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Elevation, Rootstock, and Soil Depth Affect the Nutritional Quality of Mandarin Oranges

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ABSTRACT: The effects of elevation, rootstock, and soil depth on the nutritional quality of mandarin oranges from 11 groves in California were investigated by nuclear magnetic resonance (NMR) spectroscopy by quantifying 29 compounds and applying multivariate statistical data analysis. A comparison of the juice from oranges in groves with deeper soil and trifoliate rootstock versus those with shallow soil and C-35 rootstock revealed differences in the concentrations of 4-aminobutyrate, ethanol, phenylalanine, succinate, and isoleucine. A comparison of fruit from trees grown at higher versus lower elevation revealed that those at higher elevation had higher concentrations of amino acids, succinate, and 4-aminobutyrate and lower concentrations of sugars and limonin glucoside. Such differences indicate that rootstock, soil depth, and differences in elevation influence the fruit nutrient composition. This study highlights how metabolomics coupled with multivariate statistical analysis can illuminate the metabolic characteristics of citrus, thereby aiding in the determination of the grove identity and fruit quality during orange production.

KEYWORDS: NMR, citrus, mandarin orange, Satsuma, Clementine, elevation, rootstock, soil depth, metabolomics

INTRODUCTION

Citrus is one of the most widely cultivated fruit tree crops in the world. Citrus fruits are consumed as fresh fruits, processed into fresh, frozen, and concentrated juices, and used as starting materials for the isolation of flavor, aroma, and bioactive compounds. Bioactive compounds present in citrus fruits and juices include limonoids, carotenoids, flavonoids, and others (e.g., vitamin C). As a crop, its popularity stems from two causes: first, citrus trees produce highly desirable fruits known to contribute to human health and nutrition, and second, there are multiple varieties available to choose from representing an array of fruit types, growing seasons, and varieties optimized for certain localities and growing conditions.

Citrus fruit is one of the most important horticultural crops with worldwide agricultural production over 82 million metric tons (MMT) per year.[1] Sweet orange varieties represent the largest group of cultivated citrus and are followed by mandarin/tangerine oranges, lemons and limes, and finally, grapefruits. Worldwide production of these respective groups for the 2009−2010 growing season is estimated to be at 49.8, 20.7, 6.0, and 5.4 MMT, respectively.[1] Over the past decade, sweet orange production has dropped as growers have removed sweet orange groves and replanted with mandarin and tangerine orange varieties in response to consumer demand. Properties possessed by these varieties, such as an easily peeled skin, convenient fruit size, and relative seedlessness, are thought to be the reason for increased consumer demand. In the U.S., a similar trend in mandarin and tangerine production preference has been observed, and in the 2009−2010 season, a 30% increase in production over the 2008−2009 season was reported.[1]

The major centers of commercial citrus cultivation within the United States are the coastal states of California, Florida, and Texas. Small-scale commercial production operations are also present in adjacent states, such as Arizona and Louisiana. Cultivation in Florida emphasizes varieties used for juice production, whereas the focus in California is on the fresh fruit market. However, both states cultivate mandarin types for the fresh fruit market. Production in Texas consists of a mixture of juicing and table fruit varieties, with an emphasis on grapefruits. Of the three states, California offers the most diverse geography and spans from the inland desert in southern California to the base of the Sierra Nevada mountain range located east of Sacramento in northern California. Cultivation in California is divided into four distinct growing regions: southern desert, San Joaquin Valley, central coast, and northern California. A mixture of sweet and mandarin oranges and some grapefruit and lemon varieties are cultivated in the first three areas, but production in the northern California region is focused exclusively on mandarin oranges. In contrast to the other growing regions, which are expansive, flat, frequently share common soil compositions, and are at or near sea-level, groves located in northern California are found at various elevations, soil compositions, and geographical orientations as a result of being nestled within the foothills of the Sierra Nevada mountains. Given the large variation in growing conditions and climate in California, different citrus rootstocks are required to improve cold tolerance as well as resist pests and diseases.[2,3] The combination of these varied conditions have produced unique microenvironments that afford an opportunity to evaluate their influence on fruit quality, including their effects on secondary metabolite composition.

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Table 1. Characteristics of 11 Orange Groves in Placer County

grove	elevation (m)[a]	direction	soil type	soil depth (cm)[b]	rootstock
1	180	east/west	DG[c]	76	trifoliate/rough lemon
2	207	south/southwest	DG	38	C-35
3	259	west	DG	137	trifoliate
4	137	south	clay/loam	99	trifoliate/Cleo
5	152	east	DG	66	C-35/Carrizo
6	183	south	DG	213	trifoliate/Cleo
7	282	south	DG	107	trifoliate/C-35
8	229	west	DG	107	trifoliate
9	296	west	clay/loam	61	C-35
10	122	south	DG	53	C-35
11	168	south	DG	30	C-35

[a] Average elevation. [b] Average soil depth. [c] DG = decomposed granite.

