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The Composition of Volatile Aroma Components, Flavanones, and Polymethoxylated Flavones in Shiikuwasha (*Citrus depressa* Hayata) Peels of Different Cultivation Lines

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ABSTRACT: Citrus peels are important sources of various pleasant aroma compounds and valuable bioactive substances. To investigate differences in the composition and content of Shiikuwasha (*Citrus depressa* Hayata) peels from different cultivation lines, the composition of volatile aroma components, flavanones, and polymethoxylated flavones (PMFs) in four Shiikuwasha cultivation lines was examined. The composition of volatile aroma components in cold-pressed extracts of Shiikuwasha peels was analyzed using gas chromatography−flame ionization detection and gas chromatography−mass spectrophotometry. The extracts contained mainly monoterpene hydrocarbons (93.40−97.25%), including limonene (46.52−68.26%) and γ-terpinene (21.48−30.52%). Differences in the composition of volatile aroma compounds in the Shiikuwasha cultivation lines were revealed using principal component analysis. Additionally, the composition of flavanones and PMFs was determined using high-performance liquid chromatography methods. Neohesperidin (96.58%) was the predominant flavanone in 'Izumi kugani' peel, while the other peels had high hesperidin contents (89.26−98.66%). Moreover, the PMFs of Shiikuwasha peels were composed of nobiletin (56.74−64.77%) and tangeretin (23.17−34.70%).

KEYWORDS: *Shiikuwasha peel, cultivation line, volatile aroma components, flavanones, polymethoxylated flavones (PMFs)*

INTRODUCTION

The exocarp of citrus fruits, also called peel or rind, is an important component that contributes many essential properties to the overall quality of the fruits. Citrus peels do not comprise only acceptable and pleasant flavor compounds but also bioactive substances that have beneficial effects on human health.[1,2] Hence, there is currently an upsurge of interest in the biochemical properties and functions of citrus peels, as well as their commercial application in foods, nutraceuticals, and pharmaceuticals. These chemical and biochemical characteristics may vary depending on citrus fruit origin, species, cultivation line, and degree of ripening.

Shiikuwasha (*Citrus depressa* Hayata) is a citrus fruit cultivar with about 10 kinds of cultivation lines, including 'Kaachi', 'Izumi kugani', 'Katsuyama kugani', 'Ogimi kugani', 'Ishikunibu', 'Hijyakunibu', 'Kabishi', and 'Fusubuta', which grows naturally on Okinawa Island, Japan. It has small yellowish-green fruits with a very sour taste and strong characteristic aroma.[3] The average annual commercial production of Shiikuwasha fruits was around 3000 tons from 2007 to 2009, and about 90% of its products are from 'Kaachi', 'Izumi kugani', 'Katsuyama kugani', and 'Ogimi kugani' lines.[4] Shiikuwasha is commonly consumed as fresh fruit or processed into different food products, such as beverages, confectionary items, and food additives. Shiikuwasha juice is an abundant source of ascorbic acid and flavonoids, even when subjected to filtration, sterilization, and bottling processes.[5]

A study of the characteristic aroma composition of citrus fruits has been carried out for many years, leading to investigation of the distinctiveness of each type of citrus fruit.[6] The volatile aroma components of citrus fruits, which are mainly extracted from the peels, are predominantly monoterpene and sesquiterpene hydrocarbons. Moreover, the subsequently oxygenated compounds derived from these hydrocarbons include alcohols, aldehydes, esters, ketones, ethers, phenols, and oxides of various compositions. These unique aroma compositions have been investigated using several types of extraction systems and analytical techniques.[7,8] Additionally, extracts of volatile aroma components of citrus peels, as well as other plant essential oils, have been shown to exhibit various antimicrobial, antiviral, and antioxidant activities.[9,10]

Flavonoids, along with phenolics, carotenoids, and limonoids, are known as typical and important bioactive substances from citrus peels. Common flavonoid compounds in citrus peels are flavanones, flavones, and PMFs, which possess biological activities.[11−13] Of the flavanones, citrus peel-derived hesperidin was observed to have anti-inflammatory and antialcoholic fatty liver effects,[14,15] and naringenin enhanced melanin synthesis

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Table 1. Morphological[a] and Chemical[b] Traits of Shiikuwasha Fruits

fruit traits	'Kaachi'	'Izumi kugani'	'Katsuyama kugani'	'Ogimi kugani'
average fruit weight (g)	26.74 ± 3.84	45.65 ± 7.18	26.74 ± 4.24	36.51 ± 4.51
polar diameter (mm)	29.76 ± 1.25	35.31 ± 1.38	35.32 ± 1.89	34.60 ± 1.55
equatorial diameter (mm)	41.71 ± 2.38	46.19 ± 11.23	46.49 ± 1.77	46.16 ± 2.22
skin thickness (mm)	1.68 ± 0.30	1.89 ± 0.51	1.61 ± 0.26	1.53 ± 0.24
seed number	8−12	5−7	8−12	9−12
carpel number	7−9	8−10	8−9	8−9
titratable acidity (%)	2.93 ± 0.62	1.25 ± 0.62	1.59 ± 0.11	1.28 ± 0.17
total soluble content (°Brix)	10.78 ± 0.28	7.98 ± 1.14	8.88 ± 0.69	8.66 ± 0.63

