

Analysis of flavor and other metabolites in lemon juice (*Citrus limon*) from Huanglongbing-affected trees grafted on different rootstocks

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Abstract

Flavor perception integrates sensory input from chemical receptors triggered by taste- and aroma-active metabolites to contribute to food flavor quality. Many factors alter flavor quality. Disease affects aroma and flavonoid constituents, causing off-flavors in plants. Huanglongbing (HLB) disease negatively affects citrus, although lemons are more tolerant. Lemon juice quality of HLB-affected fruits is not well studied. Lemon juice aroma-active compounds were profiled in this study using gas chromatography/mass spectrometry-olfactometry, and other metabolites contributing to overall flavor quality were investigated. Lemon juice from different rootstocks was discriminated using the metabolic profile. Flavor and other lemon juice metabolites also distinguished symptomatic from asymptomatic trees. Pathway enrichment analysis demonstrated that biosynthesis pathways of the Phosphotransferase system (PTS), and Starch and sucrose metabolism involving fructose, glucose and sucrose, were highly influenced by HLB status. This study provides the first comprehensive view of lemon juice metabolites, including alterations resulting from rootstock differences or disease severity.

Keywords: Huanglongbing, Aroma-active, Taste-active, Metabolites, Rootstocks

1. Introduction

Flavor perception is a complex integration of the sensory input transmitted by a combination of chemical receptors activated by both taste-active and aroma-active metabolites. Taste-active metabolites, which usually include primary and secondary metabolites, are non-volatile. Primary metabolites, including sugars, amino acids and organic acids, are found in high quantities, being continuously produced for use in cell growth as reactants in the biochemical reactions within an organism. These reactions produce secondary metabolites, such as aroma-active compounds, as well as plant specific flavonoids and limonoids,

and are found in much lower quantities in an organism. Identification of these individual taste- and aroma-active components is essential to determine the major contributing factors behind flavor quality of any food source. Focusing on these two flavor modalities, the taste-active, non-volatile compounds activate the gustatory sensory receptors, and the aroma-active, volatile compounds activate the olfactory sensory receptors. Together, these compounds, in very specific concentrations and combinations create the sensory perception of specific recognizable flavors. Consequently, there are many challenges in retaining the ideal concentrations and combinations in order to produce a food source with

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desirable flavor. With respect to agricultural commodities, one such challenge is disease. For example, plant diseases can result in concentration shifts of primary metabolites resulting in alterations of production of secondary metabolites directly affecting overall flavors in roots, shoot, leaves, fruits and seeds of plants. In particular, Huanglongbing (HLB), has been shown to cause off-flavors in citrus fruits, resulting in a more bitter tasting fruit than those from a healthy tree [1,2].

Commonly referred to as citrus greening disease, HLB has spread to North America [3]. In the United States (US), HLB results presumably from an infection of the bacterium, *Candidatus Liberibacter asiaticus* (CLas) [4], which is transmitted by the Asian Citrus Psyllid (ACP), *Diaphorina citri* Kuwayama. HLB was first reported in Florida in 2005, where it continues to negatively affect the cultivated citrus industry [4]. This disease has been devastating to Florida, the largest producer of citrus in the US. Although, most well-known in Florida for its damaging effects on orange and grapefruit varieties, HLB affects all citrus species to some extent, including mandarins, lemons and limes as well [5]. Current practices graft the scion cultivar onto a genetically distinct rootstock to compose the citrus trees grown for commercial uses. Rootstock selection is based on site characteristics, such as soil texture, pH, water holding capacity, site drainage and/or elevation, rootstock effects on fruit yield and quality, and tolerance to numerous diseases. Recent work in Florida has shown that some rootstocks are more supportive of tree health and productivity than others in the HLB-endemic environment [6].

Lemon (*Citrus limon*) is cultivated in many countries. Because of its sensitivity to sub-freezing temperatures, lemons are generally grown in warm or tropical climates. The top countries for lemon production include India, Argentina, Spain, Iran, and the US. In the US, California and Arizona produce the majority of commercial lemons, but warmer areas of Florida can successfully produce commercial lemons as well; because of its enhanced tolerance for HLB, more growers in Florida are planting new lemon orchards. This popular fruit is typically produced for its juice because it contains an abundance of vitamin C (ascorbic acid), yet lemon also has numerous alternate uses. The essential oils of lemon and lemon extracts are widely used in the food industry to add desirable flavor to drinks, and in the pharmaceutical industry to mask unpalatable tastes of medications [7]. Additionally, lemon is

known to provide numerous health benefits due to flavonoids, a subset of taste-active, secondary metabolites synthesized only in plants [8]. Although bitter tasting, flavonoids are major sources of fighting infection, stimulating the digestive tract, and possessing anti-oxidative activities [9].

To date, lemon fruit research focuses mainly on essential oils and their uses in flavoring and fragrances [10]. Research into lemon juice is limited, yet does include headspace solid phase micro-extraction (HS-SPME) analyses of aroma-active compounds from unspecified varieties of lemons used to flavor beverages, as well as lemon juice constituents and aroma-active compounds identified in specified healthy lemon varieties [7]. The main objective of this study is to examine flavor components, such as bitter taste-active and aroma-active secondary metabolites, as well as other metabolites, including the primary metabolites (sugars, amino acids and organic acids) that form them to better understand juice quality from lemon fruits of CLas-infected lemon trees grown on specified rootstocks. Although previous studies have reported the influence of secondary metabolites on the overall flavor of orange or mandarin juice from HLB-affected trees [1,2], no such studies have been conducted on lemon in particular how rootstocks could affect fruit quality in the HLB era. Therefore, the purpose of this research was to investigate flavor and other metabolite contributions to overall flavor quality of lemon juice from fruits of CLas-infected trees of different rootstocks. The metabolites identified in these lemon juice samples will be able to differentiate between rootstocks, and also differentiate between HLB symptomatic vs asymptomatic within a rootstock.

