbance values in the Las(−) juice samples did not show any cloud destabilization during the study period.

Total PME and TT-PME activity was significantly lower in the Las(+) (asymptomatic fruit) juice (6.93 and 0.12 mol min^{-1} L^{-1} juice, respectively) compared to Las(−) juice (8.32 and 0.36 mol min^{-1} L^{-1} juice, respectively). PME activity is known to be a causative agent for cloud loss in citrus juices (*30*−*32*). The proportion of the total PME activity due to the TT-PME isozymes in the Las(−) juice was nearly double that estimated for the Las(+) juice. Although the total- and TT-PME activity levels were significantly lower in the Las(+) juice, cloud in these samples was destabilized more rapidly than in the Las(−) juice (**Figure 3**), which is puzzling. Cameron et al. (*30*) have shown that there are multiple forms of PME present in citrus and the individual forms destabilize juice cloud at different rates. The more rapid cloud destabilization in the Las(+) juices, even though they had lower PME activity, may be a result of differing proportions of individual isozymes with the isozymes that destabilize cloud most rapidly being the dominant forms present in this juice.

Multivariate Analysis of Chemical Data. To obtain a broader picture, select chemical flavor data (SSC, TA, SSC/TA, L, and N)

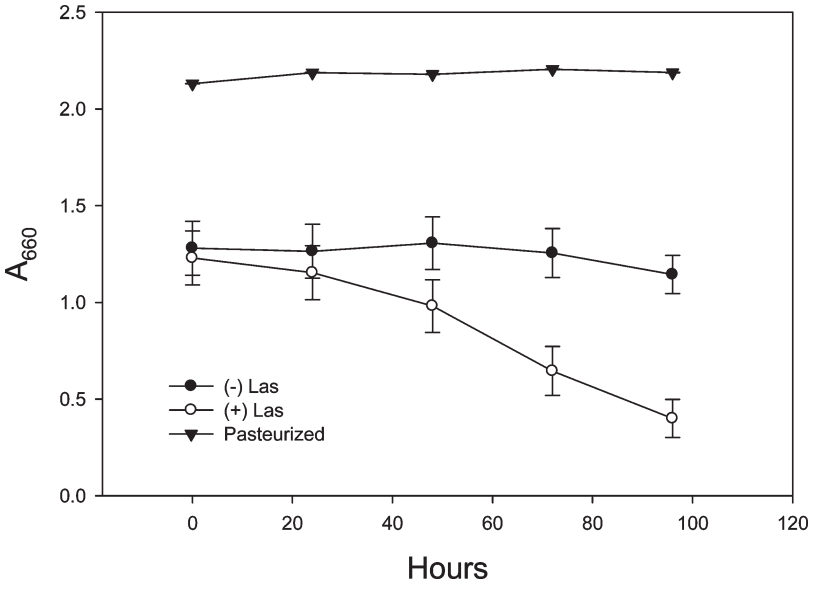

Figure 3. Cloud loss in juice from Las(+) and Las(−) commercially processed Valencia fruit harvested in June. Data for Las(+) and Las(−) are means and standard errors calculated from three replicates at each time point.