[a]Each morphological trait value is expressed as the mean ± standard deviation (n = 10). [b]Each chemical trait value (titratable acidity and total soluble content) is expressed as the mean ± standard deviation (n = 5).

and tyrosinase activity in cells.[16] On the other hand, PMFs of citrus peels are known to have a wide range of nutraceutical functions, including antiproliferative and pro-apoptotic effects in cancer cells.[17,18] Moreover, two PMF compounds in particular, nobiletin and tangeretin, from Shiikuwasha peels have been reported to have antiobesity effects,[19] and Shiikuwasha juices containing PMFs were also demonstrated to have a hepatoprotective effect against D-galactosamine-induced liver injury.[20]

To the best of our knowledge, neither the characterization of volatile aroma components nor the compositions of flavonoid compounds in Shiikuwasha peels from different cultivation lines have yet been reported. The aroma compounds were extracted by using a cold-press system for its ability to produce more authentic and unique aroma of citrus essential oils and its simplicity as compared to other extraction methods. On the other hand, flavanones and PMFs fractions were extracted through vortex and sonication. The aim of the present study, therefore, was to distinguish the four different Shiikuwasha cultivation lines from Okinawa, Japan, based on the composition and content of their volatile aroma components, as well as their flavanones and PMFs, extracted from the flavedo. The study results provide information regarding the distinctive flavors and the natural sources of bioactive substances, particularly flavonoids, for Shiikuwasha fruits.

MATERIALS AND METHODS

Plant Materials. Shiikuwasha (*C. depressa* Hayata) 'Kaachi', 'Izumi kugani', 'Katsuyama kugani', and 'Ogimi kugani' fruits were obtained from several farms located in the northern part of Okinawa Island, Japan. The Shiikuwasha trees were grown under the same climate, weather, culture, and farming conditions. The fruits that come to commercial maturity were collected during the Shiikuwasha harvesting season in December 2009. The fruit types were characterized as to average weight, size, skin thickness, titratable acidity, and total soluble content (Table 1). The total soluble content of the fruit juices was measured using a hand-held refractometer (model N-1α, Atago Co. Ltd., Tokyo, Japan) and expressed as °Brix. The outermost green surface of the peel, which is defined as flavedo, was carefully separated from the soft inner layers, or albedo, with a sharp knife.

Chemicals. Authentic chemical compounds used as standards for the analysis of volatile aroma components were purchased from Sigma-Aldrich (St. Louis, MO) and Tokyo Chemical Industry (Tokyo, Japan). Authentic compounds of *n*-hexanol and methyl myristate were purchased from Tokyo Chemical Industry. Naringenin, hesperidin, and nobiletin as standards were obtained from Wako Pure Chemical Industries (Osaka, Japan), and narirutin, neohesperidin, sinensetin, and tangeretin standards were from Funakoshi Co. Ltd. (Tokyo, Japan). A natsudaidain standard was obtained from the National Institute of Fruit Tree Science (Shizuoka, Japan). All other reagents were supplied by Wako Pure Chemical Industries and were of analytical grade unless otherwise specified.

Cold-Press Extraction of Volatile Aroma Compounds. Volatile aroma compounds of Shiikuwasha peels were extracted from the flavedo (500 g) by hand-pressing and collected in a 10 mL of saturated sodium chloride solution on ice. The collected oils were centrifuged at 2900*g* for 15 min at 4 °C. The resulting supernatant layers were dehydrated overnight with anhydrous sodium sulfate at 5 °C and were then filtered.[21] All extractions were performed in triplicate, and the yields were expressed as grams of extract per 100 g of fresh flavedo peel (%, w/w) or per kilogram of fruit. The collected extracts were stored in sealed vials at −30 °C until analysis.

Extraction of Flavanones and PMFs. Flavedo peel samples were freeze-dried and then crushed and ground using a dry blender. Briefly, 50 mg of freeze-dried Shiikuwasha flavedo powder was extracted using vortex mixing and sonication for 10 min with 600 μL of a mixture of dimethylsulfoxide and methanol (1:1, v/v) at room temperature. The mixture was then centrifuged at 15000*g* for 30 min at 4 °C, and the resulting supernatant layers were carefully collected.[22] The flavanone and PMF extracts were filtered using a 0.45 μm nitrocellulose membrane (Millipore, Bedford, MA) prior to use. All extractions were performed in triplicate.

GC-FID and GC-MS Analysis of Volatile Aroma Components. The volatile aroma components of Shiikuwasha peels were determined using an Agilent 6890N GC equipped with a bonded-phase fused silica capillary column (DB-Wax, 60 m × 0.25 mm i.d., film thickness 0.25 μm, Agilent J&W, Santa Clara, CA) and a flame ionization detector (FID), according to a previous method.[23] The GC injector and FID temperatures were both set at 250 °C. Helium was used as the carrier gas, and the linear velocity of the flow rate was 32 cm/s. Samples (1 μL) were injected using a split ratio of 1:50. The oven temperature was initially set at 40 °C for 2 min, then increased to 200 °C at a rate of 2 °C/min, and maintained at 200 °C for 38 min.

Quantitative analysis was performed using authentic aroma compounds of *n*-hexanol and methyl myristate as internal standards. The internal standards were added to the collected extract before injection into the GC. A *n*-hexanol standard was used for the peaks eluting before linalool, and a methyl myristate standard was used for those emerging after linalool in the eluted order.[21] The ratio of the neat extract and the two internal standards was 150:1:1 (w/w/w), respectively. The weight percentage of the peak was calibrated by FID responses of the internal standards, and the volatile aroma compositions were expressed as the relative concentration (%) or milligrams of volatile compound per 100 g of flavedo on a fresh-weight basis.

