Flavor Characteristics and Biological Functions of Okinawan Sugary and Citrus Materials

沖縄特産の甘味資源および柑橘資源のフレーバー特性ならびに機能性

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Abstract

Okinawa is the only prefecture that is located in the subtropical region in Japan, and has various crop and horticultural products that are different from other prefectures. Flavor characteristics and biological functions of food materials from sugary and citrus resources in Okinawa, Japan, i.e. sugarcane (*Saccharum officinarum* L.) and Shiikuwasha (*Citrus depressa* Hayata), were studied, with the intention of fostering the food and nutraceutical applications of these two economical Okinawan crops.

The composition and content differences of sugarcane wax in different sugarcane cultivars, including policosanol (a mixture of bioactive long-chain alcohols) and long-chain aldehyde were investigated. Sugarcane waxes were comprised of 55–60% aldehyde and sterol esters, and 32–40% policosanol. The composition and content of waxy materials may vary depending on sugarcane cultivar, specific part, and degree of maturity.

The potent antioxidant activity of sugarcane molasses fractions against peroxyl radicals was evaluated with chemical, cellular, DNA/biomolecular model systems. Ten phenolic constituents were identified in the fractions, such as ferulic acid, schaftoside, *p*-coumaric acid, and *p*-hydroxybenzaldehyde.

Changes in the physicochemical characteristics, aroma compounds, and Maillard reaction products (MRPs) of cane brown sugar were monitored over a 1-year storage period. The cane brown sugar lost its acidic and sulfuric odors; subsequently, the nutty and roasted aroma increased due to the volatile MRPs during storage. The browning rate of stored cane brown sugar was positively associated with the development of MRPs.

Volatile aroma composition and antioxidant activity of unripe Shiikuwasha peel oils of different extraction methods were investigated. The major constituents of the oils were limonene (43–45%) and γ-terpinene (28–29%). The cold-press extraction system may better retain phenolics of the peel and display superior antioxidant capabilities, compared to the steam distillation extraction method.

The composition and content differences of volatile aroma components, flavanones, and polymethoxylated flavones (PMFs) in Shiikuwasha peels of four cultivation lines were evaluated. The peel oils composed mainly of limonene (47−68%) and γ-terpinene (21−31%). Neohesperidin (97%) was the predominant flavanone in 'Izumi kugani' peel, while the other peels had high hesperidin contents (89−99%). Moreover, the PMFs of Shiikuwasha peels were composed of nobiletin and tangeretin.

The effect of cultivation line and peeling on food composition, taste characteristic, aroma profile, and antioxidant activity of peeled flesh and whole fruit juices of Shiikuwasha from four cultivation lines were distinguished. Shiikuwasha juice from these lines had diverse food components. The composition of volatile aroma components was influenced by fruit cultivation line, whereas its content was affected by peeling process. Peeling also caused Shiikuwasha juice to be less astringent and acidic bitter and to lose its antioxidant activity.

Sugarcane and Shiikuwasha that possess distinctive profiles of flavor components and biological functions are highly valuable food crops in Okinawa Prefecture. More food and agricultural chemical characterizations of these sugary and citrus materials revealed should promote their food, biotechnological or nutraceutical commercial uses, and therefore should endorse agribusiness development of the region.

要約

日本で唯一亜熱帯地域に位置する沖縄では，サトウキビをはじめ，果樹や野菜など他府県とは異なる多種多様な農作物が生産されている。本研究では，沖縄特産の甘味資源であるサトウキビ（*Saccharum officinarum* L.）および柑橘資源であるシークヮーサー（*Citrus depressa* Hayata）からの食品素材のフレーバー特性ならびに機能性を分析し，評価した。

種々のサトウキビ品種のワックス組成とその含量ならびにポリコサノール（高級脂肪族アルコール群）と相当する長鎖アルデヒドを分析した結果，サトウキビワックスの主要な構成成分は 55–60％のアルデヒドとステロールエステル，32–40％のポリコサノールであった。また，ワックスの組成や含量はサトウキビの品種，部位および生育ステージの影響を大きくうけることを明らかにした。

サトウキビ糖蜜およびその分画物のペルオキシラジカルに対する抗酸化活性を，常法のラジカル消去活性試験，細胞内抗酸化試験および DNA 損傷抑制試験により評価するとともに, 抗酸化活性を有するフェノール化合物として ferulic acid，schaftoside，*p*-coumaric acid，*p*-hydroxybenzaldehyde をはじめとする 10 個の化合物を単離・同定した。

沖縄黒糖の貯蔵（1 年間）に伴う物理化学的特性，香気成分およびメイラード反応生成物（MRPs）の変化を分析した。その結果，貯蔵に伴いカルボン酸や含硫化合物由来のにおいは消失し, MRPs 由来のこおばしい香りが増加することが明らかとなった。また，貯蔵に伴う黒糖の色調の褐色化と MRPs の生成には高い正の相関があることを確認した。

未熟のシークヮーサー果皮から，コールドプレス法および水蒸気蒸留法により抽出した精油の構成成分と抗酸化活性を分析した。その結果，精油の主要な構成成分は，両精油とも limonene （43–45%）と γ-terpinene （28–29%）であった。一方，コールドプレス精油は水蒸気蒸留精油に比べて，フェノール化合物含量が高く，強い抗酸化活性を示した。

シークヮーサー4 系統（適熟果）の果皮の精油，フラバノンおよびポリメトキシフラボン（PMFs）の組成ならびにその含量を分析した結果，精油の主要成分はすべての系統で limonene （47–68%）と γ-terpinene （21–31%）であったが，その含量は系統間で大きく異なっていた。またフラバノンについては，’伊豆味クガニー’では neohesperidin（97%），その他の 3 系統では hesperidin（89−99%）が主要成分であり，系統間で組成が異なっていた。一方，主要な PMFs はすべての系統で nobiletin と tangeretin であった。

シークヮーサー4 系統（適熟果）の果肉果汁（果実を剥皮後搾汁）と全果果汁（果実丸ごとの搾汁）の化学成分，フレーバー特性および抗酸化活性を分析し，比較した。その結果，シークヮーサー4 系統の両果汁において，各化学成分の含量は異なっていた。また精油の構成成分とその含量は，それぞれ系統および果実の剥皮の影響を大きくうけていた。さらに，果肉果汁は渋味や苦味が減少するものの，抗酸化活性も大きく低下することが明らかとなった。

以上のように，本研究は，沖縄特産物であるサトウキビおよびシークヮーサーのフレーバー成分や機能性成分の基礎知見だけでなく，それらの成分に関する遺伝資源情報や加工利用特性にまで言及したものである。したがって，本研究で確立した分析手法や得られた解析データは，沖縄以外の亜熱帯地域の国々における特産資源の研究やその加工利用などにも広く応用できると考える。

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

AAPH	2,2′-azobis(2-amidinopropane) dihydrochloride
BHA	butylated hydroxyanisole
CAA	cellular antioxidant activity
CP	cold-press
CSS	cane separation system
3-DG	3-deoxyglucosone
DNA-DPC	DNA-damage protective concentration
DPPH	1,1-diphenyl-2-picrylhydrazyl
ELSD	evaporative light scattering detection
FID	flame ionization detection
GAE	gallic acid equivalents
GC	gas chromatography
HPLC	high performance liquid chromatography
ICUMSA	International Commission for Uniform Methods of Sugar Analysis
LLCE	liquid-liquid continuous extraction
MRP	Maillard reaction product
MS	mass spectrophotometry
NHDF	normal human dermal fibroblasts
NIST	National Institute of Standards and Technology
ORAC	oxygen radical absorbance capacity
PCA	principal component analysis
PMF	polymethoxylated flavone
RI	retention index
SAFE	solvent-assisted flavor evaporation technique
SD	steam distillation
SPME	solid-phase microextraction
TA	total titratable acidity
TE	Trolox equivalents
TSS	total soluble solid

List of Appendixes

Chapter I

General Introduction

1.1. Sugary materials in Okinawa

Sugarcane (*Saccharum officinarum* L.) is the main cultivation crop and source of sugary materials in Okinawa Prefecture, Japan. It occupies approximately 50% of the Okinawan agriculture area. The area of sugarcane cultivation, which includes several different cultivars, is 20,970 ha in Okinawa Island and surrounding remote islands, and this area represents approximately 63% of the nationwide sugarcane cultivation area.[1] Typical sugar content for mature sugarcane plant is 10% by weight but the figure is varied, depending on the cultivar, season, and location. Correspondingly, the yield of cultivated sugarcane from the field varies considerably but a rough overall value to use in estimating sugar production is 100 tons sugarcane/ha or 10 tons sugar/ha.[2]

Sugar is made by several plants, including sugarcane, to store energy that they do not need straight away. Scientifically, sugar refers to any monosaccharide, also called simple sugar, i.e. glucose, fructose, and galactose; or disaccharide, i.e. sucrose, maltose, and lactose.[3] In non-scientific use, the term sugar is used as a synonym for sucrose, also called table sugar, a white crystalline solid disaccharide. Sugarcane is primarily cultivated for commercial sugar production, particularly granulated white table sugar. A cane brown sugar, called kokuto, has also been traditionaly produced in Okinawa by non-centrifugal method, without molasses removing process. Moreover, the sugarcane manufacturing industry also yields many kinds of by-products, such as bagasse, molasses, and wax.

1.2. Citrus materials in Okinawa

Okinawa Prefecture has about 30 types of citrus plants, such as Shiikuwasha (*Citrus depressa* Hayata), Kabuchii (*C. keraji* Hort. ex Tanaka var. *kabuchii*), Keraji (*C. keraji* Hort. ex Tanaka), Oto (*C. oto* Hort. ex Yu. Tanaka), and Tokunibu (*C. nobilis* Lour.), that have been cultivated for several hundred years.[4] Besides that, due to agribusiness and commercial purposes, there are some introduced citrus cultivars grown in Okinawa Island, including known Tankan/T-132 (*C. tankan* Hayata), Izumibeni (*C. tangerine* Hort. ex Tanaka), and Shikikitsu (*C. madurensis* Lour.). Of these Okinawan citrus fruit species, Shiikuwasha is considered to be one of the most popular local citrus fruit, and there is currently an upsurge of interest in exploration of the biological functions of this citrus cultivar used in food.

Shiikuwasha grows naturally in the northern areas of Okinawa Island and is a typical food crop of this region. There are about 10 kinds of cultivation lines of Shiikuwasha, including 'Izumi kugani', 'Katsuyama kugani', 'Ogimi kugani', 'Kaachi', 'Ishikunibu', 'Hijyakunibu', 'Kabishi', and 'Fusubuta'.[5] The term “cultivation line” refers to particular plant that resulted from the repeated self-fertilization or inbreeding of a cultivar, as defined by the International Society for Horticulture Science.[6] 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' cultivation lines.[7]

Shiikuwasha has small yellowish-green fruits with a very sour taste and strong characteristic aroma. Processed food products derived from these fruits, such as beverages and confectionary products, are regarded not only as local products from Okinawa, but also as national, Japanese products. Shiikuwasha fruits are used in many

types of food industries. In particular, unripe Shiikuwasha fruits, which are usually harvested from September to October, are used as raw material for vinegar, acidulant, and flavour enhancer productions, while ripe fruits are harvested from November to December and the fruits are utilized mainly for juice processing and for consumption as fresh fruits.

1.3. Outline of the studies

The studies of flavor characteristics and biological functions were divided into two main works based on the sugary or citrus materials, as shown in **Figure 1-1**. The sugary materials used in the studies were sugarcane wax, molasses, and cane brown sugar. First, the compositional analysis of wax, policosanol, and long-chain aldehydes was evaluated in sugarcane cultivars. Policosanol is the common name that refers to a group of long-chain (C_{20}-C_{30}) aliphatic primary alcohols that are of great interest since their effects include reduction of platelet aggregation, prevention of atherosclerosis development, and so on.[8] The study detailed the wax composition, including that of policosanol and long-chain aldehydes, of sugarcanes of different cultivars, as well as specific parts of sugarcane and maturity stages (**Chapter II**).

Second, the potent biological ability of sugarcane molasses to scavenge 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced peroxyl radicals was investigated in chemical, cellular, and DNA/biomolecular levels. The study detailed the antioxidative phenolic components of methanolic fractions of sugarcane molasses, separated via bioassay-guided fractionation (**Chapter III**). Third, changes in the quality attributes of cane brown sugar, represented by physicochemical characteristics as well as flavor components and Maillard reaction products (MRPs), were evaluated every 3

months over a 1-year storage period (**Chapter IV**).

Regarding Okinawan citrus materials, the materials used were peel part and extracted juice of Shiikuwasha fruit. First, volatile aroma composition and antioxidant activity of unripe Shiikuwasha peel oils of different extraction methods were determined. The peel oils were extracted using steam distillation or a cold-press system (**Chapter V**). Second, peels of different Shiikuwasha cultivation lines were explored in terms of their usefulness as sources of essential oils and bioactive substances with biological functions and beneficial effects on human health. The study examined and quantified the composition of volatile aroma components, flavanones and polymethoxylated flavones (PMFs) in four Shiikuwasha cultivation lines (**Chapter VI**). Third, Shiikuwasha juice from four different cultivation lines subjected to two peeling practices (with or without peeling) were discriminated in terms of their food components, taste characteristics, aroma profiles, and antioxidant capabilities. The study also detailed the dominant active components responsible for antioxidant activity of Shiikuwasha juice (**Chapter VII**).

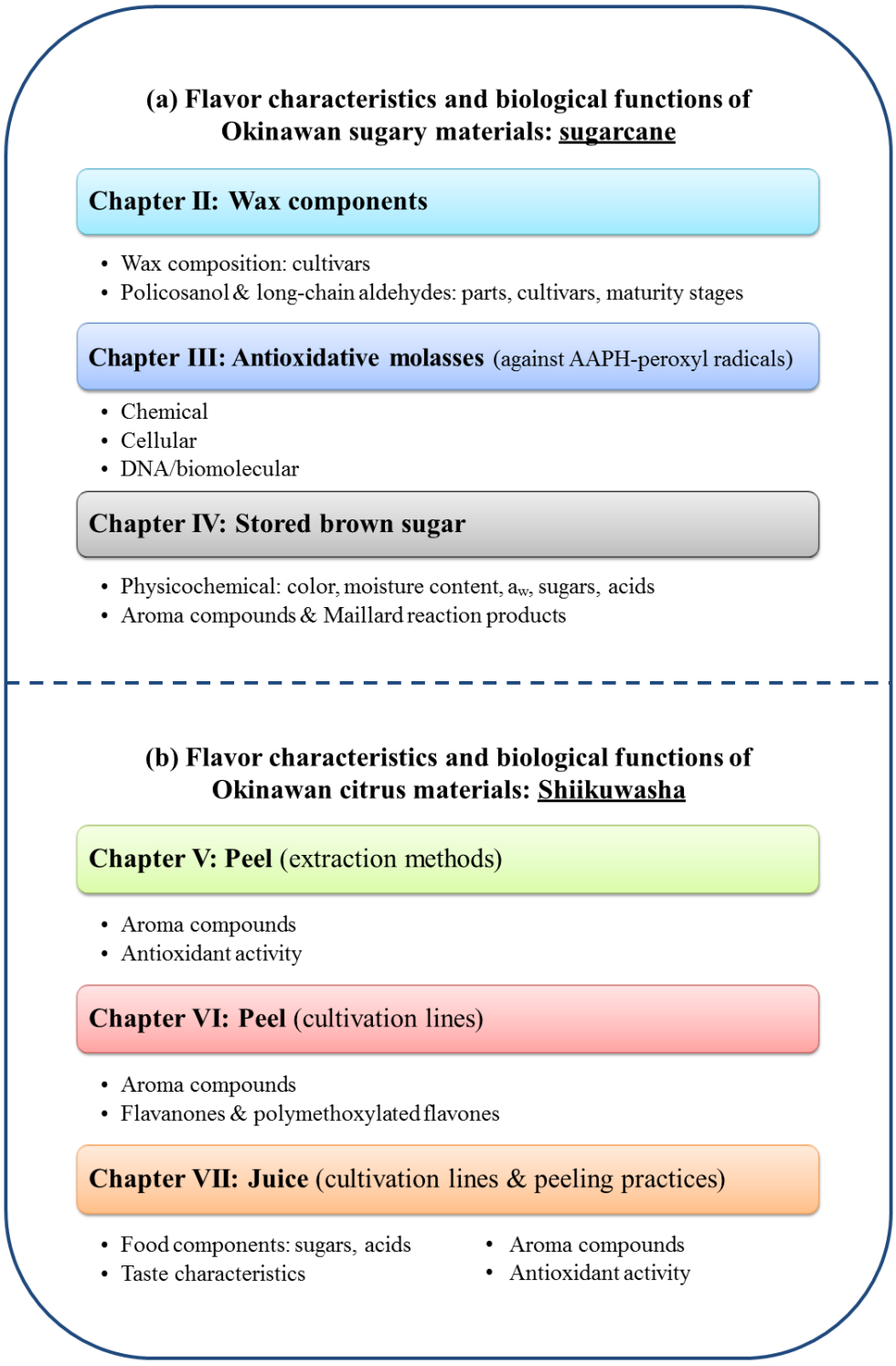

Figure 1-1. Research outlines of flavor characteristics and biological functions of Okinawan (a) sugary and (b) citrus materials.

1.4. Research objectives

The main objective of this research was to characterize the flavor characteristics and biological functions in Okinawan sugary and citrus materials, that are sugarcane and Shiikuwasha, so as to promote their agro-industrial applications. Some specific aims were applied in the studies, as follows:

a. To determine the composition and content of wax, policosanol, and long-chain aldehydes of sugarcane. Discrimination among sugarcane parts, cultivars, and maturity stages were also investigated (**Chapter II**).
b. To investigate the potent antioxidant capabilities of sugarcane molasses against AAPH peroxyl radicals in chemical, cellular, and DNA/biomolecular model systems (**Chapter III**).
c. To determine the physicochemical characteristics and flavor profiles of cane brown sugar during 1 year of storage (**Chapter IV**).
d. To determine the aroma components of the peel oil of unripe Shiikuwasha and its antioxidant capabilities. Discrimination of two extraction methods, i.e. cold-press and steam distillation methods, were also investigated (**Chapter V**).
e. To distinguish the four different Shiikuwasha cultivation lines based on the composition and content of their aroma components, as well as their flavanones and PMFs, extracted from the peel (**Chapter VI**).
f. To evaluate the effect of cultivation lines and peeling on food composition, taste characteristic, aroma profile, and antioxidant activity of Shiikuwasha juice (**Chapter VII**).

Chapter II

Wax, Policosanol, and Long-chain Aldehydes of Sugarcane Cultivars

2.1. Introduction

Sugarcane wax has been a matter of interest, due to its unique chemical composition, physical properties, and related industrial applications.[9,10] The biological activities of sugarcane wax, such as its ability to prevent atherosclerosis development, have also been studied.[11] Interestingly, long-chain (C_{20}-C_{30}) aliphatic primary alcohols that are derived from sugarcane wax and are known by the generic name policosanol, have also been a focus of much research due to their potential beneficial effects for human health. Several studies have reported that policosanol can reduce platelet aggregation and low-density lipoprotein levels in blood, and prevent atherosclerosis development and inhibit cholesterol synthesis.[12–14] Conversely, other research groups have failed to confirm nutraceutical effects of policosanol, and there is continuing controversy concerning the efficacy and mechanism of action of policosanol.[15,16] Nevertheless, policosanol is still considered to be a health-enhancing compound and interest in policosanol research is increasing. To date, the policosanol content and composition of many crop products and varieties, including sorghum, rice bran, wheat and peanuts has been investigated using various extraction methods.[17–20] Crude sugarcane wax has been reported to contain policosanol and is used as a major source of policosanol for nutritional supplement products.[21] However, differences in the policosanol content of different sugarcane cultivars have not been assessed.

In addition to policosanol, long-chain aldehydes are also major components of natural wax extracted from plants.[22] Straight chain aldehydes that are ubiquitous epicuticular wax components are responsible for the protection of plant leaves, and are lipid biomarkers of leaves and roots.[23,24] In contrast to the many reports regarding policosanol, there is very little information available regarding long-chain aldehydes in plants, particularly in sugarcane. Chemical compositions, including wax composition, of sugarcane crops vary depending on agronomic conditions, location, climate as well as cultivar. Thus, the study aimed to determine the wax composition, including the policosanol and long-chain aldehyde composition, of different sugarcane cultivars. This information may be useful for industrial production of these products, which may have many health benefits. Therefore, this study may provide information regarding the most useful sugarcane cultivar to grow in these areas for sugarcane wax or policosanol production.

In this study, the composition and content of wax, policosanol, and long-chain aldehyde of the rinds of several sugarcane cultivars were analyzed using various analytical techniques. The content of policosanol and long-chain aldehydes in different parts of the sugarcane stalk (the rind and the pith) were also examined and compared. In addition, changes in the content of policosanol and long-chain aldehydes in sugarcane rinds at two different stages of maturity were also carefully evaluated. The research flowchart can be briefly seen in **Figure 2-1**.

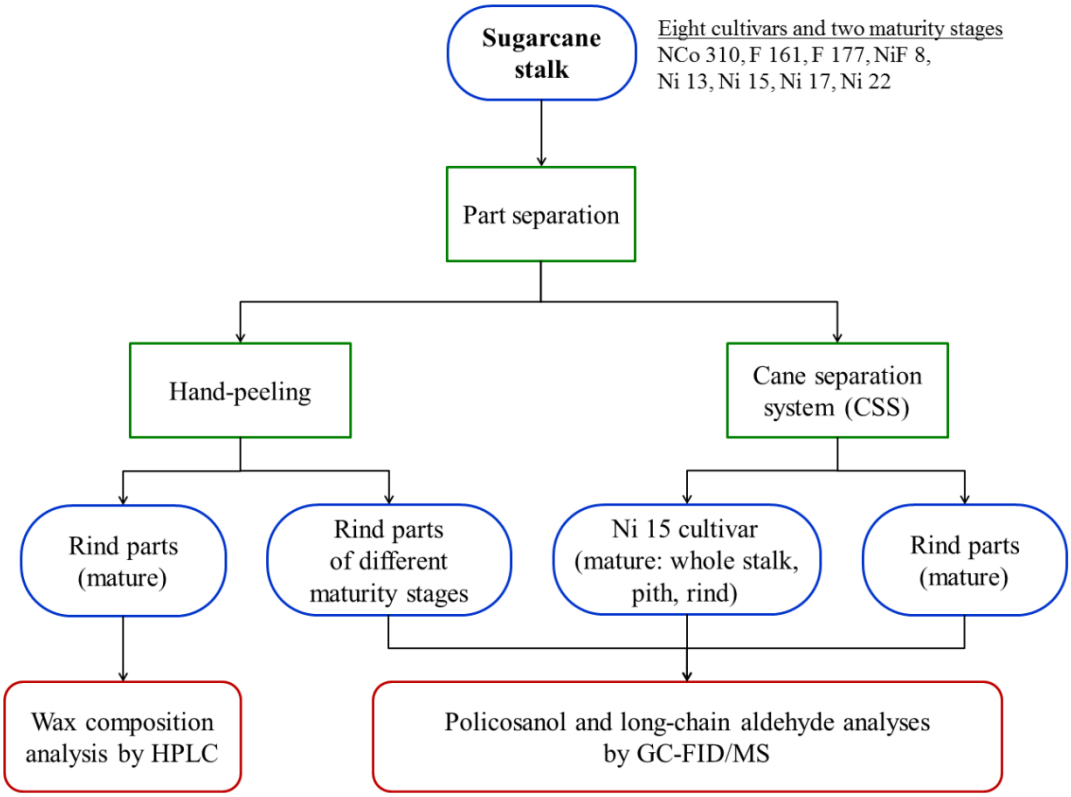

Figure 2-1. Research flowchart of wax, policosanol, and long-chain aldehydes of sugarcane cultivars.

2.2. Materials and methods

2.2.1. Standards and reagents

The policosanol standards docosanol (C_{22}-OH), tetracosanol (C_{24}-OH), hexacosanol (C_{26}-OH), octacosanol (C_{28}-OH), and triacontanol (C_{30}-OH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The derivatization reagent N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was obtained from GL Science (Tokyo, Japan). Pyridinium chlorochromate (Sigma-Aldrich) was used in the synthesis of long-chain aldehyde standards. Triacontane, cholesteryl oleate, lignoceric

acid, methyl palmitate, and stigmasterol were obtained from Sigma-Aldrich, and triolein was from Nakalai Chemicals (Kyoto, Japan). HPLC grade hexane (Wako Pure Chemical Industries, Osaka, Japan) and methyl *tert*-butyl ether (Kanto Chemicals Industries, Tokyo, Japan) were employed as mobile phases in high performance liquid chromatography (HPLC) analysis. All other reagents and chemicals were purchased from Wako Pure Chemical Industries and were of analytical grade unless otherwise specified.

2.2.2. Samples

Whole stalks of the Ni 15 sugarcane cultivar, and the rinds of seven different sugarcane cultivars (NCo 310, F 161, F 177, NiF 8, Ni 13, Ni 17 and Ni 22), were used as source materials. All cultivars were grown in the same field at Okinawa Prefectural Agricultural Research Center, Okinawa, Japan and the samples were collected at two different stages of maturity. Immature stalks (cultivated for 9 months) were obtained three months before the harvesting season and mature stalks (cultivated for 12 months) were obtained during the sugarcane harvesting season in January 2008. The outer layer rinds that coated with waxy materials and inner piths containing most of the sucrose of the sugarcane stalk were separated using a cane separation system (CSS) machine (Mitsubishi Engineering Corporation, Tokyo, Japan) or by hand peeling. In hand peeling process, rind samples were carefully trimmed from the sugarcane stalks and completely separated from soft white inner fibers, or pith, with sharp knives. All samples were crushed and ground using a dry blender and were then freeze-dried for 24 h, and kept dry at −30 °C until use.

2.2.3. Sample extraction

Briefly, 10 g of freeze-dried sugarcane rind, pith or whole stalk were placed in a thimble filter (Advantec No. 84, Tokyo, Japan) and extracted using a Soxhlet apparatus with approximately 150 mL of a mixture of hexane and methanol (20:1, v/v). Extraction time of 16 h was used for policosanol and long-chain aldehyde analysis in order to reach the optimum yields of these compounds, while for wax composition analysis, sample was extracted until obtaining a sufficient coloration of the solvent (during about 4 h). The solvent solution was completely removed from the extract using a rotary-evaporator under a vacuum at 40 °C leading to greenish-yellow wax extract. All extractions were performed in triplicate. All dry residue extract was diluted with toluene or chloroform to a volume that could be used for sample analysis.

2.2.4. Wax composition analysis using HPLC-ELSD

The wax standards used to represent the major constituents of sugarcane were: triacontane (hydrocarbon), octacosanol (alcohol), cholesteryl oleate (sterol ester), lignoceric acid (acid), methyl palmitate (methyl ester), stigmasterol (sterol), triolein (triacylglycerol), and synthesized octacosanal (aldehyde) – see **Section 2.2.5**. Standards were prepared in chloroform at different concentrations (0.05–0.50 mg/mL) that spanned the concentrations of wax constituents of the samples in HPLC analysis.

Wax components were separated and determined using a HPLC system (Shimadzu Corporation, Kyoto, Japan). The method of HPLC analysis was adapted from Adhikari et al.[25] with slight modification. The column used was a Luna silica column (250 mm × 4.6 mm i.d., 5-μm particle size) connected to a guard column (4 mm × 3 mm i.d.), (Phenomenex, Torrance, CA, USA). Two Shimadzu LC-10AD-VP pumps

were operated in combination using a Shimadzu SCL-10A-VP gradient controller. Both column and guard column were maintained at a constant temperature of 40 °C. An evaporative light scattering detector (ELSD, Shimadzu model LT) was operated at 50 °C with a nitrogen pressure of 350 kPa. Mobile phases were comprised of a gradient of hexane (solvent A) and methyl *tert*-butyl ether containing 0.2% acetic acid (solvent B), with the following gradient system: 0–2 min, 0% B; 3–10 min, 5% B; 14 min, 45% B; 23–26 min, 100% B; and then 27–40 min, 0% B. The flow rate of the mobile phases was 1 mL/min. The injection volume of samples and standards was 5 μL. The function of concentration versus peak area was calibrated by injecting each wax standard at a range of different concentrations that spanned the concentration levels of the extract samples. The concentration of the wax constituents was expressed as mg/100 g rind, and mg/kg cane stalk, on a fresh weight basis. All assays were carried out in triplicate.

2.2.5. Policosanol and long-chain aldehyde analyses using GC-FID/MS

Policosanol standard solutions were prepared in toluene for quantification of policosanol compounds by GC analysis. Samples were prepared in chloroform, and then derivatized with a silylation reagent, MSTFA, (2:1, v/v) at 50 °C for 15 min for mass spectra identification. Long-chain aldehyde standards were synthesized from their alcohol forms by oxidation using pyridinium chlorochromate.[26] The corresponding alcohol standards (final concentration 1 mM; 19.14 mg hexacosanol, 20.54 mg octacosanol, and 21.94 mg triacontanol) and 9 mM pyridinium chlorochromate (97.5 mg) were thoroughly mixed in 50 mL of dichloromethane for 1.5 h at room temperature. The reaction mixture was then eluted with dichloromethane through a short column (6 cm × 2 cm i.d.) of silica gel-60. Subsequently, the reaction products were dried under

N_2 gas and diluted in toluene. The synthesized long-chain aldehyde standards were then examined using GC-FID and their mass fragments were identified using GC-MS.

Policosanol and long-chain aldehyde constituents were determined using a Shimadzu GC 17-A, which was equipped with a fused capillary column DB-5 (30 m × 0.25 mm i.d., film thickness 0.25 μm), (J&W Scientific, Folsom, CA, USA) and a flame ionization detector, according to a previous method.[27] The GC injector and the flame ionized detector (FID) were set at 350 °C. Helium was used as the carrier gas and the flow rate used was 1 mL/min. Samples of 1 μL were injected using split mode injection (1:10). The oven temperature was initially set at 150 °C, was then raised to 320 °C at a rate of 4 °C/min, and was then maintained at 320 °C for 15 min. The function of concentration versus peak height was calibrated by injecting a mixture of policosanol and long-chain aldehyde standards of different concentrations that spanned the concentration levels of the extract samples. Policosanol and long-chain aldehyde content was expressed as mg/100 g sample on a fresh weight basis.

The mass spectra of policosanol were examined after silyl derivatization, and the mass spectra of long-chain aldehydes were examined without silylation. Policosanol compounds were identified based on their trimethylsilyl derivates. The mixed derivatization solution, which included 500 μL of sample in chloroform and 250 μL of the silylation reagent (MSTFA) was heated at 50 °C for 15 min and then chloroform was added to bring the sample to a total volume of 1 mL for analysis. Samples were analyzed using a Shimadzu GC-MS QP-2010 with a fused capillary column DB-5 MS (30 m × 0.25 mm i.d., J&W Scientific) under the same GC conditions as described above. Each sample (0.3 μL) was injected using a split ratio of 1:10. For MS detection, the electron impact (EI) ion source and transfer line temperature were programmed at

200 and 280 °C, respectively, and ionization energy was generated at 70 eV. The mass acquisition scan range and rate were (*m*/*z*) 30–500 amu and 2 scans/s, respectively. Policosanol and long-chain aldehyde components were identified by comparison of the mass spectra fragmentation pattern with the MS data of corresponding compounds obtained from the National Institute of Standards and Technology (NIST) MS Library, Version 2005, as well as by peak enrichment upon co-injection with authentic standards. All assays were carried out in triplicate.

2.2.6. Statistical analysis

The compositional analyses of wax, policosanol, and long-chain aldehydes were statistically evaluated using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA, USA). Statistical differences ($p < 0.05$) between mean values were carried out using Fisher's least significant difference post hoc analysis.

2.3. Results and discussion

2.3.1. Sugarcane samples

All sugarcane stalks were grown under the same environmental, soil composition and climatic conditions, and were collected at two different stages of maturity; immature samples were collected after 9 months cultivation and mature samples after 12 months. All sugarcane cultivars used in this study are registered cultivars released by the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan, and are currently considered as major sugarcane varieties. The cultivar stalks varied in diameter (from slightly thin to thick) and shape (cylindrical or bobbin-shaped) and large amounts of epidermal wax were easily detected by visual observation.[28] These cultivars were

selected for this study based on their economic importance and availability. The same extraction method and analytical conditions were used for all samples. It was therefore possible to compare the wax, policosanol, and long-chain aldheyde content and composition of all samples.

