



Lipids in the seeds of wild grapes native to Japan: *Vitis coignetiae* and *Vitis ficifolia* var. *ganebu*



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ABSTRACT

The seed oils of *Vitis coignetiae* (Coignetiae) and *Vitis ficifolia* var. *ganebu* (Ganebu) at véraison and the ripe stage were evaluated for their fatty acid composition, and the content and profiles of phytosterols and tocopherols/tocotrienols. The oil content in Coignetiae seeds at ripe was almost same as that in 'Merlot' and 'Muscat Bailey A' seeds. The oil content in Ganebu seeds was 73% of that in Coignetiae seeds. Although linoleic acid was >64% in 'Merlot', 'Muscat Bailey A' and Coignetiae in each developmental stage, that in Ganebu at véraison and at ripe was 46.6 and 50.3%, respectively. The stigmasterol content in Ganebu seed oil at ripe was >2.6× higher than in the other grapes. The content and composition of tocopherols/tocotrienols in Coignetiae seed oil were similar to those in 'Merlot.' Although the total tocopherol/tocotrienol levels of Ganebu at ripe showed no significant differences compared with levels in the other grapes, the α -tocopherol level was >3.3× higher than in the other grapes. Our data suggest that Coignetiae is a common source of grape seed oil, but Ganebu may be a peculiar source of grape seed oil.

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1. Introduction

The grape genus (*Vitis*) is classified into three species: Europe—West Asian species, North American species and East Asian species. Most of the East Asian species are wild, which have limited use compared with the other species. The wild grapes investigated in this study, *Vitis coignetiae* Pulliat (Coignetiae) and *Vitis ficifolia* Bunge var. *ganebu* Hatusima (Ganebu), are of the East Asian species and indigenous to Japan. Coignetiae exhibits low temperature tolerance and mainly inhabits subarctic wet climate areas of Japan. Ganebu is endemic to coastal areas of the subtropical southwestern islands of Japan. This subtropical grape has no endodormancy and can bear berries continuously in the habitat. The berry size of Coignetiae is as large as that of *Vitis vinifera*, but that of Ganebu is about a third of *V. vinifera* in terms of weight. Although there are no obvious differences in seed number per berry between the two species (average seed number per berry is ca. 2.8), the seed fresh weight of Ganebu is about 37% of that of Coignetiae (Mochioka, 1996).

Grapes, especially *V. vinifera* and the hybrids such as *V. labruscana*, are one of the important horticultural crops cultivated world widely and used for table, juice and wine. Wild grapes may be useful

as processing materials, although most of them are not suitable for table grapes owing to the many seeds per berry or the small berry size. Coignetiae is actually cultivated in vineyards and the berries are mainly used for wine and juice as local products in the northern areas of Japan. However, effective utilization of the seed has not been considered. On the other hand, the cultivation of Ganebu in vineyards and its use are currently sparse. An exception to this is use of the extract of the leaf in a cosmetic in Japan. However, Ganebu may be a useful source for wine, juice and foods because of the high productivity of resveratrol, which is a characteristic polyphenol in grape berries and wine, with antioxidant activity, in the berry skin at the ripe (Shiozaki et al., 2013).

Grape seeds in the solid residues after the production of wine or juice are often used for the production of grape seed oil. The oil can be used for several purposes, including dietetic, because it is clean, light and neutral in smell and taste. The production of grape seed oil as a by-product in wineries and the juice industry constitutes an effective utilization of resources and provides economic advantages. In addition to obtaining grape seed oil from mature grapes, immature grapes, which are eliminated during optimization of a yield and harvest quality, may also be useful for oil production. Rubio et al. (2009) have demonstrated that the seed oil from immature grapes compares favorably with respect to the oil yield from and the content of mature grapes.