The mass spectra of volatile aroma compounds were analyzed using an Agilent 7890A GC coupled with an Agilent 5975C mass spectrometer. The column and oven programs for GC-MS analysis were as described above. The MS conditions were as follows: electron impact ion source and interface temperatures of 230 °C, ionization energy of 70 eV, mass acquisition range of (m/z) 29−450 amu, and scan rate of 1.77 scans/s.

Volatile aroma components were identified by comparing their linear retention indices (RIs) and mass spectra fragmentation patterns with the MS data of corresponding authentic compounds from the National Institute of Standards and Technology (NIST) MS Library, Version 2008. Some of the identifications were confirmed by peak

enrichment on coinjection with authentic aroma standards. Linear RIs of the compounds were also calculated using a homologous series of *n*-alkanes (C7−C30). All analyses were carried out in triplicate.

HPLC Analysis of Flavanones and PMFs. The composition of flavanones and PMFs was examined using a HPLC system (SCL-10AVP, Shimadzu Corp., Kyoto, Japan). The method of HPLC analysis was adapted from Kawaii et al.[24] with slight modification. A Shim-pack VP-ODS column (150 mm × 4.6 mm i.d., 5-μm particle size, Shimadzu Corp.) was used, and the oven (Shimadzu CTO-10ASVP) was set at 40 °C. The Shimadzu LC-10A-VP pump was operated in isocratic mode at a flow rate of 1 mL/min with a mobile phase containing a mixture of methanol, acetonitrile, water, and acetic acid (15:2:2:1, v/v/v/v) for flavanone analysis and a mobile phase of 75% methanol containing 10 mM phosphoric acid for PMF analysis. The injection volume of samples and standards was 5 μL. The compounds were monitored at 340 nm using a Shimadzu UV−vis detector (model SPD-10VP). The function of concentration against peak area was calibrated by injecting each authentic standard at a range of different concentrations that covered the concentration levels of the extract samples. The concentrations of flavanones and PMFs were expressed as milligrams of flavanone or PMF compound per 100 g of flavedo on a fresh-weight basis. All analyses were carried out in triplicate.

Statistical Analysis. The compositional analyses of volatile aroma compounds, flavanones, and PMFs were statistically evaluated using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA). The mean values of the results of the analyses were subjected to analysis of variance with Fisher's least significant difference (LSD) posthoc test to evaluate the significant differences ($p < 0.05$) between Shiikuwasha cultivation lines for each individual compound. To establish the differentiation of volatile aroma compounds in the cultivation lines, principal component analysis (PCA) was also implemented.

RESULTS AND DISCUSSION

Cold-Press Extraction of Shiikuwasha Peels. In the present study, volatile aroma components of Shiikuwasha peels from different cultivation lines were produced using cold-press extraction. This extraction method is commonly used for the production of essential oils containing volatile aroma compounds from citrus peels. In the cold-press system, authentic aroma components, as well as nonvolatile compounds, pigments, and other bioactive substances, might remain in the final extracts. Moreover, Shiikuwasha cold-pressed volatile aroma extract was observed to retain more phenolic content and exhibit superior antioxidant activities as compared to a steam-distillation extract.[23]

The yield of volatile aroma components from Shiikuwasha peels of four different cultivation lines is shown in Table 2. The yield of the extracts was found to vary depending on the cultivation line. The extraction yields of both 'Izumi kugani' and 'Katsuyama kugani' (0.11%) were higher than those of 'Kaachi' and 'Ogimi kugani' (0.05%). The percentage yield of each Shiikuwasha cultivation line was measured as the extract per fresh flavedo peel. The highest extract content was found in 'Katsuyama kugani' fruit, which contained 0.24 g extract per kg fruit, while that from the other cultivation lines varied from 0.12 to 0.19 g. These data, therefore, provide useful information for the citrus flavor and essential oil industries as a basis for estimating yields from Shiikuwasha peel extracts of different cultivation lines during oil extraction.

Table 2. Yield[a] of Cold-Press Extraction from Shiikuwasha Peels

extract properties	'Kaachi'	'Izumi kugani'	'Katsuyama kugani'	'Ogimi kugani'
yield[a] (%, w/w)[b]	0.05 ± 0.02	0.11 ± 0.04	0.11 ± 0.00	0.05 ± 0.00
(g extract/kg fruit)	0.12 ± 0.04	0.19 ± 0.04	0.24 ± 0.00	0.14 ± 0.01

[a]Each value is expressed as the mean ± standard deviation ($n = 3$). [b]The ratio of extract weight to fresh flavedo peel weight.

Volatile Aroma Components of Shiikuwasha Peels. The identified volatile aroma components of Shiikuwasha peels are listed according their order of elution on a polar DB-Wax column, including their RIs, compositions, and contents (Table 3). As a whole, 39 compounds, representing approximately 99% of the total volatile aroma components in the extracts were identified by their linear RIs and mass spectra fragmentation patterns. In addition, 25 of these compounds were also specifically identified by comparing their mass spectra profiles against authentic standards. The extracts were comprised of a complex mixture of different component groups, with monoterpene hydrocarbons as the predominant component group (93.40−97.25%, 13 compounds), followed by alcohols (0.35−1.65%, nine compounds) and sesquiterpene hydrocarbons (0.30−1.50%, eight compounds). The composition of volatile aroma compounds in Shiikuwasha peels was observed to vary depending on the cultivation line. Significant differences ($p < 0.05$) in the relative concentration of almost all of the identified compounds were found in the four Shiikuwasha cultivation lines.