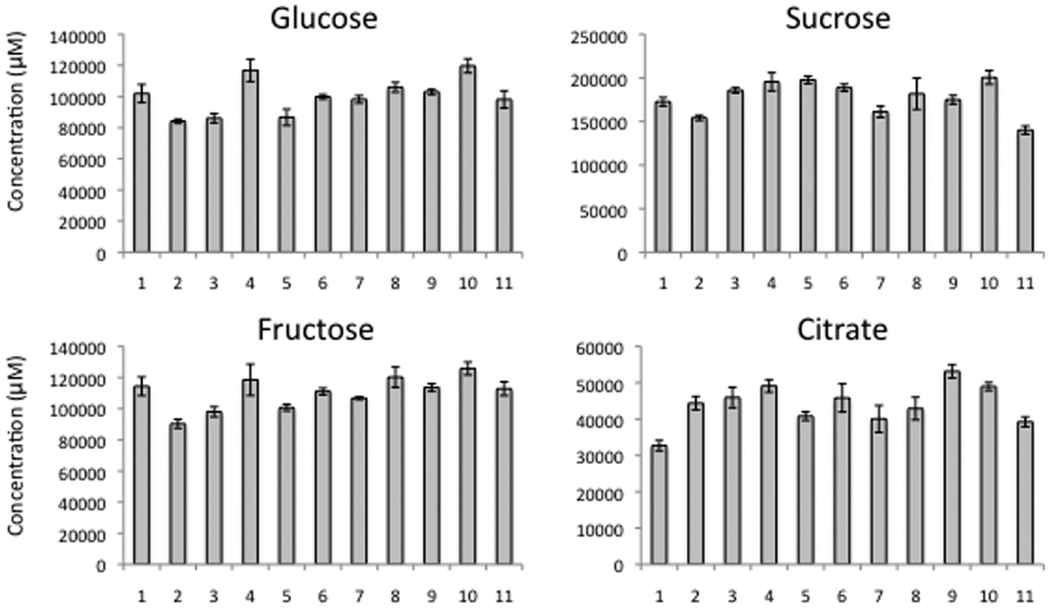

Figure 1. Comparison of average sugar and citrate concentrations between different groves.

In 2008, a study was initiated in which mandarin juice samples from 10 locations within the northern California growing region were evaluated for their synephrine content.[4] Synephrine is a nitrogen-containing bioactive compound with vasoconstrictor and bronchiectatic properties[5] and, thus, may act as a natural decongestant. Among edible plants, it is unique to *Citrus*.[6] It was shown that intergrove concentrations varied greatly, whereas the within-grove variability was less apparent. The conclusion was that, although the groves were located within the same growing region, individual differences in location, including differences in elevation, soil type, geographical orientation, as well as rootstock, resulted in unique microenvironments that influenced the final synephrine concentrations. To determine if the influence of the microenvironment is more widespread and reflected generally in fruit metabolism, a ^{1}H nuclear magnetic resonance (NMR) metabolomic study was undertaken to examine juices prepared from fruits taken from the same 10 locations, plus a related mandarin orange species from an additional location. NMR offers the advantage of simultaneously determining the identity and quantity of metabolites present in a sample.

MATERIALS AND METHODS

Plant Materials. Fruits [Owari selection of Satsuma mandarin (*Citrus unshiu* Marcovitch)] were harvested from each of 10 disparate groves located in Placer County, CA. Fruit was also harvested from an 11th location, from a grove that consisted of a mix of Satsuma and Clementine mandarin orange trees. Harvesters were instructed to pick ripe fruits of random sizes from throughout the entire grove. Fruits were harvested in mid-December 2007 and stored at 5 °C until sampling. Data on the elevation, soil type, soil depth, type of rootstock, and geographical orientation of each grove were collected and used in analysis.

Sample Preparation. For each location, healthy and undamaged fruits were randomly divided into three groups. For each group, 10 fruits were used to prepare a juice sample for analysis. Because the peels of mandarin fruits are thin, the use of a juice reamer was ruled out to avoid potentially contaminating the juice with metabolites derived from the peel. Fruits were therefore peeled manually while wearing nitrile gloves and then blended in an Osterizer Classic mixer until no chunks of pulp remained (10−20 s). For the first sample from each location, a portion of the resulting homogenate was

Table 2. Metabolites Identified and Quantified in Satsuma Mandarin Orange Juice

	present study (Satsuma mandarin)			previously quantified by NMR	
	average (μM)	low (μM)	high (μM)	Satsuma[a]	orange[b]
sugars					
fructose	110206	83108	139136	×	
glucose	100046	73492	132406	×	
sucrose	177506	130044	234973	×	
myo-inositol	11107	8661	13980		
amino acids					
alanine	2095	1126	4519	×	×
arginine	1644	786	3219	×	×
asparagine	2733	488	7371		×
aspartate	1857	629	2789		
histidine	50	18	83		
isoleucine	54	36	94	×	
leucine	51	31	97	×	
phenylalanine	142	85	271	×	
proline	4728	1741	12872	×	
threonine	253	140	412	×	×
valine	145	100	191		×
4-aminobutyrate	1607	849	2321	×	×
choline	198	102	361		
organic acids					
ascorbate	1210	761	1772		
citrate	43919	29535	56110	×	
formate	134	70	226		
succinate	117	66	200		
others					
adenosine	53	41	74		
ethanol	5621	1452	10141	×	×
synephrine	131	84	223		
limonin glucoside[c]	1988	821	4355		
methanol	1,153	448	3427		
proline betaine[c]	4890	3029	6929		×
trigonelline	226	176	310		
unknown at 2.9 ppm[c]	639	263	1086		

