sciendo

Journal of Horticultural Research 2023, vol. 31(1): 47–54
DOI: 10.2478/johr-2023-0003

VINEGAR EXTRACT PRODUCED USING RIPE FRUITS OF WILD GENOTYPE OF *CITRUS DEPRESSA* HAYATA IN OKINAWA

Takashi HANAGASAKI
Food Research Section of Okinawa Industrial Technology Center, Uruma, Okinawa, Japan

Received: March 2023; Accepted: June 2023

ABSTRACT

Shikuwasa (*Citrus depressa* Hayata) is known as Taiwan tangerine. Various local cultivars are grown, among which the most famous is the 'Kugani', which is considered a breeding cultivar. The fruits of this cultivar are used for various purposes (juices, jams, vinegar, etc.) and as a fruit for consumption. The local landrace Ishikunibu is considered wild and is not cultivated on a larger scale because it is dwarf and tastes sour even when harvested in February. This article showed the results of experiments that aimed to show that Ishikunibu fruits are suitable as an addition to the cultivation of these fruits for producing vinegar extract. The results indicate that the vinegar extract of Ishikunibu has properties similar to that of 'Kugani'. Such vinegar extracts using Ishikunibu have more ascorbic acid (115.2 $\mu g \cdot L^{-1}$ vs. 38.9 $\mu g \cdot L^{-1}$) and titratable acidity than that of 'Kugani'. The other quality parameters of such produced vinegar extracts did not differ significantly. The sensory evaluation of vinegar extracts showed no differences in aroma, green smell, and general flavor but vinegar extract of Ishikunibu tasted less bitter. Therefore, Ishikunibu, the wild genotype of shikuwasa, is deemed useful in producing vinegar extracts.

Key words: *Citrus depressa* Hayata, shikuwasa, 'Kugani', Ishikunibu, vinegar extract, polymethoxyflavone

INTRODUCTION

Shikuwasa (*Citrus depressa* Hayata) is a small citrus fruit known as Taiwan tangerine. It is distributed in the southwest part of the Japanese archipelago and the mountainous region of Taiwan (Ishikawa et al. 2016). Many locally cultivated landraces of varied phenotypes are found in the northern part of the main island of Okinawa. (Inafuku et al. 2010). 'Kugani' (*Citrus depressa* Hayata var. kuganiiz) gives birth to improved fruits of higher quality and larger size than most local genotypes (Inafuku et al. 2010). This cultivar accounts for most cultivated in commercial settings. Shikuwasa is now popular all over Japan and is used to garnish dishes and make juices and jams. As it ripens, shikuwasa changes from dark green to yellow or orange. The unripe green fruit is extremely sour and is used to make condiments. The fruit tastes best from December to the end of January when it is yellow and ripe. It is usually harvested from August to September as an acidifying agent in vinegar substitution. Fruits harvested from September to December are used in fruit juice, and fruits harvested from December to January are eaten raw. There have been previous attempts to extract the characteristic ingredients from 'Kugani' and others (Miyagi et al. 2013; Hirose et al. 2017a; Hanagasaki 2019, 2021, 2022). A citrus-based Ponzu soy sauce commonly used in Japanese cuisine is produced from vinegar extract made from wastes of 'Kugani' in juice production (Hirose et al. 2017a), which is being commercialized in Okinawa. Up until now, only commercial cultivars of shikuwasa have been focused on. Late ripening local genotype shikuwasa, also known as Ishikunibu (*Citrus depressa* Hayata form Ishikunibu), has a red-orange peel.

*Corresponding author
e-mail: hangskit@yahoo.co.jp

Its sour taste takes longer to fade (Inafuku et al. 2010). This genotype is generally less popular than other citrus fruits for commercial use because it is sour even when harvested in February. However, Ishikunibu has more potential for use in the production of vinegar extracts because of its unique sour flavor. Therefore, to improve the overall profitability of Ishikunibu fruit, vinegar extract using Ishikunibu fruit can be developed. In this study, we compared the characteristics and chemical components of Ishikunibu vinegar extract with 'Kugani' vinegar extract.

MATERIALS AND METHODS

The Ishikunibu and 'Kugani' used in this study were cultivated at the Okinawa Agricultural Research Center, Nago Branch (26°37' N, 127°59' E; 40 masl). They were harvested on December 13, 2018 and February 18, 2019. The fruit diameters are averaged from measurements of 20 fruits. The moisture content of 10 fruits was calculated by weighing them before and after freeze-drying (FD-550 from Tokyo Rikakikai, Japan).

