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Chemical Markers of Shiikuwasha Juice Adulterated with Calamondin Juice

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ABSTRACT: Detection of shiikuwasha (*Citrus depressa* Hayata) juice adulterated with calamondin (*Citrus madurensis* Lour.) juice was investigated by the analyses of (1) phloretin dihydrochalcone glucoside, 3′,5′-di-*C*-*β*-glucopyranosylphloretin (PD) detected by thin-layer chromatography and high-performance liquid chromatography (HPLC), (2) polymethoxylated flavones (PMFs), included nobiletin, tangeretin, and sinensetin, detected by HPLC, and (3) *γ*-terpinene peak percentage obtained by headspace solid-phase microextraction gas chromatography with cryofocusing. PD was detected in calamondin juice (25.5 mg/100 mL) but not in shiikuwasha juice. Shiikuwasha juice contained higher levels of nobiletin (48.8 mg/100 mL) than calamondin juice (2.4 mg/100 mL). Shiikuwasha juice was characterized by containing a higher percentage of *γ*-terpinene (12.3%) than calamondin juice (0.7%). A discrimination function obtained by a linear discriminant analysis with PMFs and a peak ratio of [nobiletin/tangeretin] and *γ*-terpinene detected the adulteration with accuracies of 91.7%. These three chemical markers were useful to detect shiikuwasha juice that is suspected of being adulterated with calamondin juice.

KEYWORDS: *Citrus depressa, Citrus madurensis, adulteration, polymethoxylated flavone, γ-terpinene, 3′,5′-di-C-β-glucopyranosylphloretin*

INTRODUCTION

Shiikuwasha (*Citrus depressa* Hayata), which belongs to *Citrus Metacitrus Acrumen*, has been a very popular fruitlet as a flavor enhancer in the Okinawa district, a southern region of Japan, and contains polymethoxylated flavones (PMFs), including nobiletin (NOB), tangeretin (TNG), and sinensetin (SIN), in large quantities in the peel part of this citrus.[1,2] Recently, nobiletin is reported to show antitumor activities[3,4] and antimetastatic activities,[5] inhibit fulminant hepatitis,[6] and improve memory impairment.[7] The market of shiikuwasha products has grown rapidly by recent health-conscious consumers, and its unit price per kilogram has been gone up by 6.5 times from 2000 to 2005. Because shiikuwasha farmers could not keep up with the supply of this fruit, shiikuwasha juice adulterated with calamondin (*Citrus madurensis* Lour.) juice produced in Taiwan and Philippines is widely commercialized. Calamondin, which belongs to *Citrus Metacitrus Pseudofortunella*, ranges from tropical to subtropical areas, including China, Philippines, Central America, Japan, Hawaii, and Florida. In addition, juice extracted from calamondin resembles juice from shiikuwasha in color and flavor. Therefore, shiikuwasha juice adulterated with calamondin is difficult to detect.

The adulteration of fruit juices has been a serious economic problem. This problem has been detrimental to consumers and the food industry for many years. Juice adulteration has changed from simple additions of water and the substitution of cheap ingredients to more sophisticated methods, including the addition of minor compounds. Most studies of citrus juice adulteration have focused on that of orange juice. To detect the adulteration of orange juice, the following methods are used: flavanone glycosides and PMFs[8] and PMFs and carotenoids[9] as chemical markers by high-performance liquid chromatography (HPLC), near-infrared (NIR) spectroscopy,[10] ^{1}H nuclear magnetic resonance (NMR) spectroscopy,[11] and polymerase chain reaction (PCR).[12] However, no information was reported to detect shiikuwasha juice adulterated with calamondin juice.

Differences between shiikuwasha and calamondin were described by the presence of 3′,5′-di-*C*-*β*- glucopyranosylphloretin (PD), a phloretin dihydrochalcone glucoside (Figure 1), and contents of PMFs. PD is present in the genus *Fortunella*, *C. madurensis*, and *Citrus halimii* but not in *C. depressa*.[13] Shiikuwasha contains larger amounts of PMFs than calamondin.[2,14] These two compounds are supposed to be useful chemical markers to detect shiikuwasha adulterated with

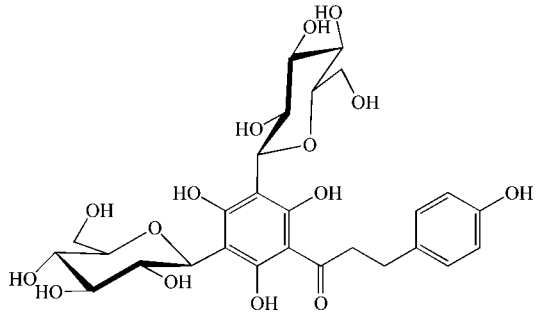

Figure 1. Structure of PD.

