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Physicochemical, Carotenoid, Metabolite, and Volatile Organic Compound Profiling of Okinawan Shiikuwasha (*Citrus depressa* Hayata) and Calamansi (*C. microcarpa* Bunge)

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Featured Application: This study assessed the physicochemical traits, total carotenoids, metabolites, and volatile profiles of Okinawan shiikuwasha and calamansi. The physicochemical characteristics and flavor components unique to shiikuwasha and calamansi fruits provide valuable insights for food processing. The discovery of the distinct characteristics of the two citrus cultivars may be beneficial to producers and consumers, thereby supporting local agro-industries.

Abstract: Shiikuwasha (*Citrus depressa* Hayata) and calamansi (*C. microcarpa* Bunge) are distinct citrus species with a strong sour taste that grow naturally in Okinawa, Japan. The present study aimed to characterize the physicochemical traits, total carotenoid content, and taste/nutrient-related metabolite and volatile organic compound (VOC) profiles of the mature fruits from three shiikuwasha cultivation lines ("Izumi kugani", "Izumi kugani-like", and "Ogimi kugani") and calamansi. The shiikuwasha lines had higher ratios of soluble solids/titratable acidity but a lower total carotenoid content than calamansi. There were positive statistical correlations between total carotenoids with red, yellow, and orange indices (a*, b*, and a*/b* color spaces, respectively). Principal component analysis (PCA) revealed that shiikuwasha had higher sucrose and choline contents, while calamansi was richer in sourness-related metabolites such as citrate and malate. PCA plots displayed differences in the VOCs between the two citrus cultivars, wherein volatile accumulation was much richer in juices prepared from the whole fruits than in juices from the edible flesh parts. The plots also showed that monoterpenes and sesquiterpenes differentiated the VOC profiles of shiikuwasha and calamansi. This study reveals differences in the flavor components of the two citrus cultivars and highlights the potential uses of each in local and regional agro-business.

Keywords: shiikuwasha; calamansi; carotenoids; taste-related metabolites; volatile organic compounds

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1. Introduction

Shiikuwasha (*Citrus depressa* Hayata) is a small citrus cultivar with strong acidity and a particular aroma that has been cultivated in Okinawa, Japan, for many years. The shiikuwasha peels contain polymethoxylated flavones, such as nobiletin and tangeretin, with various health benefits, including anticancer and antiobesity effects [1–3]. Their volatile organic compounds (VOCs) are effective for stress relief and aromatherapeutic

relaxation, and as anti-inflammatory agents [4,5]. In Okinawa, shiikuwasha fruits are used to season or decorate food or are processed into beverages and condiments such as jams and sauces [6]. Calamansi (*C. microcarpa* Bunge) is a citrus fruit widely grown in tropical to subtropical areas, including Okinawa, Taiwan, and Southeast Asian countries [7,8]. In general, shiikuwasha and calamansi juices have high acidity and a similar appearance. A chemical marker, namely 3′,5′-di-C-*β*-glucopyranosylphloretin, can be used to discriminate the two citrus cultivars; this substance has no direct contribution to the flavor properties of the two citrus cultivars [7].

In terms of the flavor characteristics of citrus juices, consumers generally focus on three factors. First, color contributes greatly to citrus fruits as the primary information that conveys attractiveness [9]. Citrus fruit pigments, known as carotenoids, such as *β*-cryptoxanthin, *β*-carotene, and lycopene, are important metabolites that become vitamin A in the body and are necessary for humans [10]. Carotenoid levels are related to color indices such as the L*a*b* color spaces [11,12]. Second, substances related to taste, such as sugars and organic acids, and the sugar–acid ratio are essential factors when considering the quality of citrus juices [13–16]. In addition to known conventional analytical methods, proton nuclear magnetic resonance (^{1}H-NMR) can be applied to analyze taste-related metabolites in a highly reproducible and convenient manner [17,18]. Multivariate analysis of taste/nutrient metabolite data is an effective approach for discriminating citrus cultivars based on metabolite composition [18]. Third, VOCs, which greatly contribute to the aroma traits of citrus, are mainly composed of monoterpene hydrocarbons. However, information on the differences in the VOC profiles of the two citrus cultivars is lacking [7,8,14,16].

This study aimed to clarify the flavor characteristics of shiikuwasha and calamansi by comparing their physicochemical traits and carotenoid, metabolite, and VOC contents. The three major cultivation lines of shiikuwasha ("Izumi kugani", "Izumi kugani-like", and "Ogimi kugani") were evaluated, as they are commonly consumed in Japan. In addition, to evaluate the impact of peeling on the VOC profile of fruit juice, the fruits were prepared, and their contents were analyzed as whole juice (with the peel) and flesh juice (without the peel); this approach contributes insights related to food processing.

2. Materials and Methods

2.1. Standards and Reagents

2,2-Dimethyl-2-silapentane-5-sulfonate (DSS) sodium salt and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Deuterium oxide (D_2O) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). 1-Hexanol was obtained from Tokyo Chemical Industry (Tokyo, Japan). All other reagents were of analytical grade.

