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Flavonoid Profiles of Wild Grapes Native to Japan: *Vitis coignetiae* Pulliat and *Vitis ficifolia* Bunge var. *ganebu* Hatusima

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Abstract

Flavonoids are a group of natural compounds in plants with versatile health benefits for humans. Grapes are a dietary source of flavonoids and the flavonoid components in grape berries can depend on the grape species and cultivar. In this experiment, proanthocyanidins, flavonols, and anthocyanins were analyzed in *Vitis coignetiae* and *V. ficifolia* var. *ganebu*, wild grapes native to Japan, and compared with those in *V. labruscana* cv. Muscat Bailey A, to evaluate the potential of the wild grapes as a grape resource. Proanthocyanidin contents in seeds were lower in the two wild grapes than in Muscat Bailey A. However, the skin of *V. ficifolia* var. *ganebu* was the richest source of proanthocyanidins. Flavonol levels in the skins of the two wild grapes were lower than that in the skin of Muscat Bailey A. Colorimetry determined that the total anthocyanin content in the skin of *V. ficifolia* var. *ganebu* was 6 times and 7 times higher, respectively, than those of *V. coignetiae* and Muscat Bailey A. Although monoglucoside anthocyanin levels analyzed by high-pressure liquid chromatography (HPLC) were in the order Muscat Bailey A > *V. ficifolia* var. *ganebu* > *V. coignetiae*, most of the diglucoside and acylated monoglucoside and diglucoside anthocyanin levels identified by HPLC-mass spectrometry were highest in *V. ficifolia* var. *ganebu*. These data suggest that *V. ficifolia* var. *ganebu* might be a novel source of flavonoids and superior to *V. coignetiae* as a source of flavonoids.

Keywords

Anthocyanin, Flavonol, Proanthocyanidin, Wild Grapes

1. Introduction

This study used *Vitis coignetiae* Pulliat (Coignetiae) and *V. ficifolia* Bunge var.

ganebu Hatusima (Ganebu), which are East Asian dioecious grape species indigenous to Japan. Coignetiae is a hardy species that mainly inhabits the subarctic wet climate areas of Japan. Ganebu is a subtropical species endemic to coastal areas of the subtropical southwestern islands of Japan. Ganebu has no endodormancy and can bear berries continuously in this habitat.

Coignetiae is cultivated in the northern areas of Japan and the cultivation of this species was initiated to promote agriculture in most areas. Nowadays, Coignetiae berries are extensively used as a source for local products such as juice and wine. In contrast, Ganebu is rarely cultivated and its uses are currently limited. Coignetiae berries are as large as most *V. vinifera* wine grapes, but the size of Ganebu berries is about a third of *V. vinifera* in terms of weight. Grapes are not generally regarded as a typical fruit for subtropical regions; therefore, cultivation of Ganebu has received little focus because of its small berries.

However, several lines of evidence on the singular characteristics of Ganebu berries were reported recently. Resveratrol is a nonflavonoid polyphenol with antioxidant activity and also a phytoalexin of grapes. High productivity of resveratrol in mature berries has been confirmed in Ganebu, but not commonly found in most *V. vinifera* cultivars and *V. ficifolia* var. *lobata* [1] [2]. The beneficial effects of resveratrol in human health have been demonstrated in numerous studies, including its antioxidant, anti-inflammatory, antiatherogenic, and antitumor activities [3] [4] [5]. Ganebu seed shows a peculiar characteristic, with higher α-tocopherol and lower linoleic acid levels than in other grape species [6]. α-Tocopherol is a molecule with the highest vitamin E validity among tocopherols and tocotrienols. Although linoleic acid is an essential oil that must be supplied in the human diet, it should not be taken excessively because of its susceptibility to oxidation and the negative effects of eicosanoids that are formed from linoleic acid [7]. Therefore, Ganebu seed might be a better source for grape seed oil than other grape species.

Other useful components in grape berries include flavonoids. Proanthocyanidins (PAs) in berry skins and seed coats, and flavonols and anthocyanins in berry skins are especially important components that vary by grape species. These flavonoids play a crucial role in the sensory properties [8] [9] and health-beneficial functions of berries, juices, and wines in the human body [10] [11] [12]. Only a few studies have investigated the profiles of these flavonoids in East Asian grape species, although a myriad of information has been provided on *V. vinifera* grapes through its worldwide cultivation and use [13] [14]. In addition, the available information about the flavonoid features in East Asian wild grapes native to Japan is restricted to anthocyanin profiles provided by high-performance liquid chromatography (HPLC) chromatograms, in which the anthocyanins identified were restricted to monoglucosides [15].

The aim of this study was to investigate the flavonoid profiles in Coignetiae and Ganebu grape berries to evaluate their potential as a grape resource. PAs, flavonols, and anthocyanins were analyzed in Coignetiae and Ganebu grape berries harvested from vines cultivated under the same climate and soil conditions

and the same cultural practices. The flavonoid components of Coignetiae and Ganebu grape berries were compared with that of *V. labruscana* Bailey cv. Muscat Bailey A, which was bred in Japan, and has been used for table, juice, and wine grapes in Japan.