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calamondin. In addition, a comparison of the characteristic flavor of each citrus is supposed to be an available method to detect the adulteration. The objective of this work is to develop a method to detect the shiikuwasha juice adulterated with calamondin juice using chemical marker compounds.

MATERIALS AND METHODS

Materials. Shiikuwasha and calamondin fruits were obtained from the Agricultural Experiment Station of Okinawa Prefecture (Nago, Japan), during the season of 2009. Hand-pressed shiikuwasha juice (sample 1) and hand-pressed calamondin juice (sample 2) were obtained by squeezing each of the whole fruits by a 260 mm long hand-press juicer with a 84 mm diameter and 32 mm depth. Shiikuwasha juice (sample 3) and concentrated calamondin juice, prepared industrially, were obtained from a company in Japan and a company in Taiwan, respectively. The concentrated calamondin juice was diluted to 9.0 ± 0.2 °Brix to adjust the concentration of industrial Shiikuwasha juice, and this juice used as sample 4. A total of 10 kinds of commercial 100% juices (samples A–J) labeled as shiikuwasha juice or shiikuwasha and calamondin juice were purchased at local markets in Okinawa, Japan.

Reagents and Standard Solution. HPLC-grade methanol was supplied by Wako Pure Chemical Industries (Osaka, Japan), and NOB, TNG, and SIN standards were provided from Extrasynthese (Lyon, France). The standards were diluted in methanol (MeOH)/dimethylsulfoxide (1:1, v/v).

PD was isolated from fresh fruits of *Fortunella margarita*.[13] NMR spectra were recorded on a DPX400 spectromater (Bruker, Japan), and mass spectra were measured using liquid chromatography–mass spectrometry (LC–MS; JMS 700, Jeol, Japan) equipped with fast atom bombardment (FAB) or atmospheric pressure chemical ionization (APCI) interfaces. Fresh fruits of *F. margarita* (225 g) had been extracted using ethanol at room temperature for 1 month. Concentrated ethanolic extract was partitioned between butanol and water. The butanol phase was concentrated, suspended in water, and subjected to HP-20 resin (Mitsubishi Kasei, Japan). The ethanol eluate (600 mg in dry weight) was applied to an ODS column (Tosoh ODS-Prep, 250 × 22 mm inner diameter, 5 μm, Tosoh, Japan) using a MeOH–0.2% acetic acid eluent (flow rate, 5.0 mL/min; gradient, 30–60% methanol in 90 min) to afford PD (220 mg).

PD: Yellow powder. $[\alpha]_D^{20}$ +83.6° (MeOH; *c* 1.0). UV λ_{max}^{MeOH} (nm) (log ε): 233 (4.3), 286 (4.1), 335 *sh*. Positive FABMS *m/z*: 599 $[M + H]^+$, 277, 249, 185. Positive APCIMS (needle, 4.0 kV; orifice, 0 V; ring lens, 20 V) *m/z*: 599, 581, 569, 479, 461, 389, 359. IR ν_{max}^{KBr} (cm^{-1}): 3550 (OH), 1620 (C=O). ^{13}C NMR (CD_3OD): phloretin moiety [δ 207.2 (C=O), 163.1 (C-4′), 162.2 (C-2′ and C-6′), 156.4 (C-4), 133.9 (C-1), 130.4 (C-2 and C-6), 116.1 (C-3 and C-5), 106.2 (C-1′), 104.4 (C-3′ and C-5′), 47.8 (C-α), 31.0 (C-β)]; glucose moiety [δ 82.7 (C-5), 79.1 (C-3), 76.7 (C-1), 74.2 (C-2), 71.1 (C-4), 61.9 (C-6)] × 2. ^{13}C NMR (DMSO-d_6): phloretin moiety [δ 205.2, 161.2, 161.1, 155.4, 131.7, 129.3, 115.2 × 2, 104.7 × 2, 104.0, 46.2, 29.3]; glucose moiety [δ 81.1, 77.8, 74.7, 72.1, 69.2, 60.0] × 2. ^1H NMR (CD_3OD): phloretin moiety [δ 7.03 (2H, *d*, *J* = 8.4 Hz, H-2 and H-6), 6.75 (2H, *d*, *J* = 8.4 Hz, H-3 and H-5), 3.34 (2H, *m*, H_2-α), 2.85 (2H, *m*, H_2-β)], glucose moiety [δ 4.94 (1H, *d*, *J* = 9.8 Hz, H-1), 3.82 (2H, *ABX*, *J* = 13, 4, and 2 Hz, H_2-6), 3.61 (1H, *dd*, *J* = 8 and 8 Hz, H-2), 3.54 (1H, *dd*, *J* = 8 and 8 Hz, H-4), 3.50 (1H, *dd*, *J* = 8 and 8 Hz, H-3), 3.42 (1H, collapsed, H-5)] × 2.

