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Characterisation of free and bound volatile compounds from six different varieties of citrus fruits

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ABSTRACT

Free volatile compounds in six varieties of citrus juices were analyzed by solid-phase microextraction–gas chromatography–mass spectrometry. Bound fractions were isolated and extracted with methanol and Amberlite XAD-2 resin and then hydrolyzed by almond β-glucosidase. A total of 43 free and 17 bound volatile compounds were identified in citrus. Free volatile contents in sweet orange were the most abundant, followed by those in grapefruits and mandarins. Among free volatiles, terpenes were the most abundant in citrus juice. Sensory analysis results showed that the flavor of the same citrus cultivars was similar, but the flavor of different cultivars varied. Among bound volatiles, benzenic compounds were the most abundant in these citrus juices. Bound volatiles also significantly differed among cultivars. In addition, only *p*-vinylguaiacol were detected in all of the samples.

1. Introduction

Citrus is one of the most abundantly fruit crops in China, with an annual production of 32,000,000 tons in 2013. Citrus is produced in large amounts in the Three Gorges Area of Central China. For instance, mandarins and Hamlin (HL) sweet oranges [*Citrus sinensis* (L.) cv. Hamlin] are commonly cultivated. Fresh mandarin fruit is produced and consumed as citrus juice because this fruit is characterised by desirable aroma, flavor, and ease of peeling. Likewise, HL sweet oranges cultivated in this area are mainly produced for their juice. A relatively small amount of grapefruit is also cultivated for its juice because grapefruit exhibits unique flavour.

Citrus juice is considered as one of the most commonly consumed beverages because citrus juice provides health benefits and yields distinctive aroma and taste (Rouseff, Perez-Cacho, & Jabalpurwala, 2009; Kelebek & Selli, 2011). The flavour of citrus juice has been extensively studied to determine aroma-producing active compounds and to evaluate product quality (Perez-Cacho, Mahattanatawee, Smoot, & Rouseff, 2007; Seideneck & Schieberle, 2011). Furthermore, the aroma of citrus juice is a complex combination of several aromatic compounds, including esters, aldehydes, alcohols, ketones, and hydrocarbons. Large amounts of limonene, linalool, γ-terpinene, β-myrcene, α-pinene, and octanal have been detected in mandarins (Qiao, Xie, Zhang, Zhou, & Pan, 2007). In addition, nootkatone, 8,9-didehydronootkatone, and 1,10-dihydronootkatone are important in grapefruit aroma and flavour (Macleod & Buigues, 1964; Stevens, Guadagni, & Stern, 1970).

Glycosidically bound volatile compounds are nonvolatile aroma precursors in many fruits, such as grape, mango, cupuacu, apple, apricot, and pineapple. After enzymatic or acid hydrolysis occurs, glycosidic precursors can yield free volatiles, mainly monoterpenes, C13-norisoprenoids, benzenic compounds, hydroxy esters, and fatty alcohols (Fan, Lu, et al., 2009a; Gunata, Bayonove, Baumes, & Cordonnier, 1985; Rodríguez-Bencomo, Cabrera-Valido, Pérez-Trujillo, & Cacho, 2011). However, glycosidic precursors in citrus fruits have seldom been investigated. For instance, Gueguen, Chemardin, Janbon, Arnaud, and Galzy (1996) reported that β-glucosidase from *Candida molischiana* can significantly increase linalool, benzyl alcohol, and 2-phenylethanol levels in orange juice. In our previous studies, bound volatile compounds in juice and peel of Jincheng oranges were investigated, and our results showed that 3-oxo-α-ionol, *p*-vinylguaiacol, and ethyl-3-hydroxybutyrate are the main bound compounds of this citrus fruit (Fan, Qiao, et al., 2009b). In addition, bound volatile compounds have been detected in orange wine. Citrus aromatic precursors should be determined to help maintain the total aroma of citrus products. To extract bound volatiles, Amberlite XAD-2 resin absorption is one of the most commonly used methods. After trifluoroacetylation or hydrolysis is completed, the obtained glucosides can be directly analyzed by gas chromatography–mass spectrometry (GC–MS).

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'Guoqing No. 1' (GN; *Citrus unshiu* Marc. cv. Guoqing No. 1), Miyagawa wase (MW; *C. unshiu* Marc. cv. Miyagawa wase), and Owari (OW; *C. unshiu* Marc. cv. Owari) are the three mandarin varieties extensively cultivated in the Three Gorges Area. These fruits harvested in September and October are characterised with good commercial quality for consumption and processing and a good balance of sweet taste and refreshing aroma. With regard to grapefruits, two varieties, namely, pink grapefruit (PG; *Citrus paradisi* Macf. cv. Star Ruby) and white grapefruit (WG; *C. paradisi* Macf. cv. Duncan), are also cultivated in this area. Pink grapefruit is well known for its characteristic colour and special flavour with slight bitterness and sourness. Thus far, glycosidically bound volatile compounds in mandarins and grapefruits have not been studied. As such, this study aimed to isolate, identify, and compare free and glycosidically bound volatile compounds in fresh mandarins, grapefruits, and Hamlin (HL) sweet oranges.

2. Materials and methods

2.1. Reagents and reference samples

The solvents (*n*-pentane, diethyl ether, and methanol) were of analytical reagent grade and redistilled before use. Amberlite XAD-2 resin (Supelco, Bellefonte, PA) was treated in accordance with Gunata et al. (1985). Almond β-glucosidase (7.7 units/mg) was obtained from Sigma Chemical Company (Saint Louis, MO). The water used in the study was purified using a Millipore-Q system (Millipore Corp., Saint-Quentin, France). An internal standard solution of cyclohexanone (99.5% purity; Buchs, Switzerland) in ethyl alcohol was prepared at a concentration of 0.946 mg/mL.