[a] From ref 13. [b] From refs 14 and 15. [c] Concentrations estimated.

transferred to a 50 mL polypropylene tube, quickly frozen with dry ice, and stored at −20 °C or less. The remaining volume of the resulting juice was clarified by centrifugation at 4700 rpm for 4 min using a Marathon 8K from Fisher Scientific, Ltd. (Waltham, MA). This clarified juice was vacuum-filtered through Whatman no. 1 filter paper (Clifton, NJ), quickly frozen with dry ice, and stored at −20 °C or less. For each location, a total of four samples were used for metabolomic analysis: three samples were clarified juice, and the fourth was a replicate of the first filtered juice sample prepared from the frozen juice homogenate.

NMR Data Collection. For NMR spectroscopy, samples were thawed and centrifuged for 15 min at a maximum speed at 4 °C to remove particulate matter. The sample supernatant was subsequently filtered through Omega-3 3000 molecular weight (MW) cutoff filters (Pall Life Sciences, Ann Arbor, MI) to remove pectin. If needed, the filtrates were diluted with H_2O to a total volume of 585 μL. An internal standard containing 5 mM 3-(trimethylsilyl)-1-propanesulfonic acid-d_6 (DSS-d_6) and 0.2% NaN_3 in 99.8% D_2O (65 μL) was added to 585 μL of the filtrates. The pH was adjusted for each sample to 6.8 ± 0.1 by adding small amounts of NaOH. Aliquots (600 μL) of each sample were transferred into 5 mm NMR tubes, and samples were stored at 4 °C until NMR data acquisition (within 24 h of sample preparation). NMR spectra were acquired using the Bruker "noesypr1d" experiment on a Bruker Avance 600 MHz NMR spectrometer equipped with a SampleJet. Acquisition parameters were 12 ppm sweep width, 2.5 s acquisition time, 2.5 s relaxation delay, and 100 ms mixing time. Water saturation was applied during the 2.5 s relaxation delay and the 100 ms mixing time. The resulting data were zero-filled to 128 000 data points, with an exponential apodization function and applied baseline correction.

Data Analysis. Metabolite identification and quantitation was accomplished through the technique of targeted profiling using Chenomx NMRSuite 6.1 (Chenomx, Inc., Edmonton, Alberta, Canada).[7] Briefly, metabolites in each sample were compared to a library of approximately 300 metabolite compounds. A total of 25 compounds were assigned on the basis of the metabolite library, and 4 more compounds, proline betaine, limonin glucoside, synephrine, and an unknown metabolite, were added to the library and quantified. In total, more than 95% of the total NMR spectral area was taken into account. Several metabolite peaks belonging to aromatic compounds that