2. Material and methods

2.1. Plant material

A total of 16 'Bearss' lemon juice samples, four samples per four different groups, were obtained from a local grower (Vero Beach, Florida). Fruit was harvested from trees grafted on three different rootstocks, Sour Orange (SO), Gou Tou (GT) and Sun Chu Sha (SCS), and juiced on a commercial extractor. Fruits collected from SO rootstock were collected from both HLB symptomatic (SO/S) and asymptomatic (SO/A) trees, whereas fruits collected from GT and SCS were only from symptomatic trees. Symptomatic and asymptomatic tree status was identified using visual evaluations conducted by independent consultants. Symptomatic trees had characteristic asymmetrical, mottled leaves, as well

as small, lopsided fruits, whereas asymptomatic trees retained healthy looking green leaves, and symmetrical, typical-sized fruits. Juice from each of the four treatment groups came from lemons randomly harvested off four different trees per rootstock, providing biological replicates. Lemon fruits were mechanically juiced using typical commercial juicing machines, delivered frozen in 3.8 L jugs and stored at $-20\text{ }^{\circ}\text{C}$ until analysis.

2.2. Chemicals and reagents

Reference compounds (Supplementary file 1) were purchased from Sigma–Aldrich (St. Louis, MO, USA, the purity of these compounds $\geq 99\%$). Aroma-active analysis chemicals, sodium chloride and octyl acetate, were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sugar, amino, acid, organic acid, flavonoid and limonoid analysis chemicals, including methanol, pyridine, *n*-methyl-*n*-(trimethylsilyl) trifluoroacetamide, methoxyamine hydrochloride, ethyl acetate, adonitol, gallic acid and catechin were also purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Determination of aroma-active components using GC/MS-O

Juice samples were thawed, and then vortexed for 1 min. Five mL of sample was added to a 40 mL SPME vial containing 1.8 g of sodium chloride and a small stir bar. Then, 5 μL of 1000 mg/L octyl acetate stock solution was added to the sample mixture as an internal standard. The vial was capped with a PTFE/silicon septum cap and incubated in a water bath at $40\text{ }^{\circ}\text{C}$ for 30 min. The SPME fiber (50/30 μm DVB/CAR/PDMS, Supelco Inc., Bellefonte, PA, USA) was then inserted into the headspace of the vial at $40\text{ }^{\circ}\text{C}$ for 30 min to collect aroma-active compounds.

The aroma-active compound analysis was carried out using a Clarus 680 gas chromatograph/Clarus SQ 8 T mass spectrometer (GC/MS) coupled with olfactometer (O) (PerkinElmer, Inc., Waltham, MA, USA), on a TR-FFAP capillary column (30 m \times 0.25 mm, 0.25 μm film thickness, Thermo Scientific, Bellefonte, PA, USA). Column temperature was programmed to rise from $40\text{ }^{\circ}\text{C}$ (1 min hold) to $100\text{ }^{\circ}\text{C}$ (2 min hold) at a rate of $30\text{ }^{\circ}\text{C}/\text{min}$, subsequently increase to $175\text{ }^{\circ}\text{C}$ (2 min hold) at a rate of $5\text{ }^{\circ}\text{C}/\text{min}$, and finally increase to $230\text{ }^{\circ}\text{C}$ (6.5 min hold) at a rate of $35\text{ }^{\circ}\text{C}/\text{min}$. Mass spectra in the electron impact mode (MS-EI) were applied at 70 eV ionization energy. The GC inlet line

temperature and source temperature were set at $250\text{ }^{\circ}\text{C}$ and $200\text{ }^{\circ}\text{C}$, respectively. Helium was used as the carrier gas (1.1 mL/min). Split-less injection mode was used for manual injections of each sample. An *n*-alkanes (C7–C30) standard was used to determine linear retention indices (RI) of each aroma-active compound. Identification of aroma compounds was based on suggestions from the NIST library and confirmed through comparison with reference compounds, comparison of retention indices (RI) on the FFAP capillary column and using mass spectra in EI modes. Semi-quantification of aroma-active compounds was calculated using an internal standard, octyl acetate. A Swafer™ was used to split the samples into the mass spectrometry and the olfactory port ($240\text{ }^{\circ}\text{C}$). Two trained panelists were used to provide the olfactory data.

2.4. GC/MS analysis of sugars, amino acids and organic acids

Five mL of thawed sample was added to a 15 mL centrifuge tube and centrifuged at 5000 rpm for 5 min at room temperature. Next, the supernatant was filtered using a 0.22 μm nylon syringe filter and 10 μL of the filtered sample was pipetted into a 300 μL insert. Then, 40 μL of a methanoic solution (1 mg/mL) of adonitol was added to the insert as an internal standard. The insert was placed into a microfuge tube, closed tightly and briefly vortexed, before being reopened and dried using a Savant RVT5105 refrigerated vapor trap (Thermo Scientific, Waltham, MA, USA) for 8 h. Next, 30 μL methoxyamine hydrochloride (20 mg/mL in pyridine) was added to the insert and vortexed for 2 h using a tube vortex (Fisher Scientific, Waltham, MA, USA) at room temperature. Finally, 80 μL methyl-*n*-(trimethylsilyl) trifluoroacetamide was added to the insert, which was placed inside a glass vial and capped. The vial was then vortexed for 30 min using a tube vortex at room temperature, before being injected into the GC/MS.