Grape seed oil is well known as one of the vegetable oils with a high amount of unsaturated fatty acids (Ahmadi and Siahfar, 2011;

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Baydar and Akkurt, 2001; Baydar et al., 2007; Demirtas et al., 2013; Hassanein and Abedel-Razek, 2009; Pardo et al., 2009). Linoleic acid, an essential fatty acid for human metabolism, is present in high levels in grape seed oil. Linolenic acid is present in low levels. A high level of linolenic acid can cause an unpleasant odor and taste in oil. Grape seed oil also contains phytosterols and tocopherols/tocotrienols, which are vitamin E active compounds and have antioxidant activity. β -Sitosterol and γ -tocotrienol have been reported, in several grape cultivars, to be the most abundant phytosterols and vitamin E active compounds (Baydar and Akkurt, 2001; Baydar et al., 2007; Beveridge et al., 2005; Crews et al., 2006; Hassanein and Abedel-Razek, 2009; Rubio et al., 2009).

Characterization of grape seed oil seems to be limited to *V. vinifera*; we have little information about the features of oil obtained from other species, including wild grapes. Characterization of the grape seed oil would provide useful information for the exploitation of low-use wild grape resources, leading the wild grapes to valuable horticultural crops.

This work describes the characteristics of the grape seed oil obtained from *Coignetiae* and *Ganebu*, both of which are promising wild grape resources in Japan. The fatty acid composition and the content and composition of phytosterols and tocopherols/tocotrienols were evaluated in the oil from the immature (at véraison) and mature (at ripe) seeds in each species, and then compared with *V. vinifera* (Merlot) and *V. labrusca* (Muscat Bailey A).

2. Materials and methods

2.1. Seeds

The grapes used in this study were grown in a research field at Osaka Prefecture University. Grape seeds were sampled from *V. coignetiae* (*Coignetiae*) and *V. ficifolia* var. *ganebu* (*Ganebu*) berries at véraison and at ripe in 2010. Seeds of *V. vinifera* cv. Merlot and *V. labrusca* cv. Muscat Bailey A, which were sampled at the same developmental stages, were used as comparison. The soluble solid content (Brix°) in the berry juice of *Coignetiae*, *Ganebu*, 'Merlot' and 'Muscat Bailey A' at ripe were 13.4°, 14.7°, 20.2° and 18.3°, respectively. Average seed fresh weight was 47.4, 76.9, 44.4 and 16.2 mg for 'Merlot', 'Muscat Bailey A', *Coignetiae* and *Ganebu*, respectively, at véraison and 36.2, 70.1, 41.8 and 15.6 mg at ripe. Seeds were pulverized in liquid nitrogen with a mortar and pestle and stored at -30°C until extraction.

2.2. Extraction of oil

Oil was extracted according to the method of Adhikari et al. (2008) and purified, with partitioning according to the method of Gómez-Brandón et al. (2008) with modification. Oil was extracted from 10 g pulverized seeds with 100 ml extraction solvent, comprising chloroform, methanol and 0.88% KCl (1:2:1 v/v). The homogenate was stirred overnight at 4°C and filtered through a Whatman No. 2 filter paper. The samples were then centrifuged for 20 min at $1800 \times g$ to separate the phases. The chloroform phase was collected. The aqueous phase was well mixed with 60 ml extraction solvent and centrifuged as described above. The chloroform phase was then collected. The aqueous residue was transferred to a separating funnel and 50 ml of 0.88% KCl was added. It was then partitioned against 50 ml chloroform and left until the two layers were clearly separated. The chloroform phase was collected and combined with the former two chloroform extracts. The combined chloroform phase was washed with 100 ml of 0.88% KCl to remove nonlipid compounds. The washed chloroform extract was dehydrated by passing it through a column containing 10 g

Na_2SO_4 and then reduced *in vacuo* to dryness. After weighing, the dry sample was redissolved in 1 ml hexane. A 100 μl aliquot was separated for fatty acid analysis and the remainder was equally divided for phytosterol and tocopherol/tocotrienol analyses. Each sample was then reduced to dryness and stored at -30°C until required for analysis.

2.3. Fatty acids: alkaline methanolysis and GC analysis

The oil sample was vortex mixed in 2 ml of 0.5 M methanolic KOH and heated for 5 min by placing a container holding the oil in boiling water. After cooling in air, the fatty acids were methylated with 1 ml of 14% methanolic boron trifluoride for 10 min at 40°C . After cooling, the sample was mixed with 10 ml saturated NaCl and partitioned three times with 5 ml hexane. The combined hexane phase was also dehydrated by passing it through a column containing 3 g Na_2SO_4 and then filtered. The dehydrated hexane was reduced *in vacuo* to dryness.