The PD standard was diluted in methanol, and all of the other solvents and reagents were of analytical grade.

Methods. *Thin-Layer Chromatography (TLC) for PD.* A total of 2 mL of juice sample was loaded onto a Waters Sep-Pak Plus C18 cartridge (360 μg), which had been preconditioned with 2 mL of methanol and then 3 mL of water. The cartridge was washed with 2 mL of water, and the PD was eluted with 1 mL of methanol.

TLC was performed on a Merck TLC plate (silica gel 60 F_{254}, 20 × 20 cm). The plates were heated for 1 h at 100 °C and placed in a desiccator before use. Each elution was applied to the plates 25 times by a capillary tube. The solvent system chloroform/methanol/1% (v/v) phosphoric acid (65:35:5, v/v/v) was used. The developed plate was dried and visualized by spraying 10% sulfuric acid (v/v) and heating the TLC plate by a gas burner.

HPLC for PD and PMFs. A total of 3 mL of juice sample was diluted to 7 mL of absolute ethanol, mixed thoroughly, and treated supersonically for 30 min. All samples were filtered through a 0.45 μm pore size syringe-driven filter (cellulose acetate) prior to an injection to HPLC.

All measurements were obtained using a Shimadzu LC-10 AD vp series. PD was analyzed under the following conditions: A C_{18} reversed-phase HPLC column (LiChrospher 100: RP-18, 250 × 4.0 mm inner diameter, 5 μm; Kanto Chemical, Tokyo, Japan) was used. The detection was achieved by monitoring at 286 nm. The flow rate was 0.9 mL/min. The column temperature was maintained at 40 °C. The sample injection volume was 5 μL. A two-solvent gradient system was used. The gradient program consisted of two periods: (1) 0–5 min, isocratic, 80% (v/v) A (aqueous 20 mM phosphoric acid) in B (methanol) and (2) 5–45 min, 80–55% A in B. All treatments were performed in triplicate, and the results presented mean values with standard deviations.

PMFs were analyzed under the following conditions: A C_{18} reversed-phase HPLC column (Hypersil ODS, 150 × 4.0 mm inner diameter, 5 μm; Agilent Technologies, Santa Clara, CA) was used. The detection was achieved by monitoring at 340 nm. A mobile phase for isocratic elution was obtained by mixing methanol/10 mM phosphoric acid aqueous solution in the ratio of 60:40 (v/v). The flow rate was 1.0 mL/min. The column temperature was maintained at 40 °C. The sample injection volume was 10 μL.

The component was identified by comparing its retention time to that of authentic compounds. The concentration of each component was calculated from the integrated peak area of the sample. All treatments were performed in triplicate, and the results presented mean values with standard deviations.

Solid-Phase Microextraction–Gas Chromatography (SPME–GC) and Gas Chromatography–Mass Spectrometry (GC–MS) for Volatile Components. A total of 1 mL of a juice sample was placed in a vial (22 × 38 mm) that contained an internal standard solution of 10 μL of 1% cyclohexanol in water. The solution was held at 40 °C for 5 min, and a Sigma-Aldrich Carboxen/Polydimethylsiloxane (PDMS) SPME fiber (fiber length, 10 mm; film thickness, 75 μm) was introduced into the septum-sealed vial and kept in the injection port for 20 min. SPME cryofocusing was carried out as follows: Heating of the GC column oven was stopped, and the column head (10 cm) of GC was dipped into liquid nitrogen to collect the volatiles in splitless mode. Then, the SPME fiber was introduced into the injector and kept there for 7 min.[15]