Production of vinegar extract

Ten grams of shikuwasa obtained from three fruits was pulverized in 40 mL of spirit vinegar (Kraft Heinz, USA) using a mill mixer (IFM-800, Iwatani, Japan) as one of three repetition samples. The mixture was blended four times for 10 s each. Consequently, the samples mixed with vinegar spirit were then centrifuged at 1190 g for 20 min and were finally filtered using No. 2 qualitative filter paper (Advantec, Japan) (Hirose et al. 2017a).

Ascorbic acid measurement

Reference samples were prepared from ascorbic acid (reduction type, Nacalai Tesque, Japan) dissolved in spirit vinegar to concentrations of 30, 40, 50, 60, 70, 80, 90, and 100 $mg \cdot L^{-1}$. A volume of 5 μL of these standard solutions was injected onto a Wakosil-5C18 HPLC column (4.6 × 250 mm, Wako Pure Chemical, Japan) at a flow rate of 1.0 $mL \cdot min^{-1}$. The solvent was 5 $mmol \cdot L^{-1}$ phosphate buffer solution. Samples were measured at the wavelength of 246 nm using an SPD-20A ultraviolet (UV) detector (Shimadzu, Japan). The ascorbic acid concentration in the samples was measured using an RQ flex reflectometer (Kanto Chemical, Japan) (Takebe & Yoneyama 1995) and was corrected using a regression formula for the relation between the HPLC and RQ flex results.

Polyphenols content

The polyphenols concentration in shikuwasa vinegar extract was determined using the Folin–Ciocalteu colorimetric method (Furuta et al. 1998). The shikuwasa vinegar samples (50 uL) were mixed with 50 μL of the Folin–Ciocalteu reagent (Wako Pure Chemical, Japan) and 50 μL of 5% Na_2CO_3 solution, and the absorbance was measured at 750 nm after 20 min of mixing at room temperature. The polyphenols contents are expressed as equivalents of mg per L gallic acid.

Measurement of titratable acidity

Ten milliliter of vinegar extract was mixed with 100 mL of ultrapure water produced by RFU424BB (Advantec), which was titrated with 0.5 $mol \cdot L^{-1}$ NaOH until pH 8.2 ± 0.3 using a pH meter by acid-base titration (Hashimoto et al. 2008).

Oxygen radical absorbance capacity assay

Oxygen radical absorbance capacity (ORAC) assay values were evaluated as described in a previous publication (Mikami et al. 2009). In short, 110.7 $nmol \cdot L^{-1}$ fluorescein sodium salt (Sigma-Aldrich, Japan) solution and 31.7 $mmol \cdot L^{-1}$ AAPH 2,2-azobis (2-amidinopropane) solution were prepared in 75 $mmol \cdot L^{-1}$ phosphate buffer solution (pH 7.4). After dilution with the assay buffer, the vinegar extract was subjected to the ORAC assay. Diluted vinegar extract and fluorescein solution were applied to a microplate. After a 10-min incubation at 37°C, fluorescence (excitation 485 nm; emission 528 nm) was recorded for the first time in a Multi-Detection Microplate Reader SH-9000 (Corona, Japan) equipped with a temperature-controlled incubation chamber.

The microplate was taken out of the SH-9000, and AAPH solution was added to the microplate, after which the plate was placed in the SH-9000 again. Fluorescence (excitation 485 nm; emission 528 nm) was then recorded every 2 min over 1.5 h using the SF6 software. Three duplicates of the measurements were expressed as μmol of Trolox equivalents (TE) per 100 mL of vinegar extract (μmol of TE per 100 mL).