GC analysis of methylated fatty acids was performed with a GC-14A (Shimadzu, Kyoto, Japan) equipped with a FID detector in split mode. The oil samples were dissolved in 500 μl acetone and the fatty acids in the samples (2 μl) were separated using a FAMEWAX capillary column (0.25 mm i.d. \times 30 m, 0.25 μm film thickness; Shimadzu, Kyoto, Japan). Nitrogen was used as the carrier gas at a flow rate of 1.4 ml min^{-1} . The split ratio was 1:50. The column temperature was initially held at 130°C for 1 min and then ramped to 225°C at a rate of $6^{\circ}\text{C min}^{-1}$ with a final hold of 10 min. The temperature of the injector and detector was 220°C and 230°C , respectively. Palmitic, stearic, oleic, linoleic and linolenic acid were identified in the samples by comparing the retention times of the standards (typical retention times of palmitic, stearic, oleic, linoleic and linolenic were 12.3 min, 15.5 min, 15.8 min, 16.5 min and 17.4 min, respectively). The amount of fatty acids was determined by comparing the peak areas to the standard curves made using each fatty acids (palmitic acid: standard curve made over the range 0–25 mM with a regression constant $r^2 = 0.995$; stearic: 0–25 mM, $r^2 = 0.983$; oleic: 0–50 mM, $r^2 = 0.989$; linoleic: 0–100 mM, $r^2 = 0.975$; linolenic: 0–1 mM, $r^2 = 0.991$). The detection ranges of each fatty acid sample were as follows: palmitic acid: 1.8–15 mM; stearic: 0.7–5 mM; oleic: 3–28 mM; linoleic: 10–92 mM; linolenic: 0.2–0.8 mM. After conversion of the molar concentration into mg per 1 g oil, the composition of each fatty acid was calculated by dividing the individual content by the total fatty acid content.

2.4. Sterols: hydrolysis, purification and GC analysis

An oil sample (0.5 g) was suspended in 5 ml of 6 N HCl and heated at 80°C for 60 min, with vortex mixing for a few seconds every 10 min. After cooling in air, hydrolyzed oil was extracted with 20 ml of 50% diethyl ether in hexane and reduced *in vacuo* to dryness. To the dry sample was added 7 ml of 0.5 M ethanolic KOH, followed by refluxing for 20 min at 80°C . After cooling, 12 ml distilled water was added and the sample partitioned against 20 ml cyclohexane. A 15 ml portion of the cyclohexane phase containing nonsaponifiables was evaporated to dryness.

The nonsaponifiables were purified using silica gel column chromatography in conjunction with a vacuum manifold (Bond Elut; Varian, Harbor City, CA, USA). The silica gel column was prepared as follows. Bondesil SI (0.5 g; Analytichem International, Harbor City, CA, USA) was packed in a Bond Elut empty reservoir (8 ml; Varian), preinstalled with a frit of 40 μm pore size. In addition, a frit of 70 μm pore size was installed on the resin to avoid disturbance of the resin. The sample flow rate was $<1 \text{ ml min}^{-1}$. Dry samples were dissolved in 1 ml hexane with ultrasonication and then loaded onto the column, which had been previously activated with 5 ml hexane. After

Table 1
Fatty acid composition of the seed oils of 'Merlot', 'Muscat Bailey A', *Vitis coignetiae* and *Vitis ficifolia* var. *ganebu* at véraison.

| Grapes | Oil content (%) | Fatty acid composition (%) | | | | | Unsaturation (%) |
|--|-----------------|-----------------------------------|---------------------|-------------------|----------------------|-----------------------|------------------|
| | | Palmitic acid (16:0) ^z | Stearic acid (18:0) | Oleic acid (18:1) | Linoleic acid (18:2) | Linolenic acid (18:3) | |
| Merlot | 10.9 | 9.7 | 4.4 | 14.6 | 70.7 | 0.6 | 85.9 |
| Muscat Bailey A | 9.0 | 8.9 | 4.9 | 19.6 | 65.6 | 0.7 | 85.9 |
| <i>V. coignetiae</i> | 10.5 | 7.6 | 3.9 | 23.6 | 64.2 | 0.7 | 88.5 |
| <i>V. ficifolia</i> var. <i>ganebu</i> | 8.2 | 15.2 | 4.8 | 32.3 | 46.6 | 1.1 | 80.0 |

^z Numerical symbol of fatty acids (carbon number: unsaturated bond number).