The volatile components of headspace gas were analyzed with a Shimadzu GC14A gas chromatograph. A 0.25 mm inner diameter × 60 m DB-WAX chemical bond type silica capillary column with 0.25 μm film thickness (Agilent Technologies, Santa Clara, CA) was employed, with the flame ionization detector (FID) at 230 °C and injection port at 260 °C. The temperature was programmed to be at 40 °C initially, increase at 3 °C/min to 230 °C, and held at a final temperature for 10 min. The helium carrier gas flow through the column was 1.0 mL/min. The headspace gas constituents were identified by GC–MS. A Varian 3400 gas chromatograph interfaced to a Finigan MAT model 800 ion trap detector (Thermo Fisher Scientific, Waltham, MA) was used. The transfer-port temperature and ion-trap temperature were kept at 230 °C. Mass units were monitored from 26 to 300 atomic mass units (amu)/s at 1250 eV. Other conditions were the same as those of GC conditions. The components were identified by comparing both mass spectra based on a library search system (Magnum Library-Search System: NIST Mass Spectra Database 62 235 compounds) and their GC retention indices (RIs) on a DB-WAX column to those of authentic compounds (Wako Pure Chemical Industries, Osaka, Japan; Tokyo Kasei Kogyo, Tokyo, Japan; and Sigma-Aldrich, St. Louis, MO) previously analyzed and stored in our private data file. The GC data were calculated by a Shimadzu GC-6R integrator. The concentration of each volatile component was determined by a comparison to the

internal standard. All treatments were performed in duplicate, and the results presented mean values.

Multivariate Analysis. The canonical discriminant analysis to differentiate shiikuwasha juice from calamondin juice was performed using IBM SPSS Statistics, version 19.0.0.

RESULTS AND DISCUSSION

Because Ogawa et al. reported that PD was contained in peels, juice sacs, and leaves of calamondin but not in those of shiikuwasha,[13] we supposed PD can be a chemical marker that can detect shiikuwasha juice adulterated with calamondin juice or other citrus fruits containing PD. PD from 14 juice samples was analyzed by TLC (Figure 2), with the black spot (R_f of 0.35) of PD being observed in the hand-pressed calamondin juice and the industrial calamondin juice. The detection sensitivity limit of PD was 1.0 mg. However, no spots were detected in the hand-pressed shiikuwasha juice and the industrial shiikuwasha juice. Samples A–C commercial juices with the black PD spots were found not to be 100% pure shiikuwasha juice and were suspected of being adulterated with calamondin juices. On the other hand, samples D–J commercial juices without the PD spots were assumed to be 100% pure shiikuwasha juices.

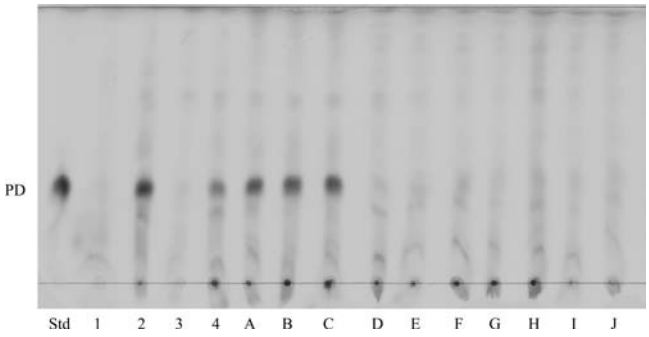

Figure 2. TLC analysis of PD. Lanes: Std, PD; 1, hand-pressed shiikuwasha juice; 2, hand-pressed calamondin juice; 3, industrial shiikuwasha juice; 4, industrial calamondin juice; and A–J, commercial juice.

Figure 3 showed the chromatograms of (c) PD standard, (a) hand-pressed shiikuwasha juice, and (b) hand-pressed calamondin juice by HPLC. The PD peak was observed in standard solution and calamondin juice at 33.9 min. The peak detected at 39.1 min from shiikuwasha and calamondin juice was identified as hesperidin by the retention time of the authentic compound. As shown in Table 1, 24.4 mg/100 mL PD is contained in calamondin hand-pressed juice, 25.5 mg/100 mL PD is contained in industrial calamondin juice, and 37.2–59.2 mg/100 mL PD is contained in samples A–C commercial juices but PD is not detected in other samples. Therefore, the PD detection by TLC and HPLC shows commercial juices (samples A–C) suspected of being adulterated with calamondin juices. In addition, HPLC data were consistent with the TLC data. TLC of PD is considered to be a rapid and useful method to detect a commercial shiikuwasha juice adulterated with calamondin juice.