2.3.2. Wax composition of sugarcane cultivars

The wax in sugarcane is present as a pale-yellowish to dark-greenish powdery deposit on the surface of the sugarcane stalks. The waxy material extracted from sugarcane has been characterized as a mixture of long-chain alkanes, wax esters, fatty acids, acids, ketones, aldehydes, and alcohols,[9,29] as well as steroids such as *β*-sitosterol, stigmasterol, ketosteroids and hydroxyketosteroids.[30]

Sugarcane wax components were analyzed, identified, and quantified using HPLC-ELSD analysis. The five peaks that were detected in the HPLC chromatogram of samples of the wax extracts, as shown by the representative chromatogram in **Figure 2-2**, were identified as aldehyde, sterol ester, triacylglycerol, acid, alcohol and sterol groups. In the representative analysis of the Ni 15 cultivar, each peak represented only a single chemical group of wax component, except for peak 1 which was a mixture of aldehyde and sterol ester groups. Adhikari et al.[25] identified this peak as a mixture of aldehyde, sterol ester and wax ester groups. The sterol ester component is known to be a minor constituent of wax, especially in sugarcane, and is naturally derived by esterification of plant sterols and fatty acids, except for free sterol compounds (i.e. *β*-sitosterol, stigmasterol, campesterol). The stigmasterol ester of palmitic acid has been reported to be a sterol ester component of sugarcane wax.[10]

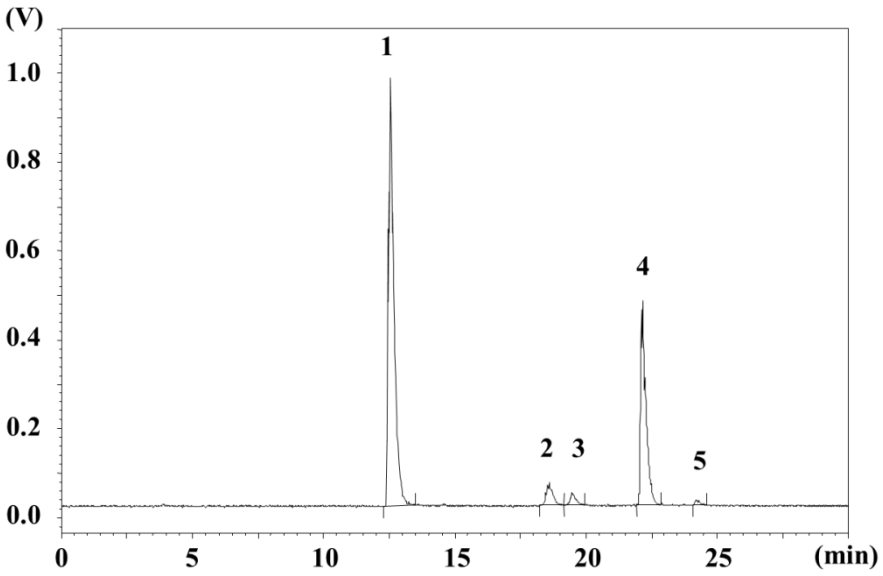

Figure 2-2. Representative HPLC chromatogram of the rind of the Ni 15 sugarcane cultivar, obtained using HPLC-ELSD.

Peak 1: A mixture of aldehyde and sterol ester; Peak 2: Triacylglycerol; Peak 3: Acid; Peak 4: Alcohol (Policosanol); Peak 5: Sterol.

Table 2-1. Wax composition[a] of hand-peeled rinds of mature sugarcane cultivars, obtained using HPLC-ELSD.

Cultivar	Aldehyde, sterol ester[b]	Triacylgycerol	Acid	Alcohol	Sterol
(mg/100 g rind, fresh weight)					
NiF 8	751.0 ± 29.4 b[c]	101.7 ± 10.7 a	62.0 ± 1.6 a	367.5 ± 8.1 b	33.3 ± 0.0 a
Ni 15	594.6 ± 32.6 c	80.2 ± 6.0 b	39.0 ± 1.4 b	216.4 ± 14.7 c	25.3 ± 0.2 b
Ni 22	1027.1 ± 66.5 a	97.0 ± 6.9 a	63.2 ± 3.1 a	462.9 ± 7.6 a	n.d.
(mg/kg cane stalk, fresh weight)					
NiF 8	541.3 ± 25.6 b	72.5 ± 7.6 a	44.2 ± 1.1 a	261.9 ± 5.7 b	23.7 ± 0.0 a
Ni 15	564.3 ± 37.3 b	74.9 ± 5.6 a	36.4 ± 1.3 b	206.1 ± 16.8 c	23.7 ± 0.2 a
Ni 22	789.5 ± 51.1 a	74.5 ± 5.3 a	48.6 ± 2.4 a	355.8 ± 5.9 a	n.d.

[a] The standards used to represent the main components of sugarcane wax (indicated in brackets) were: octacosanal (aldehyde), cholesteryl oleate (sterol ester), triolein (triacylglycerol), lignoceric acid (acid), octacosanol (alcohol; policosanol), and stigmasterol (sterol).
[b] Calculated as an aldehyde group.
[c] Data are means ± standard deviation (n = 3). Means in the same column followed by the same letter are not significantly different ($p < 0.05$). n.d. Not detected.

As shown in **Table 2-1**, the wax of sugarcane rind cultivars was mainly composed of a mixture of aldehyde and sterol ester (55–60%) and alcohol (31–39%) groups. Significant differences ($p < 0.05$) in the level of each wax component were observed in different sugar cultivars. The sugarcane rind with the highest wax content was the Ni 22 cultivar that had 1027.1 mg of a mixture of aldehyde and sterol esters per 100 g sample. The sugarcane rinds with the next highest level of wax were those of the NiF 8 and Ni 15 cultivars (751.0 and 594.6 mg/100 g, respectively). The cultivar with the highest level of alcohol groups was the Ni 22 cultivar, which had a significantly higher level of policosanol (462.9 mg/100 g rind) than the other cultivars ($p < 0.05$). This Ni 22 sugarcane cultivar was found to contain approximately 355.8 mg of a potent policosanol compounds in its stalk. These results indicate that policosanol and long-chain aldehyde groups are the main components of sugarcane wax. The higher policosanol content of the Ni 22 sugarcane cultivar compared to other cultivars might make Ni 22 a fine and valuable source of policosanol.

2.3.3. Policosanol and long-chain aldehyde content of sugarcane cultivars

The policosanol and long-chain aldehyde content of several sugarcane cultivars were further examined, with the aim of using the content and composition of these components as a method of distinguishing different cultivars, as well as their maturity stages. A typical chromatogram of sugarcane rind extract using Ni 15 as a representative cultivar is shown in **Figure 2-3**. All policosanol and long-chain aldehyde compounds were identified and completely separated into single peaks. The retention time of aldehyde compounds (hexacosanal, octacosanal, and triacontanal) was 1 min less than that of their corresponding alcohol compounds. Each policosanol and aldehyde

compound in the extracted samples was quantified based on the relationship between its retention time and the peak height of its corresponding standard.

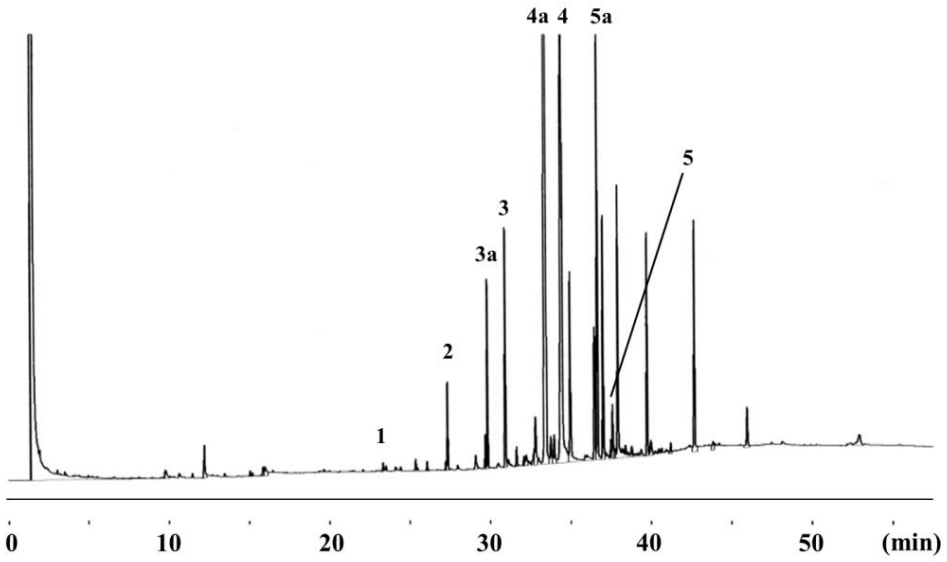

Figure 2-3. Representative gas chromatogram of policosanol and long-chain aldehydes extracted from the rind of the Ni 15 sugarcane cultivar, obtained using GC-FID.

Peak 1: C_{22}-OH; Peak 2: C_{24}-OH; Peak 3a: C_{26}-CHO; Peak 3: C_{26}-OH; Peak 4a: C_{28}-CHO; Peak 4: C_{28}-OH; Peak 5a: C_{30}-CHO; Peak 5: C_{30}-OH.

Table 2-2. Mass fragmentation pattern of trimethylsilyl policosanol derivatives and long-chain aldehydes in sugarcane rinds, obtained using GC-MS.

Compound	Molecular target ion, *m/z*	Qualifier ions, *m/z*
Docosanol (C_{22}-OH)	383	103, 384, 385
Tetracosanol (C_{24}-OH)	411	103, 412, 413
Hexacosanol (C_{26}-OH)	439	103, 440, 441
Octacosanol (C_{28}-OH)	467	103, 468, 469
Triacontanol (C_{30}-OH)	495	103, 496, 497
Hexacosanal (C_{26}-CHO)	380	334, 336, 362
Octacosanal (C_{28}-CHO)	408	362, 364, 390
Triacontanal (C_{30}-CHO)	436	390, 392, 418

Policosanol and aldehyde compounds of the samples were identified by comparison of their mass spectra with those of their respective standards. Policosanol was identified based on the mass fragment pattern of the EI spectra of its trimethylsilyl derivate as the target ion. For example, the specific molecular target ion of the trimethylsilyl ether of octacosanol (C_{28}-OH) was *m/z* 467 (**Table 2-2**). In addition, a mass fragment of *m/z* 103, the ion of $\cdot CH_2OSi(CH_3)_3$, was also confirmed in all policosanol fragments. Splitting of the $2 \times \cdot CH_3$ group from the main chain policosanol was identified in all analyzed compounds. In analysis of the long-chain aldehyde group, even through fragmentation of the C_{28} aldehyde ($C_{28}H_{56}O$) was weak, it was clearly recognized from the mass fragments of *m/z* 362 (M-46, loss of $CH_2{=}CH_2$ and H_2O from $C_{28}H_{56}O^+$); *m/z* 364 (M-44, loss of an ion $CH_2{=}CH\text{-}O^+$ from $C_{28}H_{56}O^+$), *m/z* 390 (M-18, loss of H_2O from $C_{28}H_{56}O^+$) and *m/z* 408 (M^+, $C_{28}H_{56}O^+$ ion), which are characteristic fragments of aldehyde compounds. These policosanol and aldehyde fragmentation patterns that were identified are similar to those reported in previous studies.[26,31]

Wax constituents are abundant in the rind parts of sugarcane such as in the epicuticular wax. As surface waxes, they protect plants from water loss or environmental stress.[32,33] The amounts of policosanol and long-chain aldehydes in the wax of different parts of the stalk of the Ni 15 sugarcane cultivar are shown in **Figure 2-4**. The intact stalk contained 35 mg policosanol and 24 mg aldehyde per 100 g sample. However, policosanol and aldehyde compounds were present at a much higher level in the rind than in the pith (approximately 80 versus 1 mg/100 g sample). This result is consistent with the presence of epicuticular wax in sugarcane rind.

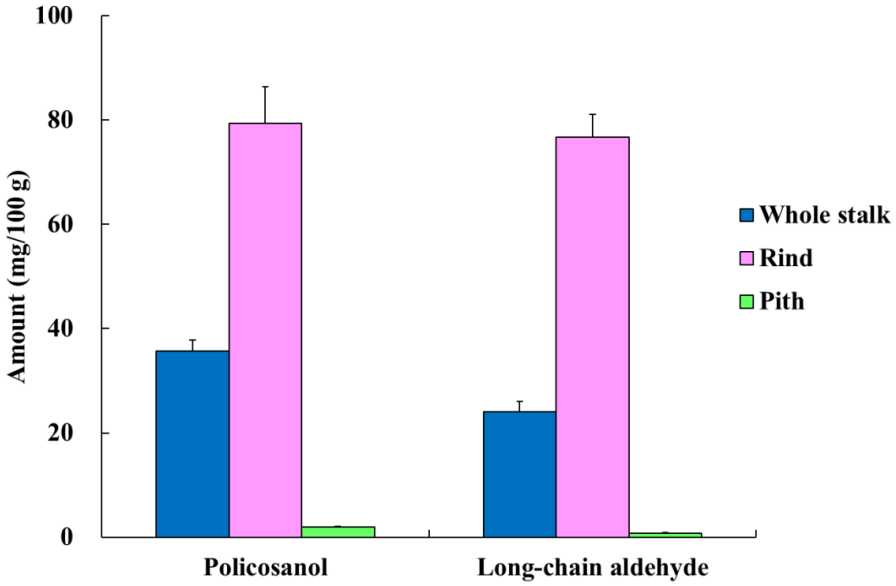

Figure 2-4. Policosanol and long-chain aldehyde content of the Ni 15 sugarcane cultivar, obtained using GC-FID.

The rinds and piths were separated using CSS.

The content and composition of sugarcane epicuticular wax, including policosanol and long-chain aldehyde groups, was clearly different between different sugarcane cultivars (**Table 2-3** and **2-4**). Thus, the total policosanol content of the Ni 17 cultivar was significantly higher (123.65 mg/100 g) than that of other cultivars (59.68–88.42 mg/100 g) ($p < 0.05$). In addition, two of the cultivars, Ni 13 and Ni 17, contained a high amount of long-chain aldehyde compounds (115.43 and 111.38 mg/100 g, respectively) compared to the level of these compounds in other cultivars, which ranged from 69 to 88 mg/100 g.

Sugarcane has been used for the production of dietary supplement of policosanol. In fact, another study reported that total policosanol content in sugarcane rind was about 27 mg/100 g, while total policosanol content of sugarcane leaves (18 mg/100 g) was found in the same level with that of the wheat straw (16 mg/100 g). Moreover, a

significant amount of policosanol was discovered in refined vegetable oils from various plant sources, such as wheat germ oil (63 mg/100 g) and olive oil (947 mg/100 g).[31,34] The total policosanol contents of the sugarcane rind samples examined in this study ranged from 60 to 124 mg/100 g. Thus, the result of the study clearly indicates that Okinawan sugarcane may be potential for policosanol production.

The composition of the policosanol and long-chain aldehyde constituents of the rinds of all of the sugarcane samples showed significant variation although octacosanol (C_{28}-OH) was the main policosanol component in each cultivar. The octacosanol content of these samples ranged from 81% to 87% of the total policosanol content. This result is in agreement with several previous reports of sugarcane policosanol levels.[31,35] Other compounds detected were hexacosanol (C_{26}-OH, 7–13%) and triacontanol (C_{30}-OH, 4–7%) as well as small amounts of docosanol (C_{22}-OH) and tetracosanol (C_{24}-OH). Regarding aldehyde group content, the octacosanal (C_{28}-CHO) level ranged from 73% to 80% of the total aldehyde group content, whereas triacontanal (C_{30}-CHO, 12–22%) and hexacosanal (C_{26}-CHO, 3–8%) were present at lower levels. Octacosanal has been suggested to be a morphogenetically active component that is involved in host plant recognition and triggering infection structure differentiation in leaf epicuticular wax.[36] Moreover, another study reported that the ratio of alcohol to aldehyde, as well as wax components of shorter carbon chain length on the surface of the sugarcane stalk might contribute towards its resistance to a sugarcane borer, *Eldana saccharina* Walker.[37]

Table 2-3. Policosanol content of CSS-separated rinds of mature sugarcane cultivars, obtained using GC-FID.

Cultivar	Policosanol content (mg/100 g sample, fresh weight)					
	C_{22}-OH	C_{24}-OH	C_{26}-OH	C_{28}-OH	C_{30}-OH	Total
NCo 310	0.10 ± 0.03 b[a]	0.20 ± 0.02 cd	4.36 ± 0.27 e	52.40 ± 4.36 d	2.61 ± 0.14 e	59.68 ± 4.63 d
F 161	0.11 ± 0.02 b	0.16 ± 0.06 d	6.42 ± 0.36 d	56.96 ± 5.36 cd	5.04 ± 0.56 bc	68.69 ± 6.20 cd
F 177	0.13 ± 0.01 b	0.26 ± 0.04 c	7.53 ± 0.53 cd	73.47 ± 5.48 b	3.70 ± 0.45 d	85.08 ± 6.31 b
NiF 8	0.12 ± 0.02 b	0.40 ± 0.06 b	12.10 ± 1.37 b	71.67 ± 6.44 b	4.14 ± 0.40 cd	88.42 ± 7.34 b
Ni 13	0.12 ± 0.01 b	0.22 ± 0.08 cd	6.66 ± 0.12 d	58.56 ± 4.21 cd	5.61 ± 0.66 ab	71.17 ± 4.38 c
Ni 15	0.13 ± 0.03 b	0.28 ± 0.06 c	8.81 ± 1.29 c	66.45 ± 5.02 bc	3.73 ± 0.79 d	79.39 ± 6.98 bc
Ni 17	0.20 ± 0.01 a	0.78 ± 0.05 a	16.04 ± 0.72 a	100.46 ± 7.06 a	6.18 ± 0.95 a	123.65 ± 7.21 a

[a] Data are means ± standard deviation (n = 3). Means in the same column followed by the same letter are not significantly different ($p < 0.05$).

Table 2-4. Long-chain aldehyde content of CSS-separated rinds of mature sugarcane cultivars, obtained using GC-FID.

Cultivar	Long-chain aldehyde content (mg/100 g sample, fresh weight)			
	C_{26}-CHO	C_{28}-CHO	C_{30}-CHO	Total
NCo 310	3.36 ± 0.09 cd[a]	61.71 ± 1.30 c	11.68 ± 1.23 d	76.75 ± 0.65 c
F 161	3.04 ± 0.42 d	53.87 ± 3.93 c	15.99 ± 0.63 c	72.89 ± 4.87 c
F 177	2.62 ± 0.10 d	56.23 ± 4.76 c	10.92 ± 1.03 de	69.78 ± 5.85 c
NiF 8	4.22 ± 0.55 c	70.57 ± 9.99 b	14.06 ± 0.99 c	88.84 ± 11.37 b
Ni 13	5.16 ± 0.24 b	84.69 ± 0.93 a	25.58 ± 1.71 a	115.43 ± 2.57 a
Ni 15	6.42 ± 1.14 a	60.72 ± 2.82 c	9.52 ± 0.75 e	76.66 ± 4.37 c
Ni 17	5.19 ± 0.27 b	85.81 ± 0.78 a	20.37 ± 1.02 b	111.38 ± 0.92 a

[a] Data are means ± standard deviation (n = 3). Means in the same column followed by the same letter are not significantly different ($p < 0.05$).

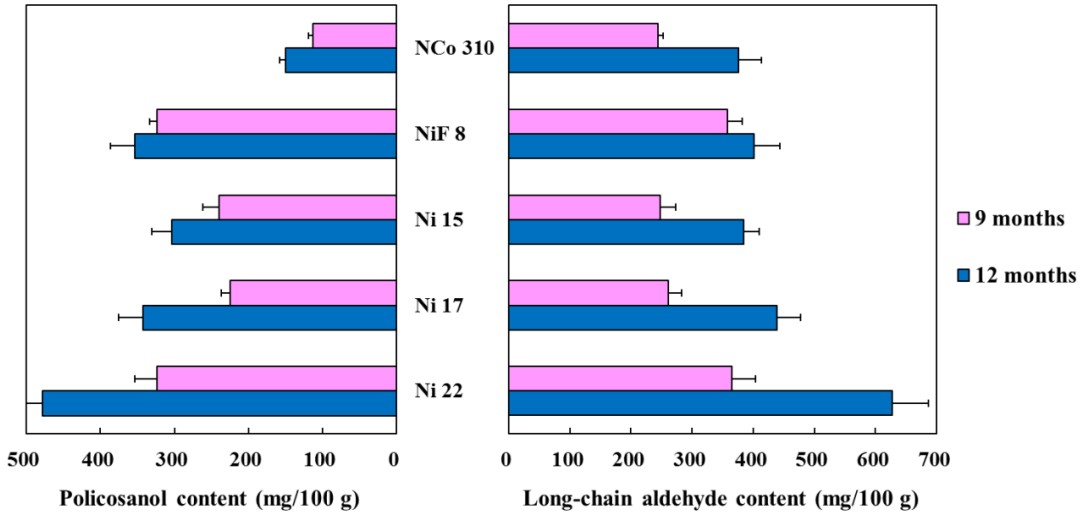

Figure 2-5. Comparison of the policosanol and long-chain aldehyde content of immature (9 months) and mature (12 months) hand-peeled sugarcane rind samples, obtained using GC-FID.

Generally, Okinawan sugarcane is harvested in the winter season after approximately 12 months cultivation, when high cane yields and sugar content are reached. The amount of sugarcane surface wax is cultivar dependent, and is also affected by the maturity stage. The total content of policosanol and long-chain aldehydes in the hand-peeled rind of the five sugarcane cultivars that were investigated is shown in **Figure 2-5**. The highest content of policosanol and aldehyde was found in the mature hand-peeled rind of the Ni 22 sugarcane cultivar, which contained approximately 500 mg policosanol and 600 mg aldehyde per 100 g of sample. The total amount of these compounds increased during the three months growth. The increase in the total amount of aldehyde compounds was greater than the increase in alcohol compounds. The increase in the policosanol content of the Ni 17 and Ni 22 cultivars was greater (53% and 48%, respectively), than that in the other cultivars. The Ni 22 cultivar displayed the greatest increase in total long-chain aldehyde compounds (72%

increase). The next highest increase was shown by the Ni 17 cultivar (68% increase) followed by the Ni 15 cultivar (55% increase). Interestingly, the smallest increases in policosanol and long-chain aldehyde content were both observed in the NiF 8 cultivar (9 and 12%, respectively). Therefore, the level of policosanol in commercial crops might be considered not only due to the sugarcane cultivar grown, but also to a function of the time of harvesting. Other research groups have reported changes in policosanol content during development of corn kernels, maturation of switchgrasses, and ripening of olive fruits.[34,38,39] The results of these studies showed that policosanol content reached a maximum level after a specific time of cultivation.

Cane separation system, or simply called CSS, is used in the sugar factory for optimum utilization of all parts of the sugarcane stalk. The CSS technology is able to separate the outer rind layer from the inner sugar-containing pith of the sugarcane stalk, as well as to provide high value added by-products of the sugarcane industry, such as fibers for paper, board, or cloth manufacture, pith residue for livestock feed, and sugarcane wax. Furthermore, it is also interesting to note that in the study, the total policosanol and long-chain aldehyde content of hand-peeled sugarcane rind samples was 3–5 times higher than that of CSS-separated sugarcane rinds (approximately 300 to 400 versus 80 mg/100 g sample of sugarcane Ni 15 cultivar – see **Figure 2-4** and **2-5**, respectively). Therefore, separation of the rinds using a CSS machine would result in the loss of many of the epicuticular waxes of the collected rinds. The loss of these waxes during CSS-separation may be due to wax falling behind the rolls and blades of the machine while the separated sugarcane pith is conveyed along the rolls, instead of the wax being separated into the epidermis wax collector.

2.4. Conclusion

Sugarcane waxes were comprised of 55–60% aldehyde and sterol esters, 32–40% alcohol, and small amounts of triacylglycerol, acid, and plant sterols. The highest content of policosanol and long-chain aldehyde compounds (500 mg and 600 mg/100 g rind, respectively) was found in the hand-peeled rind of the Ni 22 sugarcane cultivar. The content of policosanol and long-chain aldehyde compounds increased up to 72% during sugarcane maturation from October to January. This study indicated that the composition and content of wax, policosanol, and long-chain aldehydes may vary depending on cultivar and specific part of sugarcane, as well as on maturity stage. Thus, this information may provide a basis for selection of sugarcane cultivars in agricultural areas for wax or policosanol production.

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Chapter III

Antioxidant Activity of Sugarcane Molasses Against 2,2′-Azobis (2-amidinopropane) dihydrochloride-Induced Peroxyl Radicals

3.1. Introduction

The sugarcane manufacturing industry is being challenged to transform cane by-products, such as molasses, filter mud, and bagasse, from environmental burdens into economically profitable food-based products. The average annual production of sugarcane molasses in Japan was around 27,654 tons from 2007 to 2011, and it has been used mainly as feed and fertilizer.[40] Moreover, a small amount of sugarcane molasses is utilized for the production of high value-added products such as alcohol, amino acids, and yeast. It has also been reported to be a potent source of various food derivative products such as phenolic, organic acid, and pigment compounds.[41–43]

Sugarcane molasses, which are rich in phenolic and flavonoid constituents, have been reported to possess potent antioxidant, antimutation, anti-inflammatory, and tyrosinase inhibitory effects, and therefore have biofunctionality.[43,44] On the whole, these compounds are known to occur naturally in plants and crops and hold promise for application in human health because of their biological functions, including their antioxidant activities.[45] There has been an upsurge of interest in exploring the potent antioxidant activity of sugarcane molasses by using different approaches and models, such as its use in hydroxyl radical scavenging, superoxide anion radical scavenging, nitrite scavenging, β-carotene bleaching inhibition, and ferric reduction; it is also widely used for its electron or radical scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

(ABTS) radicals.[44,46–49] However, very little information is available regarding the antioxidant activity of sugarcane molasses against peroxyl radicals, particularly against azo-initiator 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) peroxyl radicals.

AAPH peroxyl radicals have been known to initiate lipid peroxidation, which leads to oxidative damage in cells and DNA molecules, and can be formed when azo-initiator compounds react with molecular oxygen.[50,51] Hence, AAPH peroxyl radicals have been effectively used to measure the antioxidant capabilities of food and bio-based materials through various analytical techniques, all of which include chemical-based antioxidant activity, total peroxyl radical trapping potential, cell-based antioxidant activity, DNA scission inhibitory activity, and other fluorescence-based lipophilic antioxidant properties.[50,52–54]

To foster the utilization of by-products from the sugarcane manufacturing industry by chemical and biochemical approaches, the antioxidant activity of sugarcane molasses against peroxyl radicals was investigated. The study thus aimed to explore the potent antioxidant capabilities of sugarcane molasses against AAPH peroxyl radicals through oxygen radical absorbance capacity (ORAC), cellular antioxidant activity (CAA), and oxidative DNA damage protective activity assays. The molasses fractions were collected through a series of fractionations. In addition, phenolic constituents of sugarcane molasses were identified using a high performance liquid chromatography (HPLC) method. The research flowchart can be briefly seen in **Figure 3-1**.

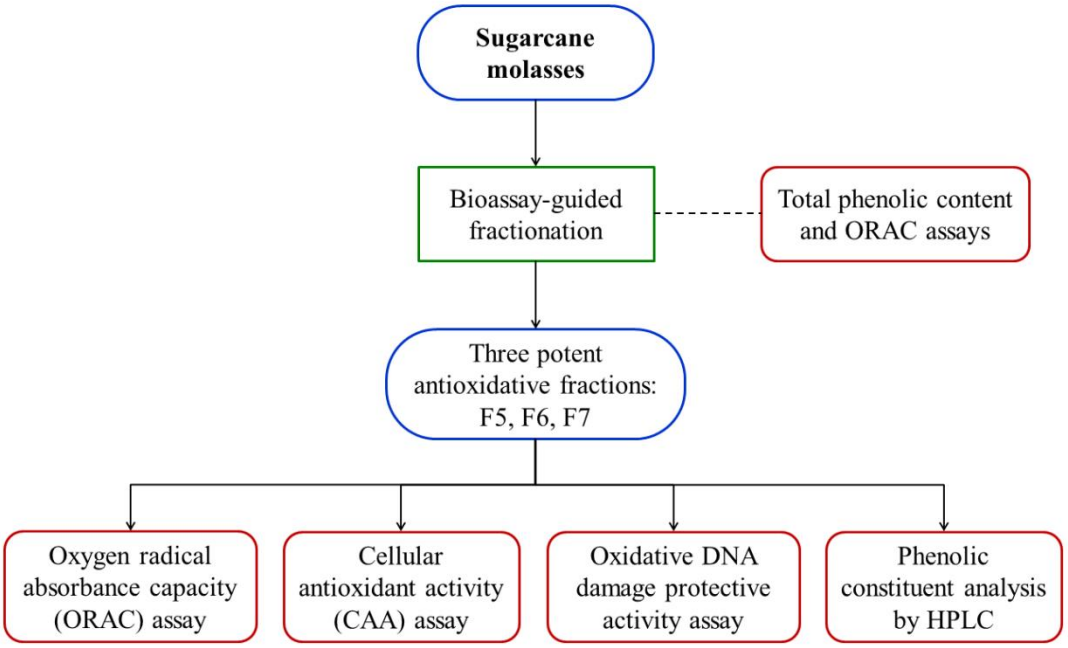

Figure 3-1. Research flowchart of antioxidant activity of sugarcane molasses against 2,2′-azobis(2-amidinopropane) dihydrochloride-induced peroxyl radicals.

3.2. Materials and methods

3.2.1. Standards and reagents

AAPH was purchased from Wako Pure Chemical Industries (Osaka, Japan). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Calbiochem (San Diego, CA, USA). Folin-Ciocalteu reagent was obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Fluorescein sodium salt and calf thymus DNA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Loading buffer and λ DNA/Hind III fragments molecular weight marker were from Wako Pure Chemical Industries. 5-(and-6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) was obtained from Invitrogen Co. (Tokyo, Japan). Normal human

dermal fibroblasts (NHDF cells) were obtained from Takara-Bio Inc. (Tokyo, Japan). Serum-free fibroblast growth medium (FGM-2) and growth-promoting additives containing fetal bovine serum (FBS), human recombinant epidermal growth factor (hEGF), bovine insulin, gentamicin, and amphotericin-B, were from Toyobo Co. Ltd. (Osaka, Japan). Gallic acid and *p*-hydroxyacetophenone were purchased from Wako Pure Chemical Industries. Ferulic acid and *p*-coumaric acid were from Tokyo Chemical Industry (Tokyo, Japan), and *p*-hydroxybenzaldehyde was from Nakalai Chemicals (Kyoto, Japan). Other phenolic compounds were from collection of Dr. Kensaku Takara (University of the Ryukyus), isolated from sugarcane products, and identified by NMR- and UV-Vis spectra in previous studies.[55,56] All other reagents were from Wako Pure Chemical Industries. Water was treated in a reverse osmosis purification system prior to use (Aquarius RFP342HA, Advantec Toyo, Tokyo, Japan).

3.2.2. Fractionation of sugarcane molasses

Sugarcane molasses materials were obtained as a by-product from sugarcane processing in the 2011–2012 production year of Shounan Seito Co. Ltd. (Okinawa, Japan). Molasses was suspended in water and was centrifuged at 8000 rpm for 20 min at 20 °C using a Hitachi CR20GIII centrifuge (Hitachi, Tokyo, Japan). The resulting supernatant was passed through Amberlite XAD-2 resin (Organo Co. Ltd., Tokyo, Japan) with 1.2 L of water, following by 1.2 L of 25, 50, 75, and 100% aqueous MeOH, respectively, as described in **Figure 3-2**. The extract with high antioxidant activity was then fractionated using a Toyopearl HW-40C gel (Tosoh Co. Ltd., Tokyo, Japan) with 20, 40, and 60% aqueous MeOH, respectively. The effluent was collected using a fraction collector (CHF161RA, Advantec Toyo, Tokyo, Japan) and its absorbance was

then monitored at 240, 270, and 300 nm using an UV-1800 Spectrophotometer (Shimadzu, Kyoto, Japan). The collected internal fractions were pooled into 7 major fractions designated fractions 1–7 (F1–F7) following the spectral profiles. The collected molasses fractions were then concentrated using a rotary vacuum evaporator and kept at −30 °C until further analysis.

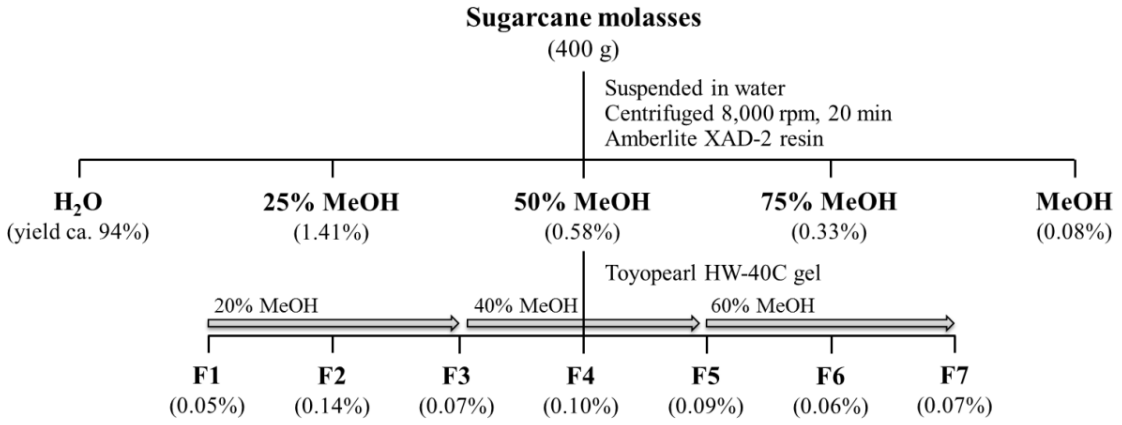

Figure 3-2. Fractionation scheme for sugarcane molasses fractions possessing antioxidant activities against AAPH peroxyl radicals.

Fraction 1 (F1) was obtained from internal fractions 1–12, F2 from fractions 13–23, F3 from fractions 24–31, F4 from fractions 32–51, F5 from fractions 52–67, F6 from fractions 68–89, and F7 from fractions 90–100. Each fractionation yield is expressed as the percentage of the solid content.