2.2. Shiikuwasha and Calamansi Fruits

Mature fruits of shiikuwasha ("Izumi kugani", "Izumi kugani-like", and "Ogimi kugani") and calamansi were harvested from a farm at the Okinawa Prefectural Agricultural Research Center Nago Branch in December 2021. The "Ogimi kugani" line was used as the control cultivation line because its wide range of food applications in Okinawa, Japan [6,14]. The fruits were randomly selected, and, either unpeeled and peeled, were cut into small pieces, then squeezed using a stainless-steel hand juicer (Minex Metal, Niigata, Japan) to produce whole and flesh juices, respectively. The pH and L*a*b* color spaces of the flesh juice were examined using a LAQUAact D-71 pH meter (Horiba, Kyoto, Japan) and an SE7700 spectrophotometer (Nippon Denshoku, Tokyo, Japan), respectively. The total soluble solids (TSSs) and titratable acidity (TA) were measured using a PAL-BX ACID F5 (Atago, Tokyo, Japan). The edible parts of 3–5 fruits were cryopulverized using a multibead shocker (Yasui Kikai, Osaka, Japan) and lyophilized for 48 h using an EYELA FDU-2000 (Tokyo Rikakikai, Tokyo, Japan). The juice and lyophilized tissues were stored at −30 °C until analysis.

2.3. Total Carotenoid Analysis

The total carotenoid content was determined using a microplate spectrophotometer [19]. Briefly, the lyophilized tissues of edible fruit parts (10 mg) were initially acclimated at room temperature and then placed in a 2 mL tube containing 5 mm diameter zirconia beads. Acetone (300 µL) was added to the tube, followed by homogenization for 10 min at 15 Hz using TissueLyser LT (Qiagen, Hilden, Germany). The mixture was centrifuged for 5 min at 15,000 rpm and 20 °C, and the supernatant (200 µL) was collected. The extraction was repeated twice, and the combined lysate (300 µL) was placed into a 96-well glass microplate (Nikkei Products, Osaka, Japan). The optical densities of the lysates were measured at 470, 645, 662, and 750 nm using a PowerWave XS2 microplate reader (BioTek, Winooski, VT, USA), and the optical density at 750 nm was used for wavelength correction. Total carotenoid content was calculated using the following equations: $C_a = (11.24A_{662} - 2.04A_{645}) \times (v/w)$; $C_b = (20.13A_{645} - 4.19A_{662}) \times (v/w)$; total carotenoid content = $[(1000A_{470} - 1.90C_a - 63.14C_b)/214] \times (v/w)$, where *Ca* is chlorophyll a; *Cb* is chlorophyll b; A_x is the absorbance at x nm; v is the volume of the solvent (mL); and w is the weight of the sample (mg).

2.4. Metabolite Component Analysis Using ^{1}H-NMR

The metabolites were determined using nontargeted ^{1}H-NMR [17]. Briefly, 140 µL of flesh juice and 560 µL of internal standard DSS (1 mM, prepared in 500 mM potassium phosphate buffer pH 7.0 in D_2O) were placed in a 2 mL tube and vortexed. The mixture was transferred into an NMR tube (5 mm diameter) and analyzed using a Bruker Avance 500 MHz NMR spectrometer (Bruker BioSpin, Karlsruhe, German). The ^{1}H-NMR spectra were acquired using the zgpr pulse program from the Bruker pulse library in digital quadrature detection mode. The acquisition parameters were as follows: proton 90° pulse, 12 µs; offset frequency, 4.7 ppm; relaxation delay, 4 s; and number of scans, 64. The metabolite signals were annotated, and the relative concentrations of the metabolites were normalized to that of DSS as an internal standard using Chenomx NMR Suite Version 8.6 (Edmonton, AB, Canada).

2.5. VOC Analysis Using SPME-GC-MS

The VOCs in whole and flesh juices were analyzed using solid-phase microextraction–gas chromatography–mass spectrometry (SPME-GC-MS) [16]. Briefly, 2 mL of juice, 1 mL of EDTA (0.1 M, pH 7.5), and 10 µL of internal standard 1-hexanol (0.5 mg/mL) were placed in a 20 mL glass vial. The mixture was heated at 40 °C for 5 min, and the volatiles were extracted using a divinylbenzene/carboxen/polydimethylsiloxane 50/30 µm SPME fiber (Supelco, Bellefonte, PA, USA) at 40 °C for 10 min on a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland). The analysis was performed using a 7890 B GC-5977A MS system (Agilent Technologies, Santa Clara, CA, USA) at a split ratio of 10:1 for 2 min. The VOCs were separated using a DB-5MS capillary column (30 m × 0.25 mm, 0.25 µm; Agilent Technologies). The GC oven temperature was initially set to 40 °C for 1 min and then increased to 200 °C at a rate of 5 °C/min. The injection and ion source temperatures were set at 250 and 230 °C, respectively. The MS electron energy was 70 eV, and the scan range and rate were set to m/z 33-350 and 1.77 scans/s, respectively. The VOCs were annotated by comparing their mass spectra with those in the NIST-MS library Version 2014 and the retention index upon measurement of the homologous series of *n*-alkanes (C_6–C_{30}). The relative concentrations of the VOCs were normalized to that of 1-hexanol as an internal standard using MS-DIAL Version 4.80 (RIKEN Center for Sustainable Resource Science, Kanagawa, Japan).