Standards of *n*-paraffins (C_6–C_{25}) were purchased from Sigma Chemical Company. Aroma standards, particularly linalyl acetate, carvyl acetate, citronellyl butyrate, neryl acetate, β-elemene, copaene, farnesal, thymol, eugenol, vanillin, farnesol, and ferulic acid, were gifts from Shenzhen Boton Flavors & Fragrances Co., Ltd. (Shenzhen, China). Ethyl butyrate, 3-carene, D-limonene, 1-octanol, terpinolene, citronellyl acetate, linalool, nonanal, α-terpineol, citral, decanal, β-myrcene, germacrene D, 3-hydroxy-β-damascone, 2-hexenal, α-pinene, geraniol, selinene, valencene, ethyl octanoate, ethyl 3-hydroxyhexanoate, benzyl alcohol, δ-cadinene, nootkatone, γ-terpinene, terpinen-4-ol, linalool oxide, carvone, farnesene, camphene, alloaromadendrene, β-gurjunene, carveol, cubebene, ethyl hexanoate, caryophyllene, *p*-vinylguaiacol, and 3-oxo-α-ionol were obtained from Sigma Chemical Company.

2.2. Plant material

Mature citrus fruits were purchased from Jingzhou City, Hubei Province, PR China. Guoqing No. 1 (GN), Miyagawa wase (MW) and Owari (OW) were harvested in September. Hamlin (HL) sweet oranges and the two cultivars of pink grapefruit (PG) and white grapefruit (WG) were harvested in November and December, respectively. The harvested citrus fruits were washed and then dried. The pulp was obtained by hand separation from the peel and was extracted into juice by using a centrifugal juice extractor. Titratable acidity (TA, as citric acid), pH, and total soluble solids (TSS) were determined. Physicochemical analysis results are shown in Table 1.

2.3. Sensory analysis of citrus juices

Descriptive sensory profile analysis was conducted by nine assessors (six females and three males) from College of Food Science and Technology, Huazhong Agricultural University. Most of the assessors were previously trained with sensory evaluation techniques and experienced in GC–olfactometry. Panelists were initially familiarised with the six citrus juices and instructed to agree on a common list of six descriptors (fruity, citrus-like, orange, grassy, floral, and sour). All of the nine assessors were trained to smell the standards of the six consensual odour descriptors. The assessors were required to memorise each odour and then describe it by using the descriptors list. Each sample (5 mL) was placed in a 10-mL coded flask for sensory tests. Approximately 20 mm of the extremity of the fragrance blotter paper (142 mm × 6 mm) was immersed in the juices for 0.5 min and then presented to the assessors. The tests were conducted at room temperature. The intensity of each characteristic was evaluated on a scale of 1–9 (1 = very weak intensity, 3 = weak intensity, 5 = moderate intensity, 7 = strong intensity, and 9 = very strong intensity) (Fan, Xu, et al., 2009c; Selli et al., 2008). All of the tests were conducted in triplicate. The results of the sensory profile analysis were averaged for each odour note and plotted in a spider web diagram.

2.4. Isolation of glycosidic precursors in citrus juices

Glycosidic precursors were isolated by adsorption on Amberlite XAD-2 resins. The citrus juices were centrifuged at 10,000*g* (4 °C) for 20 min and the supernatant was filtered. The juice (500 mL) was poured into a 50 × 1 cm i.d., column filled with solvent-washed XAD-2. The column was rinsed with 300 mL of distilled water and then eluted with 300 mL of pentane/diethyl ether (1/1) at a flow rate of 2 mL/min. Glycosidically bound fraction was subsequently eluted using 300 mL of methanol. The methanol eluate was concentrated to dryness under vacuum at 35 °C. The residue was dissolved in 20 mL of 0.06 M citric phosphate buffer solution (pH 5). The buffered mixture was washed twice with 80 mL of pentane/diethyl ether (1/1) to remove possible existing traces of free volatiles.

2.5. Extraction of free volatile compounds

A solid-phase microextraction (SPME) manual device equipped with 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre (Supelco, Bellefonte, PA) was used to extract free volatile compounds from citrus juice. The fibre was conditioned in a GC injector port at 270 °C for 1 h before use. Afterward, 10 mL of citrus juices with 3.6 g of NaCl, previously added to 50 μL of cyclohexanone (0.946 mg/mL of ethyl alcohol) as an internal standard of each sample, were placed in a 20-mL vial containing a microstirring bar. The samples were equilibrated at 40 °C for 15 min and extracted using the DVB/CAR/PDMS fibre for 40 min at the same temperature with continuous stirring. After volatiles were extracted, the fibre was inserted into the GC injection port to desorbing the analytes for 5 min. Each analytical sample was analyzed in triplicate.

2.6. Enzymatic hydrolysis of glycosidic precursors

The glycosidic precursors dissolved in the buffer solution were hydrolysed by almond β-glucosidase (40 mg, 7.7 units/mg) at 40 °C for 48 h. The free volatiles were extracted with three 40-mL portions of pentane/diethyl ether (1/1). The extracts were dried in anhydrous sodium sulfate and concentrated to a final volume of 0.5 mL in a stream of pure nitrogen.

2.7. GC–MS analysis of volatile compounds

Volatile compounds were subjected to GC analysis on an Agilent 6890 N GC coupled to an Agilent 5975B mass spectrometer and equipped with a J&W HP-5MS fused silica capillary column

Table 1
TA, TSS, pH, and TSS/TA of different citrus juices.

Sample	Hamlin (HL)	White grapefruit (WG)	Pink grapefruit (PG)	Guoqing No. 1 (GN)	Miyagawa wase (MW)	Owari (OW)
TA (%)	$0.99^{a} \pm 0.02$	$1.51^{b} \pm 0.02$	$1.43^{c} \pm 0.01$	$0.68^{d} \pm 0.01$	$0.75^{e} \pm 0.01$	$1.02^{f} \pm 0.01$
TSS (°Brix)	$12.00^{a} \pm 0.01$	$11.00^{b} \pm 0.02$	$12.00^{a} \pm 0.01$	$8.60^{c} \pm 0.01$	$11.50^{d} \pm 0.03$	$10.50^{e} \pm 0.01$
pH	$3.14^{a} \pm 0.01$	$2.68^{b} \pm 0.01$	$3.01^{c} \pm 0.01$	$3.83^{d} \pm 0.01$	$3.83^{d} \pm 0.01$	$3.43^{e} \pm 0.01$
TSS/TA	12.12	7.28	8.39	12.73	15.43	10.27

TA, titratable acid. TSS, total soluble solids. Different superscripts within the same row indicate statistical differences ($P < 0.05$) using the LSD test.