2. Materials and Methods

2.1. Chemicals and Reagents

(+)-Catechin was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Cyanidin-3-glucoside (≥98%), delphinidin-3-glucoside (Dp-3G) (≥98%), and malvidin-3-glucoside (Mv-3G) (≥98%) were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). All organic solvents of Japan industrial standard (JIS) special grade were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2. Grapes

Three vines of *V. labruscana* Bailey cv. Muscat Bailey A (X-shape-trellis, 17 years old in 2008), and two vines each (hedgerow training) of *V. coignetiae* (6 years old in 2008; Coignetiae) and *V. ficifolia* var. *ganebu* (>20 years old in 2008; Ganebu) were grown in a research field at Osaka Prefecture University. The mature berries of Muscat Bailey A, Coignetiae, and Ganebu used for PA analysis were harvested at 100 days after full bloom in 2008. The soluble solid contents (Brix) of the grape juice of Muscat Bailey A, Coignetiae, and Ganebu were 18.6, 14.0, and 14.5, respectively. The skin and seed were separated with a pair of tweezers, immediately frozen in liquid nitrogen, and stored at −30˚C until analysis.

In 2011, the mature berries of Muscat Bailey A, Coignetiae, and Ganebu were harvested at 94, 109, and 93 days after full bloom, respectively. The Brix of Muscat Bailey A, Coignetiae, and Ganebu grape juice samples were 18.3, 14.6, and 14.6, respectively. The skin for flavonols and anthocyanins analysis was separated from the berries and stored in the same manner as in 2008.

2.3. PA Analysis

Samples (5 g skin and 3 g seeds per replicate) were extracted in 100 mL of 80% (v/v) methanol using a homogenizer (T 18 ULTRA-TURRAX BS1; IKA, Staufen, Germany). The homogenate was stirred at about 200 rpm for 18 h at 4˚C and filtered through a Whatman No. 2 filter paper. The residue was washed with 100 mL of 75% (v/v) acetone. The 80% methanol extract combined with the 75% acetone wash was concentrated to the aqueous phase at 40˚C *in vacuo*. After adjusting the pH to 7.0 with 0.1 N NaOH, the volume of the aqueous extract was made up to 50 mL with distilled water.

The neutral extracts were fractionated into flavan-3-ol monomers, oligomeric PAs, and polymeric PAs through the two preconditioned Sep-Pak Plus tC-18 and C-18 cartridges (Waters; Milford, MA, USA) connected in order from top to bottom according to Sun *et al.*'s method [16]. PAs in each fraction were quanti-

fied by Sun *et al.*'s modified vanillin assay using a standard curve with (+)-catechin [17]. All determinations were conducted in triplicate.

2.4. Flavonol Analysis

Flavonols were extracted and analyzed according to a slight modification of Pena-Neira *et al.*'s method [18]. Berry skin samples were ground in liquid nitrogen with a mortar and pestle. One g of sample was extracted in 20 mL of aqueous ethanol (distilled water:ethanol = 9:1, v/v) containing tartaric acid (5 g/L) using a homogenizer. The homogenate was stirred at about 200 rpm in the dark at room temperature for 2 h. The homogenate was filtered through a Whatman No. 2 filter paper and the residue was washed once with 10 mL of extraction solvent. The combined extract and wash solvent were concentrated to the aqueous phase at 35˚C *in vacuo*. The aqueous phase was partitioned three times each against equal volumes of both ether and ethyl acetate. The combined organic phase was concentrated to dryness *in vacuo*.

The dry sample was redissolved in 1 mL methanol and centrifuged at 1800 *g* for 3 min. Twenty microliters of the supernatant were analyzed using HPLC (M600; Waters) equipped with a PDA detector (SPD-M20A; Shimadzu, Kyoto, Japan). Flavonols were detected by visible light at 360 nm. The samples were eluted at a flow rate of 1.0 mL/min from a reversed-phase column (Inertsil ODS-SP; 4.0 × 250 mm) with a guard column (Inertsil ODS-SP; 4.0 × 10 mm). The solvent system consisted of water containing 2% (v/v) acetic acid (A) and a mixture of water:acetonitrile:acetic acid at 78:20:2 (v/v) (B). Separation was carried out using a multistep linear gradient with an increasing concentration of solvent B as follows: 0% solvent B at 0 min, 20% at 15 min, 22.5% at 30 min and 25% at 50 min. Quercetin-3-rutinoside (Q3Rut), quercetin-3-glucoside (Q3Glu), and kaempferol-3-glucoside (K3Glu) were identified in the samples by comparing the retention times of the standards (typical retention times of Q3Rut, Q3Glu, and K3Glu were 27.8, 29.7, and 36.9 min, respectively). Their concentrations were quantified based on an external standard curve. Quercetin-3-glucuronide, a noncommercial flavonol, was identified by HPLC-TOF mass under the same conditions as those for anthocyanin analysis as described in section 2.5.3. The pseudomolecular ion ($[M + H]^+$) and the predominant fragment ion were 479 and 303, respectively. Quercetin-3-glucuronide was quantified by using the HPLC-PDA analysis data and the quantified data were shown as quercetin-glucoside equivalent μg/g fresh weight (gfw).

2.5. Anthocyanin Analysis

2.5.1. Extraction of Anthocyanins

Anthocyanins were extracted and analyzed according to a slight modification of Shiraishi *et al.*'s method [19]. One gram of skin powder prepared in liquid nitrogen with a mortar and pestle was homogenized in 40 mL of 50% (v/v) aqueous acetic acid with a mortar and pestle. After homogenization, the sample was placed in the dark at 4˚C for 18 h. The sample was filtered through with a

Whatman No. 2 filter paper; the residue was washed three times with 10 mL of 50% (v/v) aqueous acetic acid. The volume of the filtered sample was made up to 75 mL with 50% (v/v) aqueous acetic acid and stored at 4˚C until analysis. For mass spectrometer analysis, a 200 µL aliquot of extract was evaporated to dryness and stored at −30˚C.

2.5.2. Total Anthocyanin Analysis

Absorbance of the extract at 530 nm was measured in a quartz cell of 1 mm light length by a spectrophotometer (UV mini 1240; Shimadzu). The amount of anthocyanin was determined by comparing the absorbance to the standard curves made using Mv-3G. Total anthocyanin content in berry skins was shown as Mv-3G equivalent mg/gfw.