Polymethoxyflavone analysis

Polymethoxyflavone (PMF) extraction was performed as previously described (Ichinokiyama et al. 2012). For PMF extraction from shikuwasa fruits, 100 mg of freeze-dried powder sample with 1 mL of methanol:DMSO (1:1) were subjected to ultra-sonic wave for 10 min (M1800-J, Yamato science) and centrifuged at 2000 g for 2 min. This was performed three times and extract solutions were obtained by diluting to a volume of up to 5 mL. For PMF extraction from vinegar extract, a volume of 1 mL of vinegar extract mixed with 1 mL of ethanol was subjected to an ultrasonic wave for 30 min (M1800-J, Yamato Scientific, Japan) and the insoluble constituent was removed by centrifugation at 2000 g for 2 min. Sample solutions were prepared for HPLC after the above extract solution had been filtered (0.20 μm hydrophilic PTFE from Advantec). Sinensetin (Wako Pure Chemical), nobiletin, and tangeretin (Sigma-Aldrich, Japan) were dissolved in ethanol to a concentration of 0.1 $mg \cdot mL^{-1}$ respectively as standards. A quantitative analysis of each PMF was performed as previously described (Hirose et al. 2017a). A volume of 5 μL of the sample solutions was injected onto a Union UK-C18 HPLC column (3 × 100 mm, Imtakt, Japan) at a flow rate of 0.4 $mL \cdot min^{-1}$ at 40 °C. The solvent used was acetonitrile/water/trifluorosetic acid (50/50/0.05). Analyses were monitored at a wavelength of 340 nm using an LC-20A UV detector (Shimadzu). PMF levels were calculated as the sum of sinensetin, nobiletin, and tangeretin.

Limonoid analysis

Limonoid extraction was performed as previously described (Hirose et al. 2017a). For limonoid extraction from shikuwasa fruits, 100 mg of freeze-dried powder sample was mixed with 2 mL of acetic acid and 5 mL of ethyl acetate then vortexed for 1 min. For limonoid extraction from vinegar extracts, 0.4 mL of vinegar extract was mixed with 1 mL of ethyl acetate then vortexed for 1 min. Ethyl acetate phase was collected after centrifugation at 2000 g for 2 min. These steps were repeated three times and the supernatant was completely evaporated under reduced pressure. The extract solution was obtained by reconstituting with 0.4 mL of acetonitrile.

Sample solutions for HPLC were prepared after the above extract solution had been filtered (0.20 μm, hydrophilic PTFE from Advantec). Limonin (Wako Pure Chemical) was dissolved in acetonitrile to a concentration of 0.1 $mg \cdot mL^{-1}$ respective to their standards. Quantitative analysis of limonin was performed as previously described (Hirose et al. 2017a). A volume of 2 μL of the sample solutions were injected onto Cadenza CD-C18 HPLC column (3 × 100 mm, Imtakt) using a flow rate of 0.4 $mL \cdot min^{-1}$ at 40 °C. The solvent was acetonitrile/water/formic acid (40/60/0.1). Analyses were monitored at a wavelength of 210 nm using an LC-20A UV detector (Shimadzu).

Flavor analysis

Flavor components were evaluated as previously described (Hirose et al. 2017b). Using 1.0 mL vinegar extract, the insoluble constituent was removed by centrifugation at 2000 g for 2 min. Next, 1 mL sample was placed in a vial, and 50 μL 0.1% n-hexanol (special grade, Wako Pure Chemical) was added as an internal standard. Monotrap monolithic silica adsorbent (MonoTrap RGPS TD from Gl Sciences, Tokyo, Japan) was used to trap the odor component out of contact. Flavor components were trapped by keeping the vial heated to 50 °C for 1 h. Flavor components in the adsorbent were injected by split ratio of 1:10 onto a InertCap Pure WAX ProG 2M column (0.25 mm × 60 m, df = 0.25 μm, Gl Sciences) at a flow rate of 1.0 $mL \cdot min^{-1}$ helium at 40 °C (5 min) − (6 °C per min) − 250 °C in the GCMS-QP2010 Ultra (Shimadzu) system using thermal desorption from an OPTIC-4 multimode inlet unit (Gl Sciences, Netherlands).

As MS conditions, ion source and interface temperature were 200 °C and 250 °C and ionization method was EI at a voltage of 70 eV. Based on the data obtained from GCMS, the NIST11 Mass Spectrum Library (National Institute of Standards and Technology, USA) and FFNSC 2 Flavor and Fragrance Natural and Synthetic Compounds GCMS Library (Chromaleont, Italy) were used to identify the odor components. Results were reported as mean values of peak area ratio to an internal standard. Monoterpenes represent the sum of α-pinene, β-myrcene, and limonene. Monoterpene alcohols represent the sum of linalool, terpinen-4-ol, and α-terpineol.