(30 m × 0.25 mm i.d., 0.25 μm film thickness). The mass spectrometer was operated in electron impact mode at a voltage of 70 eV. The flow rate of helium through the HP-5 column was 1.2 mL/min. A 0.75-mm liner was used. Analysis was conducted in splitless mode. Injector temperature was 250 °C. The column was initially maintained at 40 °C for 3 min; temperature was then increased from 40 °C to 160 °C at 3 °C/min, maintained at 160 °C for 2 min, and finally increased to 220 °C at a rate of 8 °C/min. Temperature was maintained at 220 °C for 3 min.

The compounds detected by GC–MS analysis were identified by comparing the obtained mass spectra and retention indices (RI) with those of authentic standards and published data and by comparing the corresponding mass spectra with the MS libraries of Wiley 7.0 and NIST05. RIs were calculated using a mixture of *n*-paraffin (C_6–C_{25}) as standards.

Semiquantitative determinations were conducted using cyclohexanone as an internal standard. Volatile compound contents were calculated from the GC peak areas related to the GC peak area of the internal standard.

2.8. Statistical analysis

Significant differences of physicochemical properties, free and bound volatile compounds in the six citrus varieties obtained in triplicate analysis were determined by one-way ANOVA in SPSS 19.0 for Windows (SPSS Inc., Chicago, IL). Multivariate analysis was conducted by principal component analysis (PCA). Furthermore, correlations between sensory data (*X* variables) and chemical compounds (*Y* variables) were investigated using partial least squares (PLS) regression analysis in XLSTAT 2010 (Addinsoft, New York, NY).

3. Results and discussion

3.1. Sensory analysis of citrus juices

Sensory profiling, also known as descriptive analysis, is the preferred technique to relate information of aroma volatiles to sensory perception. This technique provides detailed insights into the perceptions of panellists of a number of flavour notes, which can be related to levels of individual aroma volatiles (Keenan, Brunton, Mitchell, Gormley, & Butler, 2012). The flavours of the six citrus juices were assessed by nine panellists using six descriptors (fruity, citrus-like, orange, grassy, floral, and sour), which were previously agreed as most important sensorial characteristics of juices. The nine odour descriptors used in this study were generated mainly by our panellists to characterise the odour of citrus juices and were based on previously published papers (Selli et al., 2008).

The average aroma intensity scores of the six citrus juices on the spider web diagram are shown in Fig. 1. The three mandarins presented similar profiles. Likewise, PG and WG juices showed almost the same sensory profiles. One-way ANOVA results of the mean scores of each sensory descriptor from the nine assessors indicated no significant differences ($p > 0.05$) among the three different mandarins and between the two grapefruits. The flavor profiles of

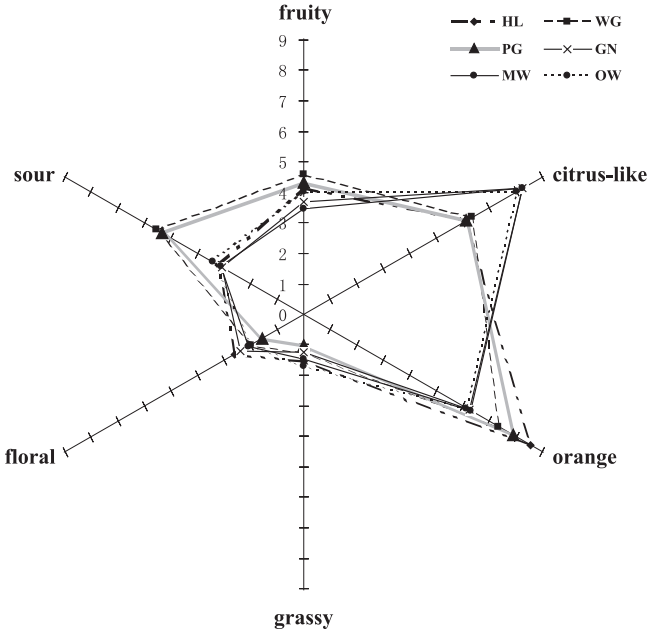

Fig. 1. Sensory descriptive analysis of citrus juices. Mean scores of nine judges (three replicates).

mandarins, grapefruits, and HL sweet orange were evidently different. This result is in agreement with the result obtained by ANOVA showing significant differences ($p < 0.05$).

Orange was the strongest flavour of the two grapefruit and HL orange juices; in contrast, orange was slightly weak in mandarin juices. Citrus-like was the descriptor with the highest score in mandarin juices but was evidently weak in grapefruit and HL orange juices. Sour was also the main flavour of the two grapefruit juices, and no significant differences were observed between these juices. The other descriptors (fruity, grassy, and floral) with low scores in the six citrus juices were perceived weak. These results showed that the flavour of the same citrus cultivars was similar, whereas the flavour between cultivars was different. Geometric means of the descriptors were calculated using frequency and intensity obtained by the panellists. The results are shown in Table 2. Citrus-like and orange were the most important descriptors of these citrus juices, followed by sour and fruity.

Table 2
Geometric means of the descriptors in sensory evaluation.