2.5.3. Identification and Quantification of Anthocyanins

The extract of 3 mL was centrifuged at 3000 g for 5 min and 10 µL of the supernatant was analyzed by HPLC (Model 576; GL Sciences, Tokyo, Japan) equipped with a PDA detector (SPD-M20A; Shimadzu). Anthocyanins were monitored from 200 nm to 600 nm. The samples were eluted at a flow rate of 1.0 mL/min from a reversed-phase column (Inertsil ODS-SP; 4.0 × 250 mm) with a guard column (Inertsil ODS-SP; 4.0 × 10 mm). The solvent system consisted of water containing 2% (v/v) acetic acid (A) and a mixture of water:acetonitrile:acetic acid at 78:20:2 (v/v) (B). Separation was carried out using a multistep linear gradient with an increasing concentration of solvent B as follows: 0% at 0 min, 20% at 15 min, 22.5% at 30 min, and 25% at 50 min. Dp-3G, petunidin-3-glucoside (Pt-3G), and Mv-3G were identified in the samples by comparing the retention times of the standards (typical retention times of Dp-3G, Pt-3G, and Mv-3G were 14.2, 18.6, and 22.2 min, respectively). Their concentrations were quantified based on an external standard curve.

Identification of diglucoside and acylated anthocyanins was conducted by HPLC (LaChrome Elite; Hitachi, Tokyo, Japan) equipped with a PDA detector (L-2455; Hitachi) and TOF mass spectrometer (NanoFrontier L; Hitachi). The dry sample was redissolved in 100 µL methanol and 100 µL water. A 10 µL sample was eluted at a flow rate of 0.19 mL/min from a Cadenza CD-C18 (2.0 × 150 mm) column using the following solvent system: solvent A: 0.1% (v/v) formic acid in water; solvent B: 0.1% (v/v) formic acid in acetonitrile; 2% solvent B at 0 min, 20% B at 19 min, and 30% B at 30 - 35 min. Positive mode of ESI was used for detection with a capillary voltage of 4 kV, a vaporizer temperature of 500˚C, and carrier gas flow (nitrogen) of 1500 L/min. The mass acquisition was carried out between 200 and 1500 m/z. Identified diglucoside and acylated anthocyanins and their molecular and characteristic fragment ions were shown in **Table 4**.

Diglucoside and acylated anthocyanins were quantified by using the HPLC-PDA analysis data (SPD-M20A; Shimadzu) and the quantified data were shown as Mv-3G equivalent µg/gfw. Diglucoside and acylated anthocyanins in the HPLC-PDA analysis were identified by comparing their relative retention times against Mv-3G between the two HPLC systems.

3. Results and Discussion

3.1. PAs and Flavan-3-ol Monomer

The contents of PAs and flavan-3-ol monomer in berry skins and seeds are shown in Table 1. The contents were in the order of polymer > oligomer > monomer, irrespective of the grape species and the materials. No significant differences in flavan-3-ol monomer content were observed between skins of grape species. The contents of flavan-3-ol oligomer and polymer were significantly higher in Ganebu skin than in Muscat Bailey A and Coignetiae skins; the levels in Ganebu skin were 4.3 and 4.7 times greater, respectively, than those in Muscat Bailey A skin.

PA content in seeds was lower in the two wild grapes than in Muscat Bailey A. The contents of flavan-3-ol monomer and oligomer PA in Coignetiae and Ganebu seeds were less than 46% and 37%, respectively, of that in Muscat Bailey A seeds. The lowest polymer PA content was found in Ganebu seeds, which was 43.2% of that in Muscat Bailey A seeds.

Cheynier *et al.* [20] and Labarbe *et al.* [21] found that the degree of PA polymerization was higher in skins than in seeds of *V. vinifera*. Our results are almost consistent with their findings; the proportions of polymer PA of the total flavan-3-ols (skins:seeds) were 76.3%:49.4%, 77.3%:59.1%, and 80.2%:71.8% in Muscat Bailey A, Coignetiae, and Ganebu grapes, respectively. However, the difference in proportions of polymer PA between skins and seeds in the two wild grapes, especially in Ganebu grapes, was not as large as that in Muscat Bailey A grapes.

Interestingly, only in Ganebu grapes did the skin have higher levels of PAs than the seed did; oligomer and polymer PAs in the skin were 1.85 and 2.36 times, respectively, higher than that in the seed. Previous studies of *V. vinifera* cultivars (albeit with a different methodology) yielded similar results; lower concentrations of PAs were found in skins compared with that in seeds [22] [23] [24] [25]. Xu *et al.* [26] analyzed the total flavan-3-ols in seeds and skins of several

Table 1. Proanthocyanidins (oligomeric PAs and polymeric PAs) and flavan-3-ol monomer content in the skin and seed of Muscat Bailey A, *Vitis coignetiae* and *Vitis ficifolia* var. *ganebu*.