Measurements of CIELAB color space

Vinegar extract was diluted 10 times with distilled water as a sample for measurement. The CIELAB color space of the diluted vinegar samples was measured using a CM-2600d spectrophotometer (Konica Minolta, Japan) using a D65 illuminant with 10° observer angle. Calibration was conducted according to the manufacturer protocol. Data were expressed in terms of L*, a*, and b* (Stintzing et al. 2003).

Sensory evaluation

The vinegar extracts were diluted 10 times with distilled water and caster sugar was added at the concentration of 4% (w/w) for taste tests. Six men and women, ages 20–50, evaluated the shikuwasa aroma, bitterness, green smell, and general flavor on a scale of 1 to 5 (Hanagasaki 2021). For the aroma, 5 represented the strongest shikuwasa aroma and 1 represented the weakest shikuwasa aroma or the strongest vinegar aroma. For bitterness, 5 represented the lowest bitterness and 1 represented the strongest bitterness. For green smell, 5 represented the lowest green smell and 1 represented the strongest green smell. For general flavor, 5 represented "like" and 1 represented "dislike".

Statistical analysis

The Tukey–Kramer test was used for statistical analysis and was run in Excel statistics 2012 (Social Survey Research Information).

RESULTS AND DISCUSSION

Shikuwasa samples

The Ishikunibu fruits tended to be smaller than the 'Kugani' fruits (Table 1). The fruit color of both genotypes was mainly green when harvested in December, then turned orange as the fruits ripened. In February, both genotypes were ripe, and their color was orange (Fig. 1).

Changes in chemical components of shikuwasa fruits

PMF and limonin in Ishikunibu fruits significantly decreased from December to February. As for the 'Kugani' fruits, PMFs, sinensetin, and tangeretin were significantly lower in February, but limonin was significantly higher in February (Table 2). According to Hanagasaki (2021), PMFs of 'Kugani' in December and February was approximately half of the value in August. Every PMF was higher in the 'Kugani' than the Ishikunibu fruits in December and February. Further, limonin in the Ishikunibu was lower than that in the 'Kugani' fruit in February and vice versa in December (Table 2).

Acidic components of vinegar extracts

The vinegar volume showed no significant difference across genotypes and harvesting time (Table 3). The titratable acidity and ascorbic acid contents in vinegar extract did not differ between genotypes and harvesting times, except that the titratable acidity of extract with 'Kugani' harvested was lower than that with Ishikunibu in February (Table 3).

Functional components of vinegar extracts

Polyphenols contents in vinegar extract did not differ between genotypes and harvesting times. ORAC assay can be used to measure antioxidant capacity (Mikami et al. 2009). Here, ORAC values did not show significant differences across genotypes and harvesting time (Table 3). The vinegar extracts had ORAC values ranging from 4.9 to 7.1 μmol TE·mL^{-1}. The PMF ORAC values are not high, as they only contain a methoxy group and lack a hydroxy group. For instance, the ORAC value of nobiletin was about 1.45 mmol TE·g^{-1}.

A nobiletin content of 7.3 mg·100 mL^{-1} in the sample from Ishikunibu harvested in February was equivalent to only 0.11 μmol TE·mL^{-1} of the total ORAC value of 5.7 μmol TE·mL^{-1}. Therefore, other components, such as polyphenols, contributed to the ORAC activity to a greater extent. The vinegar extracts showed a total polyphenol content ranging from 158.1 to 191.7 μg·mL^{-1} (Table 3). The polyphenols content and the ORAC value in extracts with Ishikunibu were higher than those with 'Kugani' harvested in December and February, yet there were no significant differences. Every PMF in vinegar extracts with 'Kugani' was significantly higher than those with Ishikunibu harvested in both December and February (Table 3).

Flavor of vinegar extracts

The limonin level in vinegar extracts with Ishikunibu was lower than that in 'Kugani' samples in both terms of harvesting, although differences were not significant because of the variance in the values (Table 3). Limonin levels were scattered because they depend on whether the sample includes more seeds. However, the bitterness of the vinegar extract made with Ishikunibu was rated "higher" than the extracts with 'Kugani' harvested in February, i.e., the vinegar extract with Ishikunibu tasted less bitter (Table 3). The monoterpene contents showed no significant differences among all categories (Table 3). However, monoterpene alcohols in the vinegar extracts made with Ishikunibu harvested in December and February were significantly higher than those with 'Kugani' (Table 3). Monoterpene alcohols are known to be important contributors to the citrus aroma as well as the sweet floral and herbal resinous aromas (Choi et al. 2002). The shikuwasa aroma and green-smell ratings of the vinegar extract made with Ishikunibu harvested in February were higher than those with 'Kugani', yet the difference was insignificant (Table 3).