3.2.3. Determination of total phenolic content

The total phenolic content of the sugarcane molasses was examined using the Folin-Ciocalteu method of Payet et al.[48] with slight modifications. Briefly, 20 µL of the molasses sample in 50% methanol, 60 µL of distilled water, and 15 µL of Folin-Ciocalteu reagent (previously diluted 2-fold with distilled water) were transferred to a 96-well microplate (Nunc, Roskilde, Denmark). The mixture was mixed well and allowed to stand at room temperature for 5 min. After that, 75 µL of sodium carbonate

(2%) was added to the well. The microplate was immediately placed and agitated in a microplate reader (PowerWave XS2, BioTek, Winooski, VT, USA) and then allowed to stand for 15 min, and the absorbance was measured at 750 nm. Total phenolic content was calculated from a calibration curve of gallic acid (10–150 μg/mL) and expressed as milligrams of gallic acid equivalents (GAE) per gram sample. All assays were carried out in triplicate.

3.2.4. Oxygen radical absorbance capacity (ORAC) assay

Antioxidant activity of sugarcane molasses against AAPH peroxyl radicals was chemically examined using an ORAC method adapted from Huang et al.[52] Briefly, various concentrations of sugarcane molasses sample (25 μL) in phosphate buffer and freshly made 90 nM fluorescein solution (150 μL) were aliquoted into the inner wells of a black 96-well microplate (Nunc, Roskilde, Denmark). The outer wells of the microplate were filled with 200 μL water. The microplate was immediately placed and agitated in a microplate reader (SH-9000 Lab, Corona Electric, Ibaraki, Japan) that had been prewarmed to 37 °C; the plates were then allowed to stand for 10 min. After this incubation, 25 μL of freshly made 160 mM AAPH was immediately added to the inner wells (final concentration, 20 mM). Fluorescence was monitored every minute for 30 min at wavelengths of excitation and emission of 485 and 530 nm, respectively, and the decrease in fluorescence was determined. A blank, using phosphate buffer instead of molasses sample, and calibration solutions of Trolox (5–40 μM) were also included in each assay. The area under the curve (AUC) of relative fluorescence value was calculated using the equation: AUC = 0.5 + *f1/f0* + ... *fi*/*f0* + ... + *f29*/*f0* + 0.5(*f30*/*f0*), where *f0* is the initial relative fluorescence reading at 0 min and fi is the relative

fluorescence reading at time *i*. The ORAC value was calculated and expressed as micromoles of Trolox equivalents (TE) per gram sample from a calibration curve of Trolox. All assays were carried out in triplicate.

3.2.5. Cellular antioxidant activity (CAA) assay (analyzed by Japan Food Research Laboratories, Osaka, Japan)

The CAA assay was performed using the approach involving exposure of NHDF cells to AAPH peroxyl radicals adapted from Dudonńe et al.[53] with slight modifications. Briefly, NHDF cells were seeded on a 96-well microplate (2.5×10^4 cells per well) and were cultivated in FGM-2 growth medium, supplemented with 2% FBS, 0.1% of hEGF, 0.1% of bovine insulin, 50 μg/mL gentamicin, and 0.05 μg/mL amphotericin-B, under a humidified atmosphere at 37 °C, 5% CO_2. After 6 d, growth medium solutions were replaced by another medium containing an ethanolic solution of sugarcane molasses fractions (0.5%, v/v) with final concentrations ranging between 1.95 and 125 μg/mL, and the cells were then cultivated for another 2 h. Control cells were cultivated in the same media lacking the molasses fractions. After cultivation, the cells were incubated with CM-H_2DCFDA (final concentration 5 μM) for 10 min, followed by AAPH (0.3 mM) for 1 h. The fluorescence intensity of the cells was measured using a SpectraMax M2e spectrofluorometer (Molecular Devices Co., Tokyo, Japan) at excitation and emission wavelengths of 493 and 523 nm, respectively. The peroxyl radical scavenging activity was expressed as CAA (%) and calculated using the equation: CAA (%) = $[(I_C - I_S)/I_C)] \times 100$, where I_S is the intensity of the cells grown in media supplemented with sugarcane molasses fractions, and I_C is the intensity of the cells in control medium solution. EC_{50} value (in μg/mL) of each sugarcane molasses sample was determined

from the semi-log plot of percentage of scavenging activity against the sample concentration that yielded 50% of the maximum peroxyl radical scavenging activity. Gallic acid was used as the positive control, at final concentrations of 0.122–7.81 μg/mL.

The viability of NHDF cells subjected to AAPH peroxyl radical treatment was also monitored by measuring the mitochondrial activity of living cells with a commercially available kit (Cell Counting Kit-8, Dojindo Lab., Kumamoto, Japan), in which 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt was used as a substrate. The relative number of living cells was determined in duplicate by setting the value of the control cells as 100%. The cell viability of the samples was greater than 90%, and the conditions were therefore considered to be nontoxic. All assays were carried out in triplicate.

3.2.6. Oxidative DNA damage protective activity assay

AAPH peroxyl radicals have the ability to induce oxidative DNA damage through scission of DNA strands.[54] The DNA damage protective activity of the sugarcane molasses was assayed *in vitro* using calf thymus DNA as a model system. Briefly, 10 μL of calf thymus DNA (1 mg/mL) in 0.1 M phosphate buffer (pH 7.4), 10 μL of various concentrations of molasses samples (10–500 μg/mL), and 10 μL of freshly made 200 mM AAPH (final concentration 20 mM, the same concentration as in the ORAC assay) were placed in 1 mL microtubes. The total volume of these mixtures was adjusted by adding the phosphate buffer to 100 μL and the samples were then vortexed well. The reaction mixtures were then incubated in a dry block at 37 °C for 4 h. Subsequently, 10 μL of loading buffer, containing 0.02% bromophenol blue, 0.02%

xylene cyanol FF, 5% glycerol, and 1% SDS, was added to the mixture to terminate the reaction. The mixtures (10 μL) were loaded onto a 1% agarose gel in 1 × TAE buffer containing ethidium bromide (0.5 μg/mL) and were run at 100 volts in an electrophoresis system (Mupid-exU, Advance, Tokyo, Japan) for 40 min. DNA bands were visualized under UV light and photographed using a FluorChem Q System (Alpha Innotech Corporation, San Leandro, CA, USA). The protective activity against oxidative DNA damage was expressed as DNA-damage protective concentration (DNA-DPC) value (in μg/mL), which referred to the minimum concentration of the sample necessary to prevent DNA damage. Trolox and the authentic phenolic compound gallic acid were used as positive controls. All assays were carried out in triplicate.

3.2.7. Phenolic constituent identification using HPLC

Phenolic constituents of the sugarcane molasses were identified using an HPLC method adapted from Guimarães et al.[47] The column used was a Develosil ODS-MG5 column (250 mm × 4.6 mm i.d., 5-μm particle size; Nomura Chemical, Seto, Japan), with a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a diode array detector (SPD-M20A type). A Shimadzu LC-20AB pump was operated in combination with a Shimadzu CBM-20A gradient system controller. The column was maintained at a constant temperature of 25 °C using a Shimadzu CTO-20AC column oven, and the injection volume was 5 μL. The mobile phases comprised (A) methanol, formic acid, and water (5.0:2.5:92.5, v/v/v); and (B) methanol, formic acid, and water (92.5:2.5:5.0, v/v/v), with the following gradients: 0–30 min, 0 to 30% B; 31–65 min, 30 to 70% B; 66–75 min, 70 to 80% B; 76–80 min, 80 to 0% B; and then 81–90 min,

0% B. The flow rate of the mobile phases was 1 mL/min. The phenolic compounds were monitored at 280 nm, and were identified by comparing the chromatographic retention times, UV spectra with those of authentic standards, and as described in the previous studies.[55–57] All assays were carried out in triplicate.

3.2.8. Statistical analysis

The results of the total phenolic content and the ORAC values of sugarcane molasses extracts or fractions were statistically analyzed using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA, USA) with analysis of variance followed by Fisher's least significant difference post hoc tests to determine the statistical differences ($p < 0.05$) between mean values.

3.3. Results and discussion

3.3.1. Sugarcane molasses extracts and fractions

Preparative separation of sugarcane molasses was first conducted using a hydrophobic cross-linked polystyrene copolymer (Amberlite XAD-2) resin, followed by elution using a water–methanol gradient (**Figure 3-2**). This chromatography technique has been successfully used in preparing antioxidative phenolic compound-rich fractions from sugarcane molasses, based on polarity and solubility of the compounds.[43] In this technique, sucrose and inorganic compounds typically eluted faster in water than in methanol. From this separation step, the extract obtained using 50% MeOH was found to have significantly higher antioxidant activity against AAPH peroxyl radicals ($p < 0.05$) than those of other extracts; this was followed by extracts obtained using 25% MeOH and 75% MeOH, as indicated by the ORAC assay (**Figure 3-3**). Regarding the

amount of phenolic compounds, the extract obtained with 50% MeOH was found to have a total phenolic content significantly higher than those in the other extracts. As seen in **Figure 3-3**, there was a direct correlation between total phenolic content and the ORAC values of the extracts with correlation coefficient R^2 = 0.87 (n = 5). This result indicated that the antioxidant activity potential of sugarcane molasses against AAPH peroxyl radicals was strongly affected by the levels of phenolic compounds. Awika et al.[58] reported that the phenolic contents of sorghum extracts also correlated positively with their ORAC values and radical-scavenging activities against 2 other agents (ABTS and DPPH). Furthermore, significant correlation between phenolic contents and antioxidant capability against both ABTS and DPPH radicals was also previously discovered in some sugarcane product extracts.[48]

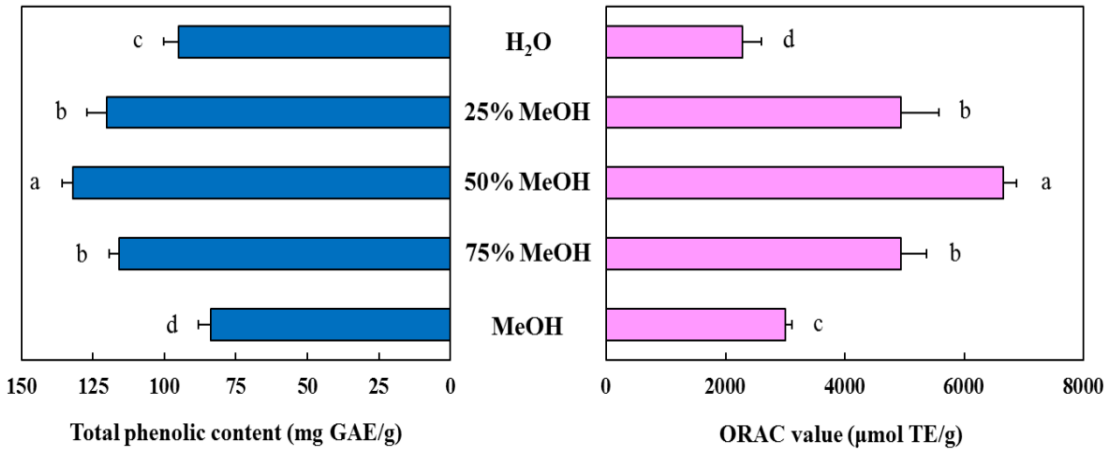

Figure 3-3. Total phenolic contents and oxygen radical absorbance capacity (ORAC) values of sugarcane molasses extracts, separated by Amberlite XAD-2 resin.

Means in the same column followed by the same letter are not significantly different (p < 0.05).

Table 3-1. Total phenolic contents of sugarcane molasses fractions, separated from the extract obtained with 50% MeOH using Toyopearl HW-40C gel.

Fraction	Total Phenolic Content (mg GAE/g)
F1	54.8 ± 2.8 e[a]
F2	69.4 ± 6.3 d
F3	68.3 ± 5.3 d
F4	90.0 ± 7.9 c
F5	105.3 ± 6.7 b
F6	121.8 ± 2.1 a
F7	114.6 ± 6.5 ab

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

As a potent source of antioxidative phenolic compounds, the extract obtained with 50% MeOH was then separated into fractions 1–7 (F1–F7) by size exclusion media using a Toyopearl HW-40C gel. The total phenolic content in the examined fractions ranged from 54.8–121.8 mg GAE/g (**Table 3-1**). Of these fractions, F6 was found to have the highest phenolic content, followed by F7 and F5. Moreover, these 3 fractions were observed to possess phenolic contents significantly ($p < 0.05$) higher than those of the fractions earlier eluted. Accordingly, the antioxidant activity against AAPH peroxyl radicals in sugarcane molasses was then examined in these 3 fractions (F5, F6, and F7).

3.3.2. Antioxidant activity of sugarcane molasses against AAPH peroxyl radicals

Azo-initiator AAPH is a clean and steady source of hydrophilic peroxyl radicals. Thermolysis of AAPH generates molecular nitrogen and 2 alkyl radicals. These alkyl radicals may combine with molecular oxygen to produce alkylperoxyl radicals that

induce the generation of peroxyl radicals.[59] The negative effects of induced peroxyl radicals on biological substances, mainly in terms of lipid peroxidation and its chain reactions, may be inhibited and then terminated by the action of antioxidative molecules via electron transfer in biradical scavenging or chain-breaking propagation reaction, that is, the so-called antioxidant activity. In the study, the antioxidant activity of sugarcane molasses was evaluated using the azo-compound AAPH in chemical and cellular model systems, as determined by employing ORAC, CAA, and oxidative DNA damage protective activity assays.

The ORAC analysis is a chemical-based method of evaluating the rate of oxidation of a fluorescent probe due to the action of AAPH peroxyl radicals and the scavenging effect of the sample tested. Among the fractions examined, the highest ORAC value was found in F7, at 6266 μmol TE/g, followed by F6 and F5, with ORAC values of 5329 and 4399 μmol TE/g, respectively (**Table 3-2**). Concentration and accumulation of many biologically active substances in sugarcane molasses from the sugar refining process may contribute to the peroxyl radical-scavenging capability it possesses. In comparison, sugarcane juices have been studied and shown to have ORAC values of no more than 24 μmol TE/mL.[60] Thus, of sugar-producing crops, sugarcane molasses exhibited ORAC values superior to those previously reported for sorghum and beet extracts. Pigmented sorghum extracts had ORAC values ranging from 140 to 870 μmol TE/g, for the grains, and 710 to 3100 μmol TE/g, for the brans, estimated on dry weight basis, while the edible part of sugar beet had an ORAC value of approximately 20 μmol TE/g.[58,61] These results indicate that sugarcane molasses is a valuable by-product with high chemical antioxidant capability against peroxyl radicals.

Table 3-2. Antioxidant activities of sugarcane molasses fractions against AAPH peroxyl radicals in oxygen radical absorbance capacity (ORAC), cellular antioxidant activity (CAA), and oxidative DNA damage protective assays.

Fraction	ORAC value (μmol TE/g)	CAA (EC_{50}, μg/mL)[a]	DNA-DPC (μg/mL)[b]
F5	4399 ± 331.0	3.9	250
F6	5329 ± 417.1	5.9	100
F7	6266 ± 242.3	3.7	100
Gallic acid	8342 ± 960.9	0.3	100

Each value is expressed as the mean ± standard deviation (n = 3).

[a] EC_{50} (μg/mL): effective concentration of the sample at which 50% of the AAPH peroxyl radicals are inhibited.

[b] The DNA-DPC is defined as the minimum concentration of the sample necessary to protect against oxidative DNA damage in the assay.

Monitoring antioxidant activity of bio-based materials using a cell-based model is important and required, due to the complexity of biological systems. In the study, the CAA of sugarcane molasses was modeled in human skin fibroblasts (NHDF cells). Oxidative stress-induced damage to NHDF cells can be initiated by free radical-derived chemicals, such as H_2O_2 and *t*-butyl hydroperoxide, as well as by physical reactive oxygen species (UVA and UVB radiation).[62] Accumulation of repetitive exposures to these oxidative stresses may accelerate the formation of peroxyl radicals and their derivatives that cause collagen cross-linking and cross-polymerization in skin cells and may also lead to mitochondrial and DNA damage.[63] Moreover, exposure of NHDF cells to AAPH peroxyl radicals has been identified as one of the key factors causing skin damage; this model is therefore an essential one in antiaging nutraceutical research.[53]

In the CAA assay, the evaluated sugarcane molasses fractions and gallic acid, as positive control, were found to be nontoxic to NHDF cell cultures, as the cell viability

of in the analysis of each of these compounds was greater than 90%. Antioxidant capability of the samples was monitored based on inhibition of peroxyl radical-mediated oxidation of a fluorescence probe, dichlorodihydrofluorescein diacetate (CM-H_2DCFDA). The EC_{50} values of the CAA assay for the sugarcane molasses fractions ranged from 3.7 to 5.9 μg/mL (**Table 3-2**). The highest activity in the CAA assay was found in F7, with an EC_{50} value of 3.7 μg/mL. F5 was found to have a similar cellular antioxidant level as F7, whereas F6 was less effective than these 2 fractions. However, these activities were 10- to 20-fold lower than that of gallic acid. The variation of cellular antioxidant levels in sugarcane molasses fractions may be affected by the physicochemical properties of its antioxidative compounds. In case of the flavonoid and phenolic components present in sugarcane molasses, the hydrophobic compounds are more likely to interact with the hydrophobic core of the cell membranes, subsequently influencing direct modulation of physical membrane properties and interrupting lipid peroxidation or other oxidative chain reactions, thus leading to chain-breaking antioxidant activity. On the other hand, the hydrophilic components may interact via hydrogen bonding with the polar head groups of membrane lipid bilayers, and thus contribute a level of protection for the cell membranes from possible external and internal oxidants.[64]

Another antioxidant method evaluated for sugarcane molasses was the protective capability against oxidative DNA damage. Principally, DNA molecules can be fragmented through chemical and physical damaging agents, including the use of chemicals (azo compounds, H_2O_2, ascorbic acid, and copper) and radiation (X- or γ-rays, UVA radiation).[47,65] Nevertheless, the use of ascorbic acid together with copper in a Fenton system for generating free radicals to examine protective activity against DNA

damage, including that of sugarcane molasses, is controversial, due to the antioxidant or pro-oxidant status of ascorbic acid. On the other hand, peroxyl radicals generated by azo compounds and other common free radicals, have been confirmed to cause peroxidation-induced DNA-based damage, primarily occurring at G-C base pairs, and decay of 8-oxodG bases, followed by cell mutation.[54,66]

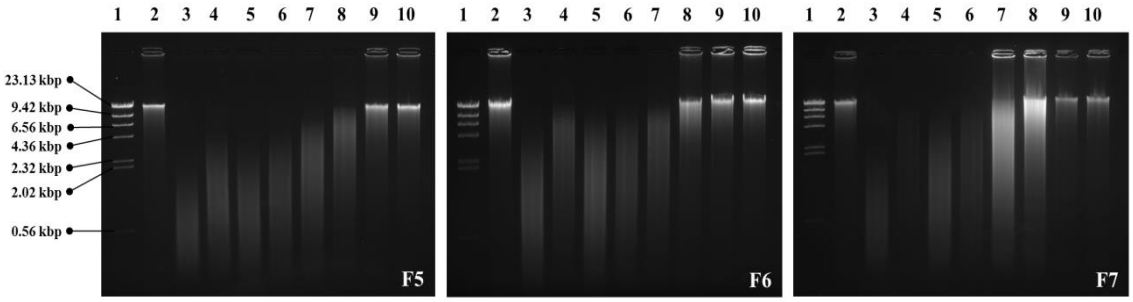

Figure 3-4. Typical results of the assay of DNA damage protective activity of sugarcane molasses fraction 5 (F5), F6, and F7 against AAPH peroxyl radicals.

The numbered lanes represent: (1) λ DNA/Hind III fragments molecular weight standard, (2) DNA only, (3) DNA exposed to AAPH peroxyl radicals, (4) DNA and AAPH peroxyl radicals + Trolox (1 mM), (5–10) DNA and AAPH peroxyl radicals + fraction sample at the following concentrations: 10, 25, 50, 100, 250, and 500 μg/mL.

In the study, the azo-initiator AAPH was used to induce peroxyl radical-mediated oxidative damage of naked DNA fragments. The DNA damage protecting effects of sugarcane molasses fractions were monitored through electrophoretic separation of the DNA fragments, as seen in **Figure 3-4**. Damaged DNA fragments were seen as faster moving, smeared bands, indicating that the DNA fragments had been randomly broken into smaller pieces. Moreover, protective activity of the molasses samples was observed in a concentration-dependent manner, in which slight protection against DNA breakage was initially found at lower concentrations (10 μg/mL). These electrophoretic patterns were then compared to those of DNA fragments not treated with peroxyl radicals,

yielding the minimum protective concentration (DNA-DPC) of the samples against oxidative DNA damage. Both sugarcane molasses F6 and F7 were found to have the ability to protect against DNA fragmentation at a DNA-DPC of 100 μg/mL (**Table 3-2**). These protective activities were identified to be as potent as that of gallic acid. The DNA-DPC of F5 was 250 μg/mL. The DNA damage-protective activity is made possible by the peroxyl radical-scavenging action of bioactive compounds contained in sugarcane molasses. The variation in capacity for DNA protection may be caused by the different chemical composition of the fractions that affected the interaction between its antioxidative constituents and DNA molecules. Nevertheless, these results are in good agreement with the high chemical antioxidant activities of the fractions.

The study revealed sugarcane molasses as a potent source of antioxidant agents against peroxyl radicals induced by the azo-initiator AAPH. Sugarcane molasses F6 and F7 have been found to possess promising antioxidant activity against AAPH peroxyl radicals in the tested chemical, cellular, and molecular model systems. These findings may thus enhance the utilization of sugarcane molasses as a valuable by-product material, with functional and beneficial effects. Additionally, this data should provide useful information for further *in vivo* studies of these antioxidative biomaterials.

3.3.3. Phenolic constituents of sugarcane molasses

Phenolic compounds are natural metabolites of plants; they are present as benzo-*y*-pyrone structures that play important roles in the plant itself and are known for their antioxidant properties; they are therefore viewed as sources of potent biofunctional food products and derivatives.[45] In this study, the phenolic constituents of sugarcane molasses fractions were detected by an HPLC method, and identified by comparing the

UV spectra with those of authentic standards, as well as isolated and NMR- and UV-Vis-spectra identified phenolic compounds from previous studies,[55–57] as shown in **Figure 3-5**. A total of 10 phenolic compounds of the 3 investigated fractions (F5, F6, and F7) were identified. Four main phenolic compounds were distinguished in sugarcane molasses F5; these were 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone, also called *β*-hydroxypropiovanillone (peak a), 3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone (peak b), schaftoside (peak c), and isoschaftoside (peak d). Phenolic compounds (a) and (b) have been reported to be present in non-centrifugal Okinawan brown cane sugar, kokuto, which is produced without molasses removal. These compounds had moderate-to-strong antioxidant capabilities, as assessed by linoleic acid autoxidation via a thiocyanate system, hydroxyl radical scavenging via deoxyribose oxidation, and rabbit erythrocyte membrane ghost methods.[55] On the other hand, the isomeric pair schaftoside and isoschaftoside, which are flavone-*C-glycosides*, have also been found in extracted cane juice with scavenging activity against DPPH radicals.[57,67]

The phenolic compound *β*-hydroxypropiovanillone (peak a) and schaftoside (peak c) were also detected in the chromatogram of F6 (**Figure 3-5**). Furthermore, F6 also contained *p*-coumaric acid (peak e). Previous studies have reported that *p*-coumaric acid is present not only in sugarcane molasses, but also remains in sugary products, like massecuite and very high polarization sugar.[46,48] *p*-Coumaric acid, one of the common dietary phenolic compounds, has been reported to possess antioxidant activity and other biofunctions such as tyrosinase inhibitory activity and antimelanogenic effects.[68]

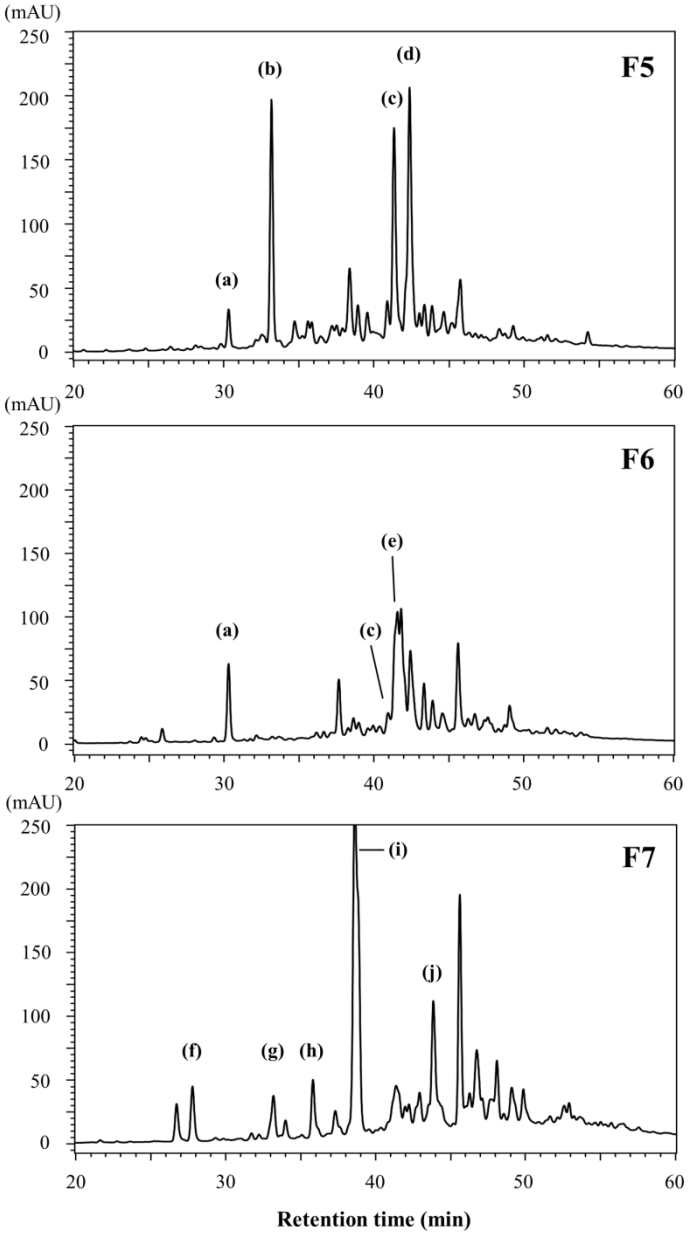

Figure 3-5. Phenolic constituent profiles of sugarcane molasses fraction 5 (F5), F6, and F7, as identified by HPLC under the detection wavelength of 280 nm. Chemical structures can be seen in **Figure A1** (appendix).

The phenolics identified were as follows: (peak a) 3-hydroxy-1-(4-hydroxy-3-methoxy-phenyl)-1-propanone; (b) 3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone; (c) schaftoside; (d) isoschaftoside; (e) *p*-coumaric acid; (f) *p*-hydroxybenzaldehyde; (g) *p*-hydroxyacetophenone; (h) 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxy-1-(*E*)-propenyl)-2-methoxyphenoxy] propyl-β-D-glucopyranoside; (i) ferulic acid; (j) luteolin-8-*C*-(rhamnosyl glucoside).

In F7, 5 phenolic constituents were detected and identified (**Figure 3-5**), including *p*-hydroxybenzaldehyde (peak f), *p*-hydroxyacetophenone (peak g), 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxy-1-(*E*)-propenyl)-2-methoxyphenoxy] propyl-β-D-glucopyranoside (peak h), ferulic acid (peak i), and luteolin-8-*C*-(rhamnosyl glucoside) (peak j). Guimarães et al.[47] reported that the antioxidative phenolic compounds *p*-hydroxybenzaldehyde and ferulic acid were also detected in their sugarcane molasses extract, whilst 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxy-1-(*E*)-propenyl)-2-methoxyphenoxy]propyl-β-D-glucopyranoside was found in a brown cane sugar product and luteolin-8-*C*-(rhamnosyl glucoside) was identified in sugarcane leaves.[56,57,67] Moreover, other research groups also reported that ferulic acid was detected not only in sugarcane molasses, but also in cane juice, cane syrup, and other sugary products.[46,48] In addition, *p*-hydroxyacetophenone has been found to be present in several brown cane sugar products.[69] Among all the compounds identified in the 3 fractions investigated in the study, the chromatographic peak of ferulic acid was higher than that of the other phenolic components.

As a whole, phenolic constituents in fractions of sugarcane molasses may contribute potent antioxidant activity against azo-initiator–induced oxidative damage, particularly against AAPH peroxyl radicals, as shown using chemical, cellular, and molecular methods. Moreover, the structural complexity of each phenolic compound, including the presence of glycosides, may also influence the antioxidant capability of these fractions, particularly at the cellular level, due to the uptake and metabolism of the compound, as well as its interaction with biomolecules in the cell.

3.4. Conclusion

Sugarcane molasses is a valuable by-product material with various potent applications in biotechnological and nutraceutical industries due to its biological functions. The results of this study indicate that sugarcane molasses F6 and F7 possess promising antioxidant capabilities against peroxyl radicals induced by the azo-initiator AAPH in chemical and cellular systems, as well as in protection against oxidative DNA damage. Ten known phenolic constituents were identified in the investigated fractions, including schaftoside, ferulic acid, *p*-coumaric acid, and *p*-hydroxybenzaldehyde. The results of this study thus provide a basis for the development of a convincing model and elucidation of the mechanism underlying the antioxidant activity of sugarcane molasses against peroxyl radicals, as well as promoting the commercial utilization of antioxidative phenolic compounds from sugarcane molasses.

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Chapter IV

Physicochemical and Flavor Profiles of Stored Cane Brown Sugar

4.1. Introduction

Cane brown sugar is a valuable nutritional product from the sugarcane industry. It is produced by dehydrating sugarcane juice without centrifugation. This cane brown sugar, also called non-centrifugal sugar, has various biological functions with beneficial effects on human health, including anticariogenic, antitoxic-cytoprotective, anticarcinogenic, and antioxidant effects.[69,70] Cane brown sugar, particularly Japanese kokuto, contains bioactive compounds, including phenolics and policosanols, which have antiatherosclerotic activities.[27,71–73]

Food storage is commercially required for preserving food materials and products prior to use. Hence, changes, both desirable and undesirable, in various food quality attributes commonly occur during storage, including changes in physicochemical properties, flavor components, and sensory characteristics.[74,75] Cane brown sugar can be stored for 1–2 years and is used either as table sugar and a snack or as raw material for the production of confectionery, beverage, and bakery products in Japan. However, very little information is available regarding the physicochemical composition and flavor constituents of stored cane brown sugar.

Non-enzymatic browning via the Maillard reaction, which involves a complex between amino acids and reducing sugars, can occur during food processing and storage. It also has critical effects on food properties, including the nutritional value, color, texture, and aroma of sugary products, coffee, and chocolate.[76,77] The aroma characteristics of Maillard reaction products (MRPs) may vary from a pleasant, flowery,

and fragrant aroma to a burnt, pungent, nutty, and caramel-like odor, depending on the amino acid and sugar composition in the food system, as well as their reaction pathways.[78,79] Besides their nutritional and organoleptic qualities, MRPs have important biological functions such as antioxidant and anti-inflammatory activities.[80] Moreover, 3-deoxyglucosone (3-DG), one of the intermediate products of the Maillard reaction that is derived from glucose through non-oxidative formation, is a precursor of 5-hydroxymethylfurfural generation, indicating the degree of browning in food systems.[75,81]

This study aimed to determine the physicochemical characteristics, including color, moisture content, water activity, sugars, and organic acids, as well as the flavor components of cane brown sugar during 1 year of storage. The volatile MRPs and 3-DG contents of cane brown sugar during storage were also evaluated. Furthermore, the influence of the Maillard reaction on the browning rate of stored brown sugar was investigated. The research flowchart can be briefly seen in **Figure 4-1**.

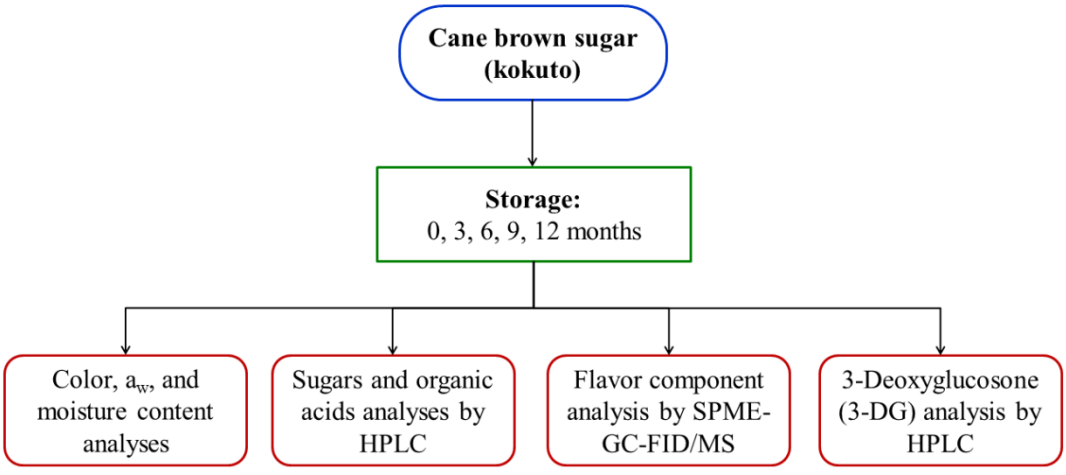

Figure 4-1. Research flowchart of physicochemical and flavor profiles of stored cane brown sugar.

4.2. Materials and methods

4.2.1. Standards and reagents

Standard sugars (sucrose, fructose, and glucose), citric acid, malic acid, and succinic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). Aconitic acid and acetic acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and lactic acid, from Kanto Chemical Co., Inc. (Tokyo, Japan). Chemicals used as standards for analyzing flavor components were obtained from Tokyo Chemical Industry (Tokyo, Japan), Sigma-Aldrich (St. Louis, MO, USA), and Wako Pure Chemical Industries. 2,3-Diaminonaphthalene (DAN) and 2-(2,3,4-trihydroxybutyl)-benzo[*g*]quinoxaline (3-DGad) were purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan), and 3-deoxyglucosone (3-DG), from Toronto Research Chemical, Inc. (Toronto, Ontario, Canada). All other reagents were from Wako Pure Chemical Industries and were of analytical grade.