Sugars, amino acids and organic acids were analyzed using an Agilent 7890 gas chromatograph/5975C mass spectrometer system (Santa Clara, CA, USA). One μL of each sample was injected at $280\text{ }^{\circ}\text{C}$ using split mode (10:1) onto an Rxi-5 MS capillary column (30m \times 0.25 mm; 0.25 μm film thickness, Restek, Bellefonte, PA, USA). Column temperature was programmed to rise from $70\text{ }^{\circ}\text{C}$ after 5 min, then increased $4\text{ }^{\circ}\text{C}/\text{min}$ to $270\text{ }^{\circ}\text{C}$ with no hold, and continued to rise $20\text{ }^{\circ}\text{C}/\text{min}$ to $320\text{ }^{\circ}\text{C}$ with a 4.5-minute hold. The MS source and MS Quad were maintained at $230\text{ }^{\circ}\text{C}$ and $150\text{ }^{\circ}\text{C}$, respectively. The *m/z* range scanned from 60 to 650 atm. Helium was used

as the carrier gas, with a flow rate maintained at 1.1 mL/min. Relative concentrations of sugars were calculated using adonitol as an internal standard.

2.5. LC/MS analysis of ascorbic acid

A 1 mL aliquot of each sample of lemon juice was centrifuged at 5000 g for 5 min at 4 °C. Supernatant was then collected and filtered through a 0.22 µm nylon filter. Next, 100 µL of filtered juice, 100 µL gallic acid (10 µg/mL dissolved in water) and 800 µL of water were combined. Each diluted juice sample was filtered through a 0.22 µm nylon filter prior to LC-MS analysis. A Thermo Ultimate 3000 HPLC equipped with a Thermo Quantiva triple quadrupole electrospray ionization tandem mass spectrometer (Thermo, Waltham, MA, USA) was used for analysis. Chromatographic separations were performed using a Phenomenex Gemini C18 column (3 µm, 3 × 150 mm) (Phenomenex, Torrance, CA, USA) with a mobile phase consisting of 0.1% formic acid aqueous solution (A) and 0.1% formic acid in acetonitrile (B). The gradient program was set as follows: 0–13 min, 2% B, 13–17 min, ramped to 95% B; 17–24 min, 95% B. The flow rate was set at 0.2 mL/min, column temperature maintained at 25 °C and the injection volume was 5 µL. The spray voltage, in negative mode, was set at 2,500 V. Other MS parameters were as follows: sheath gas, 35 arb; aux gas, 10 arb; sweep gas, 1 arb; CID gas, 2mTorr, ion transfer tube temperature: 325 °C, vaporizer temperature: 350 °C. Standard solutions of each amino acid were directly infused into the mass spectrometer at a flow rate of 200 µL/min. Product ions, collision energy and RF lens of each standard were optimized using TSQ Quantiva Tune software (Thermo Scientific). Selective reaction monitoring (MRM) in negative mode was employed at $[M-H]^-$ m/z 175 → 115 for ascorbic acid.

2.6. LC/MS analysis of flavonoids and limonoids

Five hundred µL of thawed sample was pipetted into a microfuge tube; 5 µL of 1000 ppm catechin (1 mg/1 mL MEOH) was added as an internal standard and the microfuge tube was briefly vortexed. Then, 500 µL ethyl acetate was added and samples were vortexed for 3 min. Samples were centrifuged at 5000 RPM for 5 min at room temperature and the supernatant transferred to a fresh microfuge tube. Next, an additional 500 µL ethyl acetate was added to the microfuge tube containing sample and vortexed for 3 min, before being centrifuged at 5000 RPM for 5 min at room temperature. Supernatant was again collected and added to the microfuge tube already containing

supernatant. Samples were then dried using a Savant RVT5105 refrigerated vapor trap (Thermo Scientific, Waltham, MA, USA). The dried sample was re-suspended in 500 µL methanol and then filtered through a 0.22 µm syringe filter into an amber LC glass vial for LC/MS analysis.

Lemon juice flavonoids were analyzed using a Thermo Ultimate 3000 HPLC equipped with a Thermo Quantiva triple quadrupole electrospray ionization tandem mass spectrometer (Thermo, Waltham, MA, USA). The analysis was performed following a modified procedure previously reported (Hung & Wang, 2018). Chromatographic separations were performed using a Gemini C18 column (3 µm, 3 × 150 mm) (Waters, Milford, MA, USA) with a mobile phase consisting of 0.1% formic acid aqueous solution (A) and 0.1% formic acid in acetonitrile (B). The gradient program was set as follows: 0–20 min, 5–75% B; 20–25 min, 75–95% B; 25–26 min, 95% B; 26–33 min, 95% B; 33–34 min, 95–5% B; 34–40 min, 5% B. The flow rate was set at 0.2 mL/min. The column temperature was maintained at 25 °C. The injection volume was 10 µL. The spray voltage in positive and negative modes was set at 3500 and 2,500 V, respectively. Additional MS parameters were as follows: sheath gas, 45 arb; aux gas, 20 arb; sweep gas, 1 arb; CID gas, 1.5mTorr, ion transfer tube temperature: 325 °C, vaporizer temperature: 275 °C.