Typical HPLC chromatograms for the PMF analysis of the hand-pressed shiikuwasha juice and hand-pressed calamondin juice are shown in Figure 4. Peaks at 6.5 min for sinensetin, at 9.0 min for nobiletin, and at 13.1 min for tangeretin were detected. Table 1 shows levels of PMFs and a peak ratio

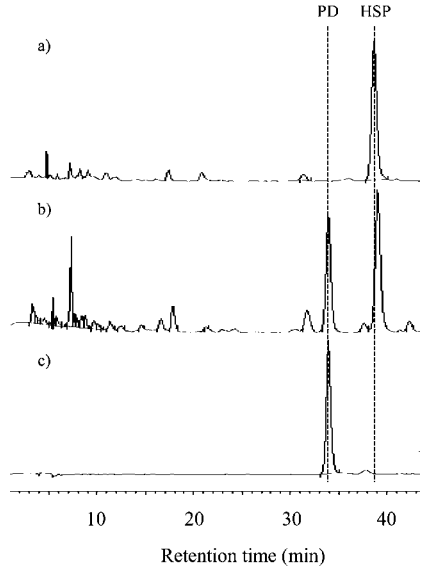

Figure 3. HPLC chromatograms for the analysis of PD of (a) hand-pressed shiikuwasha juice, (b) hand-pressed calamondin juice, and (c) PD standard. HSP = hesperidin.

[NOB/TNG], and these values were used to perform canonical discriminant analysis. The hand-pressed juices showed levels of PMFs much lower than those of industrial juices, consistent with the previous studies, indicating that more PMFs are present in juices extracted from peels under higher pressures during the industrial extraction procedures. In the shiikuwasha juice (samples 1 and 3), the contents of PMFs were more abundant than those in calamondin juice (samples 2 and 4), and these data were consistent with a previous study.[2] NOB was the major component that was detected in the commercial juices (samples A–C) in the range of 5.1–5.8 mg/100 mL and in other commercial juices (samples D–J) in the range of 11.1–143.3 mg/100 mL. Similarly, TNG and SNT showed concentration values in the ranges of 1.5–2.1 mg/100 mL and 0.2–0.5 mg/100 mL in samples A–C and 3.9–50.1 mg/100 mL and 0.1–7.4 mg/100 mL in samples D–J, respectively. Pan et al. reported that a discriminant function obtained by five peak ratios of PMF levels has detected orange juice adulterated by tangelo juice.[9] We obtained discriminant function 1 that detected the adulteration of shiikuwasha juice with the accuracy of 91.7% (11 of 12).

Figure 5 shows gas chromatograms of the volatile components in the hand-pressed shiikuwasha and calamondin juice obtained by headspace (HS)-SPME–GC. Of the detected 79 peaks, 32 aromatic components were identified in the headspace gas obtained from shiikuwasha juice. On the other hands, 30 aromatic components were identified from the detected 80 peaks of calamondin juice. The peak pattern in the gas chromatogram of shiikuwasha juice was different from that of calamondin juice. In particular, the peak of γ-terpinene detected at 17.5 min in calamondin juice was considerably smaller than that of shiikuwasha juice. Table 2 summarizes the typical composition of the headspace gas obtained from hand-pressed shiikuwasha juice and hand-pressed calamondin juice according to the functional group. Hydrocarbons, which are the most major volatile components, were almost of the equal levels between shiikuwasha and calamondin juice. Oxygenated compounds are particularly associated with the citrus flavor, and both shiikuwasha and calamondin contained middle

Table 1. Results of the Analysis of PD, PMFs, and γ-Terpinene and Evaluation of Adulteration