4.2.2. Samples

Brown sugar was obtained from a local brown sugar factory (Iriomotetougyou Co., Ltd., Okinawa, Japan) and was a new product of the 2009 production year. The hardened block of brown sugar was crushed into powder or to various sizes of pebbles (1–3 cm) by a crushing machine. The crushed brown sugar samples (20 kg each) were immediately packaged in linear low-density polyethylene-coated double-paperboard 90 gsm bags (85 cm × 42 cm × 8 cm; thickness, 60 μm) and then sealed. The samples were stored at room temperature in a closed storage room for 0, 3, 6, 9, and 12 months (mo) without humidity control. The day-night temperature and humidity changes during storage can be seen in **Figure A2** (appendix). The annual average of storage temperature

was 26.8 °C with 52.1% relative humidity. After storage, the brown sugar was milled into powder at 25,000 rpm for 3 min using a Wonder Blender WB-1 (Osaka Chemicals Co. Ltd., Osaka, Japan) to pass a screen of 1.7 mm. The powdered brown sugar samples were kept at –30 °C prior to analysis.

4.2.3. Color analysis

The color of cane brown sugar was determined in terms of the international unit of color for sugary products as per the standard ICUMSA (International Commission for Uniform Methods of Sugar Analysis) GS1/3-7 protocol.[82] Briefly, 1 g of sample was dissolved in 100 mL distilled water, and then adjusted to pH 7.0 by adding NaOH solution. The mixture was filtered through Advantec No. 2 filter paper and a 0.45-μm membrane filter (Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and its absorbance was measured at 420 nm using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corp., Kyoto, Japan). The sample color was calculated using the equation: ICUMSA color unit or IU = (absorbance × 1000)/($b \times C$), where b is the cell path length used (cm) and C is the concentration of sugar solution (g/mL). All assays were carried out in triplicate.

4.2.4. Moisture content and a_w analyses

Moisture content was evaluated based on the weight loss of a 5-g sample during oven drying at 105 °C for 1 h under atmospheric pressure and was expressed as percentage (%) of the total weight. A_w was measured with 7.5 g of sample using a Novasina IC-5000 AW-LAB water activity analyzer (Novasina AG, Lachen, Switzerland). All assays were carried out in triplicate.

4.2.5. Sugar composition analysis

The sugar composition of cane brown sugar was determined using a high performance liquid chromatography (HPLC) method adapted from Xu et al.[83] with slight modifications. Briefly, 0.3 g of sample was dissolved in 20 mL distilled water, and then filtered through a Millex-LH 0.45-μm membrane filter (Millipore Corp., Billerica, MA, USA). The columns used were Shodex SUGAR KS-801 and KS-802 columns (300 mm × 8.0 mm i.d.; 6-μm particle size; Showa Denko Co. Ltd., Tokyo, Japan) connected to a Shodex SUGAR KS-G (50 mm × 6.0 mm i.d.) guard column. A TOSO-8010 HPLC system (Toso Co. Ltd., Tokyo, Japan) equipped with a refractive index detector model RI-8010 was used. The TOSO-CCPD pump was operated in isocratic mode with distilled water at a flow rate of 1 mL/min, and the CO-8010 oven was programed at a constant temperature of 60 °C. Samples and standards (30 μL) were injected through an AS-8010 auto sampler. The composition and concentration of sugars were calibrated by plotting peak area against concentration for the respective sugar standards, and expressed as grams per 100 g cane brown sugar. All assays were carried out in triplicate.

4.2.6. Organic acid composition analysis

The organic acid composition was determined using an HPLC method adapted from Ji et al.[84] with slight modifications. Briefly, 1 g of sample was dissolved in 5 ml distilled water, centrifuged at 12,000 rpm for 5 min, and then filtered through an Advantec 0.45-μm membrane filter (Toyo Roshi Kaisha, Ltd.). Two Shim-pack SCR-102H columns (300 mm × 8 mm i.d., Shimadzu Corp.) with a guard column (50 mm × 6 mm i.d.) were maintained at 40 °C in a Shimadzu CTO-10AC oven. Two

Shimadzu LC-10AD pumps were operated to stream the mobile phase containing 8.5 mM *p*-toluenesulfonic acid and post-column detection reagent containing 8.5 mM *p*-toluenesulfonic acid, 100 μM EDTA disodium salt, and 20 mM Bis-Tris buffer in the isocratic mode at a flow rate of 0.7 mL/min. The mobile phase and post-column detection solvent were streamed to a post-column reactor and mixed at a ratio of 1:1, prior to detection with a Shimadzu CDD-6A conductivity detector. The injection volume of samples and standards was 10 μL. The composition and concentration of organic acids were calibrated by plotting peak area against concentration for the respective acid standards, and expressed as milligrams per 100 g cane brown sugar. All assays were carried out in triplicate.

4.2.7. Flavor component analysis

The flavor components of cane brown sugar were extracted using the solid-phase microextraction (SPME) technique and analyzed using gas chromatography-flame ionization detection/mass spectrophotometry (GC-FID/MS).[85] Briefly, 3 g of sample was placed in a closed vial and heated in a water bath at 60 °C for 3 min. The flavor components were then absorbed onto an SPME fiber containing divinylbenzene/ carboxen/polydimethylsiloxane (Supelco Inc., Bellefonte, PA, USA) while heating for 20 min. The GC-FID analysis was performed using an Agilent 7890A GC system equipped with a fused silica capillary column (DB-Wax, 60 m × 0.25 mm i.d., film thickness 0.25 μm, Agilent J&W, Santa Clara, CA, USA). The GC injector and the FID were both set to 250 °C. The SPME fiber was injected using a split ratio of 1:1. The oven temperature was initially set to 40 °C for 1 min, then raised to 200 °C at a rate of 3 °C/min, and then held isothermally at 200 °C for 37 min. The linear velocity of the

helium carrier gas flow rate was programed at 32 cm/s. The peak area response of the volatile compounds was monitored in order to evaluate the relative amounts (%) of the flavor components in cane brown sugar without using correction factors.

Mass spectral analysis of flavor components was performed using the same GC instrument coupled with an Agilent 5975C mass spectrophotometer, and the GC conditions were as described above. For MS detection, both electron impact ion source and interface temperatures were programed at 230 °C, and the ionization energy was set to 70 eV. The mass acquisition scan range and rate were (*m*/*z*) 29–450 amu and 1.77 scans/s, respectively. The flavor components were identified by comparison of the linear retention indices (RIs), the mass spectra fragmentation patterns with the MS data of the corresponding compounds obtained from the National Institute of Standards and Technology (NIST) MS Library, Version 2008, and the peak enrichment upon co-injection with authentic aroma standards. Linear RIs of the flavor components were determined relative to the retention times of a series of *n*-alkanes (C7–C28). All assays were carried out in triplicate.

4.2.8. 3-Deoxyglucosone (3-DG) content analysis (analyzed by Dr. Hajime Tamaki, University of the Ryukyus)

The 3-DG constituent in cane brown sugar was evaluated using HPLC methods based on formation of 3-DG/DAN adducts so-called 3-DGad.[81] Briefly, 0.1 g of sample was dissolved in 10 mL distilled water, centrifuged at 1500 rpm for 15 min, and filtered through an Advantec 0.45 μm membrane filter (Toyo Roshi Kaisha, Ltd.). The sample extract (0.5 mL) was then exposed to DAN 1.6 mM (1:1, v/v) and incubated in a water bath at 50 °C for 24 h. The 3-DG content was determined by HPLC using a Shimadzu

SCL6B (Shimadzu Corp.) equipped with a Shimadzu SPD-6A UV/Vis detector. The column used was Cosmosil 5C18-ARII (150 mm × 4.6 mm i.d., 5-μm particle size, Nacalai Tesque, Inc.), and the Shimadzu CTO-6A oven was maintained at a constant temperature of 40 °C. The mobile phase was 40% MeOH containing 0.1% formic acid and was streamed by a Shimadzu LC-6A pump at a flow rate of 1 mL/min in isocratic mode. The injection volume of the samples and standards was 10 μL. The 3-DGad peak was detected at 268 nm. The 3-DG standard at a concentration range of 6.1–100 μM was subjected to the adductive reaction that spanned the concentration levels of 3-DG in sample and used for calibrating peak area plots. The concentration of 3-DG was expressed as milligrams per 100 g cane brown sugar. All assays were carried out in triplicate.

4.2.9. Statistical analysis

The physicochemical properties of cane brown sugar during storage were statistically evaluated using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA, USA) with analysis of variance followed by Fisher's least significant difference post hoc test. Comparisons of the mean values were performed for the following periods: 0 to 3 mo, 3 to 6 mo, 6 to 9 mo, and 9 to 12 mo. Their statistical differences were determined at $p < 0.05$ or $p < 0.01$. To distinguish the effect of the Maillard reaction on browning rate in stored cane brown sugar, Pearson's correlation analysis was performed between the IU and the volatile MRPs or 3-DG contents.

4.3. Results and discussion

4.3.1. Color, moisture content, and a_w changes in stored cane brown sugar

The physical characteristics of cane brown sugar, comprising color, moisture content, and a_w, are presented in **Figure 4-2**. New cane brown sugar product at the time of initial storage (0 mo) had a brown color of IU 18,119 (**Figure 4-2.a**). The brown color in this sugar might be the result of the lack of molasses removal processes during its production, wherein phenolic and flavonoid pigments might be retained in the brown sugar.[69,73] A non-enzymatic browning reaction might also occur during or after the sugarcane stalk crushing process. The brown color of the analyzed sample was much darker than that of previously reported commercial brown and yellow sugars (IU 2375 and 636, respectively) but lighter than that of dark brown raw sugar (IU 37,080).[86,87] Moreover, the color of the analyzed cane brown sugar was found to become darker during 1-year storage. The IU of cane brown sugar was significantly raised at an average rate of 12.5% for each storage stage to IU 28,875 in the 12-mo stored sample ($p < 0.01$). The development of brown color with the progress of the Maillard reaction during food storage has also been known to occur in other food products such as Turkish white hard grape pekmez and high-protein nutritional bars.[75,77] This color change phenomenon should have an important impact on the overall quality attributes of cane brown sugar underlying variations in its food product applications with particular color preference concerns, and might be caused by chemical reactions, including the Maillard browning reaction, that occur during storage as described later.

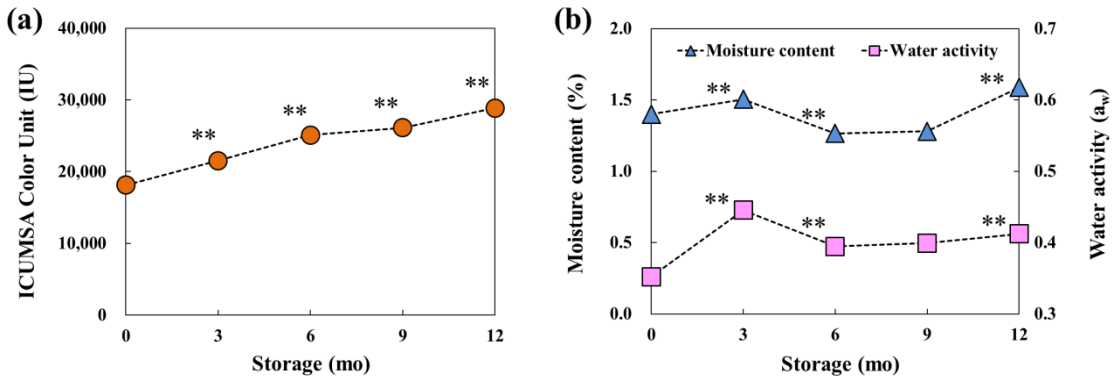

Figure 4-2. Changes in (a) color and (b) moisture content and water activity (a_w) of stored cane brown sugar.

Each value is expressed as the mean ± standard deviation (n = 3). Significant differences in these changes between different 3-mo storage stages (e.g., 0 to 3 mo, 3 to 6 mo) are indicated as follows: *, $p < 0.05$; **, $p < 0.01$.

The initial moisture content of cane brown sugar was 1.40% (**Figure 4-2.b**). It was significantly increased in the first 3 months of storage to 1.50% but then decreased in the second stage before it increased again over the final storage stage to 1.59% ($p < 0.01$). This trend was similarly observed for the a_w: the initial a_w of cane brown sugar was 0.35, and it reached 0.41 after 12 months of storage. Regarding the microbial safety of the product, nevertheless, the result of a_w values show that cane brown sugar stored up to 1 year could be considered to be protected from common microbial and oxidative spoilages and the a_w values were in the proposed range considered to contribute to enhancing browning in the Maillard reaction.[88]

4.3.2. Sugar and organic acid changes in stored cane brown sugar

On the whole, the non-reducing sugar sucrose remained at the same level of 90.9 g/100 g cane brown sugar over 1 year of storage, whereas fructose content decreased from 0.75 to 0.60 g/100 g and glucose content changed from 0.46 to 0.12 g/100 g

(**Figure 4-3.a**). Moreover, significant decreases in the levels of both fructose and glucose were found in the first and second 3-mo storage stages ($p < 0.01$ or $p < 0.05$). These 2 reducing sugars, along with amino acids, might take part in the browning development of the Maillard reaction; thus, their components are constantly degraded over time. This result is in agreement with previously reported studies on gradual degradation over time of fructose and glucose into various intermediates and advanced MRPs, including α-dicarbonyl compounds, organic acids, and melanoidins.[76,77,81]

Figure 4-3.b and **Figure 4-3.c** show the organic acid compositions of stored cane brown sugar. The initial total organic acid content was 709.5 mg/100 g, and lactic acid was found to be the predominant acid component, followed by aconitic and acetic acids. This level dropped to a relatively constant level (about 663.75 mg/100 g) over storage. The organic acid content significantly decreased during the first and second 3-mo storage stages ($p < 0.01$ or $p < 0.05$). The lactic acid content of the analyzed sample (390.8–449.5 mg/100 g) was found to be higher than that previously reported for 12 commercial samples of cane brown sugars, most likely after a period of storage (15.2–74.0 mg/100 g), in which lactic acid was also identified as the main organic acid component.[87] Variations in lactic acid as well as acetic acid content in cane brown sugar products might indicate differences in the technological processes used in pre-production and production practices, particularly in adjusting temperature and flow time conditions, wherein lactic and acetic acids in cane brown sugar are derived from fermentation reactions of lactic acid and acetic acid bacteria during the sugarcane juice crushing process, before it is heated and processed into the solidified form of brown sugar.[89,90] Moreover, aconitic acid content significantly declined in each 3-mo storage

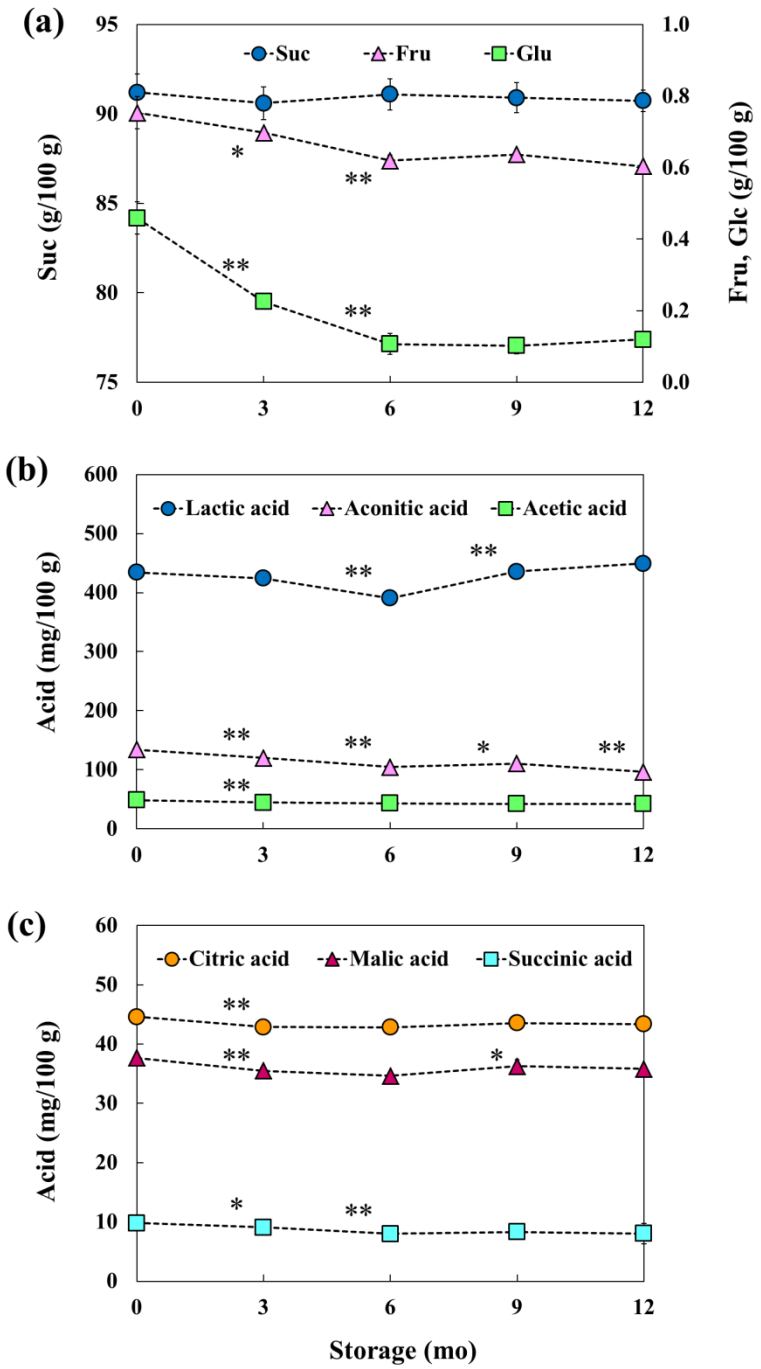

Figure 4-3. Changes in the composition of (a) sugars, (b) lactic, aconitic, and acetic acids, and (c) citric, malic, and succinic acids of stored cane brown sugar.

Each value is expressed as the mean ± standard deviation (n = 3). Significant differences in these changes between different 3-mo storage stages (e.g., 0 to 3 mo, 3 to 6 mo) are indicated as follows: *, $p < 0.05$; **, $p < 0.01$.

stage from an initial content of 134.4 to 96.3 mg/100 g ($p < 0.01$ or $p < 0.05$). The same trends were also found for citric, malic, and succinic acids. These changes during each storage stage might have important impacts on the overall quality attributes of cane brown sugar, particularly when it is used as a material in food and beverage production with acidic level concerns, particularly regarding the conditions used in their manufacturing processes and the compositions of the final products.

4.3.3. Flavor components changes in stored cane brown sugar

A total of 37 flavor components were identified in cane brown sugar from the 0-mo storage sample, including 12 Maillard reaction products (MRPs), 10 acids, and 8 alcohols (**Table 4-1**). The total intensity of the identified flavor compounds, represented by the total peak area, was 302.17.E+0.6. Cane brown sugar was observed to gradually lose its aroma intensity to 172.40.E+0.6 after 1 year of storage. Regarding its relative percentage to the total peak area of flavor compounds, more than half of the flavor components in initial cane brown sugar were determined to be acid compounds (58.70%), followed by MRPs and alcohols (26.52 and 11.96%, respectively). Butanoic acid was found to be the predominant acid constituent, followed by 2-propenoic, hexanoic, propanoic, and 3-methyl-butanoic acids. The MRP group comprised pyrazine, pyranone, pyrrole, furanone, and furan components, whereas the alcohol group was mainly composed of [R-(R*,R*)]-2,3-butanediol and its stereoisomer [S-(R*,R*)]-2,3-butanediol. In addition, marked levels of a sulfuric compound, identified as dimethyl sulfide (1.85%), 3 aldehyde compounds (0.49%), 2 monoterpene hydrocarbons (0.45%), and 1 hydrocarbon (0.03%) were found in the cane brown sugar.

Table 4-1. Flavor components of cane brown sugar (0-mo storage).

No.	RI[a]	Flavor component	Content[b] (peak area 1.E+06)	Identification[c]
1	707	Acetaldehyde	0.38 ± 0.18	RI, MS, PC
2	1054	Hexanal	0.14 ± 0.05	RI, MS, PC
3	1557	Benzaldehyde	0.98 ± 0.08	RI, MS, PC
	Relative percentage of total aldehydes (3 compounds)		***0.49%***	
4	948	Ethanol	1.19 ± 0.68	RI, MS, PC
5	1272	1-Pentanol	0.88 ± 0.18	RI, MS, PC
6	1336	1-Hydroxy-2-propanone	2.52 ± 0.43	RI, MS, PC
7	1546	2-Ethyl-1-hexanol	5.46 ± 1.90	RI, MS, PC
8	1606	[R-(R*,R*)]-2,3-Butanediol	11.89 ± 2.42	RI, MS, PC
9	1649	[S-(R*,R*)]-2,3-Butanediol	10.47 ± 2.02	RI, MS, PC
10	2286	2-Phenoxy-ethanol	0.25 ± 0.08	RI, MS, PC
11	2349	2-Methoxy-4-vinylphenol	3.62 ± 0.43	RI, MS, PC
	Relative percentage of total alcohols (8)		***11.96%***	
12	753	Dimethyl sulfide	5.63 ± 1.03	RI, MS, PC
	Relative percentage of total sulfurs (1)		***1.85%***	
13	1415	3-Methyl-tridecane	0.08 ± 0.03	RI, MS
	Relative percentage of total hydrocarbons (1)		***0.03%***	
14	1296	o-Cymene	0.01 ± 0.00	RI, MS, PC
15	1720	Menthol	1.34 ± 0.53	RI, MS, PC
	Relative percentage of total monoterpene hydrocarbons (2)		***0.45%***	
16	1292	Dihydro-2-methyl-3(2H)-furanone	1.43 ± 0.22	RI, MS, PC
17	1301	2-Methyl-pyrazine	4.01 ± 0.61	RI, MS, PC
18	1365	2,5-Dimethyl-pyrazine	39.72 ± 4.52	RI, MS, PC
19	1371	2,6-Dimethyl-pyrazine	4.17 ± 0.61	RI, MS, PC
20	1391	2,3-Dimethyl-pyrazine	2.08 ± 0.29	RI, MS, PC
21	1458	2,3,5-Trimethyl-pyrazine	7.77 ± 1.03	RI, MS, PC
22	1501	2-Ethyl-3,6-dimethyl-pyrazine	6.76 ± 0.79	RI, MS, PC

Table 4-1. Flavor components of cane brown sugar (storage 0 mo) (*continued*).

No.	RI[a]	Flavor component	Content[b] (peak area 1.E+06)	Identification[c]
23	1739	2-Furanmethanol	4.37 ± 0.73	RI, MS, PC
24	1735	5-Methyl-2-furanmethanol	0.73 ± 0.08	RI, MS
25	2098	2-Acetyl-pyrrole	2.60 ± 0.33	RI, MS, PC
26	2170	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	2.78 ± 0.40	RI, MS, PC
27	2435	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP)	3.68 ± 0.91	RI, MS
Relative percentage of total MRPs (12)			***26.52%***	
28	1595	Propanoic acid	11.44 ± 1.57	RI, MS, PC
29	1644	Butanoic acid	104.96 ± 12.97	RI, MS, PC
30	1704	2-Propenoic acid	29.80 ± 2.89	RI, MS, PC
31	1747	3-Methyl-butanoic acid	10.03 ± 1.35	RI, MS, PC
32	1809	Pentanoic acid	0.64 ± 0.09	RI, MS, PC
33	1824	2-Methyl-pentanoic acid	3.19 ± 0.36	RI, MS, PC
34	1946	Hexanoic acid	12.34 ± 1.40	RI, MS, PC
35	2066	2-Ethyl-hexanoic acid	1.48 ± 0.79	RI, MS, PC
36	2190	Octanoic acid	3.32 ± 0.06	RI, MS, PC
37	2589	Benzoic acid	0.06 ± 0.02	RI, MS, PC
Relative percentage of total acids (10)			***58.70%***	
Total identified			**302.17**	

[a] Retention indices relative to *n*-alkanes on a polar DB-Wax column.

[b] Each value is expressed as the mean ± standard deviation (n = 3).

[c] RI: identification based on retention index; MS: identification based on the NIST MS library; PC: identification based on authentic standards analyzed by mass spectrometry.

Each volatile flavor compound of cane brown sugar might contribute to the overall aroma quality and characteristic. For instance, the published literature indicates that the predominant butanoic acid might provide yogurt and papaya aromas, whereas hexanoic and octanoic acids produce sweaty or cheesy aromas, and propanoic acid produces an acidic characteristic.[91,92] Regarding the MRP components, pyrazine compounds, such as 2,5-dimethyl-pyrazine, 2,6-dimethyl-pyrazine, and 2,3,5-trimethyl pyrazine, might present nutty, woody, and roasted characteristics, and have been recognized as key aroma components in almond, coffee, and rice wine.[91,93,94] Furanones, furans, and pyranones have been known to provide sweet, burnt, pungent, and caramel-like aromas, whilst pyrroles might be associated with cereal-like flavor.[78] A mixture of other volatile components might also complement the complexity of flavor characteristics in cane brown sugar, viz. 2,3-butanediol (fruity aroma), 1-pentanol (fruity, balsamic), hexanal (fresh, green), benzaldehyde (fruity, sweet), and dimethyl sulfide (sulfurous).[91,92,95]

The relative concentration percentage of flavor components in cane brown sugar was observed to vary during storage (**Figure 4-4** and **Table A1**, appendix). From the initial storage concentration of 58.7%, acid constituents were found to be consistently and significantly down to 34.7% at 9 mo ($p < 0.01$ or $p < 0.05$), and so was the sulfuric compound dimethyl sulfide. Conversely, volatile MRPs were significantly increased from 26.5 to 43.2% in the same period ($p < 0.01$), before declining to 38.6% when reaching the final stage of storage. Like MRPs, the same trend was also observed in alcohol compounds, in which this percentage increased from 12.0 to 17.6%. However, this increase was more likely due to the decline of acid and sulfur component levels during storage. Nevertheless, this result indicated that volatile acid and sulfuric

compounds might be released due to their low boiling points, thus rendering these components more unstable and volatile during storage at room temperature, whereas the non-enzymatic browning reaction might keep occurring in stored cane brown sugar, causing relatively stable levels of volatile MRP compounds.

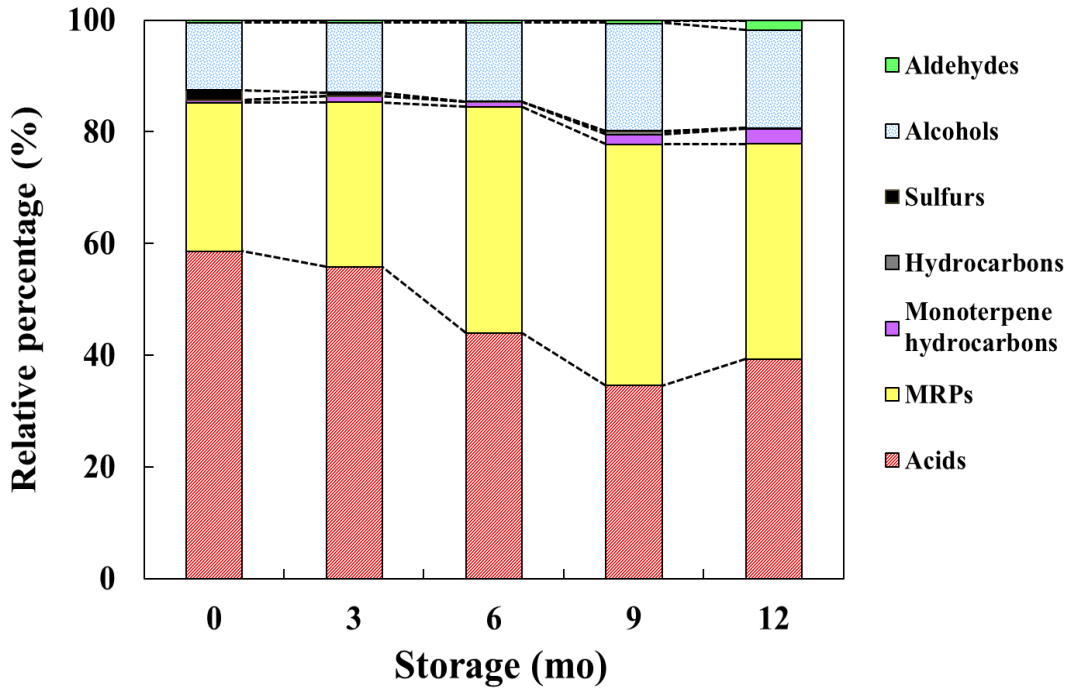

Figure 4-4. Changes in the relative concentrations (%) of flavor components of stored cane brown sugar.

Each value is expressed as the mean (n = 3). Statistical significance analysis is presented in **Table A1** (appendix).

Understanding variations in flavor composition and characteristics, as well as other physicochemical properties of stored cane brown sugar, is necessary and may play an important role in the formulation of brown sugar-based food and beverage products, especially in terms of acidic odors versus nutty or roasted characteristics. For instance,

freshly produced and up to 3-mo-stored cane brown sugar can be used in formulating confectionery or beverage products. A lighter color of the sugar with higher organic acid content and acidic aroma are considered typical characteristics of these products, and are therefore necessary as one of the key ingredients in the recipes. On the other hand, for producing bakery products like cakes and biscuits with a strong nutty or roasted flavor and a dark brown color, 9- to 12-mo-stored cane brown sugar can be an appropriate raw material. Additionally, changes in the compositions of sugars, organic acids, and flavor components can provide important information for food product development and quality control in the uses of cane brown sugar.

4.3.4. Volatile MRPs and 3-DG changes in stored cane brown sugar

The Maillard reaction occurring in stored cane brown sugar caused the composition of volatile MRPs to fluctuate at all storage stages (**Figure 4-5** and **Table A2**, appendix). In the pyrazine group, 2,5-dimethyl-pyrazine, the predominant MRP compound in cane brown sugar, showed a relative concentration percentage ranging from 2.76 to 13.16%, followed by 2,3,5-trimethyl-pyrazine, 2-ethyl-3,6-dimethyl-pyrazine, and so forth. Interestingly, all pyrazine compounds decreased significantly in the first and second 3-mo storage stages ($p < 0.01$ or $p < 0.05$), before being recorded at the lowest concentrations at the 6-mo storage stage, and then increased again toward the end of storage. On the other hand, a pyranone (DDMP) and a pyrrole (2-acetyl-pyrrole) were significantly raised in the first and second 3-mo storage stages from 1.22 to 14.11% and 0.86 to 10.04%, respectively ($p < 0.01$), before starting to lessen over 1-year storage. Moreover, the level of dihydro-2-methyl-3(2H)-furanone was increased significantly at 9-mo storage, whereas the level of the other furanone compound,

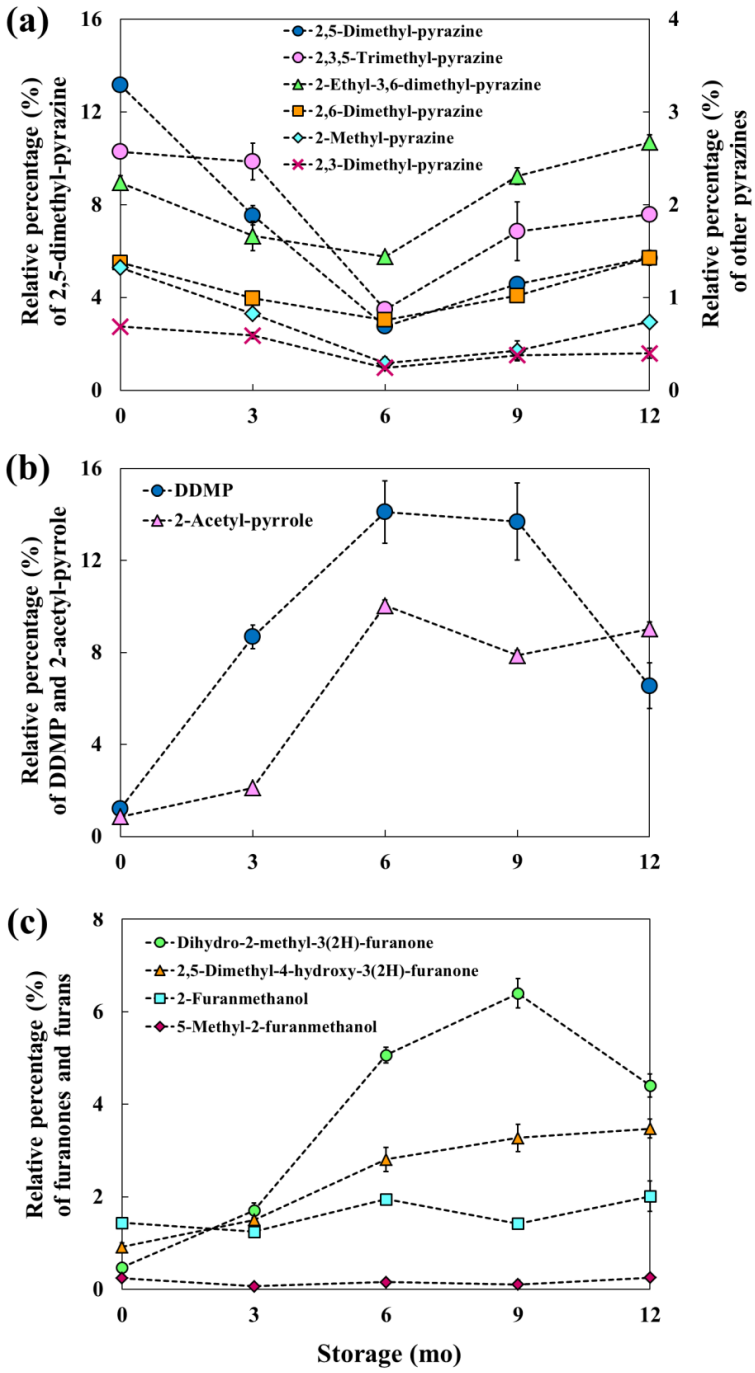

Figure 4-5. Changes in relative concentrations (%) of volatile MRP components of stored cane brown sugar: (a) pyrazines; (b) DDMP and 2-acetyl pyrrole; and (c) furanones and furans.