		Flavan-3-ol monomer		Proanthocyanidins (mg/gfw)			
Grape materials		(mg/gfw)		Oligomer		Polymer	
Skin	Muscat Bailey A	1.7	a	6.3	a	25.8	a
	V. coignetiae	2.0	a	4.3	a	21.4	a
	V. ficifolia var. *ganebu*	2.6	a	27.5	b	121.8	b
Seed	Muscat Bailey A	35.0	b	87.1	b	119.0	b
	V. coignetiae	15.8	a	31.7	a	68.5	ab
	V. ficifolia var. *ganebu*	5.4	a	14.8	a	51.4	a

Means (n = 3) with different letters in each columns of each materials (skin or seed) are significantly different by Scheffe test at 5%.

grapes, including *V. vinifera*, oriental *Vitis* species, and their hybrids (European-Asian and European-American hybrids). Higher levels of flavan-3-ols in seeds than in skins were found not only in *V. vinifera* but also in the European-American hybrid (*V. labruscana* cv. Kyoho) [26]. The difference in the PA levels between the seed and skin of Muscat Bailey A in this study is comparable with their results. Differences in the PA levels between skins and seeds of oriental grapes were also found to be very small compared with *V. vinifera* and *V. labruscana*. A slightly higher level of total flavan-3-ols in skins than in seeds was found only in *V. xunyangensis* cv. Purple grape. The much higher oligomer and polymer PA levels in the skin than in the seed appear to be a distinctive feature of Ganebu grapes among the East-Asian grape species.

3.2. Flavonols

Flavonols detected in this study included Q3Rut, quercetin-3-glucuronic acid (Q3GlcA), Q3Glc, and K3Glc (**Table 2**). The total flavonols content (sum of the content of Q3Rut, Q3GlcA [equivalent to Q3Glc], Q3Glc, and K3Glc) in the skin was highest in Muscat Bailey A and lowest in Coignetiae. Q3GlcA and Q3Glc were the predominant flavonols in each grape, but Q3Rut was only found in Coignetiae at a very low level. In Muscat Bailey A, Q3GlcA and Q3Glc accounted for 56.0% and 38.0% of the total flavonols, respectively. The Q3GlcA level in the skin of Coignetiae was significantly lower than that in Muscat Bailey A and less than 14% of that in Ganebu. The Q3Glc contents of Coignetiae and Ganebu were approximately 14.0% and 51.0%, respectively, of that of Muscat Bailey A. There were no significant differences in K3Glc content of the grapes.

The 3-*O*-glucosides (Glc), including the 3-*O*-glucuronide (GlcA), 3-*O*-galactoside, 3-*O*-(6”-rhamnosyl)-glucoside (rutinoside), 3-*O*-glucosilgalactose, and 3-*O*-glucosilxyloside structures of kaempferol, quercetin, isorhamnetin, myricetin, laricitrin, and syringetin have been detected in red grape cultivars [27]. Among them, GlcA and Glc of quercetin are dominant in the skin of red grape cultivars. Although there are some exceptions such as Shiraz and Cencibel in which Q3Glc levels are higher than Q3GlcA, a higher level of Q3GlcA than that of Q3Glc seems to be common in *V. vinifera* [28] [25]. In the skin of 344 *V. vinifera* grapes, including not only colored but also noncolored cultivars, Q3GlcA (ranging

Table 2. Flavonols content in the berry skin of Muscat Bailey A, *Vitis coignetiae* and *Vitis ficifolia var. ganebu.*

	Flavonols (μg/gfw)									
Grapes	Q3Rut	Q3GlcA[z]		Q3Glc		K3Glc		Total		
Muscat Bailey A	-	69.1	b	46.9	c	7.1	a	123.2	c	
V. coignetiae	0.6	8.3	a	6.6	a	3.5	a	19.0	a	
V. ficifolia var. *ganebu*	-	59.6	b	24.0	b	1.5	a	85.2	b	

[z]The concentration of Q3GlcA is equivalent to that of Q3Glc. Means (n = 3) with different letters in each columns are significantly different by Scheffe test at 5%.

from 0 to 71 μg/gfw; average 17 μg/gfw) accounted for an average of 40% of the total flavonols, followed by Q3Glc (ranging from 0 to 70 μg/gfw; average 14 μg/gfw) accounting for 32% of the total [29]. A similar relationship in the level of Q3GlcA and Q3Glc was found in the skin of three European-American hybrids; the average level of Q3GlcA (ranging from 35.5 to 272.5 μg quercetin equivalent (QE)/g dry weight (dw); average 121.3 μg QE/gdw) was 1.73 times higher than that of Q3Glc (ranging from 7.7 to 166.2 μg QE/gdw; average 69.8 μg QE/gdw). The results for Muscat Bailey A, a European-American hybrid, were largely in agreement with the previous data for *V. vinifera* and other European-American hybrids, with respect to the concentrations of Q3GlcA and Q3Glc and their proportions of the total flavonols.

Low contents of Q3GlcA and Q3Glc were previously reported in East-Asian grapes including *V. amurensis* cultivars, *V. ficifolia* cv. Sangye, *V. davidii* cv. Black Pearl, *V. quinquangularis* cv. Mao and *V. xunyangensis* cv. Mi compared with that of European-American hybrids, although the specific levels in each grape variety were not available in the report; Q3GlcA ranged from 11.8 to 166.9 μg QE/gdw (average 60.3 μg QE/gdw) and Q3Glc ranged from 16.9 to 155.2 μg QE/gdw (average 57.6 μg QE/gdw) [14]. The comparison of flavonol levels between Muscat Bailey A (European-American hybrid), Coignetiae and Ganebu in our experiment confirms these previous findings. The levels of Q3GlcA and Q3Glc in Coignetiae were equivalent to the relatively low levels reported in the previous study [14], taking into consideration the general water content of mature grape skins (ca. 80% for Campbell Early and Muscat Bailey A; data not shown). Coignetiae is genetically closely related to *V. amurensis*, as shown by their topological, isozymic, and genetic similarity [15]; both species are native to the high-latitude region of East Asia. Protection from ultraviolet (UV) radiation in insolation is the main conceivable role of flavonols in berry skins. The low levels of flavonols might be a common feature of East-Asian wild grapes, like Coignetiae, that are native to the northern area with low exposure to UV rays.