			PMFs (mg/100 mL)[a]					γ-terpinene[b]			
sample[c]	TLC[d]	PD (mg/100 mL)[a]	NOB	TNG	SNT	NOB/SNT	discriminant function 1[e]	ratio (%)	(ppm)	discriminant function 2[f]	adulteration[g]
1	−	nd[h]	5.9 ± 0.0	2.7 ± 0.0	0.5 ± 0.0	11.4		17.3 ± 0.1	15.6 ± 1.1		
2	+	24.4 ± 0.5	0.7 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	7.9		0.7 ± 0.0	0.8 ± 0.1		
3	−	nd	48.8 ± 1.6	25.4 ± 0.2	2.3 ± 0.0	21.5	−1.36	12.3 ± 0.1	4.1 ± 0.3	1.02	
4	+	25.5 ± 0.5	2.4 ± 0.0	1.0 ± 0.0	0.4 ± 0.0	5.6	0.45	0.7 ± 0.0	0.8 ± 0.1	−2.02	
A	+	44.1 ± 1.1	5.8 ± 0.0	2.1 ± 0.0	0.5 ± 0.0	11.7	0.69	0.1 ± 0.0	0.7 ± 0.1	−2.16	+
B	+	59.2 ± 2.4	5.7 ± 0.0	1.6 ± 0.0	0.3 ± 0.0	18.7	1.95	0.1 ± 0.0	1.2 ± 0.1	−2.16	+
C	+	37.2 ± 0.8	5.1 ± 0.0	1.5 ± 0.0	0.2 ± 0.0	20.8	1.43	0.1 ± 0.0	0.5 ± 0.1	−2.17	+
D	−	nd	14.4 ± 0.1	5.1 ± 0.0	0.7 ± 0.0	21.0	−0.01	3.4 ± 0.0	8.1 ± 0.6	−1.32	−
E	−	nd	20.0 ± 0.1	7.3 ± 0.0	1.5 ± 0.0	13.6	1.39	14.7 ± 0.1	88.4 ± 5.9	1.64	−
F	−	nd	14.3 ± 0.0	4.7 ± 0.0	0.1 ± 0.0	140.5	−1.51	12.2 ± 0.1	38.4 ± 2.6	0.98	−
G	−	nd	11.1 ± 0.0	3.9 ± 0.0	0.5 ± 0.0	21.2	0.12	11.0 ± 0.1	27.7 ± 1.9	0.66	−
H	−	nd	143.3 ± 1.5	50.1 ± 0.0	7.4 ± 0.0	19.5	−2.10	11.5 ± 0.1	130.3 ± 8.7	0.81	−
I	−	nd	34.8 ± 0.4	12.4 ± 0.0	1.6 ± 0.0	21.4	−0.71	15.9 ± 0.1	140.4 ± 9.4	1.94	−
J	−	nd	38.7 ± 0.4	12.9 ± 0.0	1.9 ± 0.0	20.4	−0.32	19.1 ± 0.1	238.7 ± 16.0	2.79	−

[a]Mean ± standard deviation (n = 3). [b]Mean ± standard deviation (n = 2). [c]Samples: 1, hand-pressed shiikuwasha juice; 2, hand-pressed calamondin juice; 3, industrial shiikuwasha juice; 4, industrial calamondin juice; and A–J, commercial juice. [d]+, black spot of R_f = 0.35; −, not detected. [e]Discriminant function 1 = −0.269NOB + 0.234TNG + 3.229SIN + 2.281NOB/TNG − 6.030. [f]Discriminant function 2 = 0.260γ-terpinene − 2.245. [g]+, adulterated with calamondin juice; −, not adulterated. [h]nd = not detected.

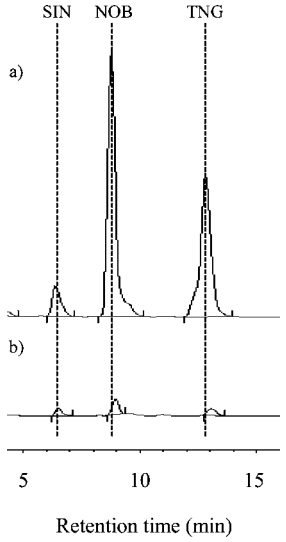

Figure 4. HPLC chromatograms for the analysis of PMFs of (a) hand-pressed shiikuwasha juice and (b) hand-pressed calamondin juice. SIN, sinensetin; NOB, nobiletin; and TNG, tangeretin.