2.7. Statistical analysis

Partial least-squares-discriminant analysis (PLS-DA) was performed using SIMCA (Version 15.0, Umetrics, Umea, Sweden). One-way analysis of variance (ANOVA) ($P < 0.05$) was performed using SPSS v.20.0 (IBM Corp., Armonk, NY) to estimate significant differences among rootstock data. After the test of homogeneity of variances, analytes indicating homoscedasticity ($p > 0.05$) were analyzed by ANOVA and Tukey, whereas Welch and Dunnett T3 (posthoc) were applied to analytes indicating heteroscedasticity ($p < 0.05$). T-tests ($P < 0.05$) and were performed using SPSS v.20.0 (IBM Corp., Armonk, NY) to estimate significant differences among HLB-symptomatic data. After the Levene's test for equality of variances, analytes indicating homoscedasticity ($p > 0.05$) were analyzed by applying equal variances assumed, whereas equal variances not assumed were applied to analytes indicating heteroscedasticity ($p < 0.05$). Metabolic pathway enrichment analysis was implemented using MBRole (<http://csbg.cnb.csic.es/mbrole2/index.php>). The purpose of metabolic pathway enrichment analysis is to identify coordinately changed Kyoto

Encyclopedia of Genes and Genomes (KEGG) pathways using metabolite data. References for pathway mapping include “Phosphotransferase system (PTS) (map02060)” and “Starch and sucrose metabolism (map 00500)” from KEGG database (<http://www.genome.jp/kegg/>).

3. Results

3.1. Identification of aroma-active compounds in lemon juice

Chromatographic separation of SPME extracts using GC/MS-O revealed a total of 33 aroma-active compounds from the four treatment groups (10 terpene hydrocarbons, 12 terpenoids, five aldehydes, two ketones, two alcohols and two esters) (Table 1).

3.2. Metabolite profile of lemon juice

A total of 84 metabolites were identified, including five sugars (sugar alcohol and sugar acids included), eight amino acids, four organic acids, 31 aroma-active compounds, and 36 flavonoids and limonoids. All analytes were semi-quantified using corresponding internal standards for comparison of the relative concentrations (Supplementary file 1).

3.3. Discrimination of metabolite profiles of lemon juice of three rootstocks

To investigate the possible rootstock effects on both primary and secondary metabolites in the lemon juice samples, partial least squares-discriminant analysis (PLS-DA) was applied to individual rootstocks using the full metabolic profile. Due to the overfitting phenomenon, rootstocks could not be discriminated. The PLS-DA score plot is presented in Supporting Information Fig S1a. Therefore, a more supervised method using PLS-DA was applied to the taste-active, primary and aroma-active, secondary metabolite profiles. In this case, rootstocks were discriminated (the y -intercepts of R^2 and Q^2 in the permutation test ($n = 999$) were 0.606 and -0.475 , respectively), demonstrated by 53.0% of the first two components (Fig. 1a). In Fig. 1a, lemon juice samples from the sour orange (SO) rootstock grouped closely together, despite being harvested from either symptomatic or asymptomatic trees. Lemon juice samples from the Sun Chu Sha (SCS) rootstock demonstrated separation, yet still grouped together, and Gou Tou (GT) samples also were

clearly distinguished from other rootstocks. Using the PLS-DA results, key metabolites dominating rootstock differentiation were identified using the variable importance for projection (VIP). Metabolites with a VIP value over 1.0 were considered potential markers distinguishing juice samples from different rootstocks. Meanwhile, one-way ANOVA ($p < 0.05$) was performed on the taste- and aroma-active compounds in lemon juice samples to estimate significant differences (Supplementary file 2). Combining VIP scores (Table 2) and ANOVA results, 15 metabolite markers were identified. Among these 15 markers, cis-carveol, caryophyllene, prenol and octanal were only identified in GT and/or SCS, whereas camphor and decanal were only present in the SO rootstock (Supplementary file 1). Juice samples from SCS rootstock possessed the largest amounts of β -pinene, nerol and sucrose, yet the least amount of p-cymene among all three rootstocks. Additional PLS-DAs were then applied individually, first to taste-active, primary metabolite compounds and second to the aroma-active, secondary metabolite compounds for each of the three different rootstocks. Results indicated that taste-active compounds alone did not discriminate lemon juice by rootstock (Supporting information Fig S1b), whereas aroma-active compounds demonstrated potential to group lemon juice samples by rootstock (Fig. 1b, the plot was validated by a 999x permutation test with $R^2 = 0.371$ and $Q^2 = -0.364$).

3.4. Discrimination of metabolite profiles among lemon juice samples obtained from HLB symptomatic and asymptomatic trees

In this study, full metabolic profile analyses were conducted on lemon juice samples of fruits harvested from symptomatic and asymptomatic trees on the SO rootstock to determine if HLB symptom severity significantly affects the overall lemon juice metabolic profile contributing to lemon juice flavor. In this instance, symptom severity was not discriminated using the full metabolic profile (Supporting information Fig S1c). However, PLS-DA applied to the taste-active, primary metabolite and aroma-active, secondary metabolite compound data of juice samples from lemons of symptomatic and asymptomatic trees clearly differentiated rootstocks by tree symptom status, supported by 95.0% using the first two components (Fig. 1c, the plot was validated by a 999x permutation test with $R^2 = 0.735$ and $Q^2 = -0.2$). A more in depth examination of the taste-active, primary metabolite and aroma-active,

Table 1. Aroma-active compounds identified in lemons juice.