2.6. Statistical Analysis

Each result is expressed as the mean value and standard deviation of four replicates. Statistical differences between groups were analyzed using Tukey's honestly significant difference test, and Pearson's correlation coefficients between two parameters were calculated

using GraphPad Prism Version 9 (GraphPad Software, Boston, MA, USA). Multivariate analysis was performed on the intensities of metabolites and VOCs following $\log_{10}$ transformation and unit variance scaling processes, and the multivariate profiles of metabolites and VOCs were visualized as principal component analysis (PCA) plots using SIMCA Version 17 (Sartorius, Göttingen, Germany).

3. Results

3.1. Physicochemical Properties of Edible Fruit Parts

The pH, TSS, and TA values of the edible fruit parts in the shiikuwasha cultivation lines ranged from 3.20 to 4.00, 8.98 to 10.03°Brix, and 0.41–1.51%, respectively (Table 1). The highest TSS value was recorded in "Izumi-kugani-like", which had a significantly lower TA value ($p < 0.05$). Conversely, calamansi had a significantly lower pH and higher TA value, at 2.55 and 3.10%, respectively. Accordingly, the order of the calculated TSS/TA ratio, a simplified index of fruit product quality, was as follows: "Izumi kugani-like" > "Izumi kugani" > "Ogimi kugani" > calamansi. The flesh juice of "Ogimi kugani" was significantly brighter in appearance owing to having the highest L* color space value compared with those in the other shiikuwasha lines and calamansi (32.32 vs. 26.69–27.80, respectively). On the other hand, calamansi had a significantly higher positive a* value (red color index, 4.64) than the shiikuwasha lines, where "Izumi kugani" and "Izumi kugani-like", which had negative a* values, appeared slightly greenish. Moreover, calamansi had the highest b* color space (yellow index) value, followed by "Ogimi kugani", "Izumi kugani", and "Izumi kugani-like" (22.81, 19.69, 17.34, and 14.80, respectively), but the difference between calamansi and "Ogimi kugani" was not significant.

Table 1. Physicochemical properties of shiikuwasha and calamansi flesh juices.

Trait	"Izumi kugani"	"Izumi kugani-like"	"Ogimi kugani"	"Calamansi"
pH	3.48 ± 0.04 b	4.00 ± 0.04 a	3.20 ± 0.09 c	2.55 ± 0.03 d
Total soluble solids (TSSs)	8.98 ± 0.30 b	10.03 ± 0.26 a	9.00 ± 0.61 b	9.75 ± 0.26 ab
Titratable acidity (TA)	0.92 ± 0.07 c	0.41 ± 0.06 d	1.51 ± 0.08 b	3.10 ± 0.38 a
TSS/TA	9.82 ± 0.63 b	25.15 ± 3.92 a	5.99 ± 0.67 bc	3.18 ± 0.42 c
Color space L*	27.80 ± 1.88 b	26.78 ± 1.34 b	32.32 ± 0.67 a	26.69 ± 0.97 b
Color space a*	−1.97 ± 0.15 c	−3.13 ± 0.05 c	0.53 ± 0.20 b	4.64 ± 0.96 a
Color space b*	17.34 ± 1.91 bc	14.80 ± 1.33 c	19.69 ± 1.29 ab	22.81 ± 2.46 a

Values are shown as mean ± standard deviations ($n = 4$). Mean in the same row followed by the same letter are not significantly different ($p < 0.05$).

3.2. Total Carotenoid Content of Edible Fruit Parts

The total carotenoid content of the edible fruit parts of the different shiikuwasha cultivation lines ranged from 0.46 to 0.71 mg/100 g FW, while calamansi had a significantly higher carotenoid content (1.43 mg/100 g FW) ($p < 0.05$) (Figure 1a). The Pearson's correlation plots exhibited linear positive relationships between the total carotenoid content of the shiikuwasha lines and calamansi with their a* and b* color spaces (red and yellow indices, respectively) (Figure 1b,c). It is noteworthy that the total carotenoids of the shiikuwasha lines and calamansi were strongly associated with the a* color space, with a linear correlation coefficient (r) of 0.9297 ($p < 0.0001$), while the pigments had a slightly weaker relationship with the b* color space (r = 0.7447; $p < 0.01$). In addition, the total carotenoids of both citrus cultivars were strongly correlated with their a*/b* ratio or orange index (r = 0.9094; $p < 0.0001$) (Figure 1d).