Each value is expressed as the mean ± standard deviation (n = 3). Statistical significance analysis is presented in **Table A2** (appendix).

2,5-dimethyl-4-hydroxy-3(2H)-furanone, kept rising until the final storage stage ($p < 0.01$ or $p < 0.05$). In addition, relatively constant levels of the 2 furans were observed. All MRPs were formed before and during the storage of cane brown sugar, depending on the interaction between different groups of sugars and amino acids (individual or in combination), kinetic behavior, and the relevant parameters, as well as the stability of intermediate products that affect various possible Maillard reaction pathways.[78,79]

An intermediate product of the Maillard reaction, 3-DG, was then evaluated to track non-enzymatic browning reactions occurring in stored cane brown sugar. The initial content of 3-DG in cane brown sugar was 4.1 mg/100 g, and this increased significantly during the first 3-mo storage period to 8.2 mg/100 g ($p < 0.01$) (**Figure 4-6.a**). The levels of this 1,2-dicarbonyl compound then consistently declined to 6.3 mg/100 g through the 12-mo storage period. The result shows that once 3-DG is produced and accumulated, it degrades slowly during storage at room temperature. This trend is in agreement with the results of a previous study by Hellwig et al.[96] who reported that during storage, 3-DG is a kinetically stable intermediate during the Maillard reaction and is degraded more slowly than other 1,2 carbonyl compounds, especially 3-deoxygalactosone (3-DGal), leading to prolonged 5-hydroxymethylfurfural generation. The level of 3-DG in stored cane brown sugar was found to be lower than those previously reported for sugary and bakery products. For instance, the 3-DG content of honey ranges from 27–164 mg/100 g, that of candies from 14–101 mg/100 g, and that of cookies is approximately 13 mg/100 g.[97]

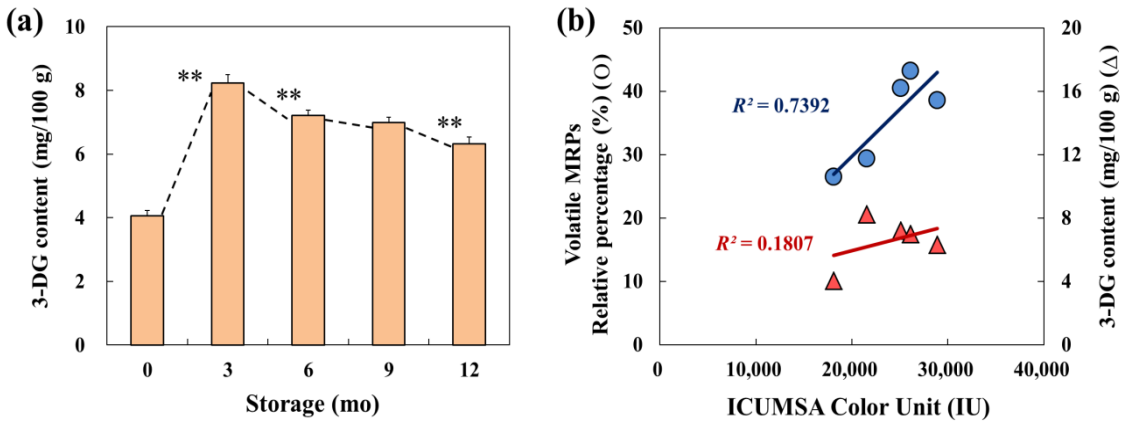

Figure 4-6. (a) Changes in the 3-DG content of stored cane brown sugar.

Each value is expressed as the mean ± standard deviation (n = 3). Significant differences in these changes between different 3-mo storage stages (e.g., 0 to 3 mo, 3 to 6 mo) are indicated as follows: *, $p < 0.05$; **, $p < 0.01$.

(b) Correlation between color (IU) and volatile MRP (○) or 3-DG (△) content of stored cane brown sugar (n = 5).

4.3.5. Correlations between browning color, volatile MRPs, and 3-DG content in stored cane brown sugar

The Maillard reaction was directly involved in the browning of stored cane brown sugar, as shown by plots of correlations between the color units (IU) and the level of volatile MRPs or 3-DG (**Figure 4-6.b**). There was a positive correlation between the browning rate and the relative percentage of volatile MRPs in the analyzed sample (R^2 = 0.739; Pearson's coefficient = 0.860, n = 5). The darker color of stored cane brown sugar might therefore be indicated by strong nutty or roasted odors from the volatile MRPs.[76,78] On the other hand, the color of stored cane brown sugar was found to have a weaker association with 3-DG content (R^2 = 0.181; Pearson's coefficient = 0.425, n = 5). The presence of 3-DG as a precursor of 5-hydroxymethylfurfural generation in the Maillard reaction was not directly correlated with brown color development in the

stored sample; therefore, 3-DG might be accumulated in the matrix sample in some storage periods, particularly in the first and second 3-mo storage periods, before it was converted into different forms via the Maillard reaction with a relatively slow average degradation rate of 6.31% per storage stage from 6- to 12-mo storage. These data should provide useful information for further studies on the kinetic behavior of the non-enzymatic browning reaction of stored cane brown sugar that influences food quality attributes, including color, aroma characteristics, and chemical composition; additional measurements on more important intermediates and co-products of the reaction pathway in kinetic modeling are still required to better comprehend their mechanisms of action.

4.4. Conclusion

Changes in the physicochemical characteristics of cane brown sugar that influence its food quality were observed over 1 year of storage. The stored cane brown sugar became darker, and its moisture content and a_w tended to increase during storage. The content of the reducing sugars fructose and glucose declined as they might take part in non-enzymatic browning via the Maillard reaction. Organic acids, mainly lactic, aconitic, and acetic acids, decreased during some storage stages. The levels of volatile acid and sulfuric compounds of flavor components tended to decrease, and volatile MRPs were generated during storage. The relative concentrations of pyrazines, furanones, furans, pyranones, and pyrroles, as products of the Maillard browning reaction, were found to vary over storage time. A browning precursor, 3-DG, was produced and accumulated in stored cane brown sugar with a relatively slow degradation rate. The browning occurring in stored cane brown sugar was positively

associated with the development of volatile MRPs, indicating the occurrence of strong nutty or roasted flavor characteristics, whereas the level of 3-DG had a weaker correlation with browning rate. These changes in the physicochemical characteristics of stored cane brown sugar may contribute to varying its specific food and beverage applications. Additionally, this information will be useful for better prediction of the storage characteristics of cane brown sugar.

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Chapter V

Aroma Components and Antioxidant Activity of the Unripe Shiikuwasha Peel Oils of Different Extraction Methods

5.1. Introduction

Shiikuwasha is a type of small citrus fruit, and has been used as raw material for beverage and food additive productions in Japan. As expected of a member of the citrus fruit group, Shiikuwasha contains an abundance of ascorbic acid, along with minerals, flavonoids, and essential oils. Shiikuwasha peels, which contain flavonoids such as polymethoxylated flavones nobiletin and tangeretin,[98] have been reported to have promising anti-obesity activities.[99] Moreover, polymethoxy flavones of Shiikuwasha juices were also demonstrated to have anti-tumor effects against gastric cancer and a hepatoprotective effect against D-galacotasamine-induced liver injury.[100,101]

Volatile aroma compounds present in citrus fruits have very important roles in the food and beverage industries. The volatile aroma components of citrus fruits, especially the components of peel, differ depending on the origin of the fruit and the cultivars and hybrids analyzed, and have been widely explored.[102,103] The peel oil of each citrus species has a different composition of hydrocarbons, sesquiterpenes, alcohols, aldehydes, esters, and other oxygenated derivatives. This unique composition suggests that the oil of each citrus fruit, including that of the Shiikuwasha fruit, may have a large number of applications and uses.

Regarding biochemical functions and food functionalities, volatile aroma components of citrus oils, as well as other essential oils, have been reported to have radical-scavenging, and therefore antioxidant, activity.[104,105] Additionally several citrus

fruits have also been shown to have other antioxidant abilities, such as inhibition of *β*-carotene bleaching and reducing power.[106,107]

Neither the composition of the volatile aroma compounds, nor the antioxidant activities of the peel oil of unripe Shiikuwasha have yet been investigated. The study aimed to explore the volatile aroma components of the peel oil of unripe Shiikuwasha 'Ogimi kugani' and to determine the potency of its antioxidant activities by assay of its ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and to inhibit *β*-carotene bleaching. Total phenolic content of the oils was examined using the Folin-Ciocalteu method. Changes in volatile aroma compounds of unripe Shiikuwasha 'Ogimi kugani' following extraction using different extraction methods [cold-press (CP) system and steam distillation (SD) method] were also compared. The research flowchart can be briefly seen in **Figure 5-1**.

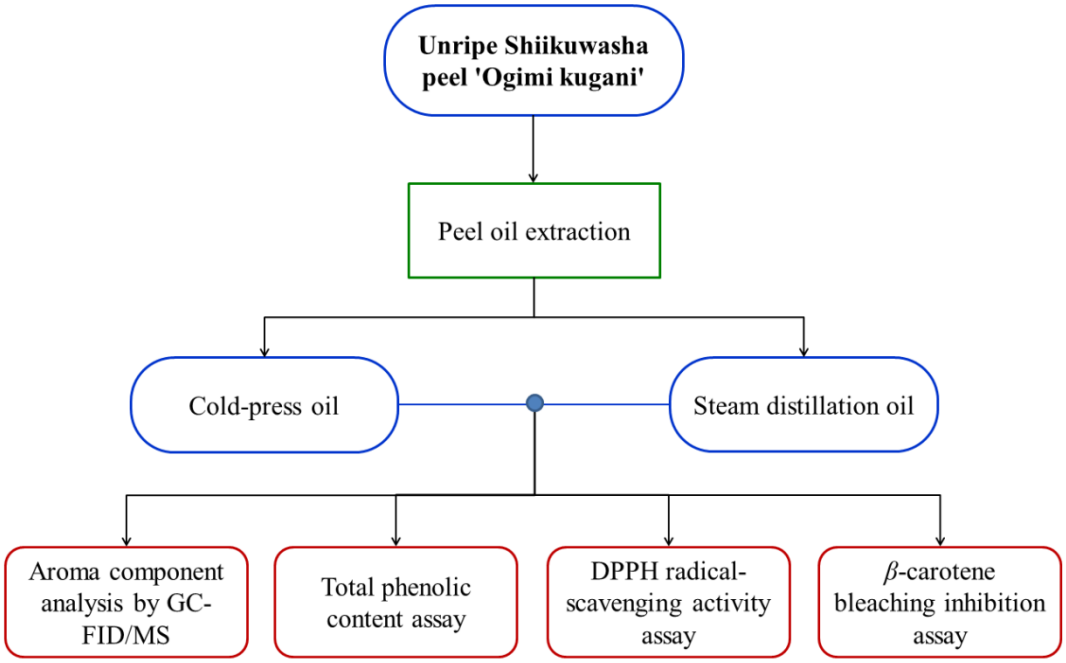

Figure 5-1. Research flowchart of aroma components and antioxidant activity of the unripe Shiikuwasha peel oils of different extraction methods.

5.2. Materials and methods

5.2.1. Standards and reagents

Chemicals used as standards for analysis of volatile aroma components were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Sigma-Aldrich (St. Louis, MO, USA). Gallic acid monohydrate, butylated hydroxyanisole (BHA), and DPPH were purchased from Wako Pure Chemical Industries (Osaka, Japan). The Folin-Ciocalteu reagent was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Linoleic acid and polyoxyethylene sorbitan monolaurate (Tween-20) were purchased from Tokyo Chemical Industry, and *β*-carotene was from Sigma-Aldrich. All other reagents were purchased from Wako Pure Chemical Industries.

5.2.2. Samples

Shiikuwasha 'Ogimi kugani' (*Citrus depressa* Hayata) fruits were obtained from a farm located in Ogimi Village, Okinawa (Japan). Fruits were collected three months before the harvesting season (September 2009). Fruit type was morphologically and chemically characterized in terms of its size, diameter, skin thickness, color, titratable acidity, and total soluble content (**Table A3**, appendix). Skin and flesh colors of the fruit were examined by visual observation. Total soluble content (expressed as °Brix) of the fruit juice was analyzed using a hand-held refractometer (Model N-1α, Atago Co. Ltd., Tokyo, Japan). The outermost surfaces of the peel, which are defined as flavedo, were separated by cutting off the albedo, or soft fiber middle layers, from the peel.

5.2.3. Extraction of volatile aroma compounds

CP-peel oils were obtained from the flavedo (500 g) by hand-pressing and collected in a 10 mL of saturated sodium chloride solution on ice. The oils were centrifuged at 2900 × g for 15 min at 4 °C. The resulting oil layers were dehydrated overnight with anhydrous sodium sulfate at 5 °C and were then filtered.[108]

Oils were also obtained using a steam distillation technique. Briefly, 50 g of flavedo peels were cut into small pieces (about 5 mm^2) added to 500 mL of distilled water, and extracted by hydro-distillation for 5 h, using a Clevenger-type apparatus. The distilled crude extract was centrifuged at 2900 × g for 15 min at 4 °C, and the upper layer was then dried overnight with anhydrous sodium sulfate at 5 °C.

CP and SD peel oils were used for further analysis. Extraction yields were determined and were expressed as grams of oil per 100 g of flavedo peel, or per kilogram of fruit. All extractions were performed in triplicate and averaged. The collected oil was weighed and stored in sealed vials at −30 °C until further use.

5.2.4. Volatile aroma components analysis using GC-FID/MS

The composition of volatile aroma components was examined 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, USA). A flame ionization detector (FID) was used for quantitative analysis of volatile aroma compounds. The GC injector and the flame ionized detector were both set at 250 °C. Samples (1 µL) were injected using a split ratio of 1:50 under a helium atmosphere. The column temperature was initially held at 40 °C for 2 min, was then increased to 200 °C, at a rate of 2 °C/min, and was maintained at 200 °C for 38 min. The linear velocity of

the helium carrier gas flow rate was 32 cm/s.

An Agilent 7890A GC coupled with an Agilent 5975C mass spectrometer was used for GC-MS analysis. The column and oven conditions for GC-MS analysis were as described above. For MS detection, both the electron impact ion source and the interface temperatures were operated at 230 °C. The mass acquisition scan range and rate were (*m*/*z*) 29–450 amu and 1.77 scans/s, respectively, and the ionization voltage was set to 70 eV.

Quantitative analysis was carried out using authentic *n*-hexanol and methyl myristate compounds as internal standards as described by Lan-Phi et al.[109] Standard *n*-hexanol was used for the peaks emerging before linalool and standard methyl myristate for the ones emerging after linalool in the relative order of elution. The neat oil and the two internal standards were prepared at a ratio of 150:1:1, respectively, before injection into the GC. The weight percentage of the peak was calibrated by the correlation factor to FID responses of the internal standards, and the chemical compositions were expressed as the relative concentration (%) or milligrams of volatile compound per 100 g of flavedo peel on a fresh weight basis. Volatile components were identified based on the linear retention index (RI), and by comparison of the mass spectra fragmentation pattern with the MS data of corresponding authentic aroma compounds from the National Institute of Standards and Technology (NIST) MS Library, Version 2008, as well as by peak enrichment upon co-injection with authentic aroma standards. Linear retention indices were also determined for all components using a homologous series of *n*-alkanes (C7–C30). All assays were carried out in triplicate.

5.2.5. Determination of total phenolic content

Total phenolic content was measured using the Folin-Ciocalteu method of Choi et al.[110] with slight modification. Briefly, 1185 µL of distilled water, 15 µL of sample, and 75 µL of Folin-Ciocalteu reagent were mixed in a 2-mL microtube, and were allowed to stand for 2-3 min at room temperature. Next, 225 µL of sodium carbonate (20%) was added to the solution. After shaking, the mixture was placed in the dark at room temperature for 20 min, and the absorbance was then read at 750 nm using an UV-1800 Spectrophotometer (Shimadzu, Kyoto, Japan). Total phenolic content was calculated from a calibration curve of gallic acid (50–500 µg/mL) and was expressed as milligrams of gallic acid equivalents (GAE) per 100 g oil. All assays were carried out in triplicate.

5.2.6. DPPH radical-scavenging activity assay (EC_{50} value)

The antioxidant activity of peel oils was evaluated using micro-scale determination of their ability to scavenge DPPH radicals.[111] Briefly, various concentrations of sample oils in ethanol (50 µL) were mixed with 150 µL of DPPH radicals (0.1 mM) in ethanol solution. The solution then was transferred into a glass 96-well microplate and shaken vigorously. The microplate was immediately placed in a microplate reader (PowerWave XS2, BioTek, Winooski, VT, USA) and left to stand for 60 min, until stable absorption values were obtained. The reduction in DPPH radicals by the oil was examined by measuring the absorption at 517 nm. A mixture of sample oils and ethanol was used as a blank for background subtraction. Radical-scavenging activity was calculated as a percentage of DPPH discoloration using the equation: %scavenging activity = $[(A_{DPPH} - A_S) / A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the oil has been added at a particular level, and A_{DPPH}

is the absorbance of the DPPH solution. The concentration of oil that produced 50% of the maximum DPPH radical-scavenging activity (EC_{50}) was calculated by interpolation from the graph of the percentage scavenging activity against oil concentration. BHA was used as a positive control. All assays were carried out in triplicate.

5.2.7. β-Carotene bleaching inhibition assay (EC_{50} value)

The *β*-carotene bleaching inhibition assay was adapted from Mikami et al.[112] with slight modification. A stock solution of *β*-carotene (1 mg/mL) was prepared in chloroform. Forty milligrams of linoleic acid, 400 mg of Tween-20, and 0.4 mL of *β*-carotene stock solution were placed into a round-bottomed flask. The chloroform was completely removed at 40 °C under vacuum, and 100 mL of distilled hot water (50 °C) was then added to the flask with vigorous shaking. The emulsion (4.8 mL) was dispensed into different test tubes containing 0.2 mL of various concentrations of the oils. Next, 1.5 mL of each oil-emulsion mixture was rapidly transferred to a 24-well microplate and shaken vigorously. The microplate was immediately placed in a microplate reader (PowerWave XS2, BioTek) and maintained at 50 °C. A blank, consisting of an emulsion without *β*-carotene, was prepared for background subtraction. The absorbance of the mixture at 470 nm was recorded every 20 min for 120 min, together with that of a positive (BHA) and a negative (ethanol) control with the same volume as that of the oils. The *β*-carotene bleaching inhibitory activity was calculated using the equation: %inhibition = $[(A_{S(120)} - A_{C(120)}) / (A_{C(0)} - A_{C(120)})] \times 100$, where $A_{S(120)}$ is the absorbance of the solution when the oil has been added at a particular level at t = 120 min, $A_{C(120)}$ is the absorbance of the negative control at t = 120 min, and $A_{C(0)}$ is the initial absorbance of the negative control. The oil concentration that produced

50% of the maximum β-carotene bleaching inhibitory activity (EC_{50}) was calculated by interpolation from the graph of percentage inhibition against oil concentration. All assays were carried out in triplicate.

5.2.8. Statistical analysis

The mean values and standard deviations of the results of the analyses were reported and the statistical differences were examined using t-test at $p < 0.05$ (Microsoft Office Excel 2007, Microsoft, Redmond, WA, USA).

5.3. Results and discussion

5.3.1. Extraction of Shiikuwasha peel oil

In this study, two extraction methods, CP and SD, were used to obtain the Shiikuwasha peel oils. The CP method is known as one of the old extraction systems for citrus aroma compounds and still used for some particular purposes, such as producing more authentic aromas of the oil and remaining some bioactive compounds, while the SD method is more widely used and economical technique. These methods then were compared in terms of their impacts on quantity, volatile aroma composition, and antioxidant activity of the peel oils.

The peel oils obtained from the unripe Shiikuwasha fruit differed in color depending on the extraction process used. Thus, the CP oil was pale yellow whereas the SD oils were colorless. The discoloration observed in the SD oil may have occurred due to heat exposure during the distillation process; thus thermally-unstable non-volatile pigments, that is carotenoids, as well as bioactive compounds that are responsible for coloration, might be easily released.[113]

Table 5-1. Extraction yield of unripe Shiikuwasha 'Ogimi kugani' peel oils.

Oil properties	CP	SD
Extraction yield[a] (%, w/w)[b]	0.56 ± 0.16	0.67 ± 0.06
(g oil/kg fruit)	0.80 ± 0.22	0.95 ± 0.09

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

The amount of peel oil that was obtained from Shiikuwasha fruit was influenced by the extraction method (**Table 5-1**). The extraction yield was greater when the SD system of flavedo peel extraction was used than when the sample was cold-pressed. Thus, the extraction yield was 0.95 g oil/kg of fruit using the SD system, compared to yield of 0.8 using the CP method. The percentage yield using the SD extraction method (measured as the oil per fresh flavedo peel of each sample) was 0.67% against 0.56% using CP method. Repeated exposure to boiling water or steam using the SD system might release almost all of the crude oil from the peel. This result is in agreement with the results of a previous study by Ferhat et al.[114] who reported that the yield of an SD oil (0.21%) was higher than that of a CP oil (0.05%).

5.3.2. Volatile aroma composition of Shiikuwasha peel oil

GC and GC-MS analysis of the peel oils of unripe Shiikuwasha fruit obtained using two different extraction methods led to the identification of 41 compounds that represented approximately 99% of the total volatile aroma composition of the fruit (**Figure 5-2** and **Table 5-2**, respectively). Unripe shiikuwasha peel oil constituents were predominantly monoterpene hydrocarbons, and 91.75–93.75% (709.32–809.05 mg/100 g of fresh flavedo peel) of the total volatile compounds in the oil belonged to this group

of compounds. The main compound was determined to be limonene [43.08–45.13% (341.46–379.81 mg/100 g of fresh flavedo peel) of the total volatile compounds] and there were also abundant amounts of γ-terpinene [27.88–29.06% (219.90–245.86 mg/100 g of fresh flavedo peel)] and *p*-cymene [8.13–11.02% (61.47–97.22 mg/100 g of fresh flavedo peel)]. Linalool, α-thujene, and myrcene, as well as two structural isomers of pinene (α-pinene and β-pinene), were present in moderate amounts.

The amount and composition of the identified compounds in the peel oils was influenced by the extraction method used. Significant differences ($p < 0.05$) in the relative concentration of 32 identified compounds were observed between SD and CP oils. The SD method, which involves circulated steam distillation, may extract more of the minor aroma constituents from peel over the distillation time than the amount extracted using the CP method. Steam distillation extracted all 41 volatile aroma constituents of the peel oils whereas γ-muurolene, (*E*)-carveol, and (*Z*)-carveol were completely absent from the CP oil. Moreover, the levels of alcohol group constituents, such as linalool, terpinen-4-ol, and α-terpineol, were found significantly higher in the SD oil than that of in the CP oil ($p < 0.05$). Linalool and terpinen-4-ol are considered to be key compounds for the aroma of citrus fruits.[115,116] Conversely, the levels of main monoterpene hydrocarbons, such as limonene and γ-terpinene, and sesquiterpene hydrocarbon (bicyclosesquiphellandrene) were extracted slightly higher by the CP method. The ratio of monoterpene hydrocarbons to sesquiterpene hydrocarbons of CP oil was 61.68, whereas this ratio was higher for steam distilled oil that was 109.23.

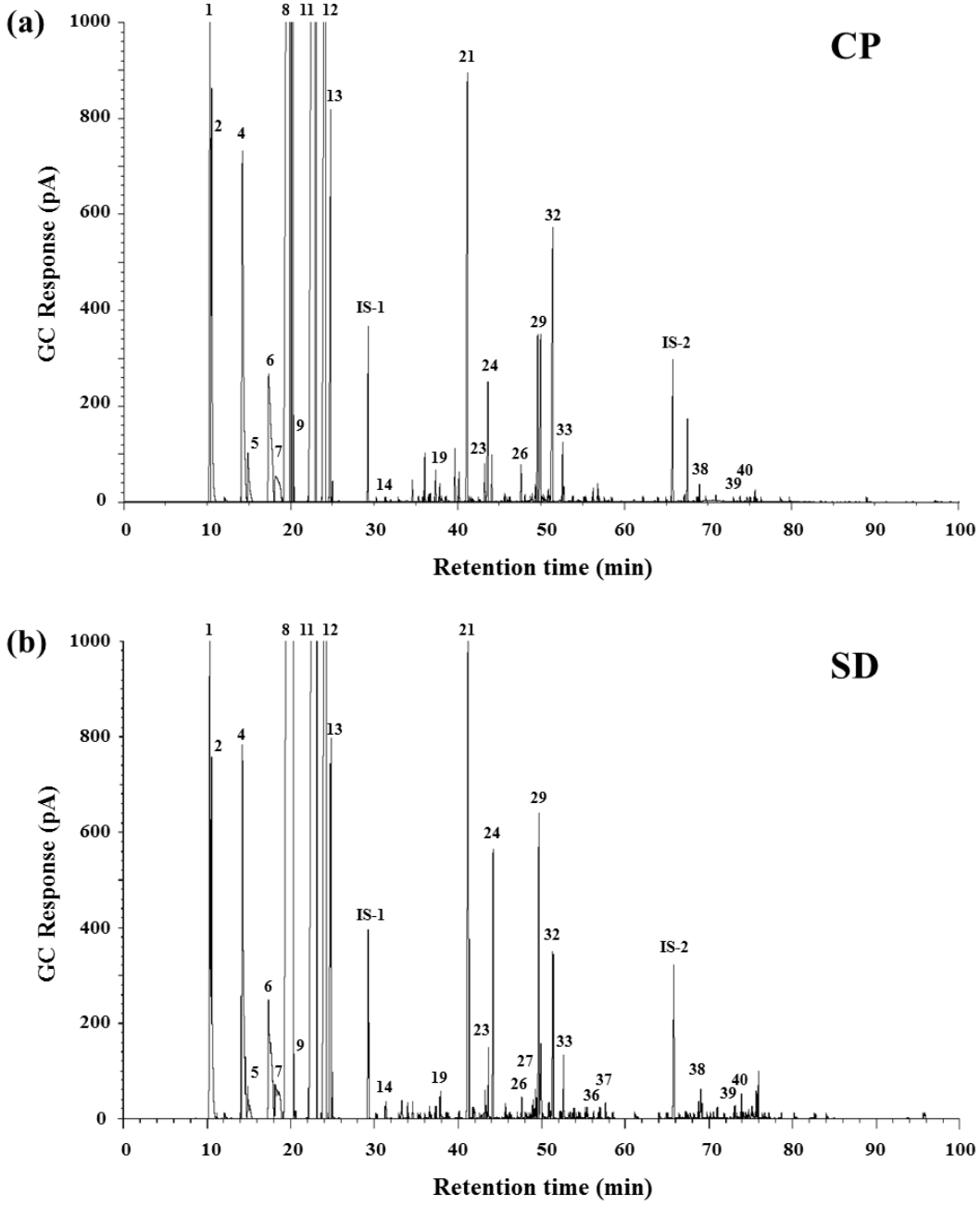

Figure 5-2. Typical gas chromatograms of unripe Shiikuwasha 'Ogimi kugani' peel oils.

The peak numbers refer to the compounds in **Table 5-2**; pA, picoampere; IS, internal standard (1: *n*-hexanol, 2: methyl myristate). The chromatograms shown were obtained using unripe fruit extracted by (a) cold-press and (b) steam distillation methods.

Table 5-2. Volatile aroma composition [relative concentration (%) and mg/100 g fresh flavedo weight] of peel oils of unripe Shiikuwasha 'Ogimi kugani'.

Peak No.	Compound	RI[a]	CP		SD		Identification[b]
			(%)	(mg/100 g)	(%)	(mg/100 g)	
1	*α*-Pinene	1021	2.66 ± 0.14 a[c]	20.08 ± 0.50	2.05 ± 0.14 b	18.12 ± 1.59	RI, MS, PC
2	*α*-Thujene	1026	1.82 ± 0.11 a	13.77 ± 0.47	1.53 ± 0.08 b	13.49 ± 0.35	RI, MS
3	Camphene	1061	0.01 ± 0.00 a	0.09 ± 0.01	0.01 ± 0.00 a	0.13 ± 0.02	RI, MS, PC
4	*β*-Pinene	1107	2.77 ± 0.09 a	20.94 ± 0.13	2.79 ± 0.06 a	24.59 ± 0.80	RI, MS, PC
5	Sabinene	1119	0.36 ± 0.01 a	2.71 ± 0.03	0.21 ± 0.01 b	1.86 ± 0.07	RI, MS
6	Myrcene	1163	1.71 ± 0.03 a	12.97 ± 0.28	1.49 ± 0.02 b	13.17 ± 0.42	RI, MS, PC
7	*α*-Terpinene	1177	0.39 ± 0.26 a	2.98 ± 1.97	0.13 ± 0.01 b	1.11 ± 0.10	RI, MS, PC
8	Limonene	1213	45.13 ± 0.28 a	341.46 ± 10.84	43.08 ± 0.19 a	379.81 ± 10.53	RI, MS, PC
9	*β*-Phellandrene	1215	0.12 ± 0.00 a	0.90 ± 0.02	0.12 ± 0.01 a	1.09 ± 0.03	RI, MS
10	1,8-Cineol	1216	0.02 ± 0.00 a	0.15 ± 0.01	0.04 ± 0.00 b	0.37 ± 0.01	RI, MS, PC
11	*γ*-Terpinene	1257	29.06 ± 0.19 a	219.90 ± 7.95	27.88 ± 0.12 a	245.86 ± 7.06	RI, MS, PC
12	*p*-Cymene	1272	8.13 ± 0.36 a	61.47 ± 2.50	11.02 ± 0.12 b	97.22 ± 3.25	RI, MS, PC
13	Terpinolene	1287	1.43 ± 0.01 a	10.85 ± 0.40	1.42 ± 0.01 a	12.52 ± 0.40	RI, MS, PC
14	Nonanal	1391	0.02 ± 0.00 a	0.13 ± 0.01	0.06 ± 0.00 b	0.57 ± 0.02	RI, MS, PC
15	*α*-Cubebene	1456	0.02 ± 0.00 a	0.12 ± 0.01	0.01 ± 0.00 b	0.37 ± 0.03	RI, MS
16	4-Carene	1468	0.16 ± 0.01 a	1.19 ± 0.07	0.01 ± 0.00 b	0.08 ± 0.01	RI, MS
17	Sabinene hydrate	1476	0.02 ± 0.00 a	0.13 ± 0.02	0.04 ± 0.00 b	0.37 ± 0.02	RI, MS, PC
18	*α*-Copaene	1489	0.09 ± 0.01 a	0.66 ± 0.05	0.04 ± 0.00 b	0.34 ± 0.03	RI, MS
19	Decanal	1497	0.05 ± 0.01 a	0.36 ± 0.04	0.07 ± 0.00 b	0.62 ± 0.04	RI, MS, PC
20	Germacrene D	1536	0.08 ± 0.00 a	0.59 ± 0.05	0.02 ± 0.00 b	0.18 ± 0.02	RI, MS
21	Linalool	1554	1.69 ± 0.06 a	12.80 ± 0.71	3.09 ± 0.09 b	27.24 ± 1.17	RI, MS, PC
22	Bornyl acetate	1579	0.01 ± 0.01 a	0.05 ± 0.04	0.01 ± 0.00 a	0.09 ± 0.02	RI, MS, PC
23	*β*-Caryophyllene	1594	0.41 ± 0.03 a	3.10 ± 0.26	0.21 ± 0.01 b	1.50 ± 0.02	RI, MS, PC
24	Terpinen-4-ol	1604	0.11 ± 0.00 a	0.84 ± 0.05	0.86 ± 0.04 b	6.26 ± 0.16	RI, MS, PC
25	(*E*)-2-Decenal	1640	0.02 ± 0.00 a	0.11 ± 0.01	0.02 ± 0.00 a	0.13 ± 0.00	RI, MS, PC
26	*α*-Caryophyllene	1664	0.10 ± 0.01 a	0.75 ± 0.06	0.06 ± 0.00 b	0.44 ± 0.01	RI, MS, PC
27	*γ*-Muurolene	1684	n.d. a	n.d.	0.01 ± 0.00 b	0.08 ± 0.00	RI, MS
28	Terpinyl acetate	1693	0.04 ± 0.01 a	0.32 ± 0.06	0.08 ± 0.00 b	0.55 ± 0.01	RI, MS, PC

Table 5-2. Volatile aroma composition [relative concentration (%) and mg/100 g fresh flavedo weight] of peel oils of unripe Shiikuwasha 'Ogimi kugani' (*continued*).