RI (FFAP)	Compound	Odor quality ^a	Method of identification ^b
1003	α -pinene	citrusy, orange peel	RI, MS, Std, O
1046	camphene	fruity, plant	RI, MS, O
1065	hexanal	grassy, leafy	RI, MS, Std, O
1088	β -pinene	musty, green	RI, MS, Std, O
1133	β -myrcene	vegetable, leafy	RI, MS, Std, O
1180	D-limonene	minty	RI, MS, Std, O
1216	(E)-2-hexenal	green, leafy	RI, MS, O
1238	γ -terpinene	chestnut, sweet fruit	RI, MS, Std, O
1263	p-cymene	citrus	RI, MS, Std, O
1277	terpinolene	woody	RI, MS, Std, O
1278	octanal	orange, citrusy	RI, MS, Std, O
1293	1-octen-3-one	raw mushroom	RI, MS, Std, O
1308	prenol	roasted nut	RI, MS, O
1329	6-methyl-5-heptene-2-one	fruity	RI, MS, Std, O
1382	(Z)-3-hexen-1-ol	grassy, preserved vegetables	RI, MS, Std, O
1386	nonanal	citrusy, green	RI, MS, Std, O
1432	p-cymenene	floral	RI, MS, Std, O
1493	decanal	fruity, candy	RI, MS, O
1513	camphor	herb, celery, camphor	RI, MS, O
1535	linalool	sweet, floral	RI, MS, Std, O
1572	fenchol	earthy, moldy	RI, MS, O
1586	β -caryophyllene	woody	RI, MS, Std
1593	terpinen-4-ol	raw nut, mushroom	RI, MS, Std, O
1594	β -terpineol	vegetable	RI, MS, O
1674	neral	citrusy, lemon	RI, MS, Std
1691	α -terpineol	moldy, earthy, woody	RI, MS, Std, O
1728	geranial	grain, lemon	RI, MS, Std, O
1744	geranyl acetate	hay, cooked grain	RI, MS, O
1745	neryl acetate	sweet, herb, floral	RI, MS, O
1753	citronellol	sweet grain	RI, MS, Std, O
1789	nerol	sweet	RI, MS, Std, O
1822	cis-carveol	sweet, fruity	RI, MS, Std, O
1835	geraniol	sweet tea	RI, MS, Std, O

^a Odor quality detected at the sniffing port.

^b MS, reference mass spectrum; RI, reference index; Std, analytical standard; O, olfactometry.

secondary metabolite profiles of lemon juice samples from symptomatic and asymptomatic trees included further PLS-DAs applied individually to first, the taste-active, primary metabolite compounds, and second, to the aroma-active, secondary metabolite compounds. Neither of these individual analyses distinguished separation of symptom status from the lemon juice samples (Supporting information Fig S1d and S1e). Using PLS-DA results, significant metabolites were identified based upon the variable importance for projection (VIP). The metabolites with a VIP value over 1.0 were identified as potential markers in distinguishing lemon juice samples from symptomatic or asymptomatic trees. Meanwhile, a t-test ($p < 0.05$) was performed to estimate significant differences among metabolite contents in lemon juice samples (Supplementary file 2). Combining VIP scores and t-test results, the following five metabolite markers were selected

(Table 2). Pathway enrichment analysis using these markers (Table 2) indicated possible biological pathways related to these metabolites (Table 3). The metabolic pathways, Phosphotransferase system (PTS) and Starch and sucrose metabolism (Fig. 2), showed high matched/total percentages with low p-values (<0.05) and false discovery rate (FDR) correction values (<0.05), indicating a close relationship with HLB progression in lemon trees. Meanwhile, this also suggests that alteration of these two pathways in lemon trees may occur after HLB infection.

4. Discussion

4.1. Profiling of aroma-active components in lemon juice

Some aroma-active compounds identified in this investigation are consistent with previous aroma-