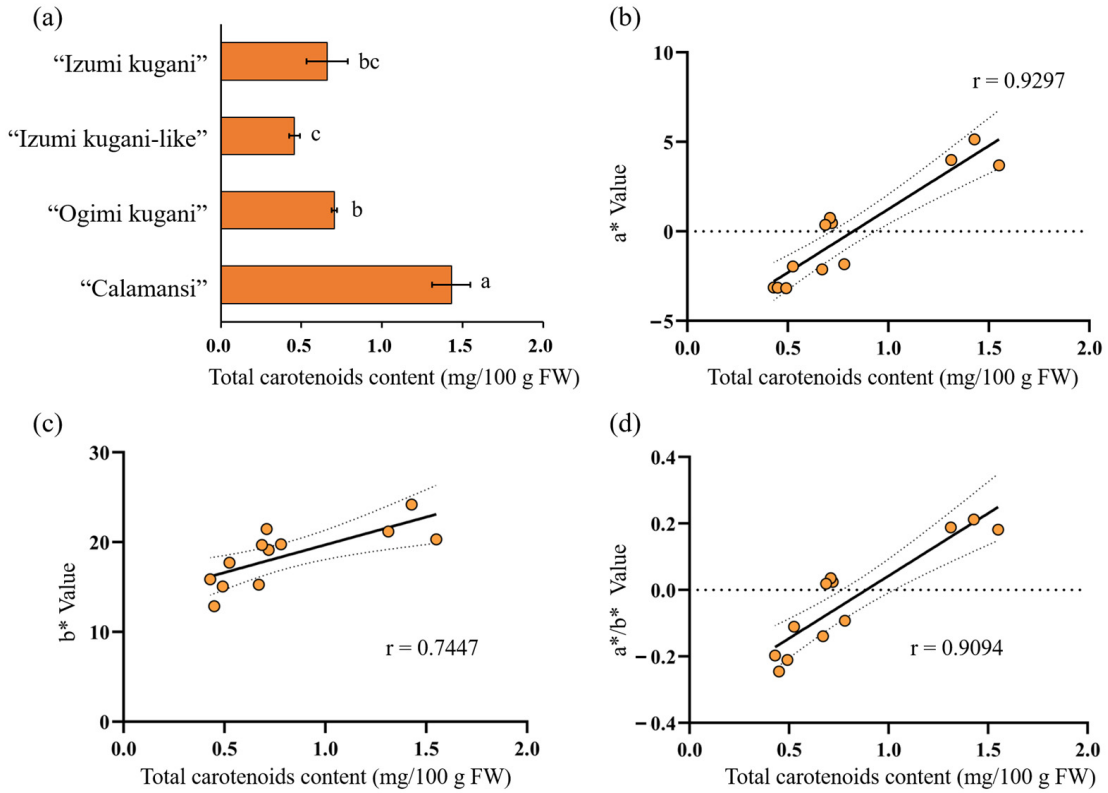

Figure 1. (**a**) Total carotenoid content in shiikuwasha and calamansi flesh (each value is expressed as the mean ± standard deviation of four replicates; means followed by the same letter are not significantly different at $p < 0.05$); Pearson's correlation plot between total carotenoid content and color spaces: (**b**) a* value (red color index), (**c**) b* value (yellow color index), and (**d**) a*/b* value (orange color index).

3.3. Metabolite Components of Edible Fruit Parts

Eleven metabolites, which were detected in the ^{1}H-NMR analysis (Supplementary Figure S1), could be used to effectively distinguish shiikuwasha and calamansi in the PCA plots (Figure 2a,b). These comprised three sugars (glucose, fructose, and sucrose), three organic acids (citrate, malate, and malonate), two amino acids (aspartate and 4-aminobutyrate), two alcohols, and choline. The shiikuwasha lines were grouped in the positive direction of the first principal component (PC1) in the PCA score plot, while calamansi occupied the opposite direction (Figure 2a). The three shiikuwasha lines were separated through the second principal component (PC2) axis and plotted from positive to negative in the order of "Ogimi kugani", "Izumi kugani", and "Izumi kugani-like". Conversely, calamansi was located around the zero axis or slightly to the negative direction in PC2. The factor loading plot indicated that choline and sucrose were both displayed in the positive PC1 direction, but differed in the PC2 axis as positive and negative, respectively (Figure 2b). Malonate was located in the positive PC2 direction near the zero coordinate of the PC1 axis, while the other metabolites were plotted in the negative direction of PC1.

The order of the relative concentration of total sugars in the two citrus cultivars was as follows: "Izumi kugani-like" > calamansi > "Izumi kugani" > "Ogimi kugani" (79.00, 71.97, 66.89, and 60.80 mM, respectively) (Figure 2c). "Izumi kugani-like" had higher sucrose levels (28.93 mM) than calamansi (11.36 mM), while calamansi contained higher glucose and fructose levels. On the other hand, the total organic acid content was higher in calamansi than in the shiikuwasha lines (59.84 vs. 9.40–20.61 mM) (Figure 2d). Citrate content, which is related to the main acidity source in citrus fruit, was 53.84 mM in calamansi. Interestingly, the malonate content in "Ogimi kugani" was much higher than that in the other lines and calamansi; this occurrence was also outlined in the PCA plots. Calamansi tended to contain

higher concentrations of aspartate and GABA, while the shiikuwasha lines had greater choline levels (Figure 2e). The relative concentrations of taste-related metabolites—sugars and organic acids—were positively correlated with the TSS and TA values, respectively (Figure 2f,g). The Pearson's correlation coefficients were r = 0.8068 ($p < 0.001$) for sugar content against TSSs, whereas an improved correlation was found for the total organic acid level with the TA value (r = 0.9770; $p < 0.0001$).