Regarding chemical composition, the ratio of monoterpene to sesquiterpene hydrocarbons was determined to describe the distinctive aroma combination in each Shiikuwasha peel extract. The ratios for 'Izumi kugani', 'Kaachi', 'Ogimi kugani', and 'Katsuyama kugani' peel extracts were 307.84, 228.17, 69.84, and 63.78, respectively (Table 3). In reference to the literature, there appears to be great variability in the ratio of monoterpene to sesquiterpene hydrocarbons among the Shiikuwasha cultivation lines, wherein the ratios of other cold-pressed citrus peel extracts were 292.62 in Mandarin orange (*C. reticulate*), 125.01 in sweet orange (*C. sinensis*), 15.11 in lemon (*C. lemon*), and 12.3−19.4 in Japanese yuzu (*C. junos*).[1,25] These chemical compositions, as well as the diversity of the terpene compounds, might differ depending on the regulation of the monoterpene and sesquiterpene hydrocarbon biosynthesis pathways in citrus plants.[26]

All compounds were quantified using the two internal standards *n*-hexanol and methyl myristate. The highest content of volatile aroma components was found in the peel of 'Izumi kugani' (141.39 mg/100 g of fresh flavedo peel), followed by 'Katsuyama kugani' (127.41 mg/100 g of fresh flavedo peel). The main volatile aroma components identified were the monoterpene hydrocarbons limonene [46.52−68.26% (32.25−87.56 mg/100 g of fresh flavedo peel)], γ-terpinene [21.48−30.52% (15.37−39.20 mg/100 g of fresh flavedo peel)], and *p*-cymene [0.57−8.98% (0.41−7.68 mg/100 g of fresh flavedo peel)]. Interestingly, the relative concentrations of limonene differed significantly among cultivation lines and follow the rank order: 'Kaachi' > 'Izumi kugani' > 'Katsuyama kugani' > 'Ogimi kugani', while the reverse sequence was observed for γ-terpinene and *p*-cymene. All Shiikuwasha peel extracts were found to contain moderate amounts of myrcene, terpinolene, and α-thujene, as well as two structural isomers of pinene (α-pinene and β-pinene), and were observed in the range of 1.02−2.36%.

Discrimination of different Shiikuwasha cultivation lines by the characteristic composition of volatile aroma components can also been seen with other moderate and low level-oxygenated compounds, such as ethers, alcohols, ketones, oxides, esters,

Table 3. Volatile Aroma Components [Relative Concentration (%) and mg/100 g Fresh Flavedo Weight][a] of Shiikuwasha Peels