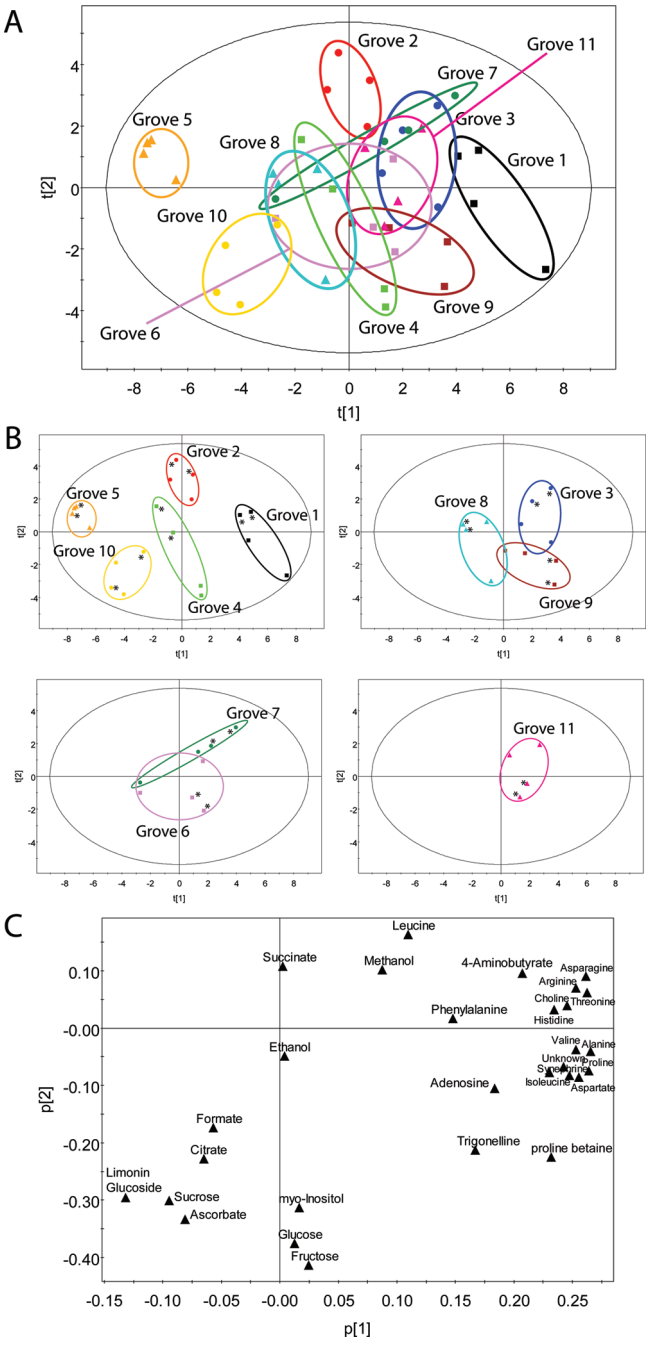

Figure 2. PCA illustrates that each grove clusters. (A) PCA showing all groves on one plot colored and circled for each grove for clarity. (B) To simplify the PCA plot in panel A, it was redrawn to clarify the position of samples from specific groves. Those samples representing repeat sampling from the same grove/area (clarified juice versus pulp homogenate) are indicated by asterisks. (C) Corresponding loadings plot for the PCA plot shown in panel A.

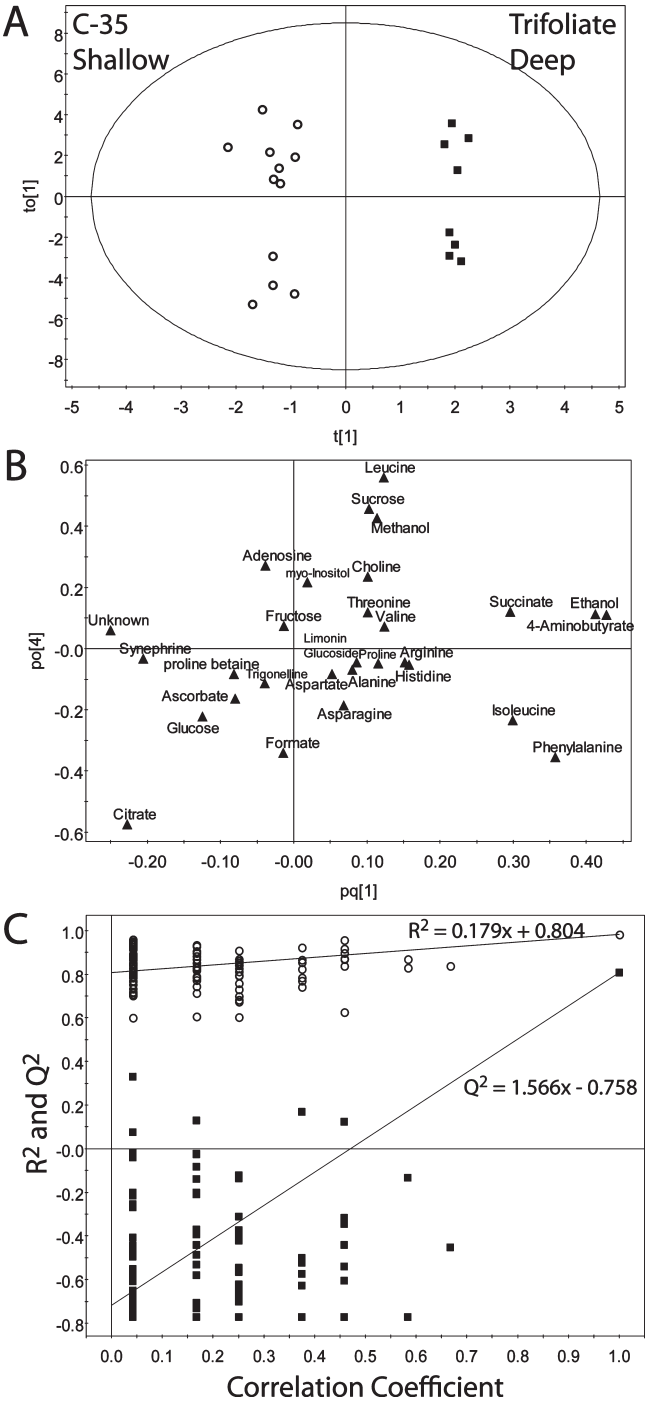

Figure 3. Comparison of metabolic composition of C-35 rootstock/shallow soil versus trifoliate rootstock/deep soil. (A) OPLS−DA plot comparing C-35 rootstock/shallow soil (groves 2, 9, and 10) (○) versus trifoliate rootstock/deep soil (groves 3 and 8) (■). (B) Loadings plot corresponding to panel A. (C) Validation of the PLS−DA in panel A using permutation testing. R^2 (○) is a measure of how well the model fits the data, and Q^2 (■) is a measure of the predictive ability of the model. Both R^2 and Q^2 have positive slopes, indicating a good model.

could not be identified were in low concentration and were, thus, not used in the analysis.