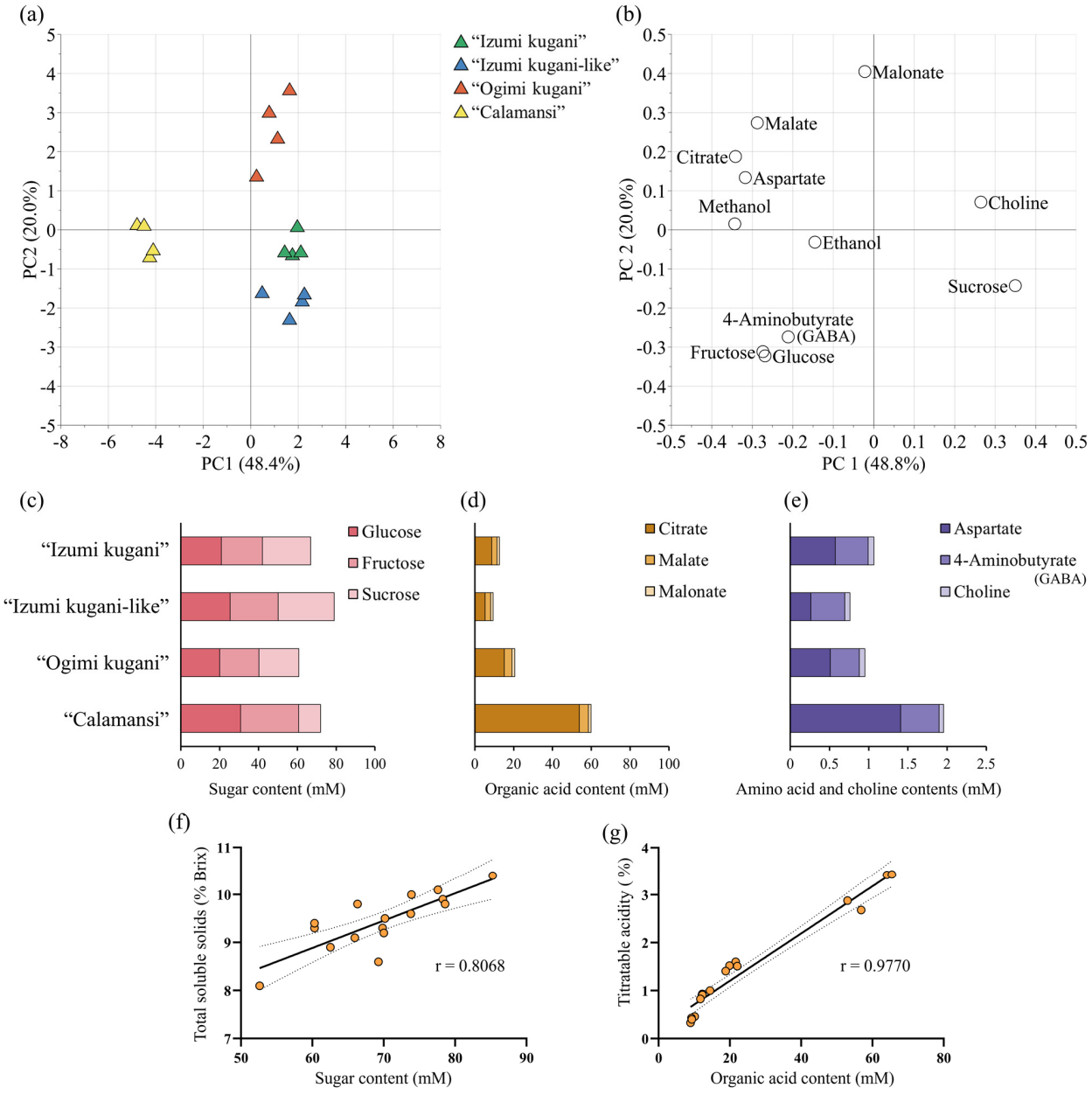

Figure 2. Metabolite components in shiikuwasha and calamansi: (**a**) PCA component score plot; (**b**) PCA factor loading plot; (**c**) concentration of sugar components; (**d**) concentration of organic acid components; (**e**) concentration of amino acid components; (**f**) Pearson's correlation plot between sugar content and total soluble solids; (**g**) Pearson's correlation plot between organic acid content and titratable acidity. PCA, principal component analysis.

3.4. VOCs of Whole and Flesh Juices

The intensity of the total chromatographic peaks, which accounted for 123 chromatographic peaks (81 identified VOCs, Supplementary Table S1, Supplementary Figure S2), was much higher in whole juice than in flesh juice, with a $\log_{10}$-normalized intensity of

approximately 2.72 against 1.24 (Figure 3a). Among the shiikuwasha lines, the whole juice of "Izumi kugani" had the greatest total VOC abundance (2.89), which was not significantly different from that in calamansi (2.83) ($p < 0.05$). Calamansi had a significantly higher VOC content in the flesh juice (1.74), followed by "Izumi kugani" and "Izumi kugani-like", and "Ogimi kugani" (1.24, 1.14, and 0.82, respectively). The PCA scores and loadings biplot confirmed the intensity differences in VOCs among shiikuwasha and calamansi juices, wherein whole and flesh juices were divided into the positive and negative directions in the first PC, respectively; most VOCs were clustered in the positive direction (Figure 3b). The biplot also indicated a clear separation of "Izumi kugani" and "Izumi kugani-like" when they were processed into whole juices.