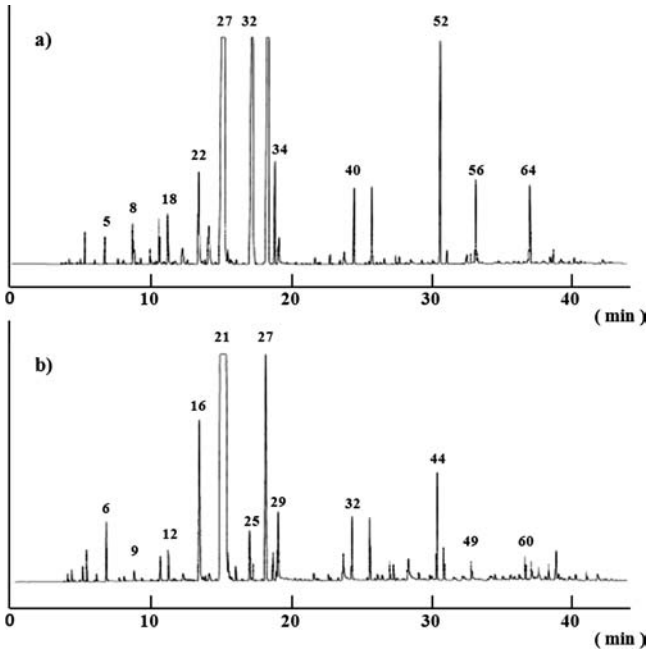

Figure 5. GC chromatograms of the (a) hand-pressed shiikuwasha juice and (b) hand-pressed calamondin juice.

percentages of alcohols and high percentages of aldehydes compared to other citrus.[16] The main volatile components in shiikuwasha juice were limonene, *p*-cymene, γ-terpinene, and linalool. In the calamondin juice, the level of limonene was the highest and the next two major volatile compounds were *p*-cymene and myrcene. The concentrations of the main volatile components for hand-pressed shiikuwasha juice compared to hand-pressed calamondin juice along with factors are as follows: 20 times for γ-terpinene, 4 times for *p*-cymene, twice for linalool, 0.6 times for myrcene, and 0.4 times for limonene. In addition, calamondin contained less amount of γ-terpinene compared to shiikuwasha. It is supposed that γ-terpinene is a useful chemical marker.

Table 1 shows that γ-terpinene ratio (γ-terpinene peak area/total peak area) and a discriminant function obtained by the γ-terpinene ratio. γ-Terpinene ratios in hand-pressed calamondin juice and industrial calamondin juice were equal, and the γ-terpinene ratio in hand-press shiikuwasha juice was larger than that in industrial shiikuwasha juice. γ-Terpinene ratios were 0.1% with regard to the commercial juices (samples A–C) and in the range of 3.4–19.1% with regard to other commercial juices (samples D–J). Moshonas and Shaw determined volatile components in calamondin peel oil and reported that the γ-terpinene area percent was 0.001%.[17] Inafuku et al. described that the γ-terpinene contents in immature shiikuwasha fruit and mature shiikuwasha fruit were 21.17 and 29.60%, respectively.[18] These results were consistent with the results obtained in this study. Discriminant function 2 detected the adulteration of shiikuwasha juice with an accuracy of 91.7% (11 of 12).

Figure 6 shows scattered plots of three cluster groups on the two canonical discriminant functions. The first group (I)

Table 2. Volatile Constituent and Concentration of Hand-Pressed Shiikuwasha and Calamondin Juice[a]