Metabolite concentrations were subjected to $\log_{10}$ transformation, and multivariate statistical data analyses [principal component analysis (PCA) and orthogonal signal correction partial least-squares−discriminant analysis (OPLS−DA)] were performed to account for non-normal distribution of the concentration data and reduce the chance of skewed variables, using SIMCA-P (version 11, Umetrics, Umeå, Sweden), with mean centering and applied unit variance scaling. Significance testing, using Student's *t* test, was performed using Microsoft Excel. Significance was set at $\alpha = 0.05$.

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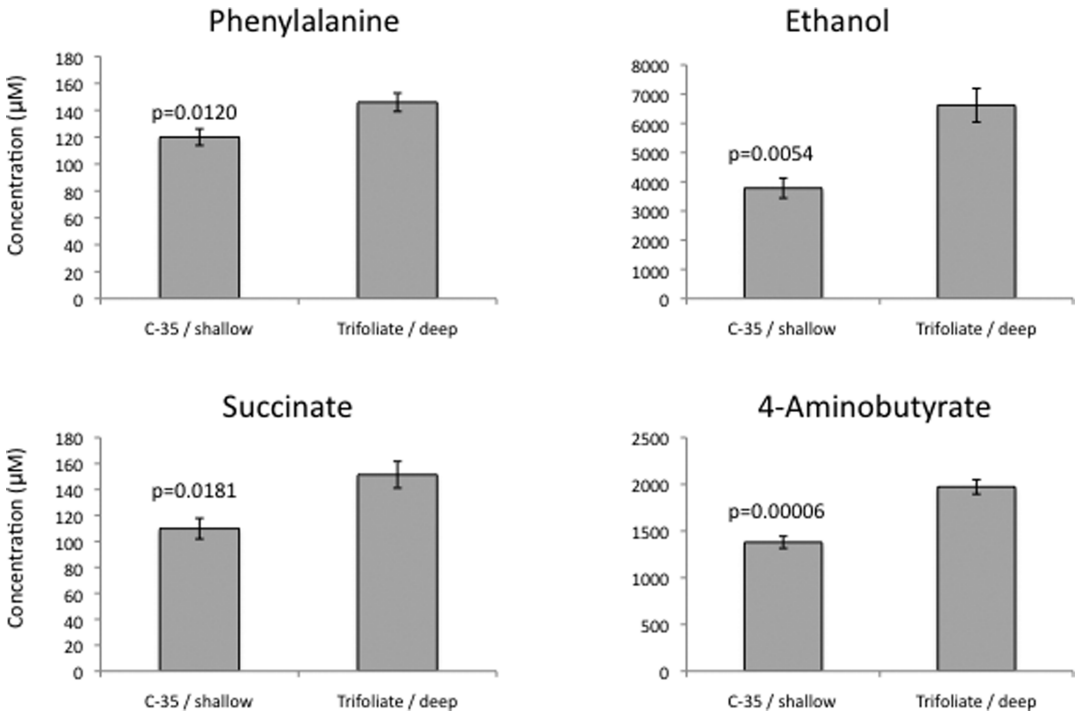

Figure 4. Comparison of metabolite concentrations between C-35 rootstock/shallow soil (groves 2, 9, and 10) and trifoliate rootstock/deep soil (groves 3 and 8). Significance (as assessed using Student's *t* test) is indicated on each graph.

RESULTS AND DISCUSSION

A total of 11 mandarin orange groves located in Placer County, California, provided samples for this study. For each grove, several samples were provided from three different areas. For one area in each grove, in addition to the pulp/juice sample provided, partially purified juice samples were provided to determine whether the presence of pulp would affect the results. Table 1 provides a summary of the characteristics of each location. Nearly all groves were either west- or south-facing, except grove 5 and part of grove 1, which were east-facing. Elevation varied from an average of 122 to 296 m above sea level, and soil depth varied from an average of 30 to 213 cm. The soil type for all groves, except 4 and 9, was decomposed granite, whereas groves 4 and 9 had clay/loam soil. Half of the groves had a single rootstock of either trifoliate or C-35, and the other half of the groves had a mixture of two types of rootstock. All groves grew Satsuma mandarin oranges; grove 11 grew Clementines as well. There was a correlation between rootstock and soil depth. In general, those groves with deeper soil used trifoliate rootstock, whereas those with more shallow soil used C-35 rootstock.