Descriptor	Fruity	Citrus-like	Orange	Grassy	Floral	Sour
Frequency (%)	94.4	100	100	75.9	83.3	96.3
Intensity (%)	44.7	79.4	78.5	14.9	23.5	43.8
Geometric means (%)	64.9	89.1	88.6	33.7	44.2	65.0

3.2. Free and bound volatile compounds in Hamlin (HL) sweet orange juice

The free volatile compounds in the six citrus juices are shown in Table 3. A total of 33 free volatile compounds were identified in HL sweet orange juice, with a concentration of 803 mg/L. Furthermore, 19 terpenes were identified in HL orange juice and were the most abundant compounds, accounting for 97.8% of the total concentration. Among these terpenes, D-limonene was the predominant compound, representing 93% of the total volatile compounds. A similar high content of D-limonene in other sweet orange cultivars was also observed. Arena, Guarrera, Campisi, and Nicolosi (2006) determined that the percentage of limonene ranged from approximately 90% to 97% to in Tarocco and Washington navel oranges, respectively. Nisperos-Carriedo and Shaw (1990) determined that limonene was the second most abundant volatile component in HL orange juice after ethanol. Ahmed, Dennison, and Shaw (1978) reported that limonene was an important contributor to the orange flavour when added at a level of 190 ppm (0.019%) to processed juice. Limonene has orange-like and fruity odour. The next most abundant terpene compound was β-myrcene, with a balsamic and geranium odour (Kelebek & Selli, 2011). Linalool was quantitatively the main terpene alcohol in HL orange juice, with a floral and green odour, representing 0.6% of the total terpenes. Although terpenes are the most abundant compounds in citrus juice, they are responsible for limited odour activity because of their high odour threshold values (Perez-Cacho & Rouseff, 2008a). However, the presence of terpenes at high concentrations may contribute to the bitter taste of juices (Bylaite & Meyer, 2006).

Esters were also abundant compounds in HL orange juice. In total, eight esters were detected in orange juice, with a concentration of 11.5 mg/L. These compounds are known to contribute to the "top note" of fruit and citrus flavours (Arctander, 1969). Ethyl butyrate was the most important ester, which is responsible for the fruity odour and is an important contributor to desirable flavour in orange products (Ahmed et al., 1978). The absence of ethyl

Table 3
Free volatile compounds in the six different citrus juices.

No.	Compounds	RI	Content (μg/L)					ID
			Hamlin (HL)	Grapefruits		Mandarins		
				White grapefruit (WG)	Pink grapefruit (PG)	Guoqing No. 1 (GN)	Miyagawa wase (MW)	Owari (OW)	
1	Ethyl butyrate	794	4190 ± 367	n.d.	n.d.	n.d.	n.d.	n.d.	a
2	2-Hexenal	844	1510^{a} ± 459	93^{b} ± 11	n.d.	62^{b} ± 23	251^{b} ± 9	141^{b} ± 7	a
3	α-Pinene	924	2800^{a} ± 263	272^{b} ± 28	267^{b} ± 7	n.d.	134^{bc} ± 18	402^{bd} ± 15	a
4	β-Myrcene	984	12,600^{a} ± 394	1850^{b} ± 456	2520^{c} ± 291	111^{d} ± 15	578^{e} ± 61	947^{e} ± 121	a
5	Ethyl hexanoate	998	3300 ± 368	n.d.	n.d.	n.d.	n.d.	n.d.	a
6	3-Carene	1009	531^{a} ± 69	40^{b} ± 11	370^{c} ± 21	n.d.	22^{b} ± 8	146^{d} ± 6	a
7	D-Limonene	1024	750,000^{a} ± 1470	99,000^{b} ± 42	115,000^{c} ± 1020	6980^{d} ± 473	34,500^{e} ± 2380	81,600^{f} ± 4590	a
8	γ-Terpinene	1051	972^{a} ± 94	207^{b} ± 40	1180^{c} ± 64	308^{b} ± 21	1640^{d} ± 98	5740^{e} ± 168	a
9	Linalool oxide	1067	n.d.	653^{a} ± 71	662^{a} ± 109	n.d.	n.d.	n.d.	a
10	1-Octanol	1069	891^{a} ± 21	n.d.	n.d.	n.d.	21^{b} ± 5	n.d.	a
11	Terpinolene	1080	599^{a} ± 68	89^{b} ± 23	160^{c} ± 7	11^{d} ± 0.9	95^{b} ± 10	57^{bd} ± 13	a
12	Linalool	1097	4890 ± 124	n.d.	n.d.	n.d.	n.d.	n.d.	a
13	Nonanal	1098	n.d.	57^{a} ± 9	93^{ab} ± 4	81^{a} ± 43	132^{b} ± 25	147^{bc} ± 33	a
14	Camphene	1109	61 ± 9	n.d.	n.d.	n.d.	n.d.	n.d.	a
15	Ethyl 3-hydroxyhexanoate	1125	2680^{a} ± 214	106^{b} ± 18	n.d.	n.d.	n.d.	n.d.	a
16	Terpinen-4-ol	1172	2490^{a} ± 154	341^{b} ± 39	74^{c} ± 16	24^{c} ± 7	76^{c} ± 30	135^{c} ± 10	a
17	α-Terpineol	1186	1140^{a} ± 81	224^{b} ± 53	10^{c} ± 2	67^{c} ± 14	202^{b} ± 30	241^{b} ± 70	a
18	Ethyl octanoate	1191	455^{a} ± 31	91^{b} ± 14	121^{b} ± 19	n.d.	n.d.	n.d.	a
19	Decanal	1199	1440^{a} ± 92	26^{b} ± 8	24^{b} ± 2	n.d.	58^{b} ± 10	78^{b} ± 9	a
20	Carvone	1240	487 ± 21	n.d.	n.d.	n.d.	n.d.	n.d.	a
21	Linalyl acetate	1250	240 ± 28	n.d.	n.d.	n.d.	n.d.	n.d.	a
22	Citral	1267	1740 ± 51	n.d.	n.d.	n.d.	n.d.	n.d.	a
23	Carveol	1332	78^{a} ± 9	n.d.	48^{ac} ± 5	201^{b} ± 45	16^{c} ± 2	n.d.	a
24	Carvyl acetate	1332	n.d.	95 ± 5	n.d.	n.d.	n.d.	n.d.	a
25	Cubebene	1342	386^{a} ± 74	170^{b} ± 21	125^{b} ± 32	n.d.	n.d.	21^{c} ± 3	a
26	Citronellyl butyrate	1347	n.d.	n.d.	175^{a} ± 14	n.d.	n.d.	30^{b} ± 4	a
27	Citronellyl acetate	1347	405 ± 24	n.d.	n.d.	n.d.	n.d.	n.d.	a
28	Geraniol	1358	313^{a} ± 21	113^{b} ± 9	123^{b} ± 22	n.d.	7^{c} ± 2	n.d.	a
29	Neryl acetate	1378	109^{a} ± 12	n.d.	134^{a} ± 15	n.d.	n.d.	n.d.	a
30	β-Elemene	1385	n.d.	n.d.	115^{a} ± 22	n.d.	n.d.	230^{b} ± 61	a
31	Caryophyllene	1414	173^{a} ± 18	26,500^{b} ± 893	53,000^{c} ± 1240	n.d.	n.d.	114^{a} ± 34	a
32	Alloaromadendrene	1431	96^{a} ± 9	94^{a} ± 11	117^{b} ± 17	n.d.	14^{c} ± 2	n.d.	a
33	Perillyl acetate	1431	107 ± 15	n.d.	n.d.	n.d.	n.d.	n.d.	b
34	Copaene	1467	405^{a} ± 23	n.d.	47^{b} ± 13	n.d.	n.d.	44^{b} ± 5	a
35	β-Gurjunene	1469	n.d.	n.d.	29^{a} ± 4	n.d.	n.d.	22^{a} ± 4	a
36	γ-Muurolene	1470	n.d.	50 ± 10	n.d.	n.d.	n.d.	n.d.	b
37	Germacrene D	1474	n.d.	9 ± 3	n.d.	n.d.	n.d.	n.d.	a
38	Selinene	1480	148^{a} ± 16	101^{b} ± 16	55^{c} ± 9	n.d.	n.d.	106^{b} ± 9	a
39	Valencene	1487	6750^{a} ± 381	162^{b} ± 23	421^{c} ± 23	399^{bc} ± 43	1100^{d} ± 56	258^{bc} ± 8	a
40	δ-Cadinene	1517	895^{a} ± 22	1020^{b} ± 21	856^{a} ± 39	n.d.	n.d.	162^{c} ± 48	a
41	Farnesal	1750	n.d.	273^{a} ± 2	405^{b} ± 23	n.d.	n.d.	n.d.	a
42	Nootkatone	1806	59^{a} ± 8	645^{b} ± 36	191^{c} ± 12	n.d.	n.d.	n.d.	a
43	Farnesene	1837	n.d.	232^{a} ± 33	n.d.	n.d.	n.d.	175^{a} ± 25	a