			'Kaachi'		'Izumi kugani'		'Katsuyama kugani'		'Ogimi kugani'		
peak no.	compound	RI[b]	%	mg/100 g	%	mg/100 g	%	mg/100 g	%	mg/100 g	identification[c]
1	α-pinene	1021	1.23 ± 0.137 d[e]	0.88 ± 0.027	1.74 ± 0.217 c	2.50 ± 0.289	2.36 ± 0.084 a	3.03 ± 0.274	2.09 ± 0.066 b	1.45 ± 0.128	RI, MS, PC
2	α-thujene	1026	1.07 ± 0.051 c	0.76 ± 0.060	1.38 ± 0.094 b	1.99 ± 0.159	1.90 ± 0.100 a	2.43 ± 0.051	1.81 ± 0.139 a	1.25 ± 0.070	RI, MS
3	camphene	1061	ND	ND	tr. b	tr.	0.01 ± 0.001 a	0.01 ± 0.002	tr. b	tr.	RI, MS, PC
4	β-pinene	1107	1.44 ± 0.057 c	1.03 ± 0.066	1.89 ± 0.118 b	2.72 ± 0.170	2.64 ± 0.010 a	3.39 ± 0.183	2.61 ± 0.060 a	1.81 ± 0.143	RI, MS, PC
5	sabinene	1119	0.21 ± 0.007 c	0.15 ± 0.014	0.94 ± 0.101 a	1.34 ± 0.146	0.35 ± 0.003 b	0.44 ± 0.022	0.34 ± 0.009 b	0.23 ± 0.018	RI, MS
6	myrcene	1163	1.71 ± 0.039 bc	1.22 ± 0.098	1.86 ± 0.035 a	2.66 ± 0.076	1.74 ± 0.005 b	2.23 ± 0.117	1.68 ± 0.013 c	1.17 ± 0.106	RI, MS, PC
7	α-terpinene	1177	0.02 ± 0.016 b	0.02 ± 0.011	0.09 ± 0.038 a	0.14 ± 0.052	0.10 ± 0.022 a	0.13 ± 0.035	0.08 ± 0.005 a	0.06 ± 0.007	RI, MS, PC
8	limonene	1213	68.26 ± 0.254 a	48.82 ± 5.158	60.97 ± 0.403 b	87.56 ± 1.814	48.47 ± 0.074 c	62.25 ± 3.394	46.52 ± 0.180 d	32.25 ± 3.096	RI, MS, PC
9	β-phellandrene	1215	0.15 ± 0.001 b	0.11 ± 0.011	0.32 ± 0.015 a	0.46 ± 0.022	0.12 ± 0.001 c	0.16 ± 0.008	0.12 ± 0.001 c	0.08 ± 0.008	RI, MS
10	1,8-cineol	1216	0.01 ± 0.000 b	0.00 ± 0.000	0.94 ± 0.080 a	1.36 ± 0.124	0.01 ± 0.000 b	0.01 ± 0.001	0.01 ± 0.000 b	0.01 ± 0.001	RI, MS, PC
11	γ-terpinene	1257	21.48 ± 0.175 c	15.37 ± 1.703	21.91 ± 0.261 b	31.47 ± 0.815	30.52 ± 0.033 a	39.20 ± 2.138	30.51 ± 0.304 a	21.16 ± 2.165	RI, MS, PC
12	p-cymene	1272	0.57 ± 0.016 d	0.41 ± 0.052	1.01 ± 0.120 c	1.45 ± 0.174	5.98 ± 0.008 b	7.68 ± 0.421	8.98 ± 0.038 a	6.23 ± 0.599	RI, MS, PC
13	terpinolene	1287	1.02 ± 0.014 d	0.73 ± 0.085	1.14 ± 0.005 c	1.63 ± 0.040	1.47 ± 0.001 b	1.88 ± 0.103	1.51 ± 0.021 a	1.05 ± 0.110	RI, MS, PC
14	nonanal	1391	0.05 ± 0.002 a	0.04 ± 0.004	0.04 ± 0.000 b	0.06 ± 0.002	0.01 ± 0.000 c	0.01 ± 0.001	0.01 ± 0.000 c	0.01 ± 0.001	RI, MS, PC
15	α-cubebene	1456	ND	ND	ND	ND	0.01 ± 0.000 b	0.02 ± 0.001	0.02 ± 0.000 a	0.01 ± 0.001	RI, MS
16	4-carene	1468	0.08 ± 0.001 d	0.06 ± 0.007	0.14 ± 0.001 a	0.20 ± 0.006	0.10 ± 0.000 b	0.12 ± 0.007	0.09 ± 0.002 c	0.06 ± 0.007	RI, MS
17	sabinene hydrate	1476	0.12 ± 0.006 b	0.08 ± 0.011	0.38 ± 0.013 a	0.54 ± 0.024	0.02 ± 0.000 c	0.03 ± 0.002	0.03 ± 0.001 c	0.02 ± 0.002	RI, MS, PC
18	α-copaene	1489	0.07 ± 0.002 c	0.05 ± 0.007	0.05 ± 0.002 d	0.07 ± 0.004	0.09 ± 0.000 b	0.11 ± 0.007	0.11 ± 0.003 a	0.07 ± 0.009	RI, MS
19	decanal	1497	0.22 ± 0.007 a	0.16 ± 0.019	0.20 ± 0.002 b	0.28 ± 0.009	0.05 ± 0.001 c	0.06 ± 0.002	0.04 ± 0.001 d	0.02 ± 0.003	RI, MS, PC
20	germacrene d	1536	0.03 ± 0.001 d	0.02 ± 0.003	0.04 ± 0.002 c	0.06 ± 0.004	0.08 ± 0.000 b	0.10 ± 0.005	0.09 ± 0.003 a	0.07 ± 0.008	RI, MS
21	linalool	1554	0.62 ± 0.124 b	0.44 ± 0.071	1.15 ± 0.027 a	1.65 ± 0.062	0.33 ± 0.000 c	0.43 ± 0.024	0.20 ± 0.004 d	0.14 ± 0.016	RI, MS, PC
22	bornyl acetate	1579	0.01 ± 0.000 b	0.00 ± 0.000	0.02 ± 0.003 a	0.03 ± 0.004	0.01 ± 0.001 b	0.01 ± 0.001	0.01 ± 0.001 b	0.01 ± 0.000	RI, MS, PC
23	β-caryophyllene	1594	0.09 ± 0.007 b	0.06 ± 0.006	ND	ND	0.42 ± 0.019 a	0.54 ± 0.049	0.38 ± 0.051 a	0.26 ± 0.019	RI, MS, PC
24	methyl thymol	1594	ND	ND	1.15 ± 0.125 a	1.65 ± 0.191	ND	ND	ND	ND	RI, MS
25	terpinen-4-ol	1604	0.06 ± 0.006 a	0.05 ± 0.005	0.04 ± 0.008 b	0.06 ± 0.012	0.06 ± 0.003 a	0.07 ± 0.007	0.04 ± 0.005 b	0.03 ± 0.002	RI, MS, PC
26	(e)-2-decenal	1640	0.03 ± 0.002 a	0.02 ± 0.002	0.03 ± 0.004 a	0.04 ± 0.005	0.02 ± 0.001 b	0.03 ± 0.003	0.01 ± 0.002 c	0.01 ± 0.001	RI, MS, PC
27	α-caryophyllene	1664	0.05 ± 0.005 b	0.04 ± 0.004	0.02 ± 0.004 c	0.03 ± 0.006	0.10 ± 0.004 a	0.12 ± 0.011	0.09 ± 0.012 a	0.06 ± 0.004	RI, MS, PC
28	terpinyl acetate	1693	0.05 ± 0.004 b	0.03 ± 0.004	0.55 ± 0.062 a	0.80 ± 0.094	0.06 ± 0.003 b	0.08 ± 0.007	0.05 ± 0.006 b	0.03 ± 0.002	RI, MS, PC
29	α-terpineol	1699	0.10 ± 0.008 a	0.07 ± 0.005	0.03 ± 0.004 c	0.05 ± 0.006	0.08 ± 0.004 b	0.10 ± 0.009	0.03 ± 0.004 c	0.02 ± 0.001	RI, MS, PC
30	bicyclosesquiphellandrene	1704	0.07 ± 0.006 c	0.05 ± 0.005	0.13 ± 0.026 c	0.18 ± 0.038	0.61 ± 0.027 a	0.79 ± 0.066	0.53 ± 0.071 b	0.36 ± 0.026	RI, MS
31	α-muurolene	1722	0.01 ± 0.001 c	0.01 ± 0.001	0.01 ± 0.001 c	0.01 ± 0.002	0.04 ± 0.002 a	0.05 ± 0.004	0.03 ± 0.004 b	0.02 ± 0.001	RI, MS
32	l-carvone	1731	0.32 ± 0.025 c	0.23 ± 0.019	0.16 ± 0.036 d	0.23 ± 0.052	1.22 ± 0.054 a	1.57 ± 0.140	1.03 ± 0.138 b	0.71 ± 0.052	RI, MS
33	δ-cadinene	1754	0.10 ± 0.009 c	0.07 ± 0.008	0.06 ± 0.010 d	0.09 ± 0.015	0.16 ± 0.007 a	0.20 ± 0.018	0.13 ± 0.018 b	0.09 ± 0.007	RI, MS
34	perilla aldehyde	1777	ND	ND	ND	ND	0.01 ± 0.000 a	0.01 ± 0.001	0.01 ± 0.001 a	0.00 ± 0.000	RI, MS, PC
35	nerol	1806	0.02 ± 0.002 a	0.01 ± 0.001	0.01 ± 0.001 b	0.01 ± 0.002	0.01 ± 0.001 b	0.01 ± 0.001	ND	ND	RI, MS, PC
36	elemol	2080	0.03 ± 0.003 b	0.02 ± 0.003	0.01 ± 0.002 c	0.01 ± 0.002	0.04 ± 0.002 a	0.05 ± 0.005	0.03 ± 0.004 b	0.02 ± 0.001	RI, MS
37	γ-eudesmol	2170	ND	ND	ND	ND	0.01 ± 0.000 a	0.01 ± 0.001	0.01 ± 0.001 a	0.00 ± 0.000	RI, MS
38	thymol	2186	0.01 ± 0.001 b	0.01 ± 0.001	0.03 ± 0.001 a	0.04 ± 0.003	0.01 ± 0.001 b	0.02 ± 0.002	0.01 ± 0.002 b	0.01 ± 0.001	RI, MS, PC
39	isothymol	2214	ND	ND	ND	ND	0.01 ± 0.000 a	0.01 ± 0.001	0.01 ± 0.001 a	0.01 ± 0.001	RI, MS, PC