Peak No.	Compound	RI[a]	CP		SD		Identification[b]
			(%)	(mg/100 g)	(%)	(mg/100 g)	
29	*α*-Terpineol	1699	0.45 ± 0.01 a[c]	3.43 ± 0.17	1.02 ± 0.06 b	7.40 ± 0.15	RI, MS, PC
30	Bicyclosesquiphellandrene	1704	0.63 ± 0.04 a	4.81 ± 0.42	0.26 ± 0.02 b	1.90 ± 0.04	RI, MS
31	*α*-Muurolene	1722	0.04 ± 0.01 a	0.30 ± 0.03	0.04 ± 0.00 a	0.28 ± 0.00	RI, MS
32	*l*-Carvone	1731	1.27 ± 0.08 a	9.65 ± 0.91	0.64 ± 0.04 b	4.67 ± 0.02	RI, MS
33	*δ*-Cadinene	1754	0.15 ± 0.01 a	1.16 ± 0.11	0.20 ± 0.01 b	1.42 ± 0.00	RI, MS
34	Perilla aldehyde	1777	0.01 ± 0.00 a	0.11 ± 0.01	0.03 ± 0.00 b	0.19 ± 0.00	RI, MS, PC
35	Nerol	1806	0.01 ± 0.00 a	0.08 ± 0.00	0.03 ± 0.00 b	0.05 ± 0.01	RI, MS, PC
36	(*E*)-Carveol	1836	n.d. a	n.d.	0.03 ± 0.00 b	0.14 ± 0.00	RI, MS, PC
37	(*Z*)-Carveol	1866	n.d. a	n.d.	0.02 ± 0.00 b	0.25 ± 0.01	RI, MS, PC
38	Elemol	2080	0.04 ± 0.01 a	0.30 ± 0.04	0.07 ± 0.01 b	0.50 ± 0.03	RI, MS
39	*γ*-Eudesmol	2170	0.01 ± 0.00 a	0.05 ± 0.01	0.06 ± 0.00 b	0.41 ± 0.02	RI, MS
40	Thymol	2186	0.01 ± 0.00 a	0.10 ± 0.01	0.06 ± 0.01 b	0.43 ± 0.02	RI, MS, PC
41	Isothymol	2214	0.01 ± 0.00 a	0.08 ± 0.00	0.03 ± 0.00 b	0.22 ± 0.01	RI, MS, PC
	Component groups						
	Monoterpene hydrocarbons		93.75	709.32	91.75	809.05	
	Sesquiterpene hydrocarbons		1.52	11.48	0.84	6.52	
	(Mon/Ses)[d]		61.68		109.23		
	Alcohols		2.35	17.81	5.29	43.28	
	Aldehydes		0.09	0.71	0.18	1.52	
	Esters & ketones		1.32	10.02	0.73	5.31	
	Oxides		0.02	0.15	0.04	0.37	
	Total identified		99.06	749.50	98.83	866.04	

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

The aroma of citrus fruits is considered to result from a complex mixture of volatile aroma components such as terpenes, sesquiterpenes, alcohols, aldehydes, esters, and ketones. Each citrus cultivar or hybrid most likely contains unique proportions of those components. By referring to literatures, the main volatile compounds of citrus essential oils were detected in the same levels during fruit development.[103,117] Thus, the volatile aroma composition of peel oil of unripe Shiikuwasha of this study to other citrus peel oils of previous reports could be evaluated. Similar to previous studies of other citrus peel oils, limonene was found to be the major compound in peel oils of Shiikuwasha.[102,109,118] However, the limonene content of Shiikuwasha (*Citrus depressa*) peel oils (43–45%) was lower than that of other citrus peels: 85–90% in sweet orange (*C. sinensis*), 74% in Mandarin orange (*C. reticulate*), 59% in lemon (*C. lemon*), and 63–68% in Japanese yuzu (*C. junos*). Conversely, the Shiikuwasha (*C. depressa*) peel oils of the study had very large amounts of *γ*-terpinene (27–29%) and *p*-cymene (8–11%) compared to their levels in other citrus peels. These different relative levels of volatile aroma components in Shiikuwasha might explain the distinctive aroma of Shiikuwasha compared to other citrus species.

5.3.3. Antioxidant activity of Shiikuwasha peel oil

Besides containing volatile aroma compounds, citrus peels have been studied to contain phenolic components that posses many antioxidant activities.[107,119] Polyphenols are known to be the main antioxidants in foods. Their antioxidant activity is due to the presence of hydroxyl substituents and to their aromatic structure, which facilitates hydrogen atom transfer that can quench most known free radicals.[120] Thus phenolic compounds in citrus peels might exhibit better antioxidant properties than other organic

components. The total phenolic content of peel oils from unripe Shiikuwasha fruit obtained using CP and SD methods was 294.2 and 225.4 mg GAE/100 g, respectively and the antioxidant activities of these oils were subsequently analyzed (**Table 5-3**).

Table 5-3. Total phenolic content[a] of peel oils from unripe Shiikuwasha 'Ogimi kugani' fruit and their EC_{50}[b,c] values in antioxidant assays.

Properties	CP	SD
Total phenolic content[a]	294.2 ± 13.2 a[d]	225.4 ± 21.3 b
DPPH (EC_{50}[b])	34.09 a	206.45 b
β-carotene (EC_{50}[c])	1.65 a	3.89 b

[a] Total phenolic content is expressed as mg GAE/100 g oil.
[b] EC_{50} (mg/mL): effective concentration at which 50% of the DPPH radicals are scavenged.
[c] EC_{50} (mg/mL): effective concentration at which 50% of β-carotene bleaching is inhibited.
[d] Each value is expressed as the mean ± standard deviation (n = 3). Means in the same row followed by the same letter are not significantly different ($p < 0.05$).

Xu et al.[105] reported that heat treatment of Ponkan (*C. poonensis* Hort. ex Tanaka) peel potently increases the amount of extracted phenolic compounds as well as the antioxidant activity of the oil, by increasing the fraction of free phenolic acids. However, the study showed that the low-temperature extraction treatment of the CP system could retain the phenolic content of peels better than the SD system. This result are consistent with the results of other research groups who have also reported that heat treatment can cause destruction of some bioactive compounds in some materials and decreases their polyphenol content.[121,122]

In agreement with the data that the low-temperature extraction method better retained the phenolic content of the oil, the CP oil was found to have significant higher antioxidant activity ($p < 0.05$) than the SD oil (**Figure 5-3** and **Table 5-3**). This result is in agreement with that of Ramful et al.,[123] who showed, using linear regression analysis,

that the antioxidant activity of peel oils of Mauritian citrus fruits correlated very well with the level of their phenolic constituents.

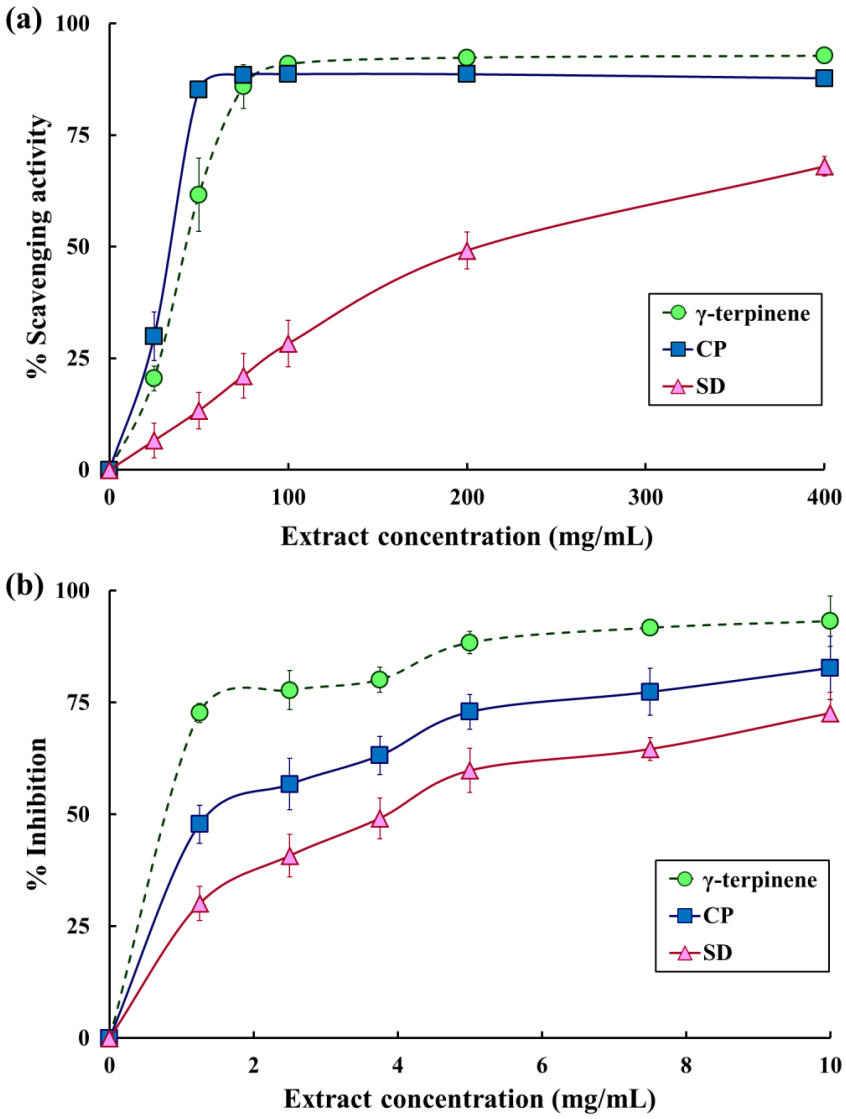

Figure 5-3. Antioxidant activities of unripe peel oils and a standard volatile compound (*γ*-terpinene).

The antioxidant activities of unripe peel oils, obtained using a cold press (CP) or a steam distillation (SD) method, as well as that of an ethanolic solution of standard volatile aromatic component, *γ*-terpinene were assayed in (a) DPPH radical scavenging assay and (b) *β*-carotene bleaching inhibition assays. Each value is expressed as the mean ± standard deviation (n = 3). BHA was used as a positive control for both assays and resulted in 96.14% scavenging activity and 98.51% inhibition respectively at a concentration of 2 mg/mL.

Peel oil scavenged DPPH radicals and inhibited *β*-carotene bleaching in a concentration-dependent manner (**Figure 5-3**). In addition, to evaluate possible factors in the oil that might be responsible for its antioxidant activity, the antioxidant effects of two main authentic, standard, volatile aroma compounds of Shiikuwasha peel, limonene and *γ*-terpinene, were also tested in these assays. Whereas *γ*-terpinene displayed antioxidant activity in these assays, the addition of limonene to the mixture of DPPH radicals in the scavenging activity assay resulted in the formation of a white emulsion that could potentially introduce a bias into the spectrophotometric reading. Additionally, limonene could not delay decolorization of the mixture in the *β*-carotene bleaching assay, suggesting that it did not inhibit *β*-carotene bleaching.

According to Choi et al.,[124] *γ*-terpinene, as well as terpinolene and geraniol, have strong DPPH radical-scavenging activity, whereas limonene, which is a major component of many citrus peels oils, has little effect on radical-scavenging. The results of the study indicate that the antioxidant activities of *γ*-terpinene are superior to the antioxidant activities of peel oils, in terms of both DPPH radical-scavenging activity and inhibition of *β*-carotene bleaching. Since *γ*-terpinene is abundant in peel oils (representing 27–29% of its volatile content), Shiikuwasha fruit may be a promising, relatively pure source of this essential oil that has strong antioxidant activity.

As shown in **Table 5-3**, the EC_{50} value for DPPH radical-scavenging activity of the oil obtained using the CP system was 6 fold lower than that for the SD oil (34.09 mg/mL in contrast to 206.45 mg/mL respectively). The superior antioxidant activity of the CP oil was further evidenced by its ability to delay *β*-carotene co-oxidation in the linoleate model system of bleaching assay. Moreover, the EC_{50} value that was determined for the oil in the *β*-carotene bleaching assay was even lower than that for the

DPPH radical-scavenging assay. The EC_{50} value for the CP oil in the *β*-carotene bleaching assay was 1.65 mg/mL, but this value was 3.89 mg/mL for the SD oil. The EC_{50} value that was determined for the Shiikuwasha SD oil in the DPPH radical-scavenging assay was higher than that reported for volatile oils of other citrus peels such as orange, lemon and lime (EC_{50} values: 95–124 mg/mL oil) obtained using an SD method as described by Guimarães et al.[106] Nevertheless, the DPPH radical-scavenging ability of the Shiikuwasha peel oil obtained using the CP system was better than that of the SD citrus peel oils. In the *β*-carotene bleaching assay, both the CP and the SD Shiikuwasha oils were found to be more effective in inhibiting decolorization than the previously reported citrus peel oils.

5.4. Conclusion

Peel oils of unripe Shiikuwasha 'Ogimi kugani' had a unique composition of volatile aroma components. The major component of the oil was limonene followed by *γ*-terpinene, *p*-cymene, *α*-pinene, and *β*-pinene. The limonene content of Shiikuwasha peels was clearly lower than that of other commonly known citrus peels, whereas the levels of *γ*-terpinene and *p*-cymene were higher. The amount and composition of volatile aroma compounds, as well as antioxidant capabilities in peels extracted from unripe Shiikuwasha was observed to vary by different extraction methods. The SD system resulted in higher extraction yield and extraction of a greater number of volatile aroma compounds than the CP method. The antioxidant activities of CP peel oil were superior to those of the SD oil. Heat exposure during steam distillation might decrease the phenolic content of the oil and lower antioxidant activities such as the ability to scavenge DPPH radicals and to inhibit *β*-carotene decolorization.

Chapter VI

Aroma Components, Flavanones, and Polymethoxylated Flavones of the Shiikuwasha Peels of Different Cultivation Lines

6.1. 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, including to that of in Shiikuwasha fruit. Citrus peels do not comprise only acceptable and pleasant flavor compounds but also bioactive substances that have beneficial effects on human health.[102,125] 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.

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.[126] 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.[114,127] Additionally, volatile aroma components of citrus peel oils, as well as other plant essential oils, have been shown to exhibit various antimicrobial, antiviral, and antioxidant activities.[118,128]

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 polymethoxylated flavones (PMFs), which possess biological activities.[129–131] Of the flavanones, citrus peel-derived hesperidin was observed to have anti-inflammatory and antialcoholic fatty liver effects,[132,133] and naringenin enhanced melanin synthesis and tyrosinase activity in cells.[134] 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.[135,136] Moreover, two PMF compounds in particular, nobiletin and tangeretin, from Shiikuwasha peels have been reported to have antiobesity effects.[99]

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 research flowchart can be briefly seen in **Figure 6-1**. The study, therefore, aimed to distinguish the four different Shiikuwasha cultivation lines ('Izumi kugani', 'Katsuyama kugani', 'Ogimi kugani', and 'Kaachi') from Okinawa, Japan, based on the composition and content of their volatile aroma components, as well as their flavanones and PMFs, extracted from the peel. The study results provide information regarding the distinctive flavors and the natural sources of bioactive substances, particularly flavonoids, for Shiikuwasha fruits.

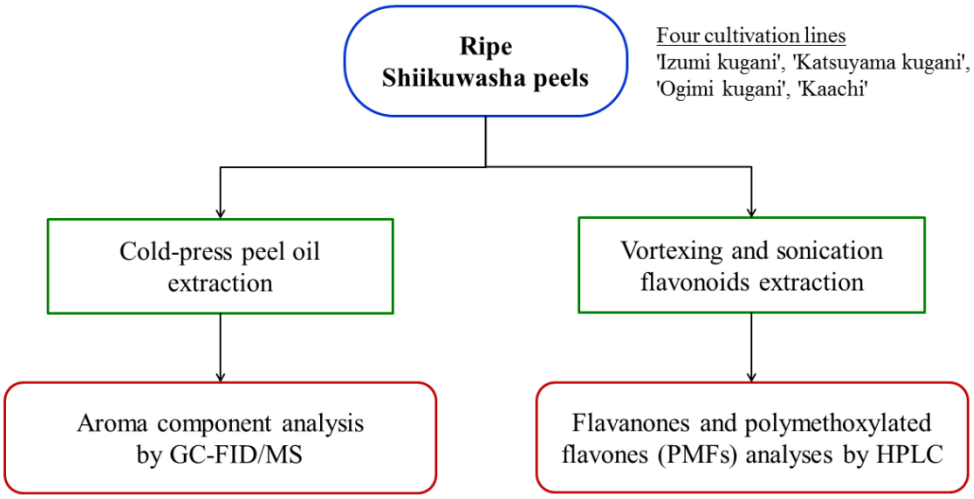

Figure 6-1. Research flowchart of aroma components, flavanones, and polymethoxylated flavones (PMFs) of the Shiikuwasha peels of different cultivation lines.

6.2. Materials and methods

6.2.1. Standards and reagents

Authentic chemical compounds used as standards for the analysis of volatile aroma components were purchased from Sigma-Aldrich (St. Louis, MO, USA) 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.

6.2.2. Samples

Shiikuwasha (*C. depressa* Hayata) 'Izumi kugani', 'Katsuyama kugani', 'Ogimi kugani', and 'Kaachi' 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 (see **Figure A3 and Table A4,** appendix). 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.

6.2.3. Cold-press extraction of volatile aroma compounds

The cold-press extraction was as described in **Section 5.2.3**.

6.2.4. Volatile aroma components analysis using GC-FID/MS

The volatile aroma components analysis was as described in **Section 5.2.4**.

6.2.5. 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

15,000 ×*g* for 30 min at 4 °C, and the resulting supernatant layers were carefully collected.[137] 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 and averaged.

6.2.6. Flavanones and PMFs analyses using HPLC

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.[138] 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 assays were carried out in triplicate.

6.2.7. Statistical analysis

The compositional analyses of volatile aroma compounds, flavanones, and PMFs were statistically evaluated using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA, USA). The mean values of the results of the analyses were subjected to analysis of variance with Fisher's least significant difference post hoc 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.

6.3. Results and discussion

6.3.1. Cold-press extraction of Shiikuwasha peels

In the 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.

Table 6-1. Yield[a] of cold-press extraction from Shiikuwasha peels.

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

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

The yield of volatile aroma components from Shiikuwasha peels of four different cultivation lines is shown in **Table 6-1**. The yield of the oils 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 oil per fresh flavedo peel. The highest extract content was found in 'Katsuyama kugani' fruit, which contained 0.24 g oil 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 oils of different cultivation lines during oil extraction.

6.3.2. 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 6-2**). As a whole, 39 compounds, representing approximately 99% of the total volatile aroma components in the oils 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 oils 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

Table 6-2. Volatile aroma components [relative concentration (%) and mg/100 g fresh flavedo weight] of Shiikuwasha peels.

Peak No.	Compound	RI[a]	'Izumi kugani'		'Katsuyama kugani'		'Ogimi kugani'		'Kaachi'		Identification[b]
			(%)	(mg/100 g)	(%)	(mg/100 g)	(%)	(mg/100 g)	(%)	(mg/100 g)	
1	α-Pinene	1021	1.74 ± 0.217 c[c]	2.50 ± 0.289	2.36 ± 0.084 a	3.03 ± 0.274	2.09 ± 0.066 b	1.45 ± 0.128	1.23 ± 0.137 d	0.88 ± 0.027	RI, MS, PC
2	α-Thujene	1026	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	1.07 ± 0.051 c	0.76 ± 0.060	RI, MS
3	Camphene	1061	tr. b	tr.	0.01 ± 0.001 a	0.01 ± 0.002	tr. b	tr.	n.d.	n.d.	RI, MS, PC
4	*β*-Pinene	1107	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	1.44 ± 0.057 c	1.03 ± 0.066	RI, MS, PC
5	Sabinene	1119	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	0.21 ± 0.007 c	0.15 ± 0.014	RI, MS
6	Myrcene	1163	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	1.71 ± 0.039 bc	1.22 ± 0.098	RI, MS, PC
7	α-Terpinene	1177	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	0.02 ± 0.016 b	0.02 ± 0.011	RI, MS, PC
8	Limonene	1213	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	68.26 ± 0.254 a	48.82 ± 5.158	RI, MS, PC
9	*β*-Phellandrene	1215	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	0.15 ± 0.001 b	0.11 ± 0.011	RI, MS
10	1,8-Cineol	1216	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	0.01 ± 0.000 b	0.00 ± 0.000	RI, MS, PC
11	*γ*-Terpinene	1257	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	21.48 ± 0.175 c	15.37 ± 1.703	RI, MS, PC
12	*p*-Cymene	1272	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	0.57 ± 0.016 d	0.41 ± 0.052	RI, MS, PC
13	Terpinolene	1287	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	1.02 ± 0.014 d	0.73 ± 0.085	RI, MS, PC
14	Nonanal	1391	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	0.05 ± 0.002 a	0.04 ± 0.004	RI, MS, PC
15	α-Cubebene	1456	n.d.	n.d.	0.01 ± 0.000 b	0.02 ± 0.001	0.02 ± 0.000 a	0.01 ± 0.001	n.d.	n.d.	RI, MS
16	4-Carene	1468	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	0.08 ± 0.001 d	0.06 ± 0.007	RI, MS
17	Sabinene hydrate	1476	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	0.12 ± 0.006 b	0.08 ± 0.011	RI, MS, PC
18	α-Copaene	1489	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	0.07 ± 0.002 c	0.05 ± 0.007	RI, MS
19	Decanal	1497	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	0.22 ± 0.007 a	0.16 ± 0.019	RI, MS, PC
20	Germacrene D	1536	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	0.03 ± 0.001 d	0.02 ± 0.003	RI, MS
21	Linalool	1554	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	0.62 ± 0.124 b	0.44 ± 0.071	RI, MS, PC
22	Bornyl acetate	1579	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	0.01 ± 0.000 b	0.00 ± 0.000	RI, MS, PC
23	*β*-Caryophyllene	1594	n.d.	n.d.	0.42 ± 0.019 a	0.54 ± 0.049	0.38 ± 0.051 a	0.26 ± 0.019	0.09 ± 0.007 b	0.06 ± 0.006	RI, MS, PC
24	Methyl thymol	1594	1.15 ± 0.125 a	1.65 ± 0.191	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	RI, MS
25	Terpinen-4-ol	1604	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	0.06 ± 0.006 a	0.05 ± 0.005	RI, MS, PC
26	(*E*)-2-Decenal	1640	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	0.03 ± 0.002 a	0.02 ± 0.002	RI, MS, PC
27	α-Caryophyllene	1664	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	0.05 ± 0.005 b	0.04 ± 0.004	RI, MS, PC
28	Terpinyl acetate	1693	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	0.05 ± 0.004 b	0.03 ± 0.004	RI, MS, PC

Table 6-2. Volatile aroma components [relative concentration (%) and mg/100 g fresh flavedo weight] of Shiikuwasha peels (*continued*).

Peak No.	Compound	RI[a]	'Izumi kugani'		'Katsuyama kugani'		'Ogimi kugani'		'Kaachi'		Identification[b]
			(%)	(mg/100 g)	(%)	(mg/100 g)	(%)	(mg/100 g)	(%)	(mg/100 g)	
29	α-Terpineol	1699	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	0.10 ± 0.008 a	0.07 ± 0.005	RI, MS, PC
30	Bicyclosesquiphellandrene	1704	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	0.07 ± 0.006 c	0.05 ± 0.005	RI, MS
31	α-Muurolene	1722	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	0.01 ± 0.001 c	0.01 ± 0.001	RI, MS
32	*l*-Carvone	1731	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	0.32 ± 0.025 c	0.23 ± 0.019	RI, MS
33	*δ*-Cadinene	1754	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	0.10 ± 0.009 c	0.07 ± 0.008	RI, MS
34	Perilla aldehyde	1777	n.d.	n.d.	0.01 ± 0.000 a	0.01 ± 0.001	0.01 ± 0.001 a	0.00 ± 0.000	n.d.	n.d.	RI, MS, PC
35	Nerol	1806	0.01 ± 0.001 b	0.01 ± 0.002	0.01 ± 0.001 b	0.01 ± 0.001	n.d.	n.d.	0.02 ± 0.002 a	0.01 ± 0.001	RI, MS, PC
36	Elemol	2080	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	0.03 ± 0.003 b	0.02 ± 0.003	RI, MS
37	*γ*-Eudesmol	2170	n.d.	n.d.	0.01 ± 0.000 a	0.01 ± 0.001	0.01 ± 0.001 a	0.00 ± 0.000	n.d.	n.d.	RI, MS
38	Thymol	2186	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	0.01 ± 0.001 b	0.01 ± 0.001	RI, MS, PC
39	Isothymol	2214	n.d.	n.d.	0.01 ± 0.000 a	0.01 ± 0.001	0.01 ± 0.001 a	0.01 ± 0.001	n.d.	n.d.	RI, MS, PC
	Component groups										
	Monoterpene hydrocarbons		93.40	134.13	95.75	122.97	96.35	66.80	97.25	69.55	
	Sesquiterpene hydrocarbons		0.30	0.44	1.50	1.93	1.38	0.95	0.43	0.30	
	(Mon/Ses)[d]		307.84		63.78		69.84		228.17		
	Alcohols		1.65	2.37	0.57	0.73	0.35	0.24	0.97	0.69	
	Aldehydes		0.27	0.38	0.09	0.11	0.06	0.04	0.30	0.21	
	Esters and ketones		0.74	1.06	1.29	1.66	1.09	0.75	0.37	0.27	
	Ethers		1.15	1.65	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
	Oxides		0.94	1.36	0.01	0.01	0.01	0.01	0.01	0.00	
	Total identified		98.45	141.39	99.21	127.41	99.25	68.79	99.32	71.02	

[a] Retention indices relative to *n*-alkanes on a polar DB-Wax column.

[b] RI: identification based on retention index; MS: identification based on the NIST MS library; PC: identification based on authentic standards analyzed by mass spectrometry.

[c] Each value is expressed as the mean ± standard deviation ($n = 3$); n.d. not detected; tr. trace amount (<0.01%). Means in the same row followed by the same letter are not significantly different ($p < 0.05$).

[d] Ratio of monoterpene hydrocarbons to sesquiterpene hydrocarbons.

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 oil. The ratios for 'Izumi kugani', 'Kaachi', 'Ogimi kugani', and 'Katsuyama kugani' peel oils were 307.84, 228.17, 69.84, and 63.78, respectively (**Table 6-2**). 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 oils 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*).[102,139] 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.[140]

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 oils 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 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.[141,142] 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 oils, 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.[109,143] 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 oils for providing a minty aroma with a relatively high flavor dilution factor[144,145] and has been known as the main volatile aroma compound in *Eucalyptus*, *Thymus*, and *Chrysanthemum* essential oils.[146−148] On the other hand, esters (bornyl acetate and terpinyl acetate) and aldehydes (nonanal, decanal, 2-decenal, and perilla aldehyde) were found in relatively small amounts in all Shiikuwasha peel oils.

By application of PCA to the relative concentrations of seven aroma compound groups in Shiikuwasha peels from four different cultivation lines (**Table 6-2**), the first two principle components (PCs: PC1 = 69.52%, PC2 = 28.79%) were identified, which accounted for 98.31% of the total variance (**Figure 6-2**). The result shows that aroma components of Shiikuwasha peels from different cultivation lines can be distinguished in a valid manner. The relations among groups of aroma compounds, as well as relations between the group compositions and the cultivation lines, can be examined from the corresponding loading plots of both PCs. 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 6-2.a**). Separation of 'Izumi kugani' and 'Kaachi' was clearly observed; however, the compositions of volatile compounds in 'Katsuyama kugani' and 'Ogimi kugani' peels were closely associated (**Figure 6-2.b**).This low differentiation ability of PCs is in agreement with the compositions of volatile aroma compounds in 'Katsuyama kugani' and 'Ogimi kugani' peel oils presented in **Table 6-2**. These differences might impact the entire aroma profile of peel oils and, consequently, the aroma characteristics of Shiikuwasha fruits from different cultivation lines.

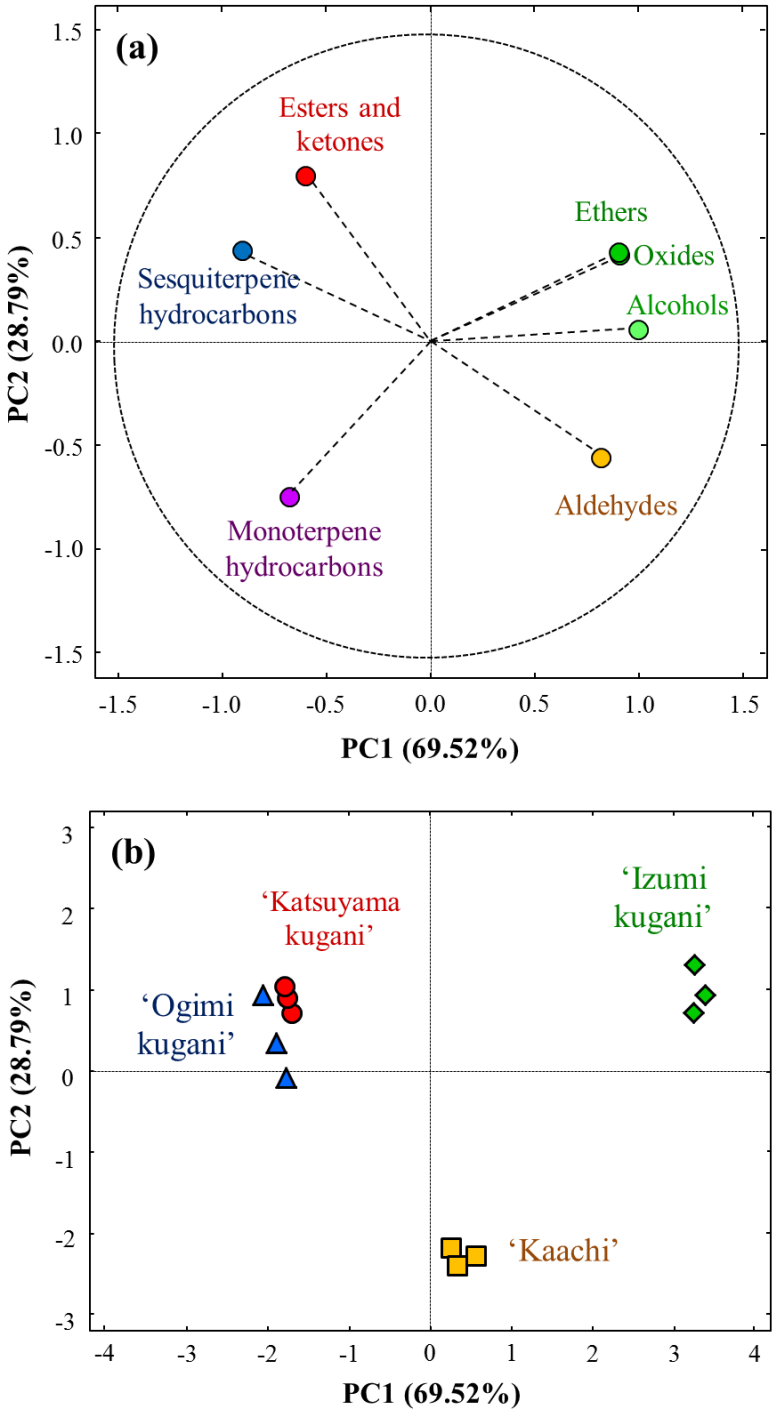

Figure 6-2. PCA plots of volatile aroma components of Shiikuwasha peels from four different cultivation lines:

(a) distribution of seven groups of volatile aroma compounds; (b) discrimination of cultivation lines based on the relative concentrations of seven groups of volatile aroma compounds.

6.3.3. 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 (chemical structures can be seen in **Figure A4**, appendix). Flavanones are known as the predominant flavonoid compounds in various citrus peels.[129,149] As shown in **Table 6-3**, 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,[150] 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.[151]

Table 6-3. Flavanone content (mg/100 g fresh flavedo weight)[a] of Shiikuwasha peels

Flavanone	'Izumi kugani'	'Katsuyama kugani'	'Ogimi kugani'	'Kaachi'
Narirutin	n.d.	49.33 ± 6.16 a	n.d.	39.18 ± 4.41 b
Naringenin	3.98 ± 1.15 b[b]	4.58 ± 0.25 b	4.35 ± 0.35 b	11.88 ± 2.50 a
Hesperidin	20.79 ± 3.80 c	474.82 ± 50.68 a	335.08 ± 15.69 b	467.31 ± 39.74 a
Neohesperidin	698.69 ± 90.70 a	n.d.	0.18 ± 0.03 b	5.16 ± 1.14 b
Total	723.46	528.73	339.62	523.53

[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 ($p < 0.05$).

Table 6-4. PMF content (mg/100 g fresh flavedo weight)[a] of Shiikuwasha peels.

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

[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 ($p < 0.05$).

In the analysis of PMFs, four compounds (sinensetin, nobiletin, natsudaidain, and tangeretin) were detected in the Shiikuwasha peels (**Table 6-4**). 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.,[150] 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*).[150] 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.[98] Therefore, the results of the study indicate that Shiikuwasha peels of different cultivation lines may be potential valuable sources of flavonoids.

6.4. Conclusion

The composition and content of volatile aroma components, flavanones, and PMFs in different Shiikuwasha cultivation lines were reported for the first time. Each Shiikuwasha peel oil 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.