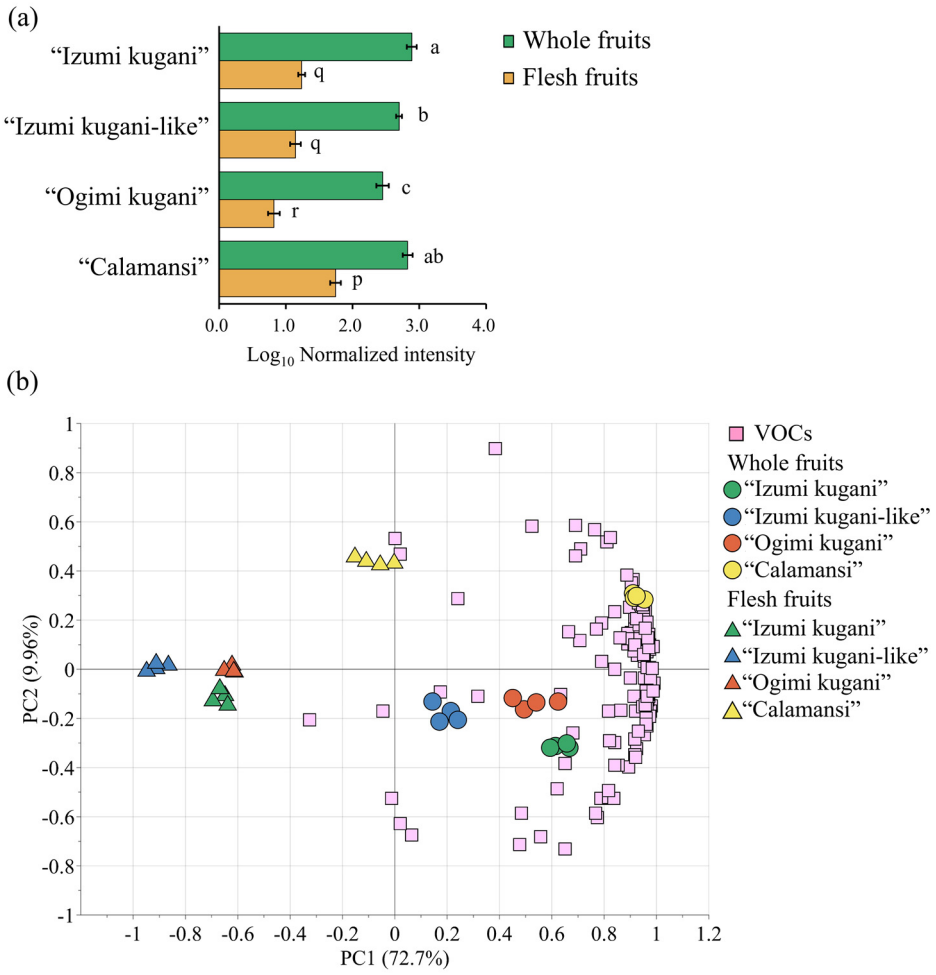

Figure 3. Volatile organic compounds (VOCs) in juices made from whole and flesh shiikuwasha and calamansi fruits: (**a**) log10-normalized intensity of total chromatographic peaks (each value is expressed as the mean ± standard deviation of four replicates; means followed by the same letter are not significantly different at $p < 0.05$); (**b**) PCA component scores and factor loading biplot.

3.5. PCA of Combined Metabolite Components and VOCs

PCA was performed by combining 11 metabolites and 123 chromatographic peaks (81 VOCs) in the data for both whole and flesh juices of shiikuwasha and calamansi fruits (Figure 4). The three shiikuwasha lines and calamansi were separately outlined in the PCA for the whole juice, wherein "Ogimi kugani" occupied the center and calamansi was located along the negative directions on both PC axes (Figure 4a). The loading plot also indicated that the metabolites and VOCs were arranged almost radially in all directions except in the quadrant of PC1 positive–PC2 negative (Figure 4b). The component score positions of the shiikuwasha lines were related to specific metabolite factors such as su-

crose and an organonitrogen compound (choline), whereas calamansi was associated with organic acids, glucose, fructose, and GABA, consistent with the data in Figure 2 regarding relative concentrations. The PCA plots also indicated that the shiikuwasha lines with lower VOC contents were characterized by the presence of monoterpenes, while calamansi was associated with sesquiterpenes, esters, and aldehydes. Lastly, a similar trend was noted in the PCA of the flesh juice, except that the component scores of "Izumi kugani" and "Ogimi kugani" tended to be closer to one another (Figure 4c,d).