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Table 3. continued

peak no.	compound	RI[b]	'Kaachi' %	'Kaachi' mg/100 g	'Izumi kugani' %	'Izumi kugani' mg/100 g	'Katsuyama kugani' %	'Katsuyama kugani' mg/100 g	'Ogimi kugani' %	'Ogimi kugani' mg/100 g	identification[c]
						component groups					
	monoterpene hydrocarbons		97.25	69.55	93.40	134.13	95.75	122.97	96.35	66.80	
	sesquiterpene hydrocarbons		0.43	0.30	0.30	0.44	1.50	1.93	1.38	0.95	
	(Mon/Ses)[d]		228.17		307.84		63.78		69.84		
	alcohols		0.97	0.69	1.65	2.37	0.57	0.73	0.35	0.24	
	aldehydes		0.30	0.21	0.27	0.38	0.09	0.11	0.06	0.04	
	esters and ketones		0.37	0.27	0.74	1.06	1.29	1.66	1.09	0.75	
	ethers		ND	ND	1.15	1.65	ND	ND	ND	ND	
	oxides		0.01	0.00	0.94	1.36	0.01	0.01	0.01	0.01	
	total identified		99.32	71.02	98.45	141.39	99.21	127.41	99.25	68.79	

[a]Each value is expressed as the mean ± standard deviation ($n = 3$); ND, not detected; tr, trace amount (<0.01%). [b]RIs relative to *n*-alkanes on a polar DB-Wax column. [c]RI, identification based on RI; MS, identification based on the NIST MS library; and PC, identification based on authentic standards analyzed by mass spectrometry. [d]Ratio of monoterpene hydrocarbons to sesquiterpene hydrocarbons. [e]Means in the same row followed by the same letter are not significantly different ($p < 0.05$).