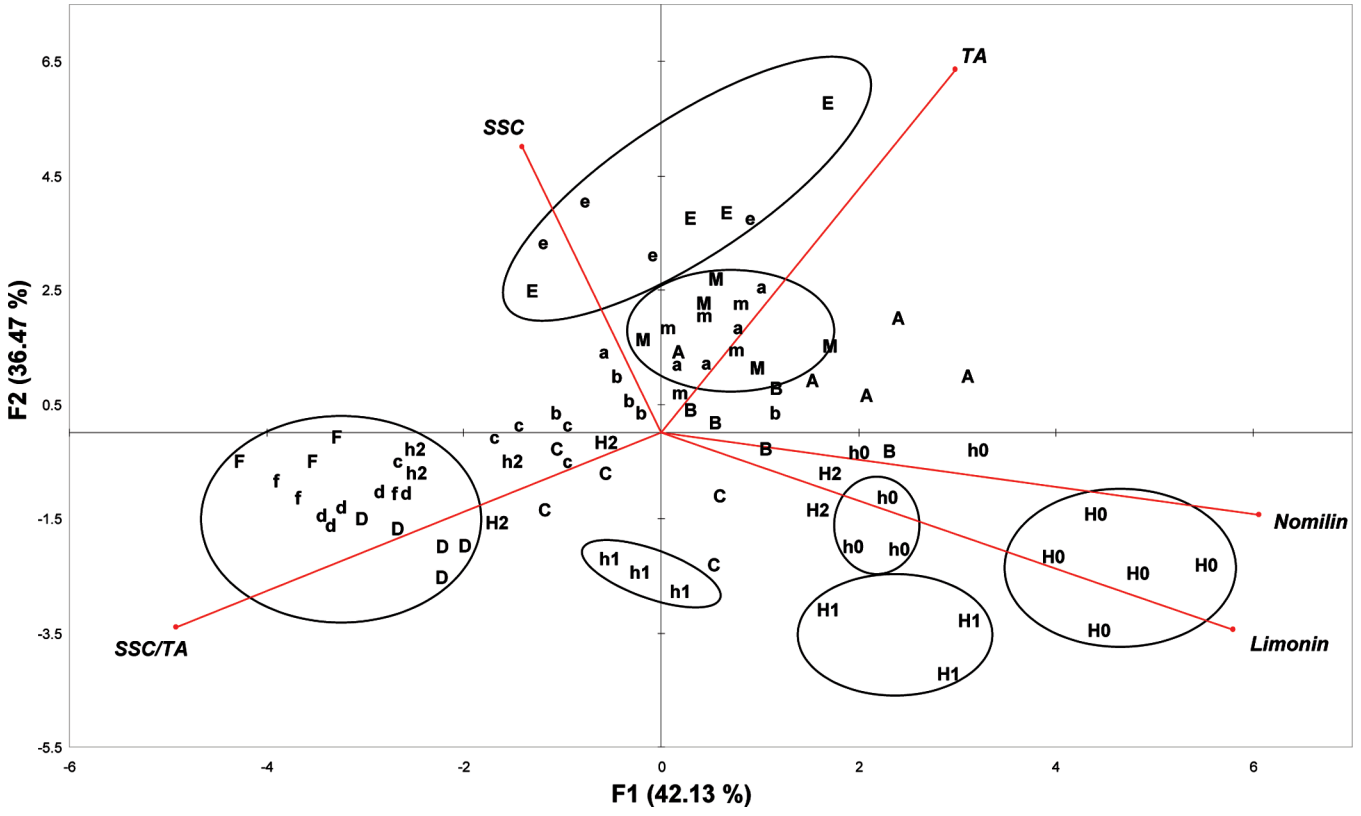

Figure 4. PCA biplot of all varieties tested in 2007 and 2008, using SSC, TA, SSC/TA, limonin, and nomilin variables. Axes, F1 and F2, account for 78.60% of the variation. a/A: Valencia March 2007, Las ±. b/B: Valencia April 2007, Las ±. c/C: Valencia May 2007, Las ±. d/D: Valencia June 2007, Las ±. e/E: Valencia April 2008, Las ±. f/F: Valencia June 2008, Las ±. h0/H0: Hamlin 2007, Las ±. m/M: Midsweet 2007, Las ±. h1/H1: Hamlin 2008 first harvest (December 2007), Las ±. h2/H2: Hamlin 2008 second harvest (February), Las ±.