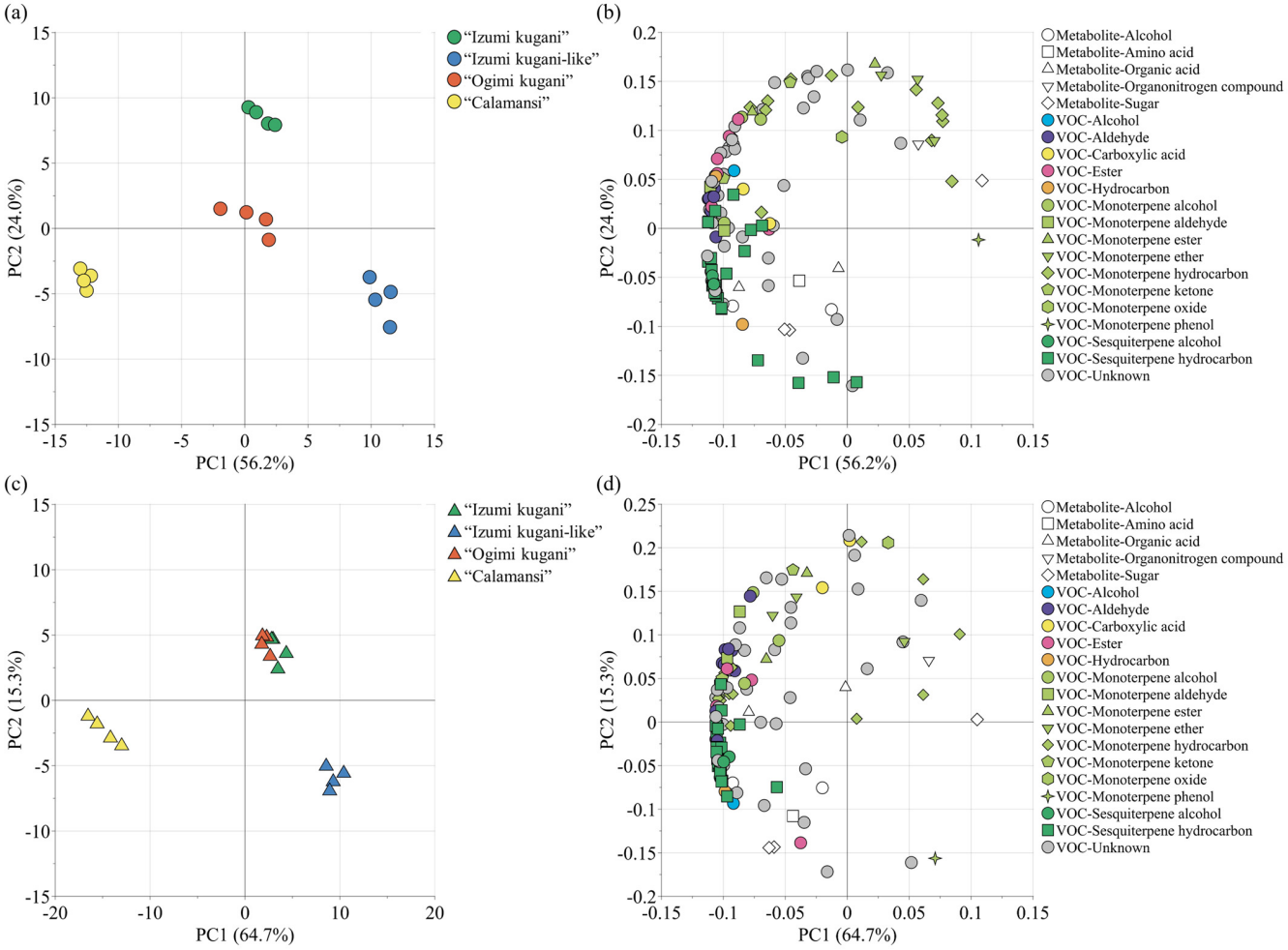

Figure 4. PCA plots of metabolites and VOCs in shiikuwasha and calamansi juices: (**a**) component scores of whole juices; (**b**) Factor loadings of whole juices; (**c**) component scores of flesh juices; (**d**) factor loadings of flesh juices.

4. Discussion

Physicochemical traits provided basic information for differentiating the primary qualities of the edible fruit parts of three major cultivated shiikuwasha lines and calamansi (Table 1). The ratio of TSS/TA in the "Izumi kugani-like" line (25.15) is comparable with that in mandarin and pummelo (26.30 and 27.78, respectively), while the ratios in "Izumi kugani" and "Ogimi kugani" are within the range recorded for grapefruit (6.32–12.00) [15]. On the other hand, the TSS/TA ratio of calamansi (3.18) is between those for lemon and grapefruit (1.79–6.32) [13]. These results indicate that shiikuwasha and calamansi can be primarily distinguished by the soluble solids and acidity in their edible parts; thus, these simple traits can be used to differentiate the basic fruit qualities of the two citrus cultivars when they are used for raw fruit or flesh juice consumption. The red indices (a* values) of the shiikuwasha and calamansi fruits were within the range of other citrus varieties such grapefruit and lemon, while their yellow index (b* value) was similar to that

of mandarin [20]. These color indices are important factors that determine the impression of consumers when evaluating the quality of fruits and fruit juices [9] and can be used to differentiate the color of shiikuwasha from that of calamansi.

The edible fruit parts of calamansi were characterized by a higher content of carotenoids and pigments for red, yellow, and orange than those in the shiikuwasha lines, and thus the two cultivars could be visually differentiated (Figure 1). Compared to the other citrus varieties, shiikuwasha features similar levels of carotenoids to those of lemon and pomelo (0.03–0.65 and 0.04–1.32 mg/100 g FW, respectively), while the carotenoid concentration of calamansi is slightly lower than that in mandarin oranges (1.85–2.49) [10]. The pigments found in most citrus varieties are β-cryptoxanthin and violaxanthin; mandarin tends to be rich in β-cryptoxanthin and orange in violaxanthin [10,21]. These carotenoids are beneficial to health because they are precursors to vitamin A, which is essential for humans, while β-cryptoxanthin may prevent osteoporosis, boost immunity, and act as an antioxidant [22]. A positive correlation between carotenoid content and a* values has also been reported for other citrus varieties, as well as vegetables such as tomatoes and pumpkins [12,23,24]. The red or orange color of the shiikuwasha and calamansi may intensify as carotenoid content increases (Figure 1b,d); thus, these pigments are responsible for distinguishing the color quality of the fruits.