Zhu *et al.* [14] reported that flavonol levels of *V. ficifolia* cv. Sangye skin were higher than that of most cultivars of *V. amurensis.* Ganebu also had higher levels of flavonoids than Coignetiae. Although the relationship of *V. ficifolia* cv. Sangye and Ganebu (*V. ficifolia* var. *ganebu*) is obscure, unlike that of *V. amurensis* and Coignetiae, and there is no information about the habitat of *V. ficifolia*, a mother plant of Sangye, relatively higher levels of flavonols in skins among East-Asian grapes is probably a characteristic of wild grapes native to the southern area.

3.3. Anthocyanin

The total anthocyanin level in Coignetiae was similar to that in Muscat Bailey A (**Table 3**). In contrast, Ganebu contained the highest total anthocyanin content in the skin; the level was 7 times higher than that in Muscat Bailey A. However, the total content of three anthocyanin monoglucosides analyzed by HPLC was the highest in Muscat Bailey A and the lowest in Coignetiae. Muscat Bailey A

Table 3. Total anthocyanin, and the monoglucoside of delphinidin (Dp-3G), petunidin (Pt-3G) and malvidine (Mv-3G) contnt in the berry skin of Muscat Bailey A, *Vitis coignetiae* and *Vitis ficifolia* var. *ganebu*.

	Total anthocyanin[z]		Anthocyanin[x] (μg/gfw)							
	(Mv-3G equivalent mg/gfw)		Dp-3G		Pt-3G		Mv-3G		total	
Muscat Bailey A	3.8	a[y]	136.6	b	156.3	c	813.6	c	1106.5	c
			(12.3)	[w]	(14.1)		(73.5)			
V. coignetiae	4.4	a	20.7	a	13.5	a	44.7	a	78.9	a
			(26.2)		(17.1)		(56.7)			
V. ficifolia var. *ganebu*	26.9	b	266.7	c	124.0	b	373.5	b	764.2	b
			(34.9)		(16.2)		(48.9)			

[z]Total anthocyanin was colorimetrically analyzed at 530 nm in a quartz cell of the 1 mm light path. [y]Means (n = 3) with different letters in each columns are significantly different by Scheffe test at 5%. [x]Each anthocyanin was analyzed by HPLC. [w]The number in the parenthesis shows percentage of the each anthocyanin in the total amount of Dp-3G, Pt-3G and Mv-3G.

contained a higher level of Mv-3G compared with Dp-3G and Pt-3G; Mv-3G accounted for 73.5% of the total anthocyanin monoglucosides. In Coignetiae and Ganebu, the proportion of Mv-3G in the three anthocyanin monoglucosides was not as high as that in Muscat Bailey A. Ganebu contained the highest level of Dp-3G in the three grape species. In both Coignetiae and Ganebu, the proportion of Dp-3G in the total anthocyanin monoglucosides was > 2 times higher than that of Muscat Bailey A.

Mv-3G is generally the most-abundant anthocyanin monoglucoside in the skin of *V. vinifera* grapes [30] [31], and also in the skin and juice of Muscat Bailey A grapes [32] [33]. Zhu *et al.* [14] reported that Mv-3G levels in East Asian grapes were lower than those in European-American hybrid grapes. Lower levels of Mv-3G and Pt-3G in Coignetiae and Ganebu grapes compared with Muscat Bailey A grapes are probably a common feature of East Asian grapes.

In general, *Vitis vinifera* grapes contain only acylated monoglucoside anthocyanin and monoglucoside anthocyanin [34]. In other grape species, diglucoside anthocyanin, acylated monoglucoside, and acylated diglucoside anthocyanin have been found, in addition to monoglucoside anthocyanin [14]. In this experiment, three diglucoside anthocyanins, two monoglucoside anthocyanins acylated with coumaric acid, and three diglucoside anthocyanins acylated with coumaric acid were identified in the samples (**Table 4**). The only diglucoside anthocyanin identified in Muscat Bailey A was Mv-3G5G. The absence of Dp-3G5G and Pt-3G5G in the juice of Muscat Bailey A was reported previously [33]. In contrast, all three diglucosides were found in Coignetiae and Ganebu. The levels of Mv-3G5G were two orders of magnitude higher in Coignetiae and Ganebu when compared with that of Muscat Bailey A. The levels of diglucoside anthocyanins were especially high in Ganebu when compared with Coignetiae; the

Table 4. Content of diglucoside and acylated anthocyanins identified by HPLC-MS in the berry skin of Muscat Bailey A, *Vitis coignetiae* and *Vitis ficifolia* var. *ganebu*.

	Anthocyanin (Mv3G equivalent μg/gfw)															
Grapes	Dp-3G5G		Pt-3G5G		MV-3G5G		Dp-3GCo5G		Pt-3GCo5G		Mv-3GCo5G		Pt-3GCo		Mv-3GCo	
	627; 465,303[Z]		641; 479,317		655; 493,331		773; 303		787; 479,317		801; 493,331		625; 317		639; 331	
Muscat Bailey A	nd		nd		60.9	a	21.4	a	31.0	a	388.9	a	75.1	b	333.7	b
V. coignetiae	55.8		107.2		1896.6	b	69.7	a	118.1	b	806.1	b	20.6	a	47.9	a
V. ficifolia var. *ganebu*	1980.3		1819.1		8696.5	c	1857.9	b	1175.7	c	3197.8	c	166.5	c	310.3	b

nd: not detected. Means (n = 3) with different letters in each columns are significantly different by Scheffe test at 5%. [Z]Molecular; Flagment ion (m/z).

levels of Dp, Pt, and Mv diglucosides in Ganebu were 35.4, 16.9, and 4.5 times higher than those in Coignetiae, respectively. Zhu *et al.* [14] reported that the levels of Dp3G5G, Pt3G5G, and Mv3G5G in nine East-Asian grapes ranged from 0 to 2378 μg/gfw, 33 to 2734 μg/gfw, and 133 to 4663 μg/gfw, respectively. Although the levels of these compounds in Coignetiae were within the range of those reported in the nine East-Asian grapes, the level of Mv3G5G in Ganebu was higher than in any previous report.