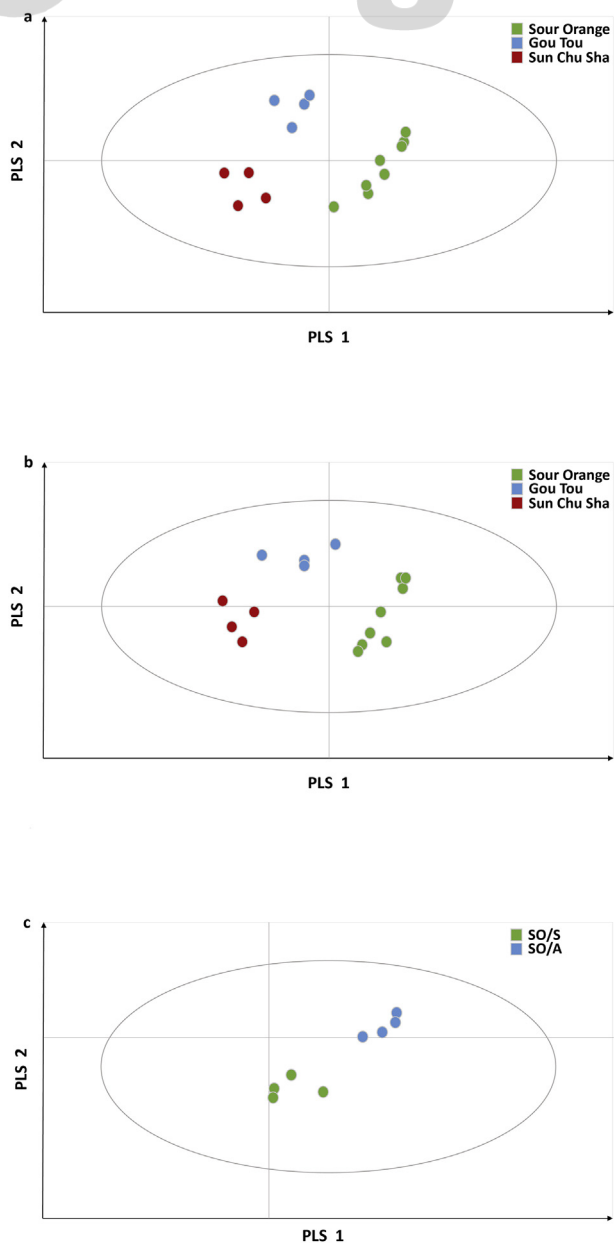


Fig. 1. PLS-DA score plots of lemon juice samples. (a) The result of the PLS-DA clearly distinguished the lemons grafted on different rootstocks using the taste-active, primary metabolite and the aroma-active, secondary metabolite profile. (b) The result of the PLS-DA clearly distinguished the lemons grafted on different rootstocks based on aroma-active, secondary metabolites. (c) The result of the PLS-DA clearly distinguished lemons from HLB symptomatic and asymptomatic trees successfully based on the overall metabolite profile using both taste-active, primary metabolites and aroma-active, secondary metabolites.

active lemon studies [11]. The odor quality of these aroma-active compounds is also described. Additionally, green aldehydes, such as hexanal and (*E*)-2-hexenal, plus a citrusy aldehyde, octanal, all previously reported as important flavor constituents in other commonly consumed citrus fruits, were

Table 2. VIP scores of metabolites in the PLS-DA model for discrimination.

Rootstock		HLB symptom	
Compound	VIP score	Compound	VIP score
cis-carveol	1.59	camphene	2.40
caryophyllene	1.56	decanal	1.26
p-cymene	1.50	glucose	1.19
valine	1.41	fructose	1.17
prenol	1.39	sucrose	1.14
camphor	1.36		
octanal	1.35		
hexanal	1.22		
6-methyl-5-hepten-2-one	1.18		
decanal	1.12		
nerol	1.11		
1-octen-3-one	1.09		
aspartic acid	1.09		
β -pinene	1.06		
sucrose	1.04		

identified in this study as well [12,13]. Lemon fruit quality investigations are mainly applicable to the food and beverage industries. Lemon studies such as those focusing on metabolites responsible for lemon juice flavor quality are rare. The current literature mainly addresses aroma-active, secondary metabolite compounds identified in lemon peel oil and leaf oil [7,14,15]. In peel oil, limonene, γ -terpinene, β -pinene, myrcene, neral, and geranial are identified as major constituents, whereas limonene, β -pinene, myrcene, neral, geranial, neryl acetate, geranyl acetate, and β -caryophyllene are important components in leaf oil.

The genus *Citrus* is comprised of numerous globally cultivated crops, the exact number of diverse species is uncertain; in fact, most species name designations are not valid [16]. Genome sequence analysis has demonstrated that lemon (*Citrus x limon*) is a hybrid of citron (*Citrus medica*) and sour orange (*Citrus x aurantium*), and sour orange itself is an F1 interspecific hybrid cross of mandarin (*Citrus reticulata*) and pummelo (*Citrus maxima*) [16]. Another important citrus crop, the sweet orange (*Citrus x sinensis*), is also an introgression hybrid of mandarin and pummelo. Thus,

Table 3. Results of metabolic pathway enrichment analysis for marker metabolites.

Pathway	Set (matched ^a)	p-Value	FDR correction
Phosphotransferase system (PTS)	5 (3)	9.50×10^{-6}	8.13×10^{-5}
Starch and sucrose metabolism	5 (3)	1.22×10^{-5}	8.13×10^{-5}

^a The number of marker compounds involved in the pathway.

one would expect the aroma profile of lemon to be quite similar to the aroma profile of its most commercially important relatives, in particular, mandarin and sweet orange. According to investigations into sweet orange juice and mandarin juice flavors [17], octanal, decanal, linalool, α -pinene and limonene are identified as key aroma compounds contributing to the characteristic orange and/or mandarin aroma profiles. These aroma-active compounds were also identified in this lemon juice study. Interestingly, a unique group of aroma-active compounds, including neral, geranial, geranyl acetate, neryl acetate, nerol and geraniol, were also observed in lemon (Table 1). Neral and geranial, a pair of isomeric monoterpene aldehydes characterized with lemon notes, are known collectively as citral. Neral and geranial accumulate in various lemon scented plants, such as lemon basil, lemongrass and lemon verbena [18]. Citral is reportedly the dominant constituent of lemon leaf oil, whereas lemon peel oil contains less, yet still a significant amount [14]. Moreover, neral and geranial are considered the most important factors for evaluating the commercial value of lemon oil [19]. Derivatives of neral and geranial include nerol, geraniol, geranyl acetate and neryl acetate. These are commonly described as sweet, floral, fruity notes that contribute pleasant attributes to the lemon aroma. Results of this study suggest that this group of lemon, sweet note odorants may predominantly contribute to the characteristic aroma of lemon, while concurrently providing significant determining factors differentiating lemon from mandarin and orange. Quantitation of these aroma-active compounds will be further discussed with additional metabolites in the following metabolomic analysis.

4.2. Discrimination of metabolic profiles among lemon juice from 'Bearss' scions grafted on different rootstocks

'Bearss', a fast growing, productive lemon variety with outstanding flavor, is well adapted to Florida growing conditions. Rootstocks chosen must be well suited for compatibility with 'Bearss' lemon, resistance to or tolerant of environmental conditions, and most importantly, make a positive contribution towards productivity and/or quality of the lemon fruits. Sour Orange rootstock, a long-established rootstock, generally demonstrates a greater degree of HLB tolerance, whereas Sun Chu Sha and Gou Tou, both minor commercial rootstocks, have undetermined HLB tolerance [6]. Monitoring the metabolite profile of lemons from various rootstocks provides a more thorough understanding of how overall lemon quality is supported by rootstock variability, as well as affected by HLB exposure. Metabolite analyses are largely applied when investigating various citrus fruit with respect to variety discrimination [20], genotype differentiation [21], geographic origin discrimination [22], and plant disease and treatment [23]. Collected data indicate significant effects from a variety of pre-harvest factors on the overall metabolic profile of citrus fruits.