Appearance of vinegar extracts

Interestingly, the vinegar extract made with Ishikunibu looked different and was more transparent and more brilliant when compared with that made with 'Kugani' (Fig. 2). The clarity of the vinegar extract improved because albedo or other parts of the Ishikunibu harvested in February might not contain substantial pectin, cellulose, and hemicellulose, which are nearly insoluble fibers (Rafiq et al. 2018). Ishikunibu harvested in February was fresher and had less fruit puffing owing to its late-ripening feature. Since the Ishikunibu harvested in February had a deep orange color (Fig. 1), the vinegar extract with the fruits harvested in February exhibited a deep and striking color (Fig. 2). Ishikunibu is known to contain a considerable amount of β-cryptoxanthin; the β-cryptoxanthin content in Ishikunibu was the highest with respect to the seven genotypes native to Okinawa when averaged, based on the harvests obtained in 2013 and 2014 (Sugawara et al. 2017). Additionally, Ishikunibu had more β-cryptoxanthin and β-carotene contents than 'Kugani' and 'Nakamoto Seedless' (Hanagasaki 2021) harvested in February 2018 (Mitsube & Sugawara 2021). Cryptoxanthin is a red crystalline solid with a metallic luster in its pure form. Further, the a* value generally represents the degree of red, which was significantly higher in the vinegar extract with Ishikunibu harvested in February than in that made with 'Kugani' (Table 3). Therefore, the Ishikunibu fruit, which was deep orange (Fig. 1), most likely contributed to the bright color of the vinegar extract because of its β-cryptoxanthin content (Sumiasih et al. 2018).

Advantage of vinegar extracts

β-cryptoxanthin performs various functions and has been reported to prevent the development of metabolic syndrome (Sugiura et al. 2015a), type-2 diabetes (Sugiura et al. 2015b), and the early pathogenesis of non-alcoholic liver disease (Sugiura et al. 2016). Vinegar is effective for treating fatigue and promoting health (Miyagi et al. 2013), and the vinegar extracts in this study contained different amounts of nutritional components. Therefore, using wild genotypes of shikuwasa, such as Ishikunibu, can yield beneficial vinegar extracts with increased sourness.

Figure 1. Photos of the Ishikunibu (left) and 'Kugani' (right) fruit harvested in December (upper row) and February (bottom row)

Figure 2. Photographs of the vinegar extracts diluted 10 times with distilled water (from left to right, the shikuwasa vinegar extracts made from Ishikunibu and 'Kugani' in December, Ishikunibu and 'Kugani' in February)

Table 1. Characteristics of Ishikunibu and 'Kugani' harvested in December and February

Harvesting date	Variety	Fruit weight (g)	Fruit diameter (cm)	Moisture content (%)
December 13, 2018	Ishikunibu	29.7	4.27	85.6
	Kugani	43.6	4.66	87.0
February 18, 2019	Ishikunibu	33.6	4.95	85.5
	Kugani	42.6	5.15	84.6

Table 2. Chemical components of Ishikunibu and 'Kugani' harvested in December and February

Harvest month	December		February	
Variety	Ishikunibu	Kugani	Ishikunibu	Kugani
PMFs (mg per g of DW)	1.97 ± 0.02^{aA}	4.20 ± 0.18^{bA}	1.34 ± 0.02^{aB}	3.70 ± 0.05^{bB}
Sinensetin	0.19 ± 0.00^{aA}	0.28 ± 0.1^{bA}	0.14 ± 0.01^{aB}	0.26 ± 0.01^{bB}
Nobiletin	1.16 ± 0.01^{aA}	2.49 ± 0.11^{bA}	0.75 ± 0.02^{aB}	2.33 ± 0.03^{bA}
Tangeretin	0.61 ± 0.01^{aA}	1.43 ± 0.06^{bA}	0.45 ± 0.01^{aB}	1.11 ± 0.02^{bB}
Limonin (mg per g of DW)	1.48 ± 0.01^{aA}	1.32 ± 0.02^{bB}	1.21 ± 0.01^{aB}	1.41 ± 0.02^{bB}