Chapter VII

Effect of Cultivation Line and Peeling on Food Composition, Taste Characteristic, Aroma Profile, and Antioxidant Activity of Shiikuwasha Juice

7.1. Introduction

Shiikuwasha, or Hirami lemon (*Citrus depressa* Hayata), is a citrus fruit cultivar with more than 10 cultivation lines, including the four main lines 'Izumi kugani', 'Katsuyama kugani', 'Ogimi kugani', and 'Kaachi', which grow naturally in the northern areas of Okinawa Island, Japan.[5] Ripe fruit of Shiikuwasha is commonly harvested during winter from November to December and processed into food products such as beverages, confectionary, and additives. Shiikuwasha juice is also abundant in ascorbic acid and flavonoids that have hepatoprotective activity against D-galactosamine-induced liver injury.[101,152]

Peeling in citrus juice production is an influential and critical practice that may affect the whole quality attributes of juice. Peels or exocarps are an essential part of citrus fruits owing to their numerous bioactive and pleasant aroma components. Citrus peels contain flavonoids, phenolics, carotenoids, and limonoids that have potent beneficial effects on human health such as anti-inflammatory and anti-atherosclerosis functions.[131,153,154] Moreover, citrus peel oils as well as other plant essential oils have been shown to exhibit various antimicrobial, antiviral, and antioxidant activities.[128] Conversely, several flavonoids with neohesperidose conjugates, particularly neohesperidin, naringin, and neoeriocitrin, as well as triterponoid limonin in citrus peels have been postulated to cause unfavorable astringent and bitter tastes.[131,155,156]

The study aimed to determine the effect of cultivation line and peeling on quality attributes, including food composition, taste characteristic, aroma profile, and antioxidant activity of Shiikuwasha juice of four cultivation lines ('Izumi kugani', 'Katsuyama kugani', 'Ogimi kugani', and 'Kaachi'). For this purpose, the sugar and organic acid composition of whole fruit and peeled flesh juices was determined and confirmed with taste sensor analysis using artificial lipid membranes. The influence of fruit cultivation line and peeling was also distinguished by observing the isolated volatile aroma components in each Shiikuwasha juice. Total phenolic content and antioxidant capabilities of the Shiikuwasha juice were examined, and the influence of the responsible active components on antioxidant activity was then investigated. The research flowchart can be briefly seen in **Figure 7-1**.

7.2. Materials and methods

7.2.1. Standards and reagents

Standard sugars (sucrose, glucose, and fructose) and organic acids (citric, malic, and ascorbic) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Chemicals used as standards to identify volatile aroma components were obtained from Tokyo Chemical Industry (Tokyo, Japan) and Sigma-Aldrich (St. Louis, MO, USA). Authentic *n*-hexanol compound was from Tokyo Chemical Industry. Gallic acid, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Wako Pure Chemical Industries. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Calbiochem (San Diego, CA, USA). Folin-Ciocalteu reagent was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), and fluorescein sodium salt was from

Sigma-Aldrich. 2-Morpholinoethanesulfonic acid (MES) buffer was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). All other reagents were purchased from Wako Pure Chemical Industries and were of analytical grade.

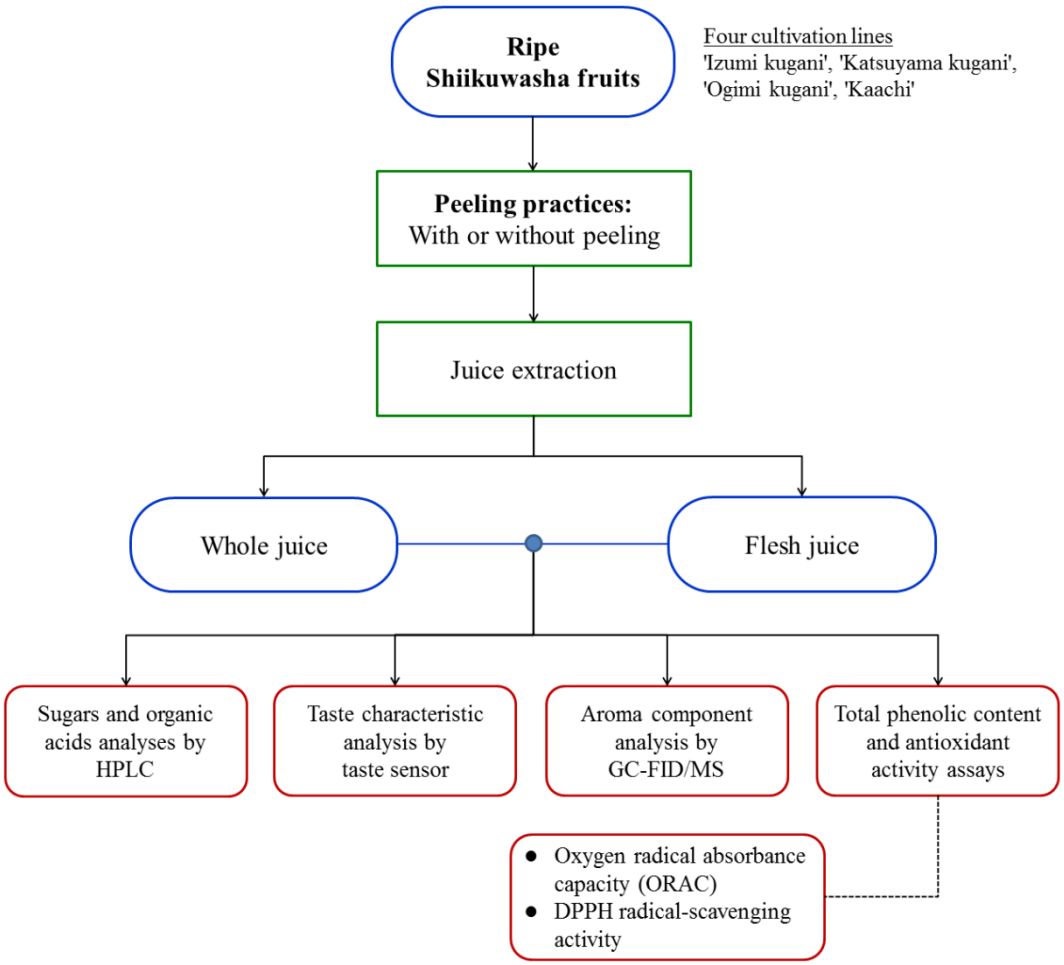

Figure 7-1. Research flowchart of effect of cultivation line and peeling on food composition, taste characteristic, aroma profile, and antioxidant activity of Shiikuwasha juice.

7.2.2. Samples

Shiikuwasha (*C. depressa* Hayata) fruits of four cultivation lines–'Izumi kugani', 'Katsuyama kugani', 'Ogimi kugani', 'Kaachi'–were collected at a commercial harvesting stage in November 2011 from several farms in the northern part of Okinawa Island, Japan. The Shiikuwasha trees were grown under the same climate, weather, and farming conditions. Shiikuwasha juice (whole juice) was obtained by centrifuging approximately 1000 g of whole fruits using a centrifugal extractor (Kokusan Enshinki Co., Ltd., Tokyo, Japan). Another 1000 g of fruits was manually hand peeled, and the juice of the flesh parts (flesh juice) was then extracted. The juice was characterized in terms of pH, total soluble solid (TSS) content, and total titratable acidity (TA, as citric acid). The TSS of Shiikuwasha juice was analyzed using an N-1α handheld refractometer (Atago Co., Ltd., Tokyo, Japan) and expressed as °Brix. All extractions and assays were performed in triplicate. The extracted juice was stored in sealed vials at –30 °C until further analysis. All extractions and assays were carried out in triplicate.

7.2.3. Sugar composition analysis

The sugar composition of Shiikuwasha juice was determined using a high-performance liquid chromatography (HPLC) method adapted from Nolasco and De Massaguer[157] with slight modification. Briefly, a sample (1 mL) was centrifuged using a CFM-200 micro-centrifuge (Iwaki Glass Co., Ltd., Chiba, Japan) at 12,000 rpm for 5 min. The supernatant was diluted before injection into a DIONEX LC-20 HPLC system equipped with electrochemical detector model ED-40 (Dionex Corp., Sunnyvale, CA, USA). The column used was a CarboPac PA1 (250 mm × 4.0 mm i.d., Dionex Corp.) connected to a guard column (50 mm × 4.0 mm i.d.). The mobile phase of 250

mM NaOH solution was streamed in isocratic mode by a DIONEX GP-50 pump at a flow rate of 1 mL/min. The injection volume of samples and standards was 50 μL. The composition and concentration of sugars were calibrated by plotting peak area against concentration for the respective sugar standards and expressed as grams per liter of juice. All assays were carried out in triplicate.

7.2.4. Organic acid composition analysis

The organic acid composition of Shiikuwasha juice, particularly citric and malic acids, was determined using an HPLC system with electroconductivity detection.[84] Briefly, a sample (1 mL) was centrifuged at 12,000 rpm for 5 min and filtered through an Advantec 0.45-μm membrane filter (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and diluted before injection. The column used was a Shim-pack SCR-102H column (300 mm × 8 mm i.d., Shimadzu Corp. Kyoto, Japan) connected to a guard column (50 mm × 6 mm i.d.). Two Shimadzu LC-10AD pumps were used to flow the mobile phase containing 5 mM *p*-toluenesulfonic acid and post-column detection reagent containing 5 mM *p*-toluenesulfonic acid, 100 μM EDTA disodium salt, and 20 mM Bis-Tris buffer in isocratic mode at a flow rate of 0.7 mL/min. The mobile phase and post-column detection solvent were streamed to a post-column reactor and mixed at a ratio of 1:1 before detection with a Shimadzu CDD-6A conductivity detector. The column, guard column, and post-column reactor were maintained at a constant temperature of 40 °C using a Shimadzu CTO-10AC oven, and the injection volume was 10 μL. The concentrations of citric and malic acids were calibrated by plotting peak area against concentration for the respective acid standards and expressed as grams per liter of juice. All assays were carried out in triplicate.

Ascorbic acid concentration was determined using an HPLC system with diode array detection.[153] Briefly, a sample (1 mL) was centrifuged at 12,000 rpm for 5 min and then filtered through an Advantec 0.45-μm membrane filter (Toyo Roshi Kaisha, Ltd.). A Develosil ODS-MG-3 column (150 mm × 3 mm i.d., 3-μm particle size; Nomura Chemical, Seto, Japan) with a guard column (10 mm × 4 mm i.d.) was maintained at 40 °C in a Shimadzu CTO-20AC oven (Shimadzu Corp.). A Shimadzu LC-20AB pump was operated in isocratic mode with 50 mM ammonium phosphate (pH 2.4) at a flow rate of 0.4 mL/min. The injection volume of samples and standards was 1 μL. Ascorbic acid peak was monitored at 250 nm with a Shimadzu SPD-M20A diode array detector, and its concentration was calibrated by plotting peak area against standard concentrations. The ascorbic acid concentration was expressed as grams per liter of juice. All assays were carried out in triplicate.

7.2.5. Taste characteristic analysis (analyzed by Institute of OISHISA Science, Kagawa, Japan)

The taste characteristic of Shiikuwasha juice was measured with a taste sensor method.[158,159] Briefly, 50 mL of reference solution of 30 mM KCl containing 0.3 mM tartaric acid, which corresponded to human saliva, was used to immerse the taste sensor and reference probes in a taste sensor system (SA402B, Intelligent Sensor Technology, Inc., Kanagawa, Japan) for 30 s at 25 °C, yielding an electrical membrane potential (V_r). The taste sensor probes used were SB2GL1 (sweetness), SB2CA0 (sourness), SB2AE1 (astringency), and SB2C00 (acidic bitterness) and consisted of various lipid/polymer membranes with an Ag/AgCl electrode and internal solutions of 3.3 M KCl and saturated AgCl. The reference probe was made of a ceramic liquid junction with core

materials the same as those in the sensor probes. The probes were then submerged into a sample (50 mL) for 30 s, and the electrical potential corresponding to the taste was measured (V_s). The difference in potential of sample and reference solution ($V_s - V_r$) was considered a relative taste value. The differentiation of relative taste values of samples was displayed in two-dimensional plots of sweetness-sourness and astringency-acidic bitterness in which the relative value of 'Ogimi kugani' whole juice was set as the corrected relative value (zero adjusted). All assays were carried out in triplicate.

7.2.6. Volatile aroma components analysis

The volatile aroma components of Shiikuwasha juice were extracted via a liquid-liquid continuous extraction/solvent-assisted flavor evaporation technique (LLCE/SAFE). Briefly, 200 mL of sample and 0.5 mg of *n*-hexanol as the internal standard followed by 200 mL of diethyl ether were placed into a LLCE system. The extract containing aroma components was obtained over a 3-h continuous extraction process. The volatile components were separated from the non-volatile fraction using a SAFE procedure under vacuum at 50 °C. The volatile aroma extract was dried with 10 g of anhydrous sodium sulfate for 12 h at 5 °C, and its volume was then reduced to 2 mL using a Vigreux column apparatus and then to 100 µL in a Kuderna-Danish concentration tube under a gentle stream of nitrogen. The isolated aroma extract was stored in sealed vials at –30 °C until analysis.

The extract was then analyzed using gas chromatography-flame ionization detection/mass spectrophotometry (GC-FID/MS) as described in **Section 5.2.4**.

7.2.7. Determination of total phenolic content

The total phenolic content of Shiikuwasha juice was examined using the Folin-Ciocalteu method.[48] Briefly, various concentrations of sample (20 μL), distilled water (60 μL), and Folin-Ciocalteu reagent (15 μL, previously diluted twofold with distilled water) were transferred to a 96-well microplate (Nunc, Roskilde, Denmark) and mixed well. The mixture was allowed to react at room temperature for 5 min, and then 75 μL of sodium carbonate (2%) was added to the well. The microplate was immediately placed in a microplate reader (PowerWave XS2, BioTek, Winooski, VT, USA), agitated, and then allowed to stand for 15 min until stable absorption values were obtained. The absorbance was then measured at 750 nm, and calibration solutions of gallic acid were included in each assay. Total phenolic content was calculated from a linear calibration curve of gallic acid and expressed as milligrams of gallic acid equivalents (GAE) per liter of juice. All assays were carried out in triplicate.

7.2.8. Oxygen radical absorbance capacity (ORAC) assay

ORAC assay was carried out according to method of Huang et al.[52] with slight modifications. Briefly, various concentrations of sample (25 μL) and 90 nM fluorescein solution (150 μL) were transferred to a black Nunc 96-well microplate. The microplate was immediately placed and agitated in a microplate reader (Synergy HT, BioTek) and then left to stand at 37 °C for 10 min. Further, 25 μL of 160 mM AAPH as the peroxyl radical generator was immediately added to the well. The reaction temperature was maintained at 37 °C, and the fluorescence was monitored kinetically with data taken every minute for 30 min with fluorescent filters set at 485 nm (excitation) and 530 nm (emission). Phosphate buffer (pH 7.0) was used as a blank, and calibration solutions of

Trolox were included in each assay. The area under the curve of relative fluorescence value was calculated using the following equation: Area under the curve = 0.5 + *f1*/*f0* + ... *fi*/*f0* + ... + *f29*/*f0* + 0.5(*f30*/*f0*), where *f0* is the initial relative fluorescence reading at 0 min and *fi* is the relative fluorescence reading at time *i*. The ORAC value was calculated from a linier calibration curve of Trolox and expressed as micromoles of Trolox equivalents (TE) per liter of juice. All assays were carried out in triplicate.

7.2.9. DPPH radical-scavenging activity assay

The antioxidant activity of Shiikuwasha juice was also evaluated in term of its capability for scavenging DPPH radicals.[160] Briefly, various concentrations of sample (50 µL) and 0.1 mM DPPH solution (50 µL) were placed into a Nunc 96-well microplate. The mixture was adjusted to a volume of 200 µL by adding MES buffer or ethanol solution. The microplate was shaken vigorously and then immediately placed in a microplate reader (PowerWave XS2, BioTek) and left to stand for 30 min until stable absorption values were obtained. The reduction in DPPH radicals by the sample was examined by measuring the absorption at 517 nm. A mixture of sample and MES buffer/ethanol was used as a blank for background subtraction, and calibration solutions of Trolox were included in each assay. The DPPH radical-scavenging activity was calculated from a linear calibration curve of Trolox and expressed as micromoles of TE per liter of juice. All assays were carried out in triplicate.

7.2.10. Statistical analysis

The differentiation of volatile aroma components in Shiikuwasha juice from the studied cultivation lines and peeling practices was evaluated using principal component

analysis (PCA) with Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA, USA). To distinguish the influence of active components on antioxidant activity of Shiikuwasha juice, we carried out Pearson's correlation analysis between the antioxidant activity (ORAC value and DPPH radical-scavenging activity) and the levels of total phenolic acid, ascorbic acid, or volatile aroma components.

7.3. Results and discussion

7.3.1. Chemical properties and sugar and organic acid compositions of Shiikuwasha juice

Compared with cultivation line, peeling had less impact on basic chemical properties in Shiikuwasha juice. The properties included pH, TSS, and TA (see **Table 7-1**). The pH of Shiikuwasha juice varied between 2.64 and 3.40, and the TSS and TA values were 7.33–9.77 °Brix and 1.09–2.69%, respectively. Regardless of peeling practice, 'Izumi kugani' fruit was less acidic than other cultivation lines as shown by higher pH and lower TA, whereas 'Kaachi' had the highest TSS and TA values in its juice. Regarding this profile, a simple ratio of TSS to TA was calculated, providing a simple figure for quality characteristic in the juice, wherein 'Izumi kugani' juice had the highest TSS/TA ratio (6.70–7.22). This data can be used to measure the TSS/TA portion of Shiikuwasha juice against other citrus fruit juices–for example, lemon (TSS/TA: 1.79–2.20), bitter orange (1.93), grapefruit (6.92), mandarin (8.00–16.87), sweet orange (8.23–14.63), and pummelo (14.44–17.09)[161,162]– revealing a distinctive balance of soluble solids and acidity in Shiikuwasha juice.

Table 7-1. Chemical properties (pH, total soluble solids, and titratable acidity) of Shiikuwasha juice.

Shiikuwasha juice	pH	TSS[a]	TA[b]	TSS/TA ratio
Flesh				
'Izumi kugani'	3.40	7.77	1.16	6.70
'Katsuyama kugani'	2.94	8.43	2.18	3.86
'Ogimi kugani'	2.94	7.33	1.90	3.86
'Kaachi'	2.64	9.53	2.69	3.55
Whole				
'Izumi kugani'	3.38	7.87	1.09	7.22
'Katsuyama kugani'	2.97	9.13	2.04	4.48
'Ogimi kugani'	2.99	7.87	1.79	4.41
'Kaachi'	2.80	9.77	2.42	4.03

Each value is expressed as the mean value (n = 3).
[a] TSS: percentage of total soluble solids (°Brix).
[b] TA: percentage of total titratable acidity (as citric acid).

The sugar and organic acid composition of Shiikuwasha juice samples were analytically obtained using HPLC methods (**Figure 7-2**), and the resulting data were similar (see **Table 7-1**). Fruit cultivation line affected the differentiation of sugar and organic acid content, whereas more similar content and compositional profiles were found in the juice after peeling. The peel of Shiikuwasha fruits might contain small amounts of sugars and organic acids but they were considerably lower than that of in the inner flesh parts. Of the sugar components, Shiikuwasha juice was found to contain 50–80 g sugars in 1 L juice, wherein the 'Kaachi' cultivation line had the highest sugar content with 75.72 and 82.31 g/L for its flesh and whole juices, respectively. Sucrose was the predominant sugar, and the ratio of sucrose, glucose, and fructose in the Shiikuwasha juice varied from 2:1:1 to 4:1:1. The ratio of these simple sugars in citrus juice is generally about 2:1:1; however, a larger amount of sucrose might be present and

varies depending on citrus fruit origin, cultivar, cultivation line, farming practice, degree of ripening, and post-harvest practices.[163,164]

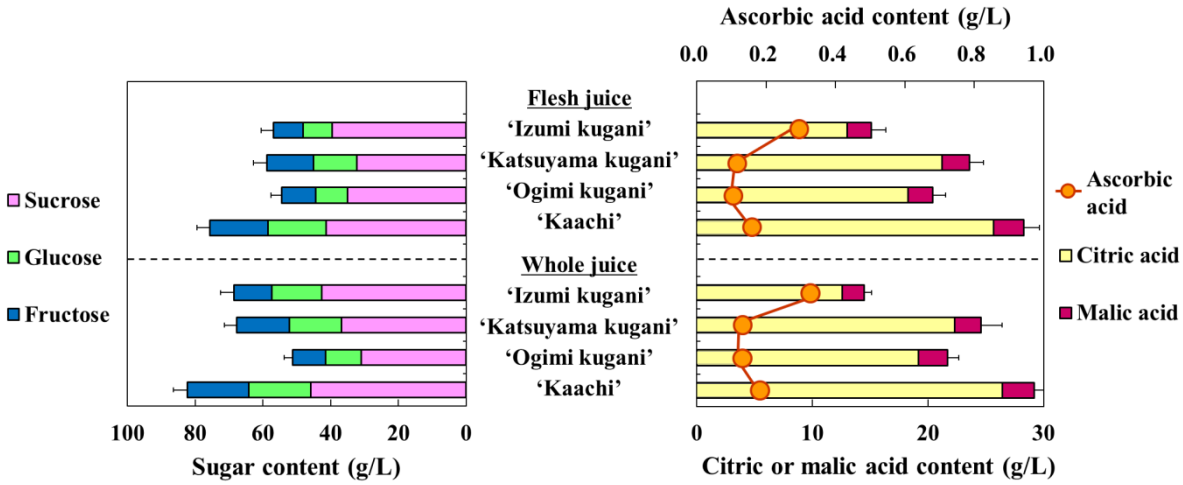

Figure 7-2. Sugar and organic acid compositions (g/L) of Shiikuwasha juice obtained through HPLC analyses.

Each value is expressed as the mean ± standard deviation (n = 3).

Regarding the organic acid composition, Shiikuwasha juice from the studied cultivation lines contained 15–30 g acids/L–mainly citric acid (84.3–90.3%) following by malic and ascorbic acids. Juices of 'Kaachi' fruit contained highest total acid content, with 28.46 and 29.36 g/L for its flesh and whole juices, respectively. Conversely, although the 'Izumi kugani' cultivation line had lower total organic acids than those in other evaluated juices, it contained the highest ascorbic acid content, with 0.29–0.32 g/L. The Shiikuwasha juice of other cultivation lines contained ascorbic acid at levels of approximately 0.10–0.18 g/L. The ascorbic acid contents of Shiikuwasha juices, particularly 'Izumi kugani', were similar to those of previously reported citrus juices such as mandarin, lemon, and pummelo but lower than those in sweet orange and grapefruit,[162,163] suggesting that Shiikuwasha juice has nutritional and health benefits.

7.3.2. Taste characteristic of Shiikuwasha juice

Taste characteristic measurements of Shiikuwasha juice were based on the change in potentials of various artificial lipid/polymer membranes induced by ionic charges from evaluated juices. Subsequently, the relative potential taste values of Shiikuwasha juice were compared to one another, wherein a difference of 1 unit relative value in each taste plot corresponded to the smallest taste difference that can be distinguished by human senses.[159] In this analysis, whole juice of 'Ogimi kugani' was set as the correction value (zero adjusted) for both sweetness-sourness and astringency-acidic bitterness plots, and taste characteristics of other evaluated juices were thus marked (**Figure 7-3**). Shiikuwasha juice from the four cultivation lines differed in sweet and sour taste attributes: 'Ogimi kugani' was relatively closed to 'Katsuyama kugani', whereas 'Izumi kugani' and 'Kaachi' were clearly separated (**Figure 7-3.a**). These taste sensor results were found to be in good agreement with those obtained from TSS and TA determinations as well as sugar and organic acid composition analyses using HPLC methods. For example, the sweet taste domination over sourness of 'Izumi kugani' juice was similar to its high TSS/TA ratio; however, the greater relative sourness value in 'Kaachi' juice was related to its high total organic acid content (see **Table 7-1, Figure 7-2**).

The astringent and acidic bitter taste differentiation of Shiikuwasha juice from the various cultivation lines was also revealed (**Figure 7-3.b**). 'Izumi kugani' juice was relatively more astringent and acidic bitter, whereas 'Kaachi' juice had the opposite taste values. Flavonoid content and composition in the fruits might have affected the occurrence of these aftertastes; whereas a previous study has reported that the peel part of 'Izumi kugani' fruit has a flavonoid content greater than that in three other cultivation

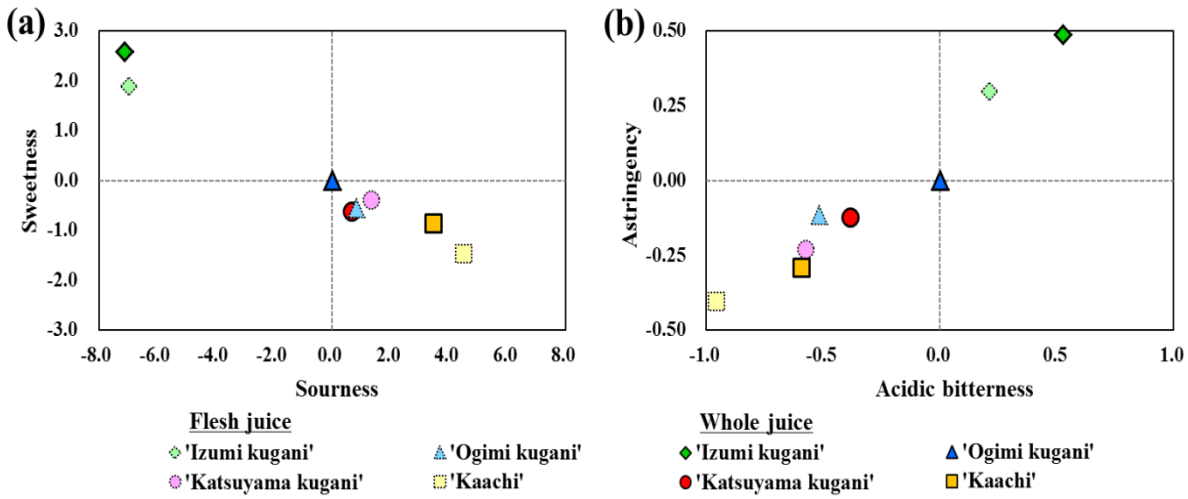

Figure 7-3. Two-dimensional plots of taste characteristics obtained through taste sensor analysis of Shiikuwasha juices: (a) sweetness-sourness, and (b) astringency-acidic bitterness plots.

lines (see **Section 6.3.3**). Moreover, it had a distinctive amount of neohesperidin, whereas other Shiikuwasha cultivation lines contained its flavanone glycoside isomer, hesperidin. The sugar neohesperidose of neohesperidin has been recognized for its bitter taste contribution to various citrus fruits, including tangelos and sour orange, whereas the sugar rutinose gives hesperidin a neutral taste.[131,156] Conversely, flesh Shiikuwasha juices clearly had lower astringency and acidic bitterness values than those in the corresponding whole juices. Bitter constituents of Shiikuwasha peel, such as limonoids and certain flavonoids, might be responsible for this phenomenon,[155] indicating that the peeling is a key processing step in reducing astringent and acidic bitter characteristics in Shiikuwasha juice, and thus might improve its palatability.

7.3.3. Volatile aroma components of Shiikuwasha juice

A total of 20 aroma compounds, representing 82.24–96.77% of the total volatile aroma components, were identified in the evaluated Shiikuwasha juices (**Table 7-2**). Aroma profiles for the Shiikuwasha juice depended on both cultivation line and peeling. Whole juices had more complex aroma profiles and content than those of flesh juices. Total aroma components in the whole juice group were 20.26–53.73 mg/L, whereas those in flesh juice were only 0.82–1.58 mg/L. Because citrus fruit peels are reportedly responsible for providing aroma components owing to oil glands in their flavedo peel layers,[128,164] peeling eliminates the aroma complexities of Shiikuwasha juices. Hence, the main volatile aroma components of both flesh and whole juices were monoterpenes linalool, limonene, terpinen-4-ol, and *γ*-terpinene. These compounds are key aroma components in various citrus fruits, imparting mostly pleasant and herbaceous characteristics such as fresh citrusy (limonene), sweet floral (linalool), herbal resinous (terpinen-4-ol), and green waxy/grassy (*γ*-terpinene).[115,161,165]

'Izumi kugani' whole juice contained the highest total content of identified volatile aroma components (53.73 mg/L) followed by 'Ogimi kugani' and 'Katsuyama kugani' whole juices. Interestingly, 'Izumi kugani' whole juice contained a distinctive amount of 1,8-cineol (13.55 mg/L), also known as eucalyptol, an oxide compound identified as a key aroma component in *Thymus* species.[147] Moreover, it differed from other evaluated whole juices in comprising methyl thymol and terpinyl acetate compounds but lacking an *l*-carvone component. Of the flesh juice group, 'Izumi kugani' juice also contained 1,8-cineol level (0.12 mg/L) approximately three times higher than those of the other Shiikuwasha juices and solely comprising *β*-phellandrene, (*Z*)-3-hexen-1-ol, and ocatanol components at amounts of 0.29, 0.03, and 0.05 mg/L, respectively.

Table 7-2. Content of volatile aroma components (mg/L)[a] obtained through GC-FID/MS analysis of Shiikuwasha juice.

Peak No.	RI[b]	Aroma component	Flesh juice				Whole juice				Identification[c]
			'Izumi kugani'	'Katsuyama kugani'	'Ogimi kugani'	'Kaachi'	'Izumi kugani'	'Katsuyama kugani'	'Ogimi kugani'	'Kaachi'	
1	1021	*α*-Pinene	–[d]	0.03 ± 0.02	–	–	0.38 ± 0.05	0.18 ± 0.10	0.19 ± 0.09	0.09 ± 0.02	RI, MS, PC
2	1026	*α*-Thujene	–	–	–	–	0.13 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	–	RI, MS
3	1107	*β*-Pinene	0.02 ± 0.00	–	–	0.02 ± 0.00	0.40 ± 0.03	0.12 ± 0.08	0.12 ± 0.08	0.04 ± 0.02	RI, MS, PC
4	1163	Myrcene	–	–	–	–	0.30 ± 0.03	0.13 ± 0.07	0.12 ± 0.07	0.11 ± 0.04	RI, MS, PC
5	1177	*α*-Terpinene	–	–	–	–	0.10 ± 0.01	0.06 ± 0.01	0.06 ± 0.03	0.03 ± 0.01	RI, MS, PC
6	1213	Limonene	0.15 ± 0.07	0.32 ± 0.15	0.08 ± 0.04	0.25 ± 0.07	9.72 ± 0.67	3.41 ± 2.08	2.98 ± 2.03	3.75 ± 1.39	RI, MS, PC
7	1215	*β*-Phellandrene	0.29 ± 0.04	–	–	–	0.04 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	RI, MS
8	1216	1,8-Cineol	0.12 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.01	13.55 ± 0.41	0.17 ± 0.01	0.18 ± 0.01	0.07 ± 0.00	RI, MS, PC
9	1257	*γ*-Terpinene	0.06 ± 0.03	0.18 ± 0.08	0.05 ± 0.03	0.06 ± 0.02	3.95 ± 0.26	1.87 ± 1.12	1.93 ± 1.31	0.88 ± 0.33	RI, MS, PC
10	1272	*p*-Cymene	–	0.06 ± 0.02	0.02 ± 0.01	–	0.32 ± 0.03	0.58 ± 0.33	0.43 ± 0.27	0.06 ± 0.00	RI, MS, PC
11	1287	Terpinolene	–	–	–	0.02 ± 0.00	0.20 ± 0.02	0.09 ± 0.06	0.10 ± 0.07	0.04 ± 0.02	RI, MS, PC
12	1291	Octanal	–	–	–	–	0.28 ± 0.03	0.05 ± 0.01	0.14 ± 0.02	0.11 ± 0.00	RI, MS, PC
13	1391	(*Z*)-3-Hexen-1-ol	0.03 ± 0.00	–	–	–	0.06 ± 0.00	–	–	0.03 ± 0.01	RI, MS
14	1554	Linalool	0.46 ± 0.03	0.34 ± 0.01	0.29 ± 0.01	0.47 ± 0.13	15.78 ± 0.29	6.78 ± 0.21	10.12 ± 0.29	9.26 ± 0.12	RI, MS, PC
15	1566	Octanol	0.05 ± 0.00	–	–	–	0.46 ± 0.02	0.06 ± 0.00	0.10 ± 0.00	0.20 ± 0.05	RI, MS, PC
16	1594	Methyl thymol	–	–	–	–	0.47 ± 0.02	–	–	–	RI, MS, PC
17	1604	Terpinen-4-ol	0.17 ± 0.00	0.30 ± 0.00	0.17 ± 0.01	0.16 ± 0.05	5.20 ± 0.12	5.06 ± 0.17	5.02 ± 0.26	2.78 ± 0.01	RI, MS, PC
18	1693	Terpinyl acetate	–	–	–	–	0.59 ± 0.02	–	–	–	RI, MS, PC
19	1699	*α*-Terpineol	0.12 ± 0.00	0.31 ± 0.01	0.17 ± 0.01	0.19 ± 0.08	1.79 ± 0.04	4.63 ± 0.39	4.93 ± 0.19	2.71 ± 0.01	RI, MS, PC
20	1731	*l*-Carvone	–	–	–	–	–	0.22 ± 0.01	0.22 ± 0.01	0.06 ± 0.00	RI, MS
Total identified			1.46	1.58	0.82	1.22	53.73	23.50	26.69	20.26	
Relative percentage (%)			92.12	96.77	90.17	82.24	96.21	93.55	95.31	92.12	

[a] Each value is expressed as the mean ± standard deviation (n = 3).
[b] Retention indices relative to *n*-alkanes on a polar DB-Wax column.
[c] RI: identification based on retention index; MS: identification based on the NIST MS library; PC: identification based on authentic standards analyzed by mass spectrometry.
[d] Not detected.

Monoterpene hydrocarbon *β*-phellandrene might provide green citrusy and floral odors, whereas alcohols (*Z*)-3-hexen-1-ol and ocatanol might contribute green grassy and sweet citrusy characteristics, respectively.[115,166] Conversely, *α*-thujene, myrcene, *α*-terpinene, octanal, methyl thymol, terpinyl acetate, and *l*-carvone compounds were absent in the evaluated flesh juices.