RI, Linear retention index on the DB-5MS column; n.d., not detected; ±, standard deviation. ID, Identification: a, comparison of mass spectra and retention index (RI) with authentic standards; b, comparison of mass spectra and RI with published data and MS library of Wiley7.0 and NIST05. Different superscripts within the same row indicate statistical differences ($p < 0.05$) using the LSD test.

butyrate leads to the missing fruity top notes in processed orange juice products (Nisperos-Carriedo & Shaw, 1990). Ethyl hexanoate is another important ester in HL orange juice. Ethyl hexanoate has a fruity and floral flavour and is an important compound in orange juice (Selli & Kelebek, 2011). Ethyl 3-hydroxyhexanoate, ethyl octanoate, linalyl acetate, citronellyl acetate, neryl acetate, and perillyl acetate are the other six esters detected in free fractions of orange juice.

In total, three aldehydes were detected in HL orange juice, with a total concentration of 4695 μg/L. These compounds are secondary metabolites formed during orange ripening and maturation, and their concentration increases with fruit maturity (Perez-Cacho & Rouseff, 2008b). Citral has the highest level among these aldehydes. This terpenic aldehyde is believed to be an important contributor to the orange flavour, with a flowery and citrus-like aroma (Ahmed et al., 1978; Selli & Kelebek, 2011). However, this terpenic aldehyde was unstable and decreased during thermal processing and storage (Perez-Cacho & Rouseff, 2008b). Decanal and 2-hexenal detected in the present study are also important compounds contributing to orange aroma. Previous studies have reported that aldehydes were important aroma active compounds in grapefruits (Lin, Rouseff, Barros, & Naim, 2002; Buettner & Schieberle, 1999, 2001).

Carvone and nootkatone were the two ketones detected in orange juice, with a combined concentration of 546 μg/L. Carvone has a minty and caraway-like flavour and can degrade the flavor quality of the juice. Carvone is an off-flavour ketone produced from the oxidation of limonene as a result of thermal treatment and oxidative storage (Perez-Cacho & Rouseff, 2008b). Nootkatone has a citrusy and grapefruit flavor and is usually detected in grapefruit. 1-Octanol was the only alcohol detected in free fractions of HL orange juice.

The bound volatile compounds in the six citrus juices are shown in Table 4. Only five bound volatile compounds, namely, two terpenic compounds, one benzenic compound, one hydroxy ester, and one C13-norisoprenoid, were detected in HL orange juice, and the total content was 461 μg/L. *p*-Vinylguaiacol was the most abundant bound volatile compound in this juice, accounting for 78% of the total concentration. Ethyl-3-hydroxyhexanoate was the only ester detected in this orange juice, and this compound was the only bound volatile compound detected in free fractions. It is considered a common bound volatile compound in many other fruits, such as grapes and pineapples.

3.3. Free and bound volatile compounds in the two grapefruit juices

As shown in Table 3, 27 free volatile compounds were detected in WG and PG, with a total concentration of 132 and 176 mg/L, respectively. Similarly, terpenes were the most abundant compounds in these free volatiles, followed by aldehydes and esters. However, most of these terpenes were not the aroma active compounds in grapefruit juices. Only several terpenes were observed to be aroma active compounds, such as limonene, which had a terpene-like aroma, and myrcene, which had a moss-like and geranium aroma (Lin et al., 2002; Buettner & Schieberle, 1999, 2001). Among these detected compounds, a total of 21 volatiles were detected as the common fractions existing in the WG and PG juices. 2-Hexenal, ethyl 3-hydroxyhexanoate, carvyl acetate, γ-muurolene, germacrene D and farnesene were only detected in WG. 2-Hexenal, limonene, nonanal, terpinen-4-ol, decanal, and nootkatone were also determined as aroma active compounds in early season WG juices (Lin et al., 2002). The results show that a relatively small difference can be observed among the aroma compounds between WG and PG. The same results were obtained in sensory analysis.