Data represent mean ± SE, n = 3; Small letters among the same harvest date indicate significant differences between varieties by Tukey–Kramer's Test ($P < 0.05$); Capital letters indicate significance by Tukey–Kramer's test ($P < 0.05$), compared with the same variety in the other month; PMFs represent the sum of sinensetin, nobiletin, and tangeretin; DW – dry weight

Table 3. Characteristics of vinegar extract from Ishikunibu and 'Kugani' harvested in December and February

Harvest month	December		February	
Variety	Ishikunibu	Kugani	Ishikunibu	Kugani
Vinegar volume (mL)	33.2±0.4^{aA}	35.5±1.5^{aA}	36.7±1.4^{aA}	33.5±0.7^{aA}
Titratable acidity (%)	4.70±0.1^{aA}	4.56±0.1^{aA}	4.83±0.1^{aA}	4.56±0.0^{bA}
Ascorbic acid (μg per mL)	69.4±15.1^{aA}	41.0±1.3^{aA}	115.2±5.2^{aA}	38.9±2.2^{bA}
Polyphenols (μg per mL)	185.1±2.0^{aA}	163.9±7.8^{aA}	191.7±8.9^{aA}	158.1±7.4^{aA}
ORAC (μmol TE^{x} per mL)	7.1±0.1^{aA}	4.9±0.8^{aA}	5.7±0.4^{aA}	5.1±0.4^{aA}
PMFs (mg per 100 mL)	14.7±1.8^{aA}	33.4±0.2^{bA}	12.5±1.8^{aA}	29.0±0.7^{bB}
Sinensetin	1.5±0.2^{aA}	2.5±0.1^{bA}	1.2±0.2^{aA}	2.0±0.1^{bB}
Nobiletin	8.9±1.4^{aA}	20.8±0.6^{bA}	7.3±1.1^{aA}	19.2±0.8^{bA}
Tangeretin	4.3±0.5^{aA}	10.0±0.6^{bA}	4.0±0.6^{aA}	7.8±0.3^{bB}
Limonin (mg per 100 mL)	17.9±5.4^{aA}	46.4±7.4^{aA}	8.7±3.9^{aA}	28.3±5.1^{aA}
Monoterpenes (IS%)^{y}	9.1±3.3^{aA}	6.5±1.1^{aA}	5.1±1.4^{aA}	7.8±1.6^{aA}
β-myrcene	0.3±0.2^{aA}	0.2±0.1^{aA}	0.1±0.1^{aA}	0.2±0.1^{aA}
Limonene	3.8±1.5^{aA}	2.2±0.5^{aA}	2.2±0.6^{aA}	2.9±0.6^{aA}
γ-terpinen	3.5±1.3^{aA}	2.7±0.4^{aA}	2.1±0.6^{aA}	3.3±0.7^{aA}
p-cymene	1.4±0.7^{aA}	1.4±0.3^{aA}	0.7±0.3^{aA}	1.5±0.4^{aA}
Monoterpene alcohols (IS%)^{y}	1.3±0.1^{aA}	0.5±0.1^{bA}	0.6±0.2^{aB}	0.1±0.1^{bB}
Linalool	0.6±0.1^{aA}	0.1±0.1^{bA}	0.1±0.1^{aB}	0.0±0.0^{bA}
Terpinen-4-ol	0.5±0.1^{aA}	0.3±0.1^{bA}	0.3±0.1^{aA}	0.1±0.1^{aB}
α-Terpineol	0.3±0.1^{aA}	0.1±0.1^{bA}	0.1±0.1^{aB}	0.0±0.0^{bB}
Sensory evaluation				
Shikuwasa aroma	2.67±0.32^{a}	3.00±0.28^{a}	3.00±0.23^{a}	2.44±0.28^{a}
Green smell	4.56±0.23^{a}	3.67±0.39^{a}	3.67±0.39^{a}	3.44±0.23^{a}
Bitterness	4.00±0.36^{ab}	3.44±0.32^{ab}	4.33±0.28^{a}	3.33±0.28^{b}
General flavor	3.44±0.28^{a}	3.78±0.21^{a}	3.78±0.27^{a}	3.67±0.23^{a}
CIELAB color space				
L*	45.6±0.3^{aA}	51.6±1.1^{bA}	44.6±0.8^{aA}	48.9±0.9^{bA}
a*	0.3±0.4^{aA}	-3.0±0.2^{bA}	5.9±1.3^{aB}	-1.0±0.4^{bB}
b*	23.4±0.9^{aA}	26.1±1.3^{aA}	29.9±1.6^{aB}	28.4±0.3^{aA}