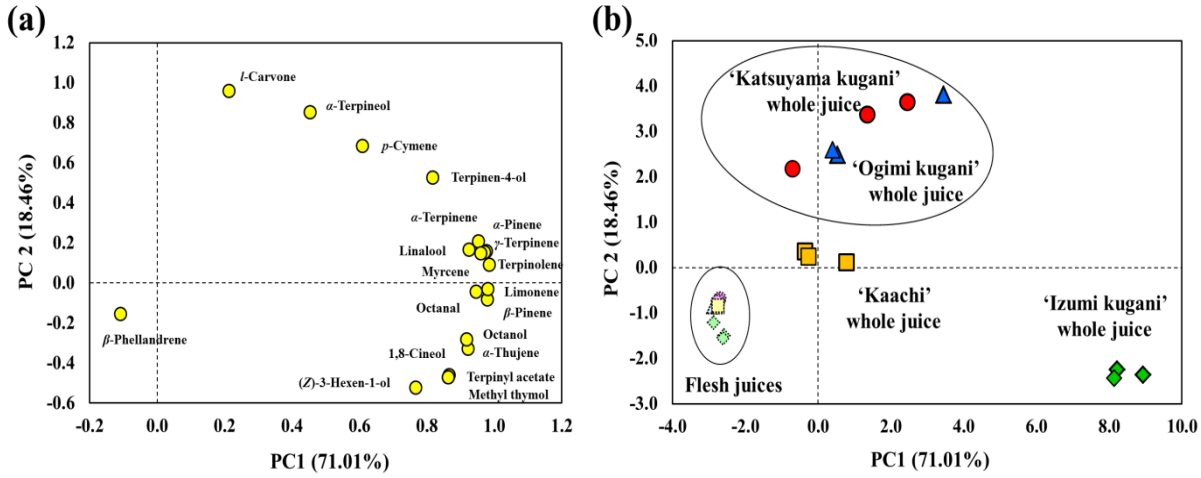

Figure 7-4. PCA plots of volatile aroma components of Shiikuwasha juice:

(a) distribution of 20 volatile aroma compounds; (b) discrimination of cultivation lines and peeling based on the content of 20 volatile aroma compounds.

Differences in the composition and content of volatile aroma components were also discovered with PCA, wherein approximately 89.47% of total variance was accounted for in the first two identified principal components (PCs; **Figure 7-4**). Accordingly, compositional discrimination of volatile aroma compounds of flesh and whole Shiikuwasha juices from four cultivation lines can be distinguished with valid results. Predominant aroma components, such as linalool, terpinen-4-ol, and *γ*-terpinene, along with *α*-pinene, myrcene, and terpinolene, were closely related to one another and

were more positively correlated to the loading plots of PC1 and PC2, whereas monoterpene hydrocarbon *β*-phellandrene was negatively associated with the PCs (**Figure 7-4.a**). Both 'Izumi kugani' and 'Kaachi' whole juices were clearly separated from the other evaluated juices, although flesh juices of the cultivation lines were all closely associated (**Figure 7-4.b**). Conversely, the volatile aroma components of 'Ogimi kugani' whole juice overlapped with those of 'Katsuyama kugani' whole juice. These compositional discrimination results agree with the volatile aroma components of Shiikuwasha juice presented in **Table 7-2**, revealing aroma profile distinctiveness in each cultivation line of Shiikuwasha whole juice. On the other hand, peeling might cause Shiikuwasha juice from the evaluated lines to have similar aroma properties and might also lead to aroma differentiation difficulties among cultivation lines.

7.3.4. Antioxidant activity of Shiikuwasha juice

Fruit cultivation line and peeling influenced the antioxidant activity of evaluated Shiikuwasha juices (**Figure 7-5**). The observed variations might be due to differences in phenolic levels, whereas the total phenolic content ranged from 101 to 434 mg GAE/L. Conversely, the ORAC value and DPPH radical-scavenging activity of Shiikuwasha juice were 2160–9415 and 125–487 μmol TE/L, respectively. Compared with literature values, the ORAC value of Shiikuwasha juice was as high as that in other citrus juices such as clementine (3900 μmol TE/L) and lime (8600 μmol TE/L) juices but lower than that in lemon or grapefruit juice, which had ORAC values of approximately 12,000 μmol TE/L.[167,168] Moreover, whole fruit juices of Shiikuwasha from various cultivation lines have antioxidant capabilities higher than those in peeled flesh juices (**Figure 7-5.b**). ORAC and DPPH radical-scavenging activity assays showed that the evaluated

whole juices were capable of scavenging free radicals at levels 53% and 34% higher, respectively, than those of flesh juices. These differences might be caused by high concentrations of various antioxidative phytochemicals in the peel tissues, especially flavanone aglycones and glycosides of flavonoids.[131,169] Thus, peeling might eliminate the antioxidant properties of Shiikuwasha juice. In term of the oxidation storage stability, a small number of external antioxidative agents might be required for flesh juice to balance the antioxidant properties of whole juice.

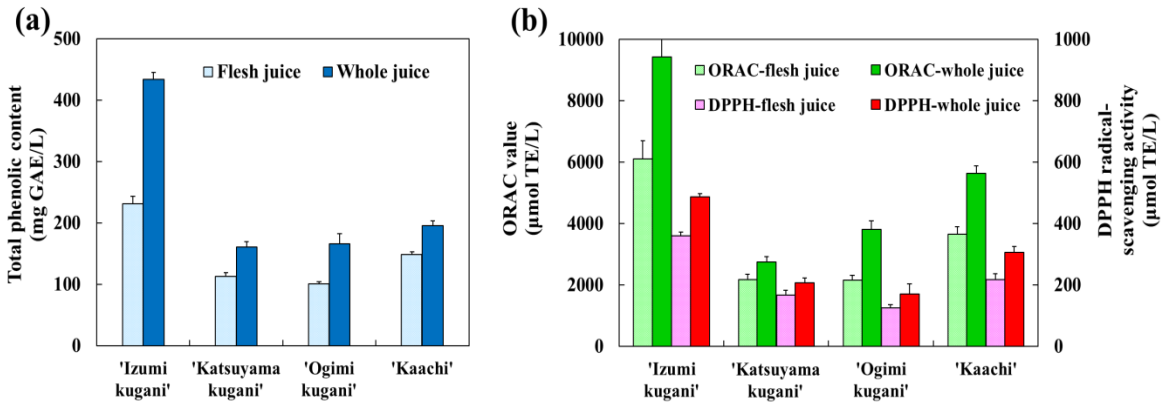

Figure 7-5. (a) Total phenolic content [as mg gallic acid equivalents (GAE)/L], and (b) antioxidant capabilities of Shiikuwasha juice, analyzed with ORAC and DPPH radical-scavenging activity assays [as μmol Trolox equivalents (TE)/L].

Each value is expressed as the mean ± standard deviation (n = 3).

Regarding cultivation line discrimination, both flesh and whole 'Izumi kugani' juices had greater amounts of phenolics than those of other cultivation lines; subsequently, 'Izumi kugani' cultivation line had the highest ORAC value and DPPH radical-scavenging activity. The total phenolic content of 'Izumi kugani' was approximately 48% and 60% higher than those in the flesh and whole juices,

respectively, of the other cultivation lines. These trends might also be related to the flavonoid compositions of the fruit peels. A previous study has reported that 'Izumi kugani' peel contains a larger amount of total flavonoid content and distinctive flavanone composition owing to the presence of neohesperidin compound, whereas peels of other Shiikuwasha cultivation lines had high hesperidin contents (see **Section 6.3.3**). Neohesperidin reportedly has greater radical scavenging abilities compared with those of hesperidin.[169,170]

Table 7-3. Pearson's correlation coefficients between antioxidant capabilities [oxygen radical absorbance capacity (ORAC) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity] and levels of phenolics (TPC), ascorbic acid (AA), and volatile aroma components (VAC) in Shiikuwasha juice.

Correlation	Flesh juice	Whole juice	Flesh and whole juices
TPC × AA	0.996 [b]	0.986 [a]	0.895 [b]
TPC × ORAC	0.996 [b]	0.954 [a]	0.960 [c]
TPC × DPPH	0.996 [b]	0.952 [a]	0.943 [c]
TPC × VAC	0.435	0.963 [a]	0.807 [a]
AA × ORAC	0.991 [b]	0.982 [a]	0.939 [c]
AA × DPPH	0.990 [a]	0.988 [a]	0.966 [c]
AA × VAC	0.414	0.905	0.485
VAC × ORAC	0.356	0.857	0.697
VAC × DPPH	0.513	0.835	0.624
ORAC × DPPH	0.985 [a]	0.967 [a]	0.972 [c]

[a] Correlation is significant at $p < 0.05$.
[b] Correlation is significant at $p < 0.01$.
[c] Correlation is significant at $p < 0.001$.

Several positive correlations were found between antioxidant capabilities and levels of total phenolic acid, ascorbic acid, and volatile aroma components in Shiikuwasha juice at $p < 0.05$, $p < 0.01$, or $p < 0.001$, respectively (**Table 7-3**). Pearson's coefficient correlations for antioxidant activity, represented by the ORAC and DPPH radical-scavenging activity assays, for total phenolic and ascorbic acid content were high in the flesh juice, whole juice, and mixed flesh and whole juices groups. However, in the mixed flesh and whole juices group, the highest correlation for ORAC value was attained with total phenolic content (Pearson's coefficient = 0.960, $p < 0.001$), whereas the ascorbic acid content of Shiikuwasha juice was highly related to its capability to scavenge DPPH radicals (Pearson's coefficient = 0.966, $p < 0.001$). This trend agrees with the results of previously reported studies on the contributions of phenolics and ascorbic acid to antioxidant activity in various citrus fruits and products.[160–162,171] Interestingly, direct correlations were demonstrated between total volatile aroma components of Shiikuwasha whole juices and both ORAC value and DPPH radical-scavenging activity at Pearson's coefficient of 0.857 and 0.835, respectively, indicating that the active components of aroma compounds are among the factors responsible for antioxidant activity. Therefore, the results of the study should provide essential information for product development and quality control in Shiikuwasha juice production and can be applied to similar small citrus fruits.

7.4. Conclusion

Shiikuwasha juice from four cultivation lines was discovered to have different basic chemical properties, sugar and organic acid compositions, taste characteristics, aroma profiles, and antioxidant capabilities. 'Izumi kugani' juice had a higher TSS/TA

ratio and was less acidic (lower TA and organic acid content) but had higher ascorbic acid levels compared with those of other cultivation lines. The volatile aroma components in the whole fruit juices of 'Katsuyama kugani' and 'Ogimi kugani' were closely associated, whereas the 'Izumi kugani' and 'Kaachi' cultivation lines were clearly separated from the other evaluated juices. Peeling caused the Shiikuwasha juice from the evaluated cultivation lines to be less astringent and acidic bitter, to have similar aroma profiles, and to lose antioxidant capabilities on ORAC and DPPH radical-scavenging activity assays. The phenolic content of the juices was highly correlated to their ORAC values, whereas ascorbic acid content highly influenced the capability to scavenge DPPH radicals. Fruit cultivation line and peeling practices in Shiikuwasha juice production are, thus, important factors to consider owing to their effects on quality attributes.

Chapter VIII

General Conclusions

8.1. Flavor characteristics and biological functions of Okinawan sugary materials, and suggestions for further studies

Sugarcane wax is a potential source of policosanol, biologically valuable long-chain aliphatic primary alcohols. The composition and content of wax, policosanol, and long-chain aldehydes may vary depending on the cultivar of the sugarcane and the specific part of the sugarcane analyzed, as well as on the degree of sugarcane maturity. The Ni 22 sugarcane cultivar was discovered to contain numerous policosanol compounds, which might have beneficial effects on human health. The results of this study have been contributing to the challenge of utilization of all parts of sugarcane for supporting the development of high value-added commercial dietary supplement derived from sugarcane wax as a potential by-product from the sugarcane industry. Furthermore, in order to gain more pure yield of sugarcane wax, application of advanced extraction systems, for instance, supercritical fluid extraction, are potential to be applied in future research (**Chapter II**).

Sugarcane molasses is a potential by-product from the sugarcane manufacturing industry that is rich in antioxidative materials. Sugarcane molasses containing phenolic compounds is able to scavenge AAPH peroxyl radicals, as demonstrated by chemical, cellular, and DNA/biomolecular approaches. The results of the study constitute important milestones for the development of a convincing model and mechanism of antioxidant activity of sugarcane molasses against peroxyl radicals. This valuable information is important not only for food industry but also for cosmetic use, wherein

antioxidant capabilities of sugarcane molasses along with its tyrosinase inhibitory effects may play essential roles in skin-related protective activities for skin care or protection products. Moreover, the isolated sugarcane molasses fractions may contain more currently-unknown potent antioxidative compounds that can be further purified and identified, and their other biological functions should also foster the utilization of sugarcane molasses in nutraceutical, biotechnological or pharmaceutical application (**Chapter III**).

The storage of cane brown sugar may change its food quality attributes, including physicochemical characteristics as well as flavor components and Maillard reaction products (MRPs). Stored cane brown sugar may become darker, and acidic-sulfuric odors may decrease but the aroma of MRPs increases. Understanding the quality attributes of stored cane brown sugar is essential for constructing a storage characteristic for cane brown sugar and better prediction of its various food applications, covering confectionery, beverage, and bakery usage. Further research on the evolutions of physicochemical properties of cane brown sugar in a longer storage period, for instance, up to 2 years, and its implication in various food and beverage product application models may be interesting to be detailed. On the other hand, applying different types of cane brown sugar, including those produced in various manufactures in Okinawa Prefecture as well as other countries, should be remarkable to be further evaluated (**Chapter IV**).

8.2. Flavor characteristics and biological functions of Okinawan citrus materials, and suggestions for further studies

Unripe Shiikuwasha fruit has a unique flavor profile in which the limonene content of its peels is lower than that of other commonly known citrus peels. The extraction process is a decisive factor that affects the composition of key citrus aroma components, as well as the antioxidant activities of the Shiikuwasha peel oil. The cold-press extraction system may better retain the total phenolic content of the flavedo peel and display superior antioxidant activities, compared to the steam distillation extraction method. The results of this study may provide a basis for selection of Shiikuwasha peel oils in food industry for citrus flavor production, as well as in product development of derivative products of Shiikuwasha, such as juice, vinegar, essential oil, and other confectionery products. Further research on changes in the aroma profiles due to Shiikuwasha fruit ripening and their odor descriptions via gas chromatography with olfactometry detection (GC-O) may be valuable to be detailed. In addition, antioxidative contributions of more volatile aroma constituents to antioxidant activity of Shiikuwasha peel oil may reveal the origin of its biological functions, and may also be applied in other citrus essential oils (**Chapter V**).

Peels of different Shiikuwasha cultivation lines are potential sources of essential oils and bioactive substances with beneficial effects on human health. The composition and content of volatile aroma components and flavonoids may vary depending on the cultivation line of the Shiikuwasha ripe fruit. The peel oils were discovered to contain mainly monoterpene hydrocarbons, including limonene and γ-terpinene. Moreover, the aroma profile and flavanone composition of 'Izumi kugani' peel distinctively differed from other evaluated Shiikuwasha lines. The results of this study draw attention to the

potential utilization of Shiikuwasha peels from different cultivation lines in foods, nutraceuticals, and other related industries. Explorations in flavor and flavonoid components of other sub-tropical Okinawan citrus as well as tropical citrus species by application of advanced analytical systems, for instance, electronic nose (e-nose) system, should further distinguish exclusivity of each citrus fruit material (**Chapter VI**).

Shiikuwasha juices of four cultivation lines have distinct food compositions, taste characteristics, and aroma profiles. Peeling in Shiikuwasha juice production may reduce astringent and acidic bitter aftertastes but also lower aroma complexity and antioxidant activity. Eliminating aftertaste characteristics in Shiikuwasha juice is critically important that may thus improve its palatability. Furthermore, distinctiveness of 'Izumi kugani' line for its particular flavor and functionality properties can be further evaluated through genetic diversity approach. Comprehensive information on the effect of cultivation line and peeling on juice quality attributes will be useful for product development and quality control in Shiikuwasha juice production, and can also be applied in juice production of similar small citrus fruits (**Chapter VII**).

8.3. Concluding Remarks

Sugarcane and Shiikuwasha that possess distinctive profiles of flavor components and biological functions are important and highly valuable food crops in Okinawa Prefecture, Japan. More food and agricultural chemical characterizations of these sugary and citrus materials revealed should promote their food, beverage, biotechnological, or nutraceutical commercial uses, and therefore should endorse agribusiness development of the region.

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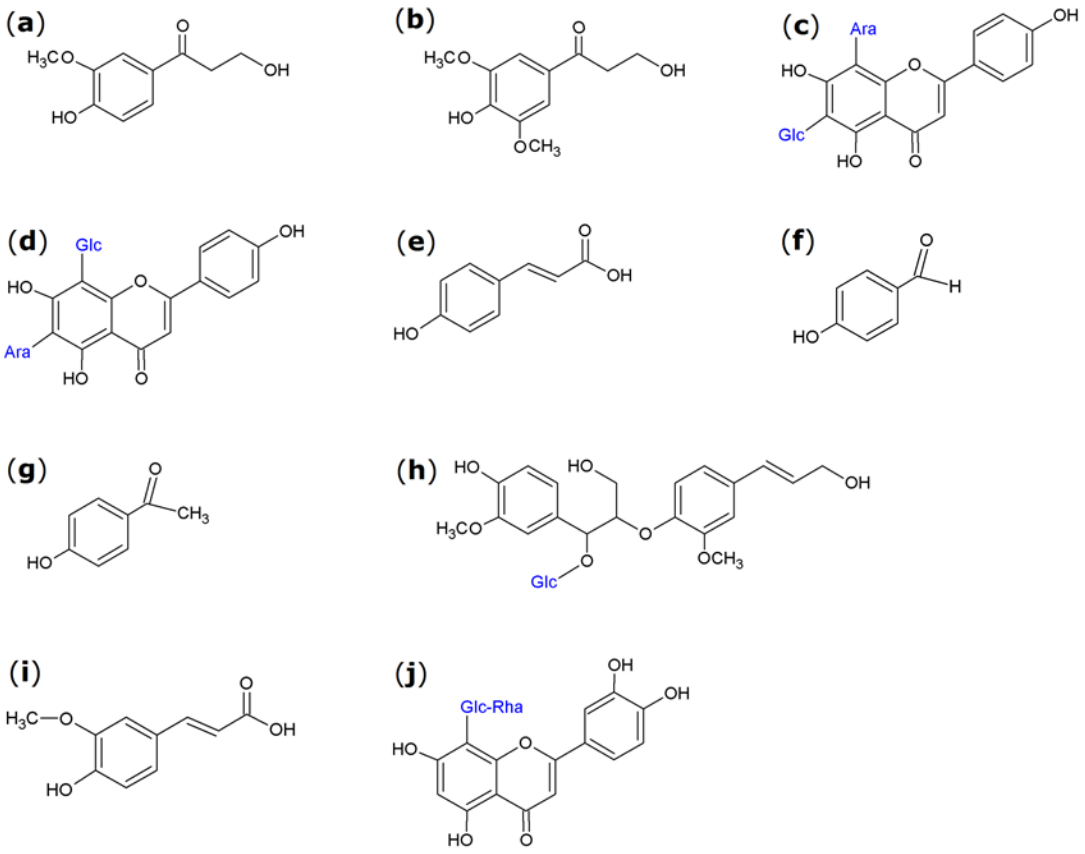
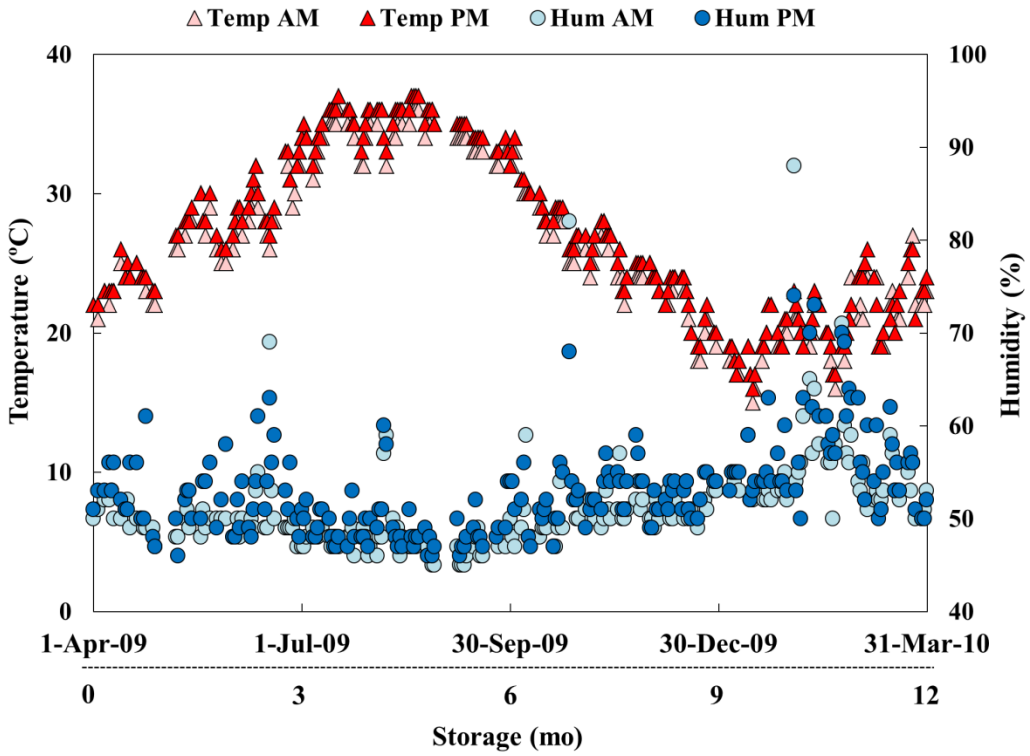
APPENDIX

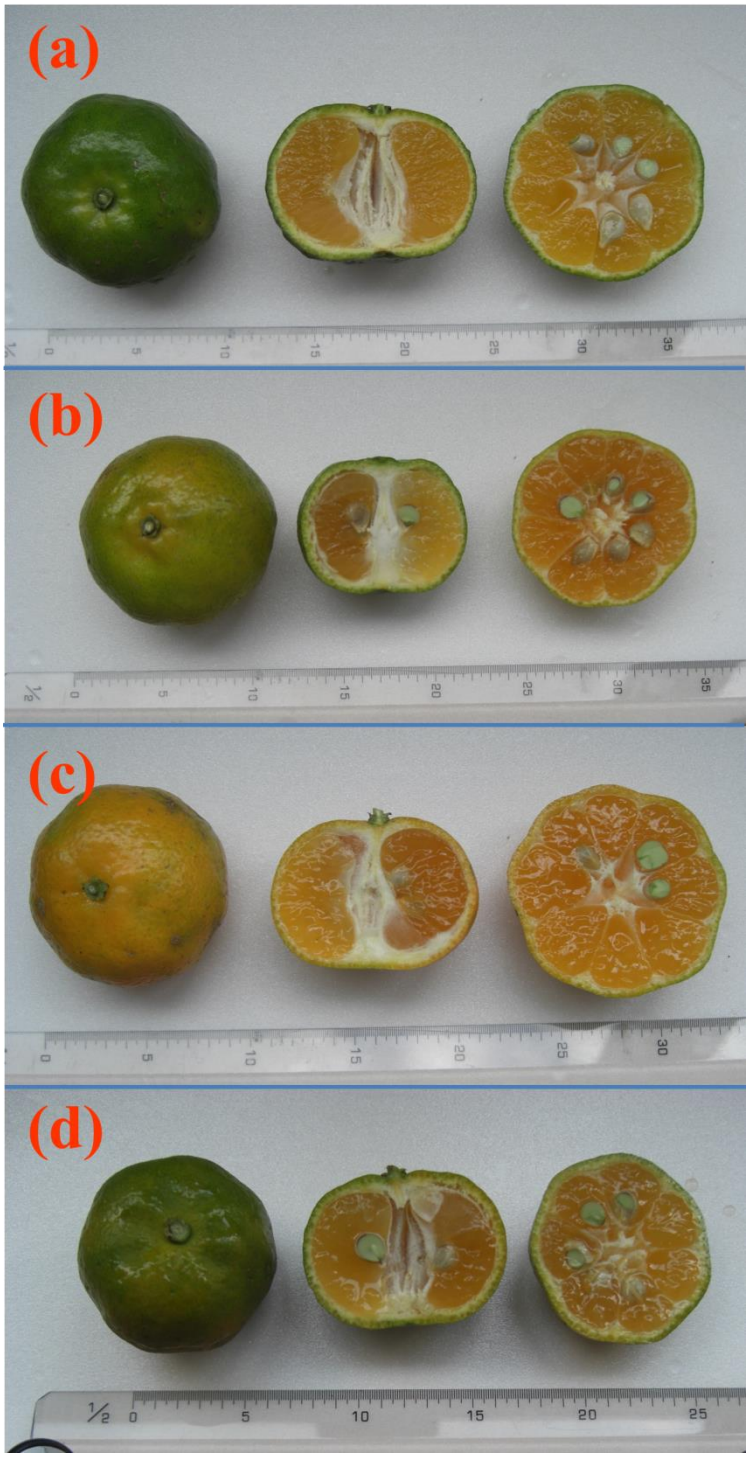


Appendix Figure 1. Chemical structures of phenolic constituents of sugarcane molasses fraction 5 (F5), F6, and F7, as identified by HPLC under the detection wavelength of 280 nm (**Chapter III**).

The phenolics were as follows: (peak/compound a) 3-hydroxy-1-(4-hydroxy-3-methoxy-phenyl)-1-propanone; (b) 3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone; (c) schaftoside; (d) isoschaftoside; (e) *p*-coumaric acid; (f) *p*-hydroxybenzaldehyde; (g) *p*-hydroxyacetophenone; (h) 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxy-1-(*E*)-propenyl)-2-methoxyphenoxy] propyl-β-D-glucopyranoside; (i) ferulic acid; (j) luteolin-8-*C*-(rhamnosyl glucoside).

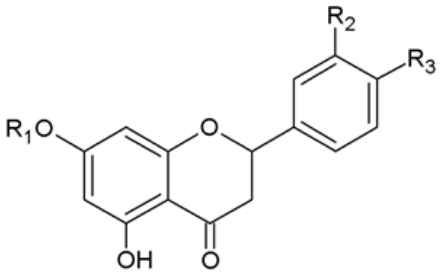


Appendix Figure 2. Storage temperature (°C) and relative humidity (%) conditions of cane brown sugar (**Chapter IV**).



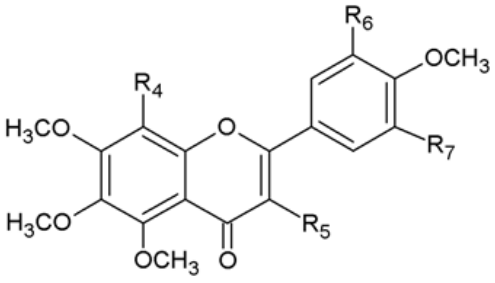
Appendix Figure 3. Ripe Shiikuwasha fruits of different cultivation lines: (a) 'Izumi kugani', (b) 'Katsuyama kugani', (c) 'Ogimi kugani', and (d) 'Kaachi' (**Chapter VI**).

Flavanones:



(a)	narirutin	R_1 = rutinoside,	R_2 = H,	R_3 = OH
(b)	naringenin	R_1 = H,	R_2 = H,	R_3 = OH
(c)	hesperidin	R_1 = rutinoside,	R_2 = OH,	R_3 = OCH_3
(d)	neohesperidin	R_1 = neohesperidose,	R_2 = OH,	R_3 = OCH_3

PMFs:



(a)	sinensetin	R_4 = H,	R_5 = H,	R_6 = OCH_3,	R_7 = H
(b)	nobiletin	R_4 = OCH_3,	R_5 = H,	R_6 = OCH_3,	R_7 = H
(c)	natsudaidain	R_4 = OCH_3,	R_5 = OH,	R_6 = H	R_7 = OCH_3
(d)	tangeretin	R_4 = OCH_3,	R_5 = H,	R_6 = H	R_7 = H

Appendix Figure 4. Chemical structures of flavanones and polymethoxylated flavones identified in Shiikuwasha peels (**Chapter VI**).

The flavonoids were as follows: (compound a) narirutin; (b) naringenin; (c) hesperidin; (d) neohesperidin; (e) sinensetin; (f) nobiletin; (g) natsudaidain; (h) tangeretin.

Appendix Table 1. Significant differences in changes in the relative concentrations of flavor components of stored cane brown sugar (data is presented in **Figure 4-3**, **Chapter IV**).

Aroma component	Storage stage	Change
Aldehydes	0–3 mo	
	3–6 mo	
	6–9 mo	
	9–12 mo	**
Alcohols	0–3 mo	
	3–6 mo	*
	6–9 mo	**
	9–12 mo	*
Sulfurs	0–3 mo	**
	3–6 mo	**
	6–9 mo	
	9–12 mo	
Hydrocarbons	0–3 mo	
	3–6 mo	
	6–9 mo	**
	9–12 mo	**
Monoterpene hydrocarbons	0–3 mo	
	3–6 mo	
	6–9 mo	
	9–12 mo	
MRPs	0–3 mo	**
	3–6 mo	**
	6–9 mo	**
	9–12 mo	**
Acids	0–3 mo	*
	3–6 mo	**
	6–9 mo	**
	9–12 mo	**

Significant differences in changes between 3-mo storage stages are indicated as follows: *, $p < 0.05$; **, $p < 0.01$.

Appendix Table 2. Significant differences in changes in the relative concentrations of volatile MRP components of stored cane brown sugar (data is presented in **Figure 4-4**, **Chapter IV**).

MRP component	Storage stage	Change
2,5-Dimethyl-pyrazine	0–3 mo	**
	3–6 mo	**
	6–9 mo	**
	9–12 mo	**
2,3,5-Trimethyl-pyrazine	0–3 mo	
	3–6 mo	**
	6–9 mo	**
	9–12 mo	
2-Ethyl-3,6-dimethyl-pyrazine	0–3 mo	**
	3–6 mo	*
	6–9 mo	**
	9–12 mo	**
2,6-Dimethyl-pyrazine	0–3 mo	**
	3–6 mo	**
	6–9 mo	**
	9–12 mo	**
2-Methyl-pyrazine	0–3 mo	**
	3–6 mo	**
	6–9 mo	*
	9–12 mo	**
2,3-Dimethyl-pyrazine	0–3 mo	*
	3–6 mo	**
	6–9 mo	**
	9–12 mo	
DDMP	0–3 mo	**
	3–6 mo	**
	6–9 mo	
	9–12 mo	**
2-Acetyl-pyrrole	0–3 mo	**
	3–6 mo	**
	6–9 mo	**
	9–12 mo	**
Dihydro-2-methyl-3(2H)-furanone	0–3 mo	**
	3–6 mo	**
	6–9 mo	**
	9–12 mo	**

Appendix Table 2. Significant differences in changes in the relative concentrations of volatile MRP components of stored cane brown sugar (data is presented in **Figure 4-4**, **Chapter IV**) (*continued*).

MRP component	Storage stage	Change
2,5-Dimethyl-4-hydroxy-3(2H)-furanone	0–3 mo	**
	3–6 mo	**
	6–9 mo	*
	9–12 mo	
2-Furanmethanol	0–3 mo	
	3–6 mo	**
	6–9 mo	**
	9–12 mo	**
5-Methyl-2-furanmethanol	0–3 mo	**
	3–6 mo	
	6–9 mo	
	9–12 mo	*

Significant differences in changes between 3-mo storage stages are indicated as follows: *, $p < 0.05$; **, $p < 0.01$.

Appendix Table 3. Morphological[a] and chemical[b] traits of unripe Shiikuwasha 'Ogimi kugani' (**Chapter V**).

Traits	Value/description
Average fruit weight (g)	18.61 ± 1.66
Polar diameter (mm)	28.23 ± 1.44
Equatorial diameter (mm)	33.96 ± 1.12
Skin thickness (mm)	1.34 ± 0.22
Skin color	Deep yellow green
Flesh color	Light greenish yellow
Seed number	4–7
Carpel number	8–9
Titratable acidity (%)	4.08 ± 0.37
Total soluble content (°Brix)	7.3 ± 0.4

[a] Each morphological trait value is expressed as the mean ± standard deviation (n = 10). Colors were determined by visual observation.

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

Appendix Table 4. Morphological[a] and chemical[b] traits of ripe Shiikuwasha fruits (**Chapter VI**).

Fruit traits	**'Izumi kugani'**	**'Katsuyama kugani'**	**'Ogimi kugani'**	**'Kaachi'**
Average fruit weight (g)	45.65 ± 7.18	26.74 ± 4.24	36.51 ± 4.51	26.74 ± 3.84
Polar diameter (mm)	35.31 ± 1.38	35.32 ± 1.89	34.60 ± 1.55	29.76 ± 1.25
Equatorial diameter (mm)	46.19 ± 11.23	46.49 ± 1.77	46.16 ± 2.22	41.71 ± 2.38
Skin thickness (mm)	1.89 ± 0.51	1.61 ± 0.26	1.53 ± 0.24	1.68 ± 0.30
Seed number	5–7	8–12	9–12	8–12
Carpel number	8–10	8–9	8–9	7–9
Titratable acidity (%)	1.25 ± 0.62	1.59 ± 0.11	1.28 ± 0.17	2.93 ± 0.62
Total soluble content (°Brix)	7.98 ± 1.14	8.88 ± 0.69	8.66 ± 0.63	10.78 ± 0.28

[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).