Table 2
Fatty acid composition of the seed oils of 'Merlot', 'Muscat Bailey A', *Vitis coignetiae* and *Vitis ficifolia* var. *ganebu* at ripe.

| Grapes | Oil content (%) | Fatty acid composition (%) | | | | | Unsaturation (%) |
|--|-----------------|-----------------------------------|---------------------|-------------------|----------------------|-----------------------|------------------|
| | | Palmitic acid (16:0) ^z | Stearic acid (18:0) | Oleic acid (18:1) | Linoleic acid (18:2) | Linolenic acid (18:3) | |
| Merlot | 10.9 | 9.9 | 4.3 | 15.1 | 70.2 | 0.5 | 85.8 |
| Muscat Bailey A | 11.5 | 9.3 | 4.7 | 19.6 | 65.8 | 0.6 | 86.0 |
| <i>V. coignetiae</i> | 11.5 | 7.3 | 4.0 | 23.4 | 64.7 | 0.6 | 88.7 |
| <i>V. ficifolia</i> var. <i>ganebu</i> | 8.4 | 15.4 | 4.7 | 28.6 | 50.3 | 1.0 | 79.9 |

^z Numerical symbol of fatty acids (carbon number: unsaturated bond number).

washing with 5 ml hexane and 5 ml of 10% diethyl ether in hexane, sterols were eluted in 5 ml of 50% diethyl ether in hexane. An aliquot of the eluate was evaporated to dryness in a test tube, which had been previously silylated with 5% dichlorodimethylsilane in toluene.

The dry samples, redissolved in 50 μ l pyridine, were silylated with 50 μ l BSTFA-TMCS (90:1, v/v) for 30 min at 60 °C. A silylated sample (2 μ l) was analyzed by GC (GC-14A; Shimadzu, Kyoto, Japan) in split mode. The column used was a CBP1 capillary column (0.22 mm i.d. \times 25 m, 0.25 μ m film thickness; Shimadzu). Nitrogen was used as the carrier gas at a flow rate of 1.2 ml min⁻¹. The column temperature was programmed as follows: holding at 70 °C for 1 min, heating at a rate of 40 °C min⁻¹ to 245 °C, holding at 245 °C for 1 min, heating at a rate of 3 °C min⁻¹ to 275 °C and holding at 275 °C for 35 min. The injector temperature was set at 250 °C and the split ratio was 1:50. The detector temperature was 300 °C. Campesterol, stigmasterol and β -sitosterol were identified in the samples by comparing the retention times of the standards (typical retention time: campesterol, 29 min; stigmasterol, 31 min; β -sitosterol, 34 min). These sterols were quantified using each calibration curves of the standard peak areas (campesterol: standard curve made over the range 0–200 μ g with a regression constant $r^2 = 0.995$; stigmasterol: 0–200 μ g, $r^2 = 0.994$; β -sitosterol: 0–400 μ g, $r^2 = 0.973$). The sterol in an analysis sample was detected in the following ranges: campesterol, 23–93 μ g; stigmasterol, 48–173 μ g; β -sitosterol, 204–462 μ g.

2.5. Tocopherols and tocotrienols: purification and HPLC analysis

To an oil sample (0.5 g) was added 2 ml of 5% pyrogallol in ethanol and 0.75 ml of 60% KOH. This was refluxed for 30 min at 80 °C. After cooling, 20 ml distilled water and 30 ml diethyl ether were added. The mixture was transferred to a separating funnel. After agitation, the sample was allowed to settle until the solvent layers had clearly separated, then the diethyl ether phase was collected. The aqueous phase was partitioned two more times against 30 ml diethyl ether. The diethyl ether fractions were combined and washed three times against 100 ml distilled water in a separating funnel. After dehydration by passing it through a column containing 10 g Na₂SO₄, the diethyl ether fraction, containing tocopherol/tocotrienol, was evaporated to dryness at 30 °C.