Simple sugars such as monosaccharides and disaccharides are the main sources of sweetness in citrus fruits, while organic acids such as citric, malic, and malonic acids provide a sour taste [14,25]. The shiikuwasha lines were primarily associated with higher concentrations of sucrose and choline (Figure 2a–e). "Izumi kugani" and "Izumi kugani-like", which were positioned close to one another on the PCA component scores plot, had overall similar profiles regarding metabolite composition, while the lower sucrose and higher malonate contents in "Ogimi kugani" were the main reasons for its separation from the other two lines. Accordingly, "Izumi kugani" may be perceived sweeter and less sour than "Ogimi kugani", as reported previously [14]. In contrast, calamansi, which had higher citric and malic acid contents, could be distinguished by its high sourness intensity. The two citrus cultivars likely provide different levels of beneficial nutrients, that is, choline in shiikuwasha and GABA in calamansi (Figure 2e). Choline is required in cell membranes, neurotransmitter systems, and metabolic cycles, while GABA is an amino acid that has been garnering attention for its ability to inhibit stress and elevated blood pressure [26,27]. The ^{1}H-NMR technique for measuring taste- and nutrient-related metabolites is a relatively new approach in food analysis [17,18]. The relative concentration of these taste-related substances from the ^{1}H-NMR analysis corresponded the TSS and TA data (Figure 2f,g), suggesting the effectiveness of the method in evaluating the taste and nutrient qualities of citrus fruits.

Shiikuwasha and calamansi juices had different VOC profiles, with whole fruit juices characterized by greater volatile concentrations and more complex profiles than juices made from the edible parts only (flesh juices) (Figure 3). The aroma qualities of shiikuwasha and calamansi could thus be distinguished even when the juice was derived from the edible parts or when consumed as raw fruits. However, the inclusion of fruit peels, a source of essential oils [14,28,29], in the production of whole juice facilitated the distinction between the two cultivars. The VOCs in both cultivars are mainly composed of monoterpenes and sesquiterpenes (Supplementary Table S1, Supplementary Figure S3), and their overall compositions are consistent with those reported previously [7,8,14,16]. Consistently, high amounts of limonene (citrus-fruity odor), γ-terpinene (woody-oily), and *p*-cymene (green) are found in the three shiikuwasha cultivation lines, while limonene is the sole predominant volatile in calamansi [28–30]. "Izumi kugani" and "Ogimi kugani" could be distinguished based on volatile content only when examining the prepared whole juice (Supplementary Figure S3), suggesting the importance of peel parts in constructing the aroma quality of each fruit juice. In addition, "Izumi kugani-like" can feature woody-green and citrus-floral aromas from thymol and (*E*)-β-farnesene, respectively [28]. On the other hand,

calamansi, which contains greater amounts of sesquiterpenes, esters, and aldehydes, may have additional fruity and grassy aromas [28–30].

The combined PCA plots allowed the visualization of the effects of taste- and aroma-responsible compounds in shiikuwasha and calamansi (Figure 4). The balance between sugars and organic acids in citrus is important, and strong or moderate sweetness along with typical fruity or fresh citrus odors are generally preferred [9]. The two cultivars had different flavor characteristics, with shiikuwasha containing higher sucrose levels and calamansi being rich in organic acids; the mixed fruit juice of the two cultivars can thus incorporate the best features of both. Moreover, the whole juices of all three shiikuwasha lines and calamansi were clearly separated in the PCA plots, suggesting the importance of the organic substances in the peel parts for distinguishing the overall aroma quality [29,30]. In addition to having a richer aroma, the juice from whole fruits can be perceived as more bitter and astringent than that of flesh juice because it may include limonoids and flavonoids from the peels [14,25]. Accordingly, the peeling process greatly affects the composition of taste and aroma compounds in shiikuwasha and calamansi juices, thereby altering their flavor characteristics.

5. Conclusions

The TA values and the TSS/TA ratio are convenient traits for differentiating shiikuwasha and calamansi; we found that the TA value was significantly higher in calamansi, while the TSS/TA ratio was lower. Positive correlations were found between total carotenoid content and red, yellow, and orange indices; the total carotenoid contents were highest in calamansi. Shiikuwasha juice was rich in sucrose and choline, while fructose, glucose, and organic acids were more abundant in the calamansi juice, indicating that differentiation is possible according to taste-related metabolites such as sugars and organic acids. VOCs were more abundant in the whole juice than in the flesh juice from shiikuwasha and calamansi. The composition of the VOCs in the juices may indicate different aroma qualities. The PCA of the combined taste-related metabolite and VOC data elicited a clear separation of the three shiikuwasha cultivation lines from calamansi according to the intensity of the flavor-contributing substances when unpeeled fruits were processed into foods and beverages. Taken together, the results of the present study reveal the physicochemical traits and flavor components of Okinawan shiikuwasha and calamansi fruits. The findings also provide valuable information for producers and consumers when making choices for processing and purchasing the two citrus cultivars.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14156746/s1, Figure S1: Representative ^{1}H-NMR spectra of metabolite components in shiikuwasha and calamansi; Figure S2: Representative GC-MS chromatograms of the VOCs in shiikuwasha and calamansi; Figure S3: PCA plots of VOCs in shiikuwasha and calamansi juices; Table S1: Composition of volatile organic compounds in shiikuwasha and calamansi juices.

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