were analyzed using PCA so that trends and covariance could be discerned, although false positive correlations can occur in multivariate analyses of large and diverse data sets (*33*). The first two factors explained a total of 78.60% of the variation (**Figure 4**). Factor 1 (F1) accounts for 42.13% of the variation and is explained by SSC/TA on the negative side and N and L on the positive side. Factor 2 accounts for another 36.47% of the variation and is explained by SSC and TA. Hamlin Las(+) data from the 2007 and 2008 seasons were generally low in SSC/TA and high in L and N (lower right quadrant of figure), while Valencia (±) Las 2007 and 2008 data from later harvests are the opposite (lower left quadrant of figure). Hamlin Las(−) control data are closer to the center of the plot in the lower half (less L and N than Las+ Hamlin), while Las(±) Midsweet is in the center upper half, having shown less differences due to disease. The earlier Valencia 2008 harvest is clustered high on F2, indicating

these samples were high in SSC and TA but low in SSC/TA (due to high TA) than later harvested Valencia samples, clustered in the lower left quadrant. So this reiterates that differences between the Las(±) fruit were influenced by variety and harvest date.

There seems to be significant variation in juice quality attributes and chemical compositions due to tree, location, and harvest date, which make interpretation difficult. It is not known whether, as the disease progresses, more chemical differences will become evident or more consistent, or how to determine the severity or duration of infection.

Generally, Las(+) asymptomatic fruit tended to be slightly smaller and greener, but not as much as symptomatic fruit. When differences between the levels of chemical flavor compounds for Las(+) versus Las(−) control juice were found, they seem to be more prevalent in Hamlin and more evident and consistent in the secondary metabolites, especially L, N, and their glucosides for all varieties. Levels of L and N, however, are at or below reported threshold in nonsymptomatic Las(+) fruit (*34*, *35*), indicating there should be minimal impact on flavor for these fruits, especially if incorporated into commercial juice blends that include fruit from healthy trees. Nevertheless, for commercially processed juice made from asymptomatic fruit in 2008, in the companion sensory paper, a model could explain some of the flavor differences between Las(+) and Las(−) juice with the L data (*9*). Sugars were lower in Las(+) Hamlin but not generally different in Valencia, which is also reflected in the companion sensory study (*9*) where more sensorial differences were found for asymptomatic Hamlin fruit than for Valencia, and much more for symptomatic fruit than for asymptomatic fruit of both varieties. Where differences were found, bitterness, metallic, saltiness, and sourness were often the descriptors (typical of some secondary metabolites such as alkaloids, L, and N) (*35*−*40*). In conclusion, symptomatic fruit generally exhibited higher levels of L relative to nonsymptomatic fruit or healthy controls and N compared to healthy controls (**Figure 2**) and showed flavor differences in trained panels (*9*). However, in a commercial situation where juices from different varieties, locations, and seasons are blended, the off-flavor of Las(+) symptomatic fruit would likely be diluted (as evidenced in most of the pooled samples for the 2008 season; see the Supporting Information), and since these fruit generally abscise (drop off the tree) in any case not many should enter the juice stream.

ACKNOWLEDGMENT

Joao Amador, JBT Food Technology, and Doug Van Strijp, US Sugars, are acknowledged for organizing and processing the juice.