The dry tocopherol/tocotrienol samples were redissolved in 1 ml hexane and a 20 μ l aliquot was analyzed using HPLC (Model 576; GL Sciences, Tokyo, Japan) equipped with a fluorescence detector (RF-550; Shimadzu). The excision and emission wavelengths were 290 nm and 323 nm, respectively. Samples were isocratically eluted from the normal phase HPLC column (Inertsil SIL, 4.0 \times 250 mm) attached to the guard column (Inertsil SIL, 4.0 \times 10 mm) with a solvent mixture of hexane:dioxane:isopropanol (985:10:5) at a flow rate of 1 ml min⁻¹. α -Tocopherol, γ -tocopherol and δ -tocopherol were identified in the samples by comparing the retention times of the standards (typical retention time: α -Tocopherol, 6.4 min; γ -tocopherol, 11.5 min; δ -tocopherol, 18.8 min) and quantified using each calibration curves of the standard peak areas (α -Tocopherol: standard curve made over the range 0 to 1 mM

Table 3
Sterols content in the seed oils of 'Merlot', 'Muscat Bailey A', *Vitis coignetiae* and *Vitis ficifolia* var. *ganebu* at véraison.

| Grapes | Sterol (mg/100 g oil) | | | Total |
|--|--|------------------|---------------------|---------|
| | Campesterol | Stigmasterol | β -Sitosterol | |
| Merlot | 43.5 a ^z (13.2) ^y | 47.4 b (14.4) | 238.6 a (72.4) | 329.5 a |
| Muscat Bailey A | 40.7 a (13.6) | 35.6 b (12.1) | 210.8 a (74.3) | 287.1 a |
| <i>V. coignetiae</i> | 36.7 a (12.0) | 37.5 b (12.2) | 231.6 a (75.7) | 305.8 a |
| <i>V. ficifolia</i> var. <i>ganebu</i> | 31.7 a (9.0) | 77.9 a (22.3) | 237.1 a (68.7) | 346.7 a |

^z Means ($n = 3$) with different letters in each columns are significantly different by Fisher's PLSD test at 5%.

^y The values in the parenthesis show the component ratio of the sterols in each type of grape seeds.

Table 4
Sterols content in the seed oils of 'Merlot', 'Muscat Bailey A', *Vitis coignetiae* and *Vitis ficifolia* var. *ganebu* at ripe.

| Grapes | Sterol (mg/100 g oil) | | | Total |
|--|--|------------------|---------------------|---------|
| | Campesterol | Stigmasterol | β -Sitosterol | |
| Merlot | 34.6 a ^z (13.8) ^y | 32.5 b (13.1) | 183.6 a (73.1) | 250.7 a |
| Muscat Bailey A | 36.5 a (14.0) | 32.3 b (12.4) | 192.2 a (73.6) | 261.0 a |
| <i>V. coignetiae</i> | 30.1 a (11.5) | 29.0 b (11.1) | 202.0 a (77.4) | 261.1 a |
| <i>V. ficifolia</i> var. <i>ganebu</i> | 32.2 a (8.2) | 85.5 a (22.4) | 255.0 a (69.4) | 372.7 a |

^z Means ($n=3$) with different letters in each columns are significantly different by Fisher's PLSD test at 5%.

^y The values in the parenthesis show the component ratio of the sterols in each type of grape seeds.