From the analysis of the ^{1}H NMR spectra of samples from each grove, 25 compounds were initially identified and quantified. Included within this list of compounds were sugars, amino acids, organic acids, and other metabolites (see Table 2). Proline betaine, limonin glucoside, synephrine, and an unknown compound at 2.9 ppm were subsequently identified and added to the library of compounds to give a total of 29 compounds used for the statistical analyses. There were also several peaks in the aromatic region that likely correspond to polyphenolics or flavanoids,[14] but these peaks could not be accurately assigned and, thus, were not included in the analysis.

On a grove-to-grove basis, the concentrations of the 29 compounds variedly greatly and, in some instances, a 5-fold or more difference between the high and low concentrations was observed (e.g., aspartic acid, proline, methanol, and ethanol). In contrast, intragrove variability for metabolites in the millimolar range was less than 5% coefficient of variation (CV), and intragrove variability for metabolites in the micromolar range was less than 10% CV. As expected, the most abundant metabolites (Figure 1) were glucose, sucrose, fructose, and citrate. Concentrations of the sugars varied from 84 mM (grove 2) to 120 mM (grove 10) for glucose, 140 mM (grove 11) to 200 mM (grove 10) for sucrose, and 90 mM (grove 2) to 126 mM (grove 10) for fructose. Sucrose concentrations were consistently 1.5−2 times greater than that of glucose and fructose for each location, and this observation is in-line with what has been previously reported for Satsuma[8] and sweet orange[9,10] juices. Citrate concentrations ranged from 33 mM (grove 1) to 53 mM (grove 9). Results obtained by NMR for the sugar and acid contents of the juices directly correlated with those obtained with physicochemical measurements of °Brix and total titratable acids (data not shown). Another important nutritional component of citrus juices is vitamin C (ascorbate). The largest difference between two groves was nearly 2-fold (1.5 mM for grove 9 and 0.9 mM for grove 2). Sugars, acids, and chemical aromaticity are considered to be major components of fruit quality,[11,12] and the fact that metabolite concentrations can vary so greatly depending upon the growth conditions of a grove suggests that each grove may have a particular taste profile.

Chemical shifts associated with the most abundant primary and secondary metabolites present in citrus juices have been identified; however, most of the work applying NMR techniques to the analysis of juices has focused on pattern recognition rather than quantification of metabolites. Table 2 compares the identities of the metabolites quantified in this study to those previously quantified by NMR for both Satsuma [13] and sweet orange[14,15] juices. Analysis of Satsuma juice by