Supporting Information Available: Additional information on fruit size, weight, and color as well as on the effect of blending juice from the 2008 harvests which reduced differences due to HLB disease. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Bové, J. M. Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. *J. Plant Pathol.* **2006**, *88*, 7–37.
(2) da Graça, J. V. Citrus greening disease. *Annu. Rev. Phytopathol.* **1991**, *29*, 109–136.
(3) Jagoueix, S.; Bové, J. M.; Garnier, M. PCR detection of the two '*Candidatus*' liberibacter species associated with greening disease of citrus. *Mol. Cell. Probes* **1996**, *10*, 43–50.
(4) Bassanezi, R.; Montesino, L.; Stuchi, E. Effects of huanglongbing on fruit quality of sweet orange cultivars in Brazil. *Eur. J. Plant Pathol.* **2009**, *125* (4), 565–572.
(5) Halbert, S. E.; Manjunath, K. L. Asian citrus psyllids (sternorrhyncha: psyllidae) and greening disease of citrus: a literature review and assessment of risk in florida. *Fla. Entomol.* **2004**, *87*, 330–353.
(6) Polek, M. V. G.; Godfrey, K. *Citrus bacterial canker disease and Huanglongbing* (*citrus greening*); University of California Publication, 8218, ANR; University of California: Davis, CA, 2007.
(7) FDOACS. Florida Department of Agriculture & Consumer Services, Division of Plant Industry. *Disease Detection Maps − Known Distribution of Citrus Canker*/*Citrus Greening* (*HLB*), http://www.doacs.state.fl.us/pi/chrp/ (accessed 2 July 2008).
(8) Plotto, A.; Baldwin, E. A.; McCollum, T. G.; Narciso, J. A.; Irey, M. Effect of early detection Huanglongbing on juice flavor and chemistry. *Proc. Fla. State Hortic. Soc.* **2008**, *121*, 265–269.
(9) Plotto, A.; Baldwin, E. A.; McCollum, G.; Manthey, J.; Narciso, J.; Irey, M. Effect of Liberibacter infection (Huanglongbing disease) of citrus on orange juice flavor quality by sensory evaluation. *J. Food Sci.* **2009**, submitted for publication.
(10) Li, W.; Hartung, J. S.; Levy, L. Quantitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus huanglongbing. *J. Microbiol. Methods* **2006**, *66*, 104–115.
(11) Baldwin, E. A.; Nisperos-Carriedo, M. O.; Baker, R.; Scott, J. W. Quantitative analysis of flavor parameters in six Florida tomato cultivars (*Lycopersicon esculentum* Mill). *J. Agric. Food Chem.* **1991**, *39*, 1135–1140.
(12) Nisperos-Carriedo, M. O.; Shaw, P. E. Comparison of volatile flavor components in fresh and processed orange juices. *J. Agric. Food Chem.* **1990**, *38*, 1048–1052.
(13) Wheaton, T. A.; Stewart, I. Feruloylputrescine: Isolation and identification from citrus leaves and fruit. *Nature* **1965**, *206*, 620–621.
(14) Albach, R. F.; Redman, G. H. Composition and inheritance of flavanones in citrus fruit. *Phytochemistry* **1969**, *8*, 127–143.
(15) Kumamoto, H.; Matsubara, Y.; Iizuka, Y.; Okamoto, K.; Yokoi, K. Structure and hypotensive effect of flavonoid glycosides in Kinkan (*fortunella japonica*) peelings. *Agric. Biol. Chem.* **1985**, *49*, 2613–2618.
(16) Horowitz, R. M.; Gentili, B. Flavonoid constituents of citrus. In *Citrus Science and Technology*; Nagy, S., Shaw, P. E., Velduis, M. K., Eds.; AVI Publishing: Westport, CT, 1977; Vol. 1, pp 397−426.
(17) Manners, G. D.; Breksa, A. P.; Schoch, T. K.; Hidalgo, M. B. Analysis of bitter limonoids in citrus juices by atmospheric pressure chemical ionization and electrospray ionization liquid chromatography-mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 3709–3714.
(18) Hasegawa, S.; Bennett, R. D.; Herman, Z.; Fong, C. H.; Ou, P. Limonoid glucosides in citrus. *Phytochemistry* **1989**, *28*, 1717–1720.
(19) Ohta, H.; Fong, C. H.; Berhow, M.; Hasegawa, S. Thin-layer and high performance liquid chromatographic analysis of limonoids and limonoid glucosides in Citrus seeds. *J. Chromatogr.* **1993**, *639*, 295–302.
(20) Krop, J. The mechanism of cloud loss phenomena in orange juice. Ph.D. Thesis, Agricultural University, Wageningen, The Netherlands, 1974.
(21) Pitifer, L. A.; McLellan, M. R.; Van Buren, J. P. Analysis of pectin content and degree of polymerization in orange juice. *Food Chem.* **1994**, *50*, 29–32.
(22) Luzio, G. A. Microwave release of pectin from orange peel albedo using a closed vessel reactor system. *Proc. Fla. State Hortic. Soc.* **2008**, *121*, 315–319.
(23) Grohmann, K.; Baldwin, E. A. Hydrolysis of Orange Peel with Pectinase and Cellulase Enzymes. *Biotechnol. Lett.* **1992**, *14*, 1169–1174.
(24) Cameron, R. G.; Grohmann, K.; Hotchkiss, A. T. Separation and Detection of Oligogalacturonides. *Proc. Fla. State Hortic. Soc.* **2003**, *116*, 413–417.
(25) McGuire, R. G. Reporting of objective color measurements. *HortScience* **1992**, *27*, 1254–1255.
(26) Francis, F. J.; Clydesdale, F. M. *Food Colorimetry: Theory and Applications*; AVI Publ. Co.: Westport, CT, 1975; p 477.
(27) Shaw, P. I.; Ahmed, E. M.; Dennison, R. A. Orange juice flavor: contribution of certain volatile components as evaluated by sensory panels. *Proc. Int. Soc. Citriculture* **1977**, *3*, 804–807.
(28) Perez-Cacho, P. R.; Rouseff, R. L. Fresh squeezed orange juice odor: A review. *Crit. Rev. Food Sci. Nutr.* **2008**, *48*, 681–695.
(29) Plotto, A.; Margaría, C. A.; Goodner, K. L.; Baldwin, E. A. Odour and flavour thresholds for key aroma components in an orange juice