Data represent mean ± SE, n = 3 (except sensory evaluation); Small letters among the same harvest date indicate significant differences between varieties (except sensory evaluation) by Tukey–Kramer's Test ($P < 0.05$); Capital letters indicate significance by Tukey–Kramer's test ($P < 0.05$), compared with the same variety in the other month (except sensory evaluation); For sensory evaluation, small letters indicate significant differences between all vinegar samples within column; x represents Trolox equivalent; PMFs represent the sum of sinensetin, nobiletin and tangeretin; y represents the peak area ratio to the internal standard of 1-hexanol; Monoterpenes represent the sum of β-myrcene, limonene, γ-terpinen, and p-cymene; Monoterpene alcohols represent the sum of linalool, terpinen-4-ol, and α-terpineol

CONCLUSION

Wild shikuwasa, Ishikunibu, contains a considerable amount of β-cryptoxanthin, features dwarf fruits, and tastes sour. Comparison of vinegar extracts obtained with the addition of already-known 'Kugani' and the Ishikunibu studied here does not show differences in the chemical components and sensory characteristics. Therefore, vinegar production with using Ishikunibu could find a niche in the market. Since opinions about the health and cosmetic value of fruit vinegar are growing more favorable, experiments on processing technology should be continued and research on other fruits characteristic of a given geographical location should be expanded.

Acknowledgments

Author would like to thank Mr. Mitsube for providing shikuwasa fruits, Mr. Hirose of Okinawa Industrial Technology Center for his advice, and Mr. Kadekawa and Mr. Yogi of the Okinawa Agricultural Research Center for their excellent technical assistance.

REFERENCES

Choi H.S., Sawamura M., Kondo Y. 2002. Characterization of the key aroma compounds of *Citrus flaviculpus* Hort. ex Tanaka by aroma extraction dilution analysis. Journal of Food Science 67(5): 1713–1718. DOI: 10.1111/j.1365-2621.2002.tb08711.x.

Furuta S., Suda I., Nishiba Y., Yamakawa O. 1998. High *tert*-butylperoxyl radical scavenging activities of sweet potato cultivars with purple flesh. Food Science and Technology International 4(1): 33–35. DOI: 10.3136/fsti9596t9798.4.33.

Hanagasaki T., Hirose N., Maeda G., Onda S., Wada K. 2019. Vinegar extract of fruit waste from juice production using tankan (*Citrus tankan* Hayata) native to Okinawa, Japan. Food Science and Technology Research 25(5): 667–676. DOI: 10.3136/fstr.25.667.

Hanagasaki T. 2021. Vinegar extraction from unripe shikuwasa (*Citrus depressa* L.), an Okinawan citrus fruit. Foods and Raw Materials 9(2): 310–316. DOI: 10.21603/2308-4057-2021-2-310-316.

Hanagasaki T. 2022. Vinegar extraction from the acerola fruit (*Malpighia emarginata*) cultivated in Okinawa, Japan. Fruits 77(2): 1–6. DOI: 10.17660/th2022/007.

Hashimoto Y., Chuda Y., Suzuki T., Yasui A. 2008. Method validation for determination of total acid in vinegar based on potentiometric titration by interlaboratory study. Bunseki Kagaku 57(6): 453–459. DOI: 10.2116/bunsekikagaku.57.453. [in Japanese with English abstract]

Hirose N., Maeda G., Onda S., Shoda M., Miyagi K., Wada K., Ohta H. 2017a. Development of vinegar extract from the waste peels of Shiikuwasha. Nippon Shokuhin Kagaku Kogaku Kaishi 64(2): 81–89. DOI: 10.3136/nskkk.64.81. [in Japanese with English abstract]

Hirose N., Maeda G., Miyagi K., Wada K., Ohta H. 2017b. Simplified analysis of flavor in Shiikuwasha products using monolithic silica adsorbents "MonoTrap" and GC-MS. Technical Report, C146-E335, Shimadzu. www.glsciences.eu/monotrap/app/td/monotrap-shiikuwasha.pdf