and aldehydes. For instance, methyl thymol was solely observed in 'Izumi kugani' peel [1.15% (1.65 mg/100 g of fresh flavedo peel)]. This ether compound is somewhat rare in citrus peels but is one of the main volatile aroma components in the essential oils seafennel (*Crithmum maritimum* L.) and juniper berry (*Juniperus drupacea* L.), comprising 25 and 22%, respectively.[27,28] Moreover, a significantly higher relative level of linalool was also found in 'Izumi kugani' peel [1.15% (1.65 mg/100 g of fresh flavedo peel)] as compared to the other peel extracts, while 'Katsuyama kugani' peel showed a significantly higher level of a ketone compound, *l*-carvone [1.22% (1.57 mg/100 g of fresh flavedo peel)]. Linalool is a key compound in citrus peels, contributing citrusy, floral, fresh, and sweet aromas, while *l*-carvone produces a minty aroma.[21,29] In addition, 'Izumi kugani' peel contained 1,8-cineol [0.94% (1.36 mg/100 g of fresh flavedo peel)] more than 90 times higher as compared to other Shiikuwasha lines. This oxide compound was recognized as one of the key aroma components in Pontianak orange (*C. nobilis* Lour. var. *microcarpa* Hassk.) peel extracts for providing a minty aroma with a relatively high flavor dilution factor[30,31] and has been known as the main volatile aroma compound in *Eucalyptus*, *Thymus*, and *Chrysanthemum* essential oils.[32−34] On the other hand, esters (bornyl acetate and terpinyl acetate) and aldehydes

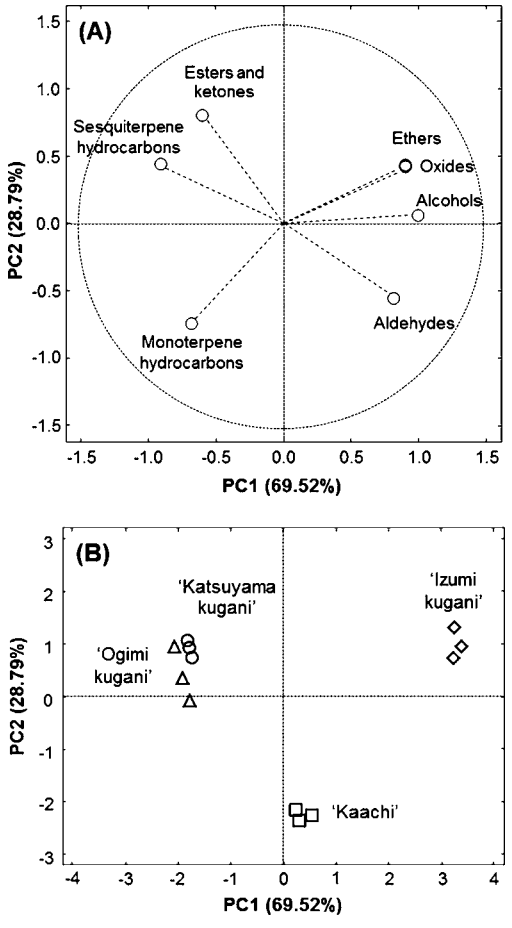

Figure 1. PCA plots of volatile aroma components of Shiikuwasha peels from four different cultivation lines: (A) distribution of seven groups of volatile aroma compounds and (B) discrimination of cultivation lines based on the relative concentrations of seven groups of volatile aroma compounds.

Table 4. Flavanone Content (mg/100 g Fresh Flavedo Weight)[a] of Shiikuwasha Peels

flavanone	'Kaachi'	'Izumi kugani'	'Katsuyama kugani'	'Ogimi kugani'
narirutin	39.18 ± 4.41 b[b]	ND	49.33 ± 6.16 a	ND
naringenin	11.88 ± 2.50 a	3.98 ± 1.15 b	4.58 ± 0.25 b	4.35 ± 0.35 b
hesperidin	467.31 ± 39.74 a	20.79 ± 3.80 c	474.82 ± 50.68 a	335.08 ± 15.69 b
neohesperidin	5.16 ± 1.14 b	698.69 ± 90.70 a	ND	0.18 ± 0.03 b
total	523.53	723.46	528.73	339.62

[a]Each value is expressed as the mean ± standard deviation (n = 3); ND, not detected. [b]Means in the same row followed by the same letter are not significantly different (p < 0.05).

Table 5. PMF Content (mg/100 g Fresh Flavedo Weight)[a] of Shiikuwasha Peels

PMF	'Kaachi'	'Izumi kugani'	'Katsuyama kugani'	'Ogimi kugani'
sinensetin	15.24 ± 0.30 c[b]	26.29 ± 2.94 a	19.17 ± 1.41 b	19.29 ± 0.98 b
nobiletin	128.63 ± 6.09 b	168.37 ± 4.20 a	169.53 ± 5.89 a	163.65 ± 9.20 a
natsudaidain	5.30 ± 0.26 a	5.07 ± 0.13 a	3.67 ± 0.08 b	3.24 ± 0.20 c
tangeretin	77.52 ± 7.63 b	60.22 ± 8.87 c	102.22 ± 5.89 a	95.33 ± 4.27 a
total	226.70	259.95	294.59	281.50

[a]Each value is expressed as the mean ± standard deviation (n = 3). [b]Means in the same row followed by the same letter are not significantly different (p < 0.05).

(nonanal, decanal, 2-decenal, and perilla aldehyde) were found in relatively small amounts in all Shiikuwasha peel extracts.