Glycosylation at C3 of the ring-C in the flavan skeleton, the first step in glycosylation of anthocyanidins just after their synthesis in plants, is necessary for stability in the acidic environment of the vacuole. In addition, an increase in the glycosyl number of anthocyanins is positively correlated with their stability [35]. Because of the high levels of glycosylation of anthocyanidins in Coignetiae and Ganebu, these grape species have more stable anthocyanins compared with Muscat Bailey A grapes. In particular, it is worthy of special mention that MV-3G5G was found at a very high level in Ganebu grapes among East Asian grapes.

Considering the acylated anthocyanins, malvidin-3-(6"-p-coumaroyl)-glucoside-5-glucoside (Mv-3GCo5G) was commonly the most-abundant acylated diglucoside anthocyanin in the grapes, similar to the diglucoside derivatives. Although the levels of each acylated diglucoside anthocyanin (Dp-3GCo5G, Pt-3GCo5G, and Mv-3GCo5G) were lowest in Muscat Bailey A, the levels of acylated monoglucoside anthocyanins (Pt-3GCo and Mv-3GCo) were lowest in Coignetiae. The levels of acylated diglucoside anthocyanin in Ganebu were notable; it contained 86.8, 37.9, and 8.2 times higher levels of Dp-3GCo5G, Pt-3GCo5G, and Mv-3GCo5G, respectively, than those in Muscat Bailey A.

All anthocyanins show common light absorptivity with two distinctive absorption peaks: one in the UV-C region (260 - 280 nm) and the other in the visible region (490 - 550 nm). The spectrum of anthocyanins can be altered by the amount and position of glycosylation in the molecule, and by the amount of acylation [36]. Diglycosylation at C3 and C5 of anthocyanin decreases the ratio between the absorbance at 440 nm and the absorbance at the $\lambda_{vis\text{-}max}$ of that in C3 glycoside anthocyanin. Although diglycosylation contributes little to the absorbance of UV-A and UV-B [37], acylation with cinnamic acid as a glycosidic substitute alters the anthocyanin to be able to absorb UV-B (310 - 360 nm) [38]. Acylation with p-coumaric acid, a hydroxyl derivative of cinnamic acid, also

modifies the absorbance of anthocyanin in a similar manner [27]. Therefore, the acylated anthocyanins probably contribute to the attenuation of UV radiation in the berry as with flavonols. It is reasonable that Ganebu contains high levels of acylated anthocyanins, as its habitat, the southwest region of Japan, has high UV-B in insolation.

4. Conclusions

All of flavonoids analyzed in this study were not higher in *V. coignetiae* Pulliat (Coignetiae) and *V. ficifolia* Bunge var. *ganebu* Hatusima (Ganebu) than that in *V. labruscana* Bailey cv. Muscat Bailey. However, specific flavonoid components that were characteristic of the species, especially for Ganebu, were determined in this study, which provides information on their potential application and utility. Ganebu berry skin contains considerably high levels of total anthocyanins and specifically, high levels of diglucoside and acylated anthocyanins. Highly glycosylated and acylated anthocyanins are more stable in response to changes in pH and heat, which might indicate good potential of Ganebu berries for processing.

Higher levels of oligomer and polymer PAs in Ganebu grape skins compared with other grapes is a specific characteristic of flavonoids in this species. Although Ganebu contains lower levels of PA in the seed and lower levels of flavonols in the skin than those in the Muscat Bailey A seed and skin, respectively, Ganebu PA levels were not significantly different from those in Coignetiae and the flavonol levels were higher than those in Coignetiae. Ganebu berries are a better source of PA and flavonols than Coignetiae berries. Overall, we can conclude that Ganebu berries are a useful source for flavonoids.

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References

[1] Jeandet, P., Bessis, R. and Gautheron, B. (1991) The Production of Resveratrol (3,5,4'-trihydroxystilbene) by Grape Berries in Different Developmental Stages. *American Journal of Enology and Viticulture*, **42**, 41-46.

[2] Shiozaki, S., Nakamura, T. and Ogata, T. (2013) Resveratrol Productivity of Wild Grapes Native to Japan: *Vitis ficifolia* var. *lobata* and *Vitis ficifolia* var. *ganebu*. *American Journal of Enology and Viticulture*, **64**, 163-168. https://doi.org/10.5344/ajev.2012.12066

[3] Fremont, L. (2000) Biological Effects of Resveratrol. *Life Sciences*, **66**, 663-673. https://doi.org/10.1016/S0024-3205(99)00410-5

[4] Pirola, L. and Frojdo, S. (2008) Resveratrol: One Molecule, Many Targets. *IUBMB Life*, **60**, 323-332. https://doi.org/10.1002/iub.47

[5] Ramprasath, V.R. and Jones, P.J.H. (2010) Anti-Atherogenic Effects of Resveratrol. *European Journal of Clinical Nutrition*, **64**, 660-668. https://doi.org/10.1038/ejcn.2010.77

[6] Shiozaki, S. and Murakami, M. (2016) Lipids in the Seeds of Wild Grapes Native to

Japan: *Vitis coignetiae* and *Vitis ficifolia* var. *ganebu*. *Scientia Horticulturae*, **201**, 124-129. https://doi.org/10.1016/j.scienta.2016.01.038

[7] Matthäus, B. (2008) Virgin Grape Seed Oil: Is It Really a Nutritional Highlight? *European Journal of Lipid Science and Technology*, **110**, 645-650. https://doi.org/10.1002/ejlt.200700276

[8] Lesschaeve, I. and Noble, A.C. (2005) Polyphenols: Factors Influencing Their Sensory Properties and Their Effects on Food and Beverage Preferences. *The American Journal of Clinical Nutrition*, **81**, 330S-335S.