Ichinokiyama H., Maegawa T., Goto M. 2012. Flavonoid contents of whole fruit and various tissues of a new acid citrus, 'Niihime'. Horticultural Research 11(3): 387–391. DOI: 10.2503/hrj.11.387. [in Japanese with English abstract]

Inafuku-Teramoto S., Yamamoto M., Kinjyo H., Kitajima A., Wada K., Kawamitsu Y. 2010. Local citrus genetic resources and their polymethoxyflavones content in northern part of Okinawa Island. Horticultural Research 9(3): 263–271. DOI: 10.2503/hrj.9.263. [in Japanese with English abstract]

Ishikawa R., Badenoch N., Miyagi K., Medoruma K., Osada T., Onishi M. 2016. Multi-lineages of Shiikuwasha (*Citrus depressa* Hayata) evaluated by using whole chloroplast genome sequences and its bio-diversity in Okinawa, Japan. Breeding Science 66(4): 490–498. DOI: 10.1270/jsbbs.15151.

Mikami I., Yamaguchi M., Shinmoto H., Tsushida T. 2009. Development and validation of a microplate-based β-carotene bleaching assay and comparison of antioxidant activity (AOA) in several crops measured by β-carotene bleaching, DPPH and ORAC assays. Food Science and Technology Research 15(2): 171–178. DOI: 10.3136/fstr.15.171.

Mitsube F., Sugawara T. 2021. Seasonal variation of polymethoxyflavones and carotenoids contained in Shiikuwasa fruit (*Citrus depressa*, Hayata). Research for Tropical Agriculture 14(2): 3–4. [in Japanese]

Miyagi K., Arakaki E., Teruya R., Wada K., Ohta H., Hirose N. 2013 Extraction of nobiletin and synephrine from the waste peels of Shiikuwasha (*Citrus depressa* Hayata) by using vinegar. Food Preservation Science 39(6): 337–341. DOI: 10.5891/jafps.39.337. [in Japanese with English abstract]

Rafiq S., Kaul R., Sofi S.A., Bashir N., Nazir F., Nayik G.A. 2018. Citrus peel as a source of functional ingredient: A review. Journal of the Saudi Society of Agricultural Sciences 17(4): 351–358. DOI: 10.1016/j.jssas.2016.07.006.

Stintzing F.C., Schieber A., Carle R. 2003. Evaluation of colour properties and chemical quality parameters of cactus juices. European Food Research and Technology 216(4): 303–311. DOI: 10.1007/s00217-002-0657-0.

Sugawara T., Nishiba Y., Medoruma K., Matsumura M. 2017. Varietal differences in β-cryptoxanthin content of citrus fruits native to Okinawa. 80th Meeting of the Association of the Kyusyu Agricultural Research Institution. [in Japanese]

Sugiura M., Nakamura M., Ogawa K., Ikoma Y., Yano M. 2015a. High serum carotenoids associated with lower risk for the metabolic syndrome and its components among Japanese subjects: Mikkabi cohort study. British Journal of Nutrition 114(10): 1674–1682. DOI: 10.1017/s0007114515003268.

Sugiura M., Nakamura M., Ogawa K., Ikoma Y., Yano M. 2015b. High-serum carotenoids associated with lower risk for developing type 2 diabetes among Japanese subjects: Mikkabi cohort study. BMJ Open Diabetes Research and Care 3(1); e000147; 8 p. DOI: 10.1136/bmjdrc-2015-000147.

Sugiura M., Nakamura M., Ogawa K., Ikoma Y., Yano M. 2016. High serum carotenoids are associated with lower risk for developing elevated serum alanine aminotransferase among Japanese subjects: Mikkabi cohort study. British Journal of Nutrition 115(8): 1462–1469. DOI: 10.1017/s0007114516000374.

Sumiasih I.H., Poerwanto R., Efendi D., Agusta A., Yuliani S. 2018. β-cryptoxanthin and zeaxanthin pigments accumulation to induce orange color on citrus fruits. IOP Conference Series: Materials Science and Engineering 299; 012074; 8 p. DOI: 10.1088/1757-899x/299/1/012074.

Takebe M., Yoneyama T. 1995. An analysis of nitrate and ascorbic acid in crop exudates using a simple reflection photometer system. Japanese Journal of Soil Science and Plant Nutrition 66(2): 155–158. DOI: 10.20710/dojo.66.2_155. [in Japanese]