A total of six and three bound volatile compounds were detected in WG and PG, with total concentrations of 1690 and 3010 μg/L, respectively. A significant difference was observed between the bound fractions of these two grapefruits. More bound volatile compounds were found in WG than in PG. Only linalool oxide and *p*-vinylguaiacol were detected in the two grapefruits. Benzenic compounds were the most abundant compounds. Almost the same contents of *p*-vinylguaiacol were detected in these two juices. Benzenic compounds were also observed as bound fraction in many other fruits and other orange varieties (Fan, Lu, et al., 2009a). Benzenic compounds have a spicy and woody aroma and are odour active compounds in young Fiano wine (Ugliano & Moio, 2008).

Linalool oxide was the only compound both in free and bound fractions in these two grapefruits. The content of this compound in bound form was more than twice as much as in free form in PG, and this indicated that the release of this compound might be a contributor to the overall aroma of PG. A relative low concentration of this compound was found in WG.

3.4. Free and bound volatile compounds in the three mandarin juices

With regard to mandarins, a total of 10, 16, and 21 free volatile compounds were detected in GN, MW, and OW, with total

Table 4
Bound volatile compounds in the six different citrus juices.

No.	Compounds	RI	Content (μg/L)						ID
			Hamlin	Grapefruit White	Grapefruit Red	Guoqing	Miyagawa Wase	Owari satsuma	
1	Benzyl alcohol	1038	n.d.	$0.5^{a} \pm 0.1$	n.d.	$1.2^{a} \pm 0.3$	$5.7^{b} \pm 1.3$	n.d.	a
2	Linalool oxide	1067	n.d.	$63^{a} \pm 8$	$1360^{b} \pm 34$	$330^{c} \pm 5$	$52^{a} \pm 21$	n.d.	a
3	Ethyl 3-hydroxyhexanoate	1126	76 ± 9	n.d.	n.d.	n.d.	n.d.	n.d.	a
4	Thymol	1304	n.d.	n.d.	n.d.	5 ± 1.1	n.d.	n.d.	a
5	*p*-Vinylguaiacol	1311	$362^{a} \pm 12$	$1420^{b} \pm 65$	$1580^{c} \pm 95$	$72^{d} \pm 23$	$48^{d} \pm 0.3$	$684^{e} \pm 19$	a
6	Eugenol	1355	n.d.	n.d.	n.d.	$1.7^{a} \pm 1$	$8^{b} \pm 2$	$5.9^{c} \pm 1.6$	a
7	Vanillin	1409	n.d.	n.d.	n.d.	$0.8^{a} \pm 0.4$	$7^{b} \pm 1.4$	n.d.	a
8	Isoeugenol	1453	n.d.	n.d.	$66^{a} \pm 7$	n.d.	n.d.	$150^{a} \pm 57$	b
9	*p*-Menth-1-en-9-ol	1492	$3^{a} \pm 0.6$	n.d.	n.d.	$6.7^{b} \pm 0.0$	n.d.	n.d.	b
10	*p*-Mentha-2,8-dienol	1610	$14^{a} \pm 2$	n.d.	n.d.	$22.2^{b} \pm 0.2$	$4^{c} \pm 1.6$	n.d.	b
11	3-Hydroxy-β-damascone	1615	n.d.	n.d.	n.d.	n.d.	3.4 ± 1.2	n.d.	a
12	3-Oxo-α-ionol	1654	$6.4^{a} \pm 0.9$	$60^{b} \pm 8$	n.d.	$381^{c} \pm 43$	$154^{d} \pm 16$	$104^{e} \pm 9$	a
13	Zingerone	1663	n.d.	7 ± 2	n.d.	n.d.	n.d.	n.d.	b
14	Vanillyl ethyl ether	1688	n.d.	n.d.	n.d.	$77^{a} \pm 5$	n.d.	$38^{b} \pm 11$	b
15	Farnesol	1734	n.d.	n.d.	n.d.	$2.8^{a} \pm 1.8$	$3.1^{a} \pm 3.0$	n.d.	a
16	Ferulic acid	1915	n.d.	142 ± 6	n.d.	n.d.	n.d.	n.d.	a
17	Methoxyeugenol	2202	n.d.	n.d.	n.d.	$24.6^{a} \pm 3.3$	$26^{a} \pm 10$	n.d.	b

RI, Linear retention index on a DB-5MS column; n.d., not detected; ±, standard deviation. ID, Identification: a, comparison of mass spectra and retention index (RI) with authentic standards; b, comparison of mass spectra and RI with published data and MS library of Wiley7.0 and Nist05. Different superscripts within the same row indicate statistical differences ($p < 0.05$) using the LSD test.