[9] Preys, S., Mazerolles, G., Courcoux, P., Samson, A., Fischer, U., Hanafi, H., Bertrand, D. and Cheynier, V. (2006) Relationship between Polyphenolic Composition and Some Sensory Properties in Red Wines Using Multiway Analyses. *Analytica Chimica Acta*, **563**, 126-136. https://doi.org/10.1016/j.aca.2005.10.082

[10] Peralbo-Molina, A. and Luque de Castro, M.D. (2014) Grape Phenols and Their Healthy Properties. In: Câmara, J.S., Ed., *Grapes*: *Production*, *Phenolic Composition and Potential Biomedical Effects*, Nova Science Publishers, Inc., New York, 181-209.

[11] Yao, L.H., Jiang, Y.M., Shi, J., Tomás-Barberán, F.A., Datta, N., Singanusong, R. and Chen, S.S. (2004) Flavonoids in Food and Their Health Benefits. *Plant Foods for Human Nutrition*, **59**, 113-122. https://doi.org/10.1007/s11130-004-0049-7

[12] Zafra-Stone, S., Yasmin, T., Bagchi, M., Chatterjee, A., Vinson, J.A. and Bagchi, D. (2007) Berry Anthocyanins as Novel Antioxidants in Human Health and Disease Prevention. *Molecular Nutrition & Food Research*, **51**, 675-683. https://doi.org/10.1002/mnfr.200700002

[13] Baiano, A. (2014) Influence of Genotype, Pedoclimatic Conditions, Viticultural Practices and Ripening on the Phenolic Composition of Grapes: A Review. In: Câmara, J.S., Ed., *Grapes*: *Production*, *Phenolic Composition and Potential Biomedical Effects*, Nova Science Publishers, Inc., New York, 3-26.

[14] Zhu, L., Zhang, Y. and Lu, J. (2012) Phenolic Contents and Compositions in Skins of Red Wine Grape Cultivars among Various Genetic Backgrounds and Originations. *International Journal of Molecular Sciences*, **13**, 3492-3510. https://doi.org/10.3390/ijms13033492

[15] Yamashita, H. and Mochioka, R. (2014) Wild Grape Germplasms in Japan. *Advances in Horticultural Science*, **28**, 214-224.

[16] Sun, B., Leandro, C., Ricardo da Silva, J.M. and Spranger, I. (1998) Separation of Grape and Wine Proanthocyanidins According to Their Degree of Polymerization. *Journal of Agricultural and Food Chemistry*, **46**, 1390-1396. https://doi.org/10.1021/jf970753d

[17] Sun, B., Ricardo da Silva, J.M. and Spranger, I. (1998) Critical Factors of Vanillin Assay for Catechins and Proanthocyanidins. *Journal of Agricultural and Food Chemistry*, **46**, 4267-4274. https://doi.org/10.1021/jf980366j

[18] Pena-Neira, A., Duenas, M., Duarte, A., Hernandez, T., Estrella, I. and Loyola, E. (2004) Effects of Ripening Stages and of Plant Vegetative Vigor on the Phenolic Composition of Grapes (*Vitis vinifera* L.) cv. Cabernet Sauvignon in the Maipo Valley (Chile). *Vitis*, **43**, 51-57.

[19] Shiraishi, M., Yamada, M., Mitani, N. and Ueno, T. (2007) A Rapid Determination Method for Anthocyanin Profiling in Grape Genetic Resources. *Journal of the Japanese Society for Horticultural Science*, **76**, 28-35. https://doi.org/10.2503/jjshs.76.28

[20] Cheynier, V., Prieur, C., Guyot, S., Rigaud, J. and Moutounet, M. (1997) The Struc-

tures of Tannins in Grapes and Wines and Their Interaction with Proteins. In: Watkins, T.R., Ed., *Wine*: *Nutritional and Therapeutic Benefits*, ACS Symposium Series 661, American Chemical Society, Washington DC, 81-93.

[21] Labarbe, B., Cheynier, V., Brossaud, F., Souquet, J.-M. and Moutounet, M. (1999) Quantitative Fractionation of Grape Proanthocyanidins According to Their Degree of Polymerization. *Journal of Agricultural and Food Chemistry*, **47**, 2719-2723. https://doi.org/10.1021/jf990029q

[22] Fujita, A., Soma, N., Goto-Yamamoto, N., Mizuno, A., Kiso, K. and Hashizume, K. (2007) Effect of Shading on Proanthocyanidin Biosynthesis in the Grape Berry. *Journal of the Japanese Society for Horticultural Science*, **76**, 112-119. https://doi.org/10.2503/jjshs.76.112

[23] Harbertson, J.F., Kennedy, J.A. and Adams, D.O. (2002) Tannin in Skins and Seeds of Cabernet Sauvignon, Syrah, and Pinot Noir Berries during Ripening. *American Journal of Enology and Viticulture*, **53**, 54-59.