PLS-DA was used to investigate possible rootstock influence on lemon juice metabolites. Lemon juice samples from trees grown on different rootstocks were clearly distinguished by taste-active, primary metabolite and aroma-active, secondary metabolite compound profiles. Also analyzed, flavonoid and limonoid constituents did not aid in discrimination of rootstocks. However, the bitter tasting flavonoids have been demonstrated as potential markers for differentiation of citrus fruits [24], including certain

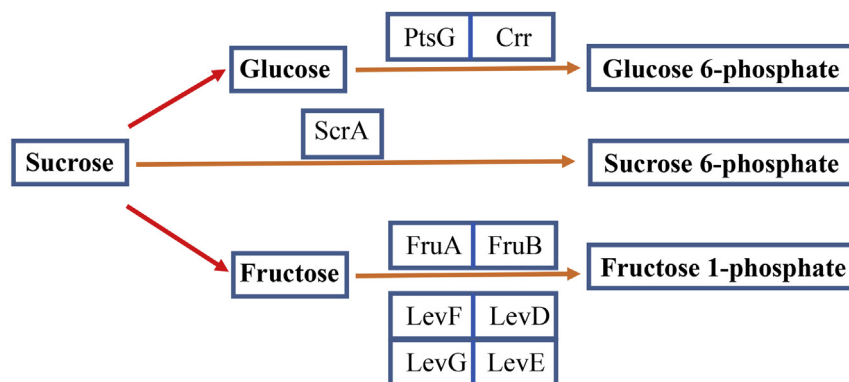


Fig. 2. Phosphotransferase system (PTS) and Starch and sucrose metabolism pathways closely related to discrimination of HLB symptom status (orange arrow indicates Phosphotransferase system (PTS) pathway and red arrow indicates Starch and sucrose metabolism pathway with related enzymes).

mandarin varieties [20]. This suggests genotype as a key factor for determining flavonoid profiles of citrus fruits. Moreover, it is possible that rootstock alone does not significantly influence characteristic flavonoids, particularly among a single scion (lemon variety). Additional application of PLS-DA using the taste-active, primary metabolites alone could not discriminate lemon juice by rootstocks, whereas using the aroma-active, secondary metabolites alone indicated potential for rootstock discrimination. Since turnover of secondary metabolites is slower than primary metabolites, alterations in their compositions or concentrations might be easier to detect because the frequency of change is low [25]. Conversely, alterations to the composition or concentration of primary metabolites may be harder to detect because a higher rate of turnover means faster replacement of altered compounds. Due to high quantities of primary metabolites producing secondary metabolites in lower quantities, any alteration at the primary metabolite level becomes concentrated in the resulting secondary metabolites, which may explain why the aroma-active, secondary metabolites could differentiate rootstock difference [25]. These results indicate that rootstock differences in lemon may not influence alterations at the primary metabolite level, but may induce changes at the subsequent secondary metabolite level, even within the same scion. Similarly, a previous study on mandarin fruits grafted on different rootstocks drew the same conclusion [20].

Sugars, a critically important subset of taste-active, primary metabolites, strongly influences the flavor quality of citrus fruits. Formed in the leaves during photosynthesis, sugar is transported throughout the plant, to develop fruits. Additionally, citric acid, another taste-active, primary metabolite, found in the highest concentration among all organic acids in these lemon juice samples, is the main compound responsible for fruit acidity [26,27]. Citric acid is endogenously synthesized in the mitochondria via the citric acid cycle during cell metabolism [28]. The content level of sugar and organic acids in fruits depends not only on endogenous factors, but also on horticultural factors as well. Sugar levels in a fruit are limited by genetics. However, environmental factors, such as sunlight, water availability, soil conditions and fertilizer all contribute to the extent at which that level can be achieved [29,30]. Similar to sugar, organic acid levels in fruit also vary according to a variety of pre-harvest factors. Sugar and organic acid concentrations in fruits have been shown to differ among trees on various citrus rootstocks [31]. However, any significant detected differences in primary metabolite

levels is determined by the magnitude of variation among the rootstocks. Furthermore, currently, there is insufficient evidence to support a single rootstock factor conclusively affects any primary metabolite. However, in contrast to the taste-active, primary metabolites, the aroma-active, secondary metabolites showed greater ability for rootstock differentiation in this study. Effects of different rootstocks on the aroma profile of various fruits have previously been discussed [32]. The horticultural focus regarding rootstocks has historically been on overall yield. However, recent research has uncovered rootstock choice may affect the attributes of fruit quality. Due to the importance of aroma-active compounds on overall fruit flavor perception, this study suggests that more attention should be paid to the selection of the rootstock during tree planting so that high quality fruits with favored aroma profiles can be achieved.