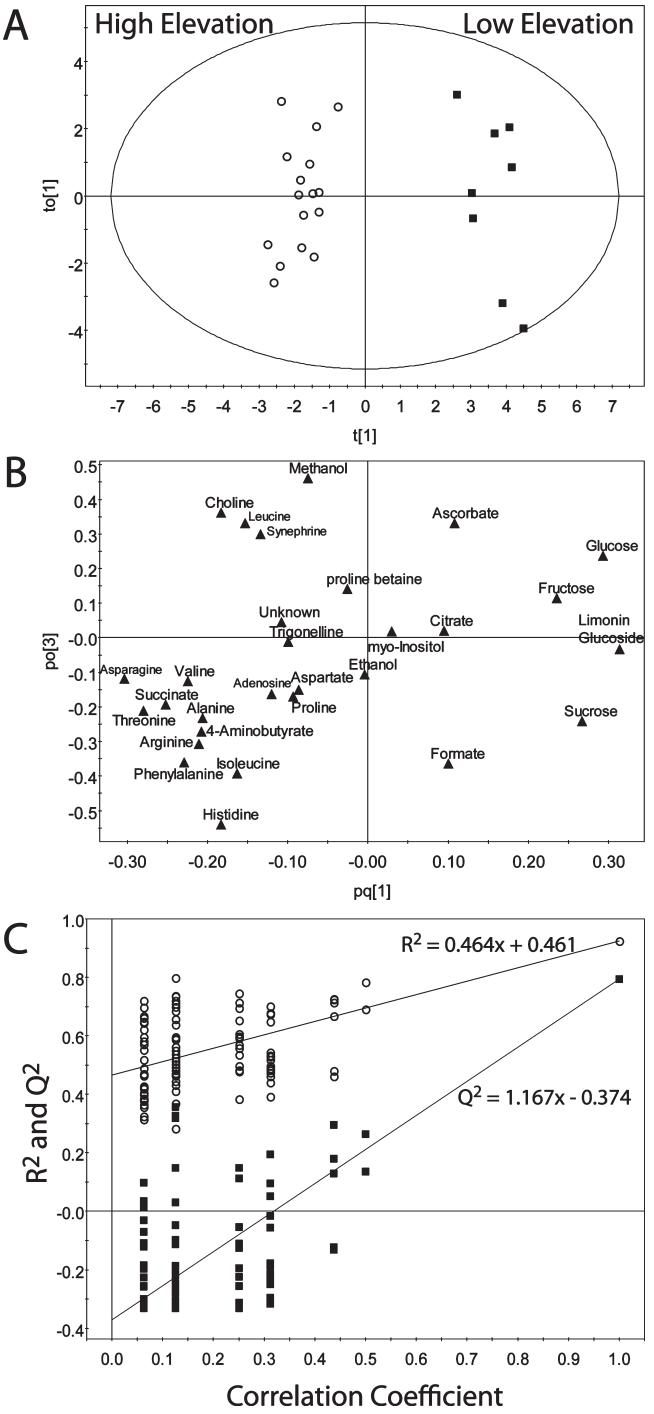

Figure 5. Comparison of metabolic composition of oranges in high elevation versus low elevation. (A) OPLS−DA plot comparing high elevation (groves 2, 3, 7, and 9) (○) and low elevation (groves 4 and 10) (■). Loadings plot corresponding to panel A. (C) Validation of the PLS−DA in panel A using permutation testing. R^2 (○) is a measure of how well the model fits the data, and Q^2 (■) is a measure of the predictive ability of the model. Both R^2 and Q^2 have positive slopes, indicating a good model.

Tachiyashiki et al.,[13] using ^{1}H NMR spectroscopy at 270 MHz, revealed the identification of many of the compounds that we identified (Table 2). However, our analysis was able to identify more metabolites.

In an attempt to determine whether there was a correlation between factors such as elevation, soil type, soil depth, and rootstock on the composition of metabolites in each grove, we applied multivariate statistical analysis. Figure 2 is a PCA plot of all 11 groves based on metabolite data. As may be observed, most groves tended to cluster together, indicating similar metabolite composition across each grove. In addition, whether the samples were provided as juice or a homogenate containing pulp (which was separated out before analysis), the results of PCA were similar (Figure 2). Indeed, for most groves (except grove 10), the juiced samples were in a similar position on the plot as the samples provided as homogenates, indicating that the majority of metabolites observed were from the juice and not the pulp. Interestingly, the groves with trees having more than one type of rootstock, groves 1, 4, 6, and 7 (Table 1), and grove 11, which has both Satsuma and Clementine scions, are more disperse in the PCA plot. Grove 5 contains two rootstocks that are very similar: C-35 (cross between trifoliate and ruby sweet orange) and Carrizo (cross between trifoliate and the Washington navel). Interestingly, this grove has the least dispersion in the plot.

To further explore the relationship between soil depth/rootstock and metabolite concentration, we divided the groves into those with the most shallow soil and, hence, C-35 rootstock (groves 2, 9, and 10) and those with the deepest soil and, hence, trifoliate rootstock (groves 3 and 8). The elevation for C-35 rootstock ranged between 122 and 296 m, with a south/west exposure, primarily decomposed granite soil, and an average soil depth of 46 cm. For the trifoliate rootstock, the elevation ranged between 229 and 259 m, with a west exposure, decomposed granite soil, and an average soil depth of 122 cm. Figure 3 shows an OPLS−DA score plot and corresponding loadings plot, illustrating the differences between the rootstock and soil depth and metabolite differences. The largest differences appear to be 4-aminobutyrate, ethanol, phenylalanine, isoleucine, and succinate, which are all higher in the oranges taken from trees grown on trifoliate rootstock. In the C-35 rootstock/shallow soil, the average ethanol concentration was just under half the concentration in the trifoliate rootstock/deep soil (Figure 4) and 4-aminobutyrate (GABA) and succinate were approximately $^3/_4$ of the concentration of the oranges grown on trifoliate rootstock/deep soil. The isoleucine concentration was not significantly different (data not shown). Differences in the metabolite concentration related to rootstock are not unexpected. In a study on the effects of rootstock on the composition of bergamot essential oil, it was determined that the use of trifoliate rootstock produced the lowest levels of bergamot oils compared to sour orange rootstock.[16] In addition, differences in the inorganic composition of fruit grown on different rootstocks have also been reported.[17]

To explore the relationship between elevation and metabolite concentration in the samples, we divided the groves into low elevation (groves 4 and 10) and high elevation (groves 2, 3, 7, and 9). Groves 4 and 10 had either decomposed granite or clay/loam soil of a depth ranging from 30 to 99 cm. The rootstock was either C-35 or trifoliate/Cleo, with groves pointing south. Groves 2, 3, 7, and 9 had similar soil characteristics, with a depth ranging from 53 to 137 cm. The rootstock was either C-35 or trifoliate. Figure 5 shows an OPLS−DA score plot and corresponding loadings plot, illustrating the metabolite differences between the different elevations. Analysis of the differences in the metabolite concentration because of elevation revealed many changes (Figure 6). In general, trees grown at a higher elevation tended to have higher concentrations of amino acids (specifically asparagine, threonine,