[24] Hernandez-Jimenez, A., Gomez-Plaza, E., Martinez-Cutillas, A. and Kennedy J.A. (2009) Grape Skin and Seed Proanthocyanidins from Monastrell × Syrah Grapes. *Journal of Agricultural and Food Chemistry*, **57**, 10798-10803. https://doi.org/10.1021/jf903465p

[25] Rodrigues-Montealegre, R., Romero-Peces, R., Chacon-Vozmediano, J.L., Martinez-Gascuena, J. and Garcia-Romero, E. (2006) Phenolic Compounds in Skins and Seeds of Ten Grape *Vitis vinifera* Varieties Grown in a Warm Climate. *Journal of Food Composition and Analysis*, **19**, 687-693. https://doi.org/10.1016/j.jfca.2005.05.003

[26] Xu, C., Zhang, Y., Cao, L. and Lu, J. (2010) Phenolic Compounds and Antioxidant Properties of Different Grape Cultivars Grown in China. *Food Chemistry*, **119**, 1557-1565. https://doi.org/10.1016/j.foodchem.2009.09.042

[27] Lago Vanzela, E.S., Alves Baffi, M., Machado de Castilhos, M.B., Moacir Ribeiro Pinto, M.R., Del Bianchi, V.L., Mota Ramos, A., Cesar Stringeta, P., Hermosin-Gutierrez, I. and Da-Silva, R. (2014) Phenolic Compounds in Grapes and Wines: Chemical and Biochemical Characteristics and Technological Quality. In: Câmara, J.S., Ed., *Grapes*: *Production*, *Phenolic Composition and Potential Biomedical Effects*, Nova Science Publishers, Inc., New York, 47-105.

[28] Downey, M.O., Harvey, J.S. and Robinson, S.P. (2003) Synthesis of Flavonols and Expression of Flavonol Synthase Genes in the Developing Grape Berries of Shiraz and Chardonnay (*Vitis vinifera* L.). *Australian Journal of Grape and Wine Research*, **9**, 110-121. https://doi.org/10.1111/j.1755-0238.2003.tb00261.x

[29] Liang, Z., Owens, C.L., Zhong, G.-Y. and Cheng, L. (2011) Polyphenolic Profiles Detected in the Ripe Berries of *Vitis vinifera* Germplasm. *Food Chemistry*, **129**, 940-950. https://doi.org/10.1016/j.foodchem.2011.05.050

[30] Cook, M.G., Zhang, Y., Nelson, C.J., Gambetta, G., Kennedy, J.A. and Kurtural, S.K. (2015) Anthocyanin Composition of Merlot Is Ameliorated by Light Microclimate and Irrigation in Central California. *American Journal of Enology and Viticulture*, **66**, 266-278. https://doi.org/10.5344/ajev.2015.15006

[31] Kallithraka, S., Abdel-Azeem Mohdaly, A., Makris, D.P. and Kefalas, P. (2005) Determination of Major Anthocyanin Pigments in Hellenic Native Grape Varieties (*Vitis vinifera* sp.): Association with Antiradical Activity. *Journal of Food Composition and Analysis*, **18**, 375-386. https://doi.org/10.1016/j.jfca.2004.02.010

[32] Matsuyama, S., Tanzawa, F., Kobayashi, H., Suzuki, S., Tanaka, R. and Saito, H. (2014) Leaf Removal of Accelerated Accumulation of Delphinidin-Based Anthocyanins in Muscat Bailey A [*Vitis* × *labruscana* (Bailey) and *Vitis vinifera* (Muscat

Hamburg)] Grape Skin. *Journal of the Japanese Society for Horticultural Science*, **83**, 17-22. https://doi.org/10.2503/jjshs1.CH-062

[33] Oh, Y.S., Lee, J.H., Yoon, S.H., Oh, C.H., Choi, D.S., Choe, E. and Jung, M.Y. (2008) Characterization and Quantification of Anthocyanins in Grape Juices Obtained from the Grapes Cultivated in Korea by HPLC/DAD, HPLC/MS, and HPLC/MS/MS. *Journal of Food Science*, **73**, C378-C389. https://doi.org/10.1111/j.1750-3841.2008.00756.x

[34] Ribereau-Gayon, P., Glories, Y., Maujean, A. and Dubourdieu, D. (2006) Phenolic Compounds In: Ribereau-Gayon, P., Glories, Y., Maujean, A. and Dubourdieu, D., Eds., *Handbook of Enology Volume* 2. *The Chemistry of Wine Stabilization and Treatments*, 2nd Edition, John Wiley & Sons, Ltd., Chichester, 141-204. https://doi.org/10.1002/0470010398.ch6

[35] Zhao, C.L., Chen, Z.J., Bai, X.S., Ding, C., Long, T.J., Wei, F.G. and Miao, K.R. (2014) Structure-Activity Relationships of Anthocyanidin Glycosylation. *Molecular Diversity*, **18**, 687-700. https://doi.org/10.1007/s11030-014-9520-z

[36] Giusti, M.M. and Wrolstad, R.E. (2001) Characterization and Measurement of Anthocyanins by UV-Visible Spectroscopy. In: Giusti, M.M. and Wrolstad, R.E., Eds., *Current Protocols in Food Analytical Chemistry*, John Wiley and Sons, Inc., Hoboken, F1.2.1.-F1.2.13.

[37] Woodall, G.S. and Stewart, G.R. (1998) Do Anthocyanins Play a Role in UV Protection of the Red Juvenile Leaves of *Syzygium*? *Journal of Experimental Botany*, **49**, 1447-1450.

[38] Burger, J. and Edwards, G.A. (1996) Photosynthetic Efficiency, and Photodamage by UV and Visible Radiation, in Red versus Green Leaf *Coleus* Varieties. *Plant Cell Physiol.*, **37**, 395-399. https://doi.org/10.1093/oxfordjournals.pcp.a028959