4.3. Discrimination of metabolic profiles from lemon juice of fruits from HLB symptomatic and asymptomatic trees

Candidatus Liberibacter asiaticus (CLAs) infects all citrus crops. Taste-active, primary metabolites, such as sugars, amino acids and organic acids, plus aroma-active compounds, such as terpenes, aldehydes, ketones, alcohols and esters; and other secondary metabolites, including flavonoids and limonoids all contribute to the characteristic flavor profile of citrus fruits. The characteristic sweet or sour taste of citrus comes from sugar and citric acid, respectively, and flavonoids and limonoids are responsible for any bitter, tart or harsh tastes. Diseases, such as HLB have been reported to induce off-flavors [33], as well as disrupt the balance of sugars and organic acid content [34]. Using PLS-DA, effects of HLB progression on the taste-active, primary and aroma-active, secondary metabolites could be observed in the lemon juice samples, whereas individual analysis of taste-active, primary metabolites or aroma-active, secondary metabolites, could not differentiate symptomatic from asymptomatic trees. This result was not surprising due to the complexity of HLB disease progression and symptom development, as well as the subjective process used to identify 'symptomatic' trees during sample collection. The incubation period for HLB ranges from a few months to many years [35]. Time frame variations have been observed between CLAs infection and the onset of visual symptoms. Uneven distribution of CLAs within each tree, prolonged incubation periods before the onset of symptoms and multiple asymptomatic infections make

diagnosis of actual HLB status a challenge [35]. Therefore, although changes in the overall metabolic profile of lemon juice due to HLB progression in lemon trees were observed in this study, the resulting effects of the disease on taste-active, primary metabolites or aroma-active, secondary metabolites in particular were not significant.

Five potential biomarkers were identified for monitoring HLB progression, of these, only camphene, a monoterpene with a pungent camphor smell, was detected in lemon juice of fruits from symptomatic trees. Aroma-active terpenes in plants, known to be upregulated as a stress response and used as defense mechanisms [36], include camphene. Therefore, the presence of camphene in the lemon juice from fruits of symptomatic trees would correlate with initiation of defense mechanisms in trees as the disease progresses. Decanal, a pleasant, sweet fruity aroma in citrus fruits [17], decreases in concentration in HLB-affected orange juice [33]. This is supported by significantly higher levels of decanal detected in the lemon juice samples from the asymptomatic trees in this study.

Using pathway enrichment analysis, three taste-active, primary metabolites (fructose, glucose and sucrose) were involved in both pathways. This study indicated fructose, glucose and sucrose at significantly higher levels in lemon juice from symptomatic trees compared to the asymptomatic trees. Studies researching HLB affected lemon fruit are limited. However, as one of the most commercially important citrus crops, orange fruit has been intensively studied, especially once a tree is HLB-affected, particularly for alterations in overall quality and overall metabolite profile. Commercially processed Valencia orange juice from HLB-positive trees has been reported to present with stronger sweet notes compared to HLB-negative trees, depending on harvest time [1]. Valencia juice from healthy, asymptomatic and symptomatic oranges was compared [37]. Results indicated that juice from healthy and asymptomatic trees had higher levels of fructose, glucose and sucrose compared to juice from symptomatic trees, in which only sucrose revealed significant differences. Similarly, in an investigation of the metabolic profile of Hamlin sweet orange, significantly higher levels of sucrose were measured in juice from asymptomatic trees, whereas there was no significant difference in the levels of fructose or glucose between juice from symptomatic and asymptomatic trees [38]. Furthermore, there were significantly lower levels of sucrose, yet higher levels of fructose and glucose detected in HLB-affected Valencia juice compared

to juice from healthy trees [39]. Taken as a whole, results from the investigations previously referred to suggest that the taste-active, primary metabolite sugars (fructose, glucose and sucrose) may contribute to metabolic variations to be used in monitoring the HLB progression in citrus trees. Moreover, the sugar alterations noted between the symptomatic and the asymptomatic lemon juice samples may play a role in the development of secondary metabolite alterations identified in the pathway analyses. Unfortunately, to date, no detectable pattern regarding such level changes has been identified. Furthermore, the sugar alterations noted between the symptomatic and the asymptomatic lemon juice samples may play a role in the development of secondary metabolite alterations identified by the pathway analyses.

5. Conclusion

Current literature on lemon juice quality is rather limited. It does include investigations into essential oils and their uses in fragrances and flavoring. This investigation identified and, for the first time, thoroughly described aroma-active compounds in lemon juice. Neral, geranial and their derivatives were proposed as potential aromatic components determining characteristic lemon flavor. In addition, findings from this study revealed rootstock can affect the metabolic profile of fruit, as aroma-active, secondary metabolites demonstrated discrimination of lemon juice from fruit harvested off trees grown on different rootstocks. However, taste-active, primary metabolites were not useful as an independent factor in discriminating fruit from different rootstocks, possibly resulting from variations in pre-harvest factors. Examining data regarding rootstock effect on the profile of lemon juice aroma-active, secondary metabolites, may provide insight into the importance of rootstock selection for producing lemon fruit with desirable flavor qualities, thus influencing future option selections for ideal planting regimens. Investigating the effects of HLB progression on the overall metabolic profile in lemon juice indicated significant alterations in the lemon juice samples from symptomatic trees compared to samples from asymptomatic trees. However, the complexity of HLB disease progression, and symptom onset, as well as the difficulties associated with accurately evaluating HLB status makes differentiating symptomatic and asymptomatic trees based solely on primary or secondary metabolites a challenge. Taste-active, primary metabolite sugars, (fructose, glucose and sucrose)

demonstrated potential as biomarkers for monitoring HLB progression in lemon trees. Additionally, using pathway enrichment analysis, both Phosphotransferase system (PTS) and Starch and sucrose metabolism pathways presented as important altered pathways based on HLB status in lemon trees.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.38212/2224-6614.1060>.

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