matrix: esters and miscellaneous compounds. *Flavour Fragrance J*. **2008**, *23*, 398–406.
(30) Cameron, R. G.; Baker, R. A.; Grohmann, K. Multiple forms of pectinmethylesterase from citrus peel and their effects on juice cloud stability. *J. Food Sci*. **1998**, *63*, 253–256.
(31) Ackerley, J.; Corredig, M.; Wicker, L. Clarification of citrus juice is influenced by specific activity of thermolabile pectinmethylesterase and inactive PME-pectin complexes. *J. Food Sci*. **2002**, *67*, 2529–2533.
(32) Versteeg, C.; Rombouts, F. M.; Spaansen, C. H.; Pilnik, W. Thermostability and orange juice cloud destabilizing properties of multiple pectinesterases from orange. *J. Food Sci*. **1980**, *45*, 969–971.
(33) Goodner, K. L.; Dreher, J. G.; Rouseff, R. L. The dangers of creating false classifications due to noise in electronic nose and similar multivariate analyses. *Sens. Actuators, B* **2001**, *80*, 261–266.
(34) Guadagni, D. G.; Maier, V. P.; Turnbaugh, J. G. Effect of some citrus juice constituents on taste thresholds for limonin and naringin bitterness. *J. Sci. Food Agric*. **1973**, *24*, 1277–1288.
(35) Rouseff, R. L.; Matthews, R. F. Nomilin, taste threshold and relative bitterness. *J. Food Sci*. **1984**, *49*, 777–779.
(36) Baldwin, E. A. Citrus Fruit. In *Biochemistry of Fruit Ripening*; Seymour, G. B., Taylor, J. E., Tucker, G. A., Eds.; Chapman and Hall: New York, 1993; pp 107−149.
(37) Kefford, J. F. The chemical constituents of citrus fruit. *Adv. Food Res*. **1959**, *9*, 285–372.
(38) Maier, V. P.; Bennett, R. D.; Hasegawa, S. Limonin and other limonoids. In *Citrus Science and Technology*; Nagy, S., Shaw, P. E., Veldhuis, M. K., Eds.; AVI: Westport, CT, 1977; Vol. 1, pp 355−396.
(39) Fong, C. H.; Hasegawa, S.; Herman, Z.; Ou, P. Limonoid glucosides in commercial citrus juices. *J. Food Sci*. **1989**, *54*, 1505–1506.
(40) Ting, S. V.; Rouseff, R. L. *Citrus Fruits and Their Products: Analysis and Technology*; Marcel Dekker: New York, 1986; p 312.

Received for review September 9, 2009. Revised manuscript received November 30, 2009. Accepted December 5, 2009.