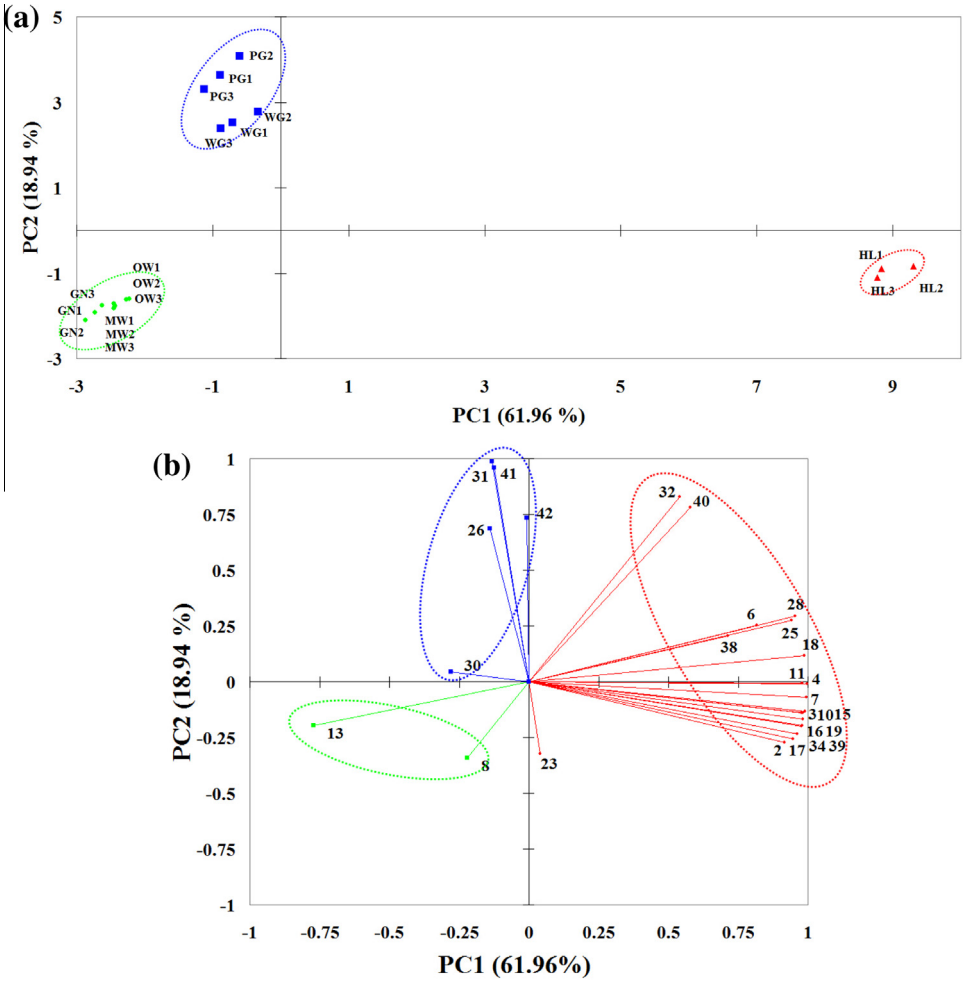

Fig. 2. PCA of free volatile compounds in six varieties of citrus juices [(Δ) HL sweet orange; (□) grapefruits; (○) mandarins]. (a) Score plot of PC2 against PC1; (b) variable plot of PC2 against PC1. Variables are identified as volatile compounds listed in Table 2.

concentrations of 8.2, 38.8, and 90.7 mg/L, respectively. Similarly, terpenes were also the predominant compounds in these mandarins, followed by aldehydes. Only 2-hexenal, β-myrcene, D-limonene, γ-terpinene, terpinolene, nonanal, terpinen-4-ol, α-terpineol, and valencene were detected in all of the three different mandarins. A significant difference was observed among the free volatile compounds of these mandarins. The concentration and number of aroma active compounds in OW were the most abundant, followed by MW and GN. Cubebene, citronellyl butyrate, β-elemene, caryophyllene, copaene, β-gurjunene, selinene, δ-cadinene and farnesene were the nine compounds found only in OW, while 1-octanol and geraniol were only detected in MW.

A total of 12, 10, and 5 bound volatiles were detected in GN, MW, and Owari satsuma, with total concentrations of 925, 311, and 982 μg/L, respectively. The bound fractions in GN were almost the same as that in MW. Nine bound volatiles were detected in these two mandarins. The concentration of the bound fraction in GN was approximately three times more than that in MW. Only five bound volatiles were detected in Owari satsuma, although the total contents of these compounds were almost the same as that in GN. Among these compounds, only *p*-vinylguaiacol, eugenol, and 3-oxo-α-ionol were also detected in the other two mandarins. None of these bound compounds were found as free forms in these three mandarins.

3.5. Comparison of free and bound volatile compounds between the three varieties

The concentration and type of free volatile compounds in sweet oranges were the most abundant, followed by grapefruits and mandarins. No great significant differences in concentration and type of most of the free volatiles were observed between the two grapefruits. The same results were also observed among the three mandarins. This finding indicated that a significant difference was observed among the free volatiles of the different citrus varieties. Different results were observed between the different citrus strains. The HL sweet orange juice had the highest level and the most abundant free volatiles, followed by grapefruits and mandarins. Totally 19 free volatiles were found in all the three citrus varieties, while ethyl butyrate, ethyl hexanoate, linalool, camphene, carvone, linalyl acetate, citral, citronellyl acetate and perillyl acetate were only found in the HL sweet orange juice. These compounds might be the main contributors to the orange flavour in HL which scored higher than that in grapefruits and mandarins according to sensory analysis. Linalool oxide, carvyl acetate, γ-muurolene, germacrene D and farnesal were the five free volatiles only detected in grapefruits.

Based on the results of bound volatile compounds listed in Table 4, significant differences were observed among the bound volatiles of cultivars. Only *p*-vinylguaiacol existed in all of the orange juices. The levels of this compound and linalool oxide in

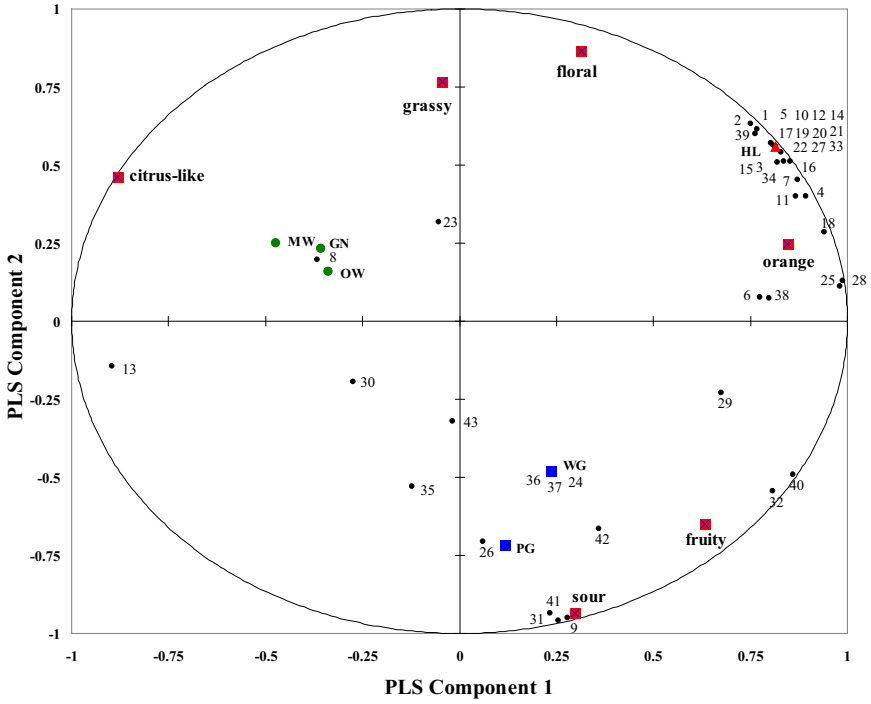

Fig. 3. PLS regression loading plots of sensory data correlating with chemical compounds.