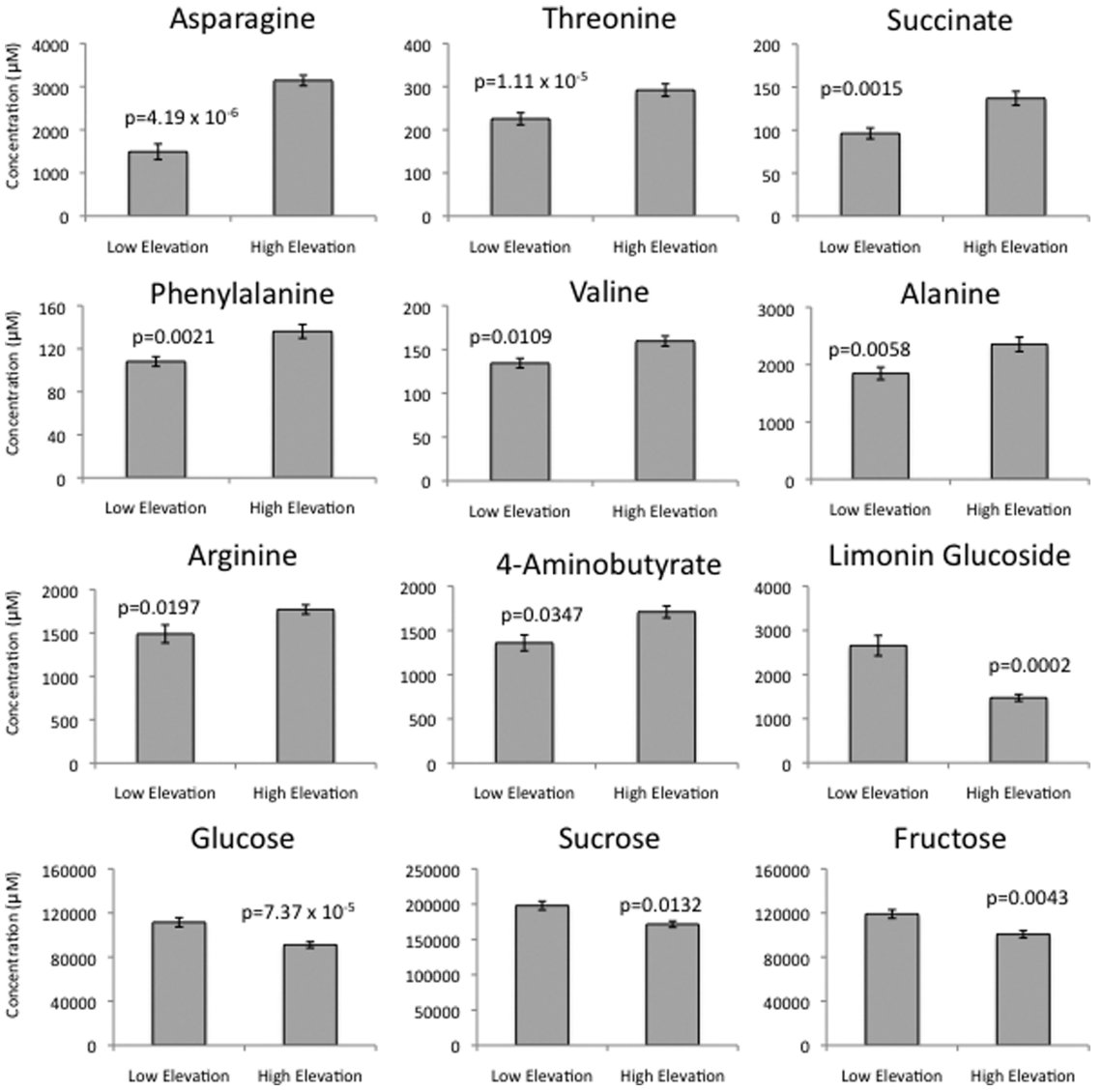

Figure 6. Comparison of the metabolite concentration of oranges in high elevation (groves 2, 3, 7, and 9) versus low elevation (groves 4 and 10). Significance (as assessed using Student's *t* test) is indicated on each graph.

succinate, phenylalanine, valine, alanine, and arginine), succinate, and 4-aminobutyrate but lower concentrations of sugars (glucose, fructose, and sucrose) and limonin glucoside.

In this study, we used NMR to compare the metabolite profiles obtained for Satsuma mandarin juices prepared from fruit harvested from 11 separate locations. In descending order, the metabolites at the highest concentrations were sucrose, glucose, fructose, and citrate. Other components, such as the amino acids, limonin glucoside, 4-aminobutyrate, synephrine, trigonelline, and proline betaine, ranged from under 1 mM to several millimolars. Metabolomic data coupled with statistical analysis demonstrated that rootstock, soil composition, and elevation all influence the nutritional composition of mandarin orange fruit. The fact that metabolite concentrations can vary so greatly depending upon the growth conditions of a grove indicate that each grove may have a particular taste profile and that NMR analysis can be harnessed as a tool in developing precise agricultural practices that result in fruits optimized for consumer preferences.

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ABBREVIATIONS USED

NMR, nuclear magnetic resonance; PCA, principal component analysis; OPLS−DA, orthogonal signal correction partial least-squares−discriminant analysis; MMT, million metric tons; CV, coefficient of variation

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