peak[b]	component	RI	hand-pressed shiikuwasha juice	hand-pressed calamondin juice	peak	component	RI	hand-pressed shiikuwasha juice	hand-pressed calamondin juice
4/5	ethyl acetate	886	0.05 (0.05)	0.06 (0.07)	47/−	α-copaene	1502	0.06 (0.05)	nd[c]
5/6	ethanol	933	0.26 (0.24)	0.45 (0.53)	48/39	decanal	1501	0.13 (0.11)	0.68 (0.79)
8/9	α-pinene	1025	0.45 (0.41)	0.15 (0.17)	52/44	linalool	1550	3.54 (3.19)	1.34 (1.57)
9/−	α-thujene	1029	0.16 (0.14)	nd	53/30	1-octanol	1560	0.21 (0.19)	0.41 (0.48)
10/10	toluen	1037	0.08 (0.08)	0.05 (0.05)	55/−	β-caryophyllene	1616	0.21 (0.19)	nd
15/11	hexanal	1081	0.64 (0.58)	0.29 (0.34)	56/49	terpinene-4-ol	1608	1.41 (1.27)	0.30 (0.36)
18/12	β-pinene	1114	0.88 (0.80)	0.38 (0.45)	−/52	myrtenal	1638	nd	0.10 (0.12)
22/16	myrcene	1168	1.99 (1.79)	2.72 (3.20)	61/53	α-caryophyllene	1685	0.08 (0.08)	0.14 (0.17)
25/19	α-terpinene	1182	1.23 (1.11)	0.18 (0.21)	63/56	p-mentha-(E)-2,8-dien-1-ol	1633	0.06 (0.06)	0.12 (0.15)
26/20	heptanal	1185	0.07 (0.06)	0.04 (0.04)	64/60	α-terpineol	1702	1.39 (1.25)	0.34 (0.40)
27/21	limonene	1205	39.60 (35.67)	77.80 (91.41)	65/61	germacrene D	1724	0.06 (0.05)	0.36 (0.42)
32/25	γ-terpinene	1255	17.27 (15.55)	0.65 (0.76)	−/62	D-carvone	1742	nd	0.06 (0.07)
33/27	p-cymene	1277	20.27 (18.26)	3.81 (4.47)	67/64	β-bisabolene	1748	0.16 (0.15)	0.09 (0.11)
34/28	terpinolene	1291	1.52 (1.36)	0.29 (0.34)	−/66	β-elemol	2086	nd	0.05 (0.05)
35/29	octanal	1290	0.70 (0.63)	1.30 (1.53)	75/−	τ-cadinol	2180	0.13 (0.12)	nd
39/31	nonanal	1395	0.24 (0.22)	0.62 (0.73)	76/−	thymol	2185	0.14 (0.12)	nd
44/35	1-heptanol	1458	0.09 (0.08)	0.10 (0.12)	77/78	isothymol	2215	0.27 (0.24)	0.07 (0.09)
45/37	octyl acetate	1477	0.11 (0.10)	0.18 (0.21)					

[a]Numbers indicate constituent (%; mean; $n = 2$), and numbers in parentheses indicate concentration (ppm; mean; $n = 2$). [b]Peak number a/b in Figure 5. [c]nd = not detected.

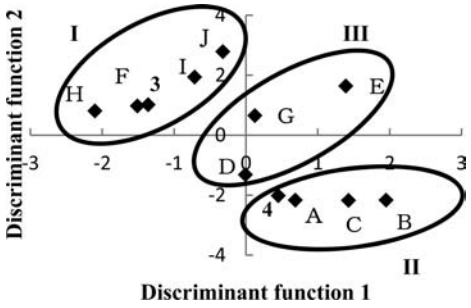

Figure 6. Scattered plots of the three cluster groups on the two canonical discriminant functions. The calculated discriminant functions were shown in Table 1. Samples: 1, hand-pressed shiikuwasha juice; 2, hand-pressed calamondin juice; 3, industrial shiikuwasha juice; 4, industrial calamondin juice; and A–J, commercial juice.

included five samples: 3, F, H, I, and J. It was defined by high levels of PMFs and γ-terpinene, and this group was supposed to consist of pure shiikuwasha juices. The second group (II) included four samples: 4, A, B, and C. This group was characterized by lower amounts of PMFs and γ-terpinene, and this group was supposed to consist of shiikuwasha juice adulterated with calamondin juice. The third group (III) included three samples: D, E, and G. This group was situated between the first group (I) and the second group (II). Sample D was characterized by middle levels of PMFs and lower levels of γ-terpinene. Sample E was characterized by middle levels of PMFs and γ-terpinene. Sample G was characterized by high levels of γ-terpinene and lower amounts of PMFs. Although samples D, E, and G were incorrectly judged with only one canonical discriminant function, they are expected to decrease wrong judgements with two other canonical discriminant functions. Therefore, these results assumed that this method is more accurate than that using a single canonical discriminant function.

In conclusion, we have developed a method to detect shiikuwasha juice that is suspected of being adulterated with calamondin juice using chemical marker compounds. PD was detected in calamondin juice but not in shiikuwasha juice, and PD detection by TLC is a rapid and useful method to detect a commercial shiikuwasha juice that is suspected of being adulterated with calamondin juice. Both of the two discrimination functions obtained by PMFs and a peak ratio and γ-terpinene detected the adulteration of shiikuwasha juice with the accuracy of their analysis at a level of 91.7%. Scattered plots on two canonical discriminant functions were more accurate than a judgment with a single canonical discriminant function. These chemical markers were useful to detect shiikuwasha juice that is suspected of being adulterated with calamondin juice.

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Notes

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