grapefruits were the highest. Benzenic compounds were the major bound volatiles in these citrus fruits. *p*-Vinylguaiacol was the only compound detected in all six citrus juices, while the highest content of this compound was found in grapefruits. Most of these benzenic compounds, such as benzyl alcohol, *p*-vinylguaiacol, eugenol, and vanillin, were also detected in many other fruits, such as cherry (Wen et al., 2014), kiwifruit (Garcia, Stevenson, Atkinson, Winz, & Quek, 2013), and strawberry (Ubeda et al., 2012). Terpenoids were considered the most important and abundant bound aroma compounds in grapes, whereas only linalool oxide, *p*-menth-1-en-9-ol, *p*-mentha-2,8-dien-1-ol, and farnesol were detected in the present study. *p*-Menth-1-en-9-ol and *p*-mentha-2,8-dien-1-ol were detected both in oranges and mandarins, while ethyl-3-hydroxyhexanoate was only detected in orange juice. 3-Hydroxy-β-damascone and 3-oxo-α-ionol were the only two C13-norisoprenoids detected in these citrus juices. 3-Oxo-α-ionol was found in all three citrus varieties. GN had the highest levels of 3-oxo-α-ionol, Owari satsuma had relatively high levels of isoeugenol, and MW had the highest concentration of benzyl alcohol. Some compounds, such as thymol, 3-hydroxy-β-damascone, zingerone, and ferulic acid, were only detected in one of the citrus juices. In addition, thymol, eugenol, vanillin, 3-hydroxy-β-damascone, vanillyl ethyl ether, farnesol, and methoxyeugenol were only detected in mandarins. Among these detected bound fractions, linalool oxide and ethyl 3-hydroxyhexanoate were the only compounds also detected in free fractions.

3.6. Principal component analysis

PCA was conducted to understand the correlation and segregation among those free volatile compounds that are significantly different among cultivars. Based on the PCA results, four principal components (PCs) were obtained, which accounted for 96.8% of the total variance. PC1 accounted for 61.9% of the total variance and PC2 accounted for 18.9% of the total variance. Fig. 2 shows the correlations between chemical variables and the first two dimensions using PCA conducted on the normalised variables. As shown in the PCA score plot (Fig. 2), the aroma profiles of mandarins, grapefruits, and sweet orange juices showed significant differences, and clearly separate all the three varieties from one another due to their volatile profile. Thus, complete separation between these citrus samples was achieved. This result was similar to that of the sensory descriptive analysis of the six citrus juices. Three clusters were observed in the segregation of volatile compounds based on their geographical origins (Fig. 2(a)). The HL sweet orange juice was grouped in the lower right quadrant, indicating that it was strongly and positively correlated with PC1, whereas the grapefruit juice was negatively correlated with PC1. The mandarin juice exhibited a negative correlation with PC1 and PC2. This indicated that a complete separation on the volatiles among HL sweet orange, grapefruits and mandarin juice was achieved. Upon further analysis of variable loadings, we observed that the dense loading of variables was located at the lower right quadrant of the PCA plot (Fig. 2(b)), indicating their positive correlations with PC1 and negative correlations with PC2.

Most of the major terpenes (e.g., α-pinene, β-myrcene, D-limonene, terpinen-4-ol, α-terpineol, and valencene) and other oxygenated compounds (e.g., 2-hexenal, decanal and ethyl 3-hydroxyhexanoate) had positive correlations with PC1. These compounds were the major contributors to the higher volatile contents in the citrus juices, and were closer to the HL sweet orange juice due to their significantly higher concentrations. Similar results were also found on the volatile compounds in the peel of different calamansi (Cheong et al., 2012). Citronellyl butyrate, β-elemene, caryophyllene, farnesal, and nootkatone were positively correlated with PC2. This finding indicated that these compounds were the important aroma compounds in grapefruit juice that was different from the other two citrus juices, while in the lower left quadrant, the mandarins had fewer volatiles overall. Similar results were also obtained by Miyazaki, Plotto, Goodner, and Gmitter (2010).

3.7. Partial least square regression analysis

The correlations between sensory data and chemical compounds were investigated using PLS regression analysis, which is

based on the hypothesis that sensory perception is influenced by the volatile flavor profile. The PLS regression analysis was performed using the 43 free volatiles and the 6 sensory descriptors.

As shown in Fig. 3, orange flavour was located closely to HL orange juices, which was in accordance with the sensory analysis. Many potent aroma compounds, such as terpenes (α-pinene, β-myrcene, 3-carene, D-limonene, linalool, terpinen-4-ol, α-terpineol, and geraniol), aldehydes (decanal and citral) and esters (ethyl butyrate, ethyl hexanoate, and ethyl 3-hydroxyhexanoate) were grouped. This indicated that these compounds were associated with the "orange" sensory attribute. Similarly, γ-terpinene and nonanal were associated with the "citrus-like" sensory attribute. The sour and fruity flavours were located more closely to the two grapefruit juices and might be influenced by linalool oxide, carvyl acetate, caryophyllene, farnesal, neryl acetate, alloaromadendrene, citronellyl butyrate, and nootkatone. Grassy and floral flavours were located far from the citrus juices and those volatile compounds, and this indicated that these two notes were not important to the citrus juice flavor due to the absence of related volatiles.

4. Conclusions

This study focused on free and bound volatile compounds in six different varieties of citrus juice. Sensory analysis results showed that the flavour of the same citrus cultivars was similar, whereas the flavour between cultivars was different. SPME–GC–MS analysis results revealed 43 free and 17 bound volatile compounds identified in these citrus juices. The free volatiles of different citrus varieties differed. Likewise, varied results were observed among different citrus strains. The first two PCs in a PCA plot of the free volatiles accounted for 61.9% and 18.9% of the total variance and, on this plot, the six citrus juices can be differentiated.

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