By application of PCA to the relative concentrations of seven volatile aroma compound groups in Shiikuwasha peels from four different cultivation lines (Table 3), the first two principle components (PCs) were identified, which accounted for 98.31% of the total variance (Figure 1). The result shows that volatile aroma components of Shiikuwasha peels from different cultivation lines can be distinguished in a valid manner. The relations among groups of volatile aroma compounds, as well as relations between the group compositions and the cultivation lines, can be examined from the corresponding loading plots of PC1 and PC2. Of the volatile aroma compounds, the monoterpene hydrocarbons group was found to be negatively correlated to the PCs, while the alcohol, ether, and oxide groups were closely related to each other and were more positively associated to the PCs (Figure 1A). Separation of 'Izumi kugani' and 'Kaachi' was clearly observed; however, the compositions of volatile aroma compounds in 'Katsuyama kugani' and 'Ogimi kugani' peels were closely associated (Figure 1B). This low differentiation ability of PCs is in agreement with the compositions of volatile aroma compounds in 'Katsuyama kugani' and 'Ogimi kugani' peel extracts presented in Table 3. These differences might impact the entire aroma profile of peel extracts and, consequently, the aroma characteristics of Shiikuwasha fruits from different cultivation lines.

Flavanone and PMF Compositions of Shiikuwasha Peels. The flavanone and PMF contents of four Shiikuwasha cultivation lines were examined, with the aim of using the composition and content of these components as an approach for distinguishing different cultivation lines. Flavanones are known as the predominant flavonoid compounds in various citrus peels.[11,35] As shown in Table 4, the flavanones of Shiikuwasha peels were comprised of narirutin, naringenin, hesperidin, and neohespiridin. These compounds are the most common flavanones in citrus peels. The highest flavanone content was found in 'Izumi kugani' (723.46 mg/100 g of fresh flavedo peel), followed by 'Katsuyama kugani' and 'Kaachi' (528.73 and 523.53 mg/100 g of fresh flavedo peel, respectively). The composition and content of flavanones differed between the four Shiikuwasha cultivation lines. Interestingly, except for 'Izumi kugani' peel, which contained a significant amount of neohesperidin [96.58% (698.69 mg/100 g of fresh flavedo peel)], the other Shiikuwasha peels were observed to have high hesperidin contents [89.26−98.66% (335.08−474.82 mg/100 g of fresh flavedo peel)]. In reference to the reported literature,[36] the amount of neohesperidin in 'Izumi kugani' peel was as high as in sour orange (*C. aurantium*; 569 mg per 100 g of fresh flavedo peel), while the amount of hesperidin in the other Shiikuwasha peels was at the same level as other citrus varieties (in 100 mg of fresh flavedo peel), such as Japanese Yatsushiro (*C. yatsushiro*, 355 mg), Japanese Iyo (*C. iyo*, 363 mg), and Tahitian lime (*C. latifolia*, 462 mg). Moreover, narirutin was not detected in either 'Izumi kugani' or 'Ogimi kugani' peel, while neohesperidin was completely absent from the 'Katsuyama kugani' peel. The distribution of these flavonoid compounds in citrus fruits is affected by genetic variation and differential expression of genes in flavonoid biosynthesis.[37]

In the analysis of PMFs, four compounds (sinensetin, nobiletin, natsudaidain, and tangeretin) were detected in the Shiikuwasha peels (Table 5). Significant differences in the level of each PMF component were also observed in the different Shiikuwasha cultivation lines. The total PMF contents of Shiikuwasha peels examined in this study ranged from 226.70 to 294.59 mg per 100 g of fresh flavedo peel. The PMFs of Shiikuwasha peels were mainly composed of nobiletin [56.74−64.77% (128.63−169.53 mg/100 g of fresh flavedo peel)] and tangeretin [23.17−34.70% (60.22−102.22 mg/100 g of fresh flavedo peel)]. This result is in agreement with Nogata et al.,[36] who reported that the amounts of nobiletin and tangeretin in an undefined cultivation line of Shiikuwasha were approximately 122 and 71 mg per 100 g of fresh flavedo peel, respectively. The observed amounts were at the same level as other Japanese citrus fruits (in 100 mg of fresh flavedo peel): 139 mg of nobiletin and 65 mg of tangeretin in Shunkokan (*C. shunkokan*) and 118 mg of nobiletin and 108 mg of tangeretin in Kishu (*C. kinokuni*).[36] Furthermore, application of advanced extraction systems, for instance, supercritical fluid extraction, has been developed and is reported to allow optimum yield of these valuable PMFs from Shiikuwasha

peel.[38] Therefore, the results of the present study indicate that Shiikuwasha peels of different cultivation lines may be potential valuable sources of flavonoids.

To our knowledge, this is the first report on the composition and content of volatile aroma components, flavanones, and PMFs in different Shiikuwasha cultivation lines. Each Shiikuwasha peel extract had a distinctive composition of aroma compounds, resulting in different aroma profiles. Particularly, 'Katsuyama kugani' and 'Ogimi kugani' were observed to have a similar aroma compound grouping, while the aroma constituents of 'Kaachi' and 'Izumi kugani' peels differed from those of the former cultivation lines. In addition, the Shiikuwasha peels contained numerous flavonoid compounds with possible beneficial impacts on human health. Remarkably, unlike the other Shiikuwasha peels with high flavanone hesperidin contents, 'Izumi kugani' peel contained mainly neohesperidin. Moreover, the Shiikuwasha peels contained the PMFs nobiletin and tangeretin. Thus, this information provides a basis for the utilization of Shiikuwasha peels from different cultivation lines in foods, nutraceuticals, and other related areas.

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Notes

The authors declare no competing financial interest.

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