Table 5
Tocopherol and tocotrienol (vitamine E) content in the seed oils of 'Merlot', 'Muscat Bailey A', *Vitis coignetiae* and *Vitis ficifolia* var. *ganebu* at véraison.

| Grapes | Tocopherol and tocotorienol (mg/100 g oil) | | | | | | | | Total |
|--|--|-----------------------------|----------------|----------------|----------------|------------------|----------------|----------------|---------|
| | α -Toc | α -Toc3 ^z | β -Toc | β -Toc3 | γ -Toc | γ -Toc3 | δ -Toc | δ -Toc3 | |
| Merlot | 10.8 b ^y (13.3) ^x | 16.9 c (20.8) | 0.2 c (0.3) | 0.2 b (0.3) | 1.9 b (2.3) | 49.3 b (60.9) | 0.2 a (0.2) | 1.5 a (1.9) | 81.0 b |
| Muscat Bailey A | 32.5 a (28.9) | 7.6 d (6.7) | 0.3 b (0.3) | – | 6.1 a (5.4) | 64.7 a (57.4) | 0.1 a (0.1) | 1.2 b (1.1) | 112.6 a |
| <i>V. coignetiae</i> | 17.7 ab (20.9) | 22.7 b (26.7) | 0.2 c (0.3) | 0.2 b (0.2) | 1.0 c (1.2) | 42.0 b (49.6) | 0.1 a (0.1) | 0.8 c (1.0) | 84.8 b |
| <i>V. ficifolia</i> var. <i>ganebu</i> | 36.1 a (50.9) | 26.7 a (37.5) | 0.8 a (1.2) | 0.6 a (0.8) | 2.0 b (2.8) | 4.2 c (6.0) | 0.3 a (0.4) | 0.4 d (0.5) | 71.0 b |

^z β -Tocopherol and tocotrienols are shown as α -tocopherol equivalents.

^y Means ($n=3$) with different letters in each columns are significantly different by Fisher's PLSD test at 5%.

^x The values in the parenthesis show the component ratio of Toc and Toc3 in each type of grape seeds.

with a regression constant $r^2=0.999$; γ -tocopherol: 0–0.2 mM, $r^2=0.998$; δ -tocopherol: 0–0.2 mM, $r^2=0.999$) The detection ranges of each tocopherol sample were as follows: α -Tocopherol, 0.03–0.62 mM; γ -tocopherol, 0.01–0.10 mM; δ -tocopherol, 0.0005–0.0383 mM.

The other tocopherols and tocotrienols were identified by comparing the relative retention time between α -tocopherol, γ -tocopherol and δ -tocopherol, and the other tocopherols and tocotrienols with reference to a previous report (Abe and Matsumoto, 1993). β -Tocopherol and tocotrienols were quantified as α -tocopherol equivalents. The molar concentration of tocopherols and tocotrienols was converted into mg per 100 g oil.

2.6. Statistic analysis

Data on each of the lipids were analyzed by analysis of variance and means were compared by Fisher's PLSD test at $P<0.05$ using StatView 5.0 (SAS Institute Inc.).

3. Results and discussion

3.1. Oil content

The oil content of grape seeds of 'Merlot' and 'Muscat Bailey A' at ripe was 10.9 and 11.5%, respectively, per unit fresh weight of the seeds (Table 2). The oil content of the two grape cultivars was in good agreement with the data previously reported; the content ranged from 9.9–19.6% (Ahmadi and Siahshar, 2011; Baydar et al., 2007; Ohnishi et al., 1990). Although the oil content of *V. coignetiae* (Coignetiae) seeds was comparable to that of 'Merlot' and 'Muscat Bailey A' at ripe, that in *V. ficifolia* var. *ganebu* (Ganebu) seeds was 2.5–3.1% lower than that of the other cultivars.

3.2. Fatty acids: composition

In all oil samples, palmitic and stearic acids were evaluated as saturated fatty acids, and oleic, linoleic and linolenic acids as unsaturated fatty acids (Tables 1 and 2). There were no obvious differences in fatty acid composition in the seed oils of all grape species tested, between véraison and the ripe stage. In terms of the fatty acid composition of grape seed oil, linoleic is the most abundant, followed in order by oleic, palmitic and stearic, while linolenic is the least abundant (Ahmadi and Siahshar, 2011; Baydar et al., 2007; Crews et al., 2006). Beverage et al. (2005) reported the composition of these fatty acids in eight cultivars of *V. vinifera*, including 'Merlot': 6.35–8.62% palmitic, 3.64–5.62% stearic, 12.63–18.95% oleic, 66.76–73.61% linoleic and 0.5–0.79% linolenic acids, in addition to myristic, palmitoleic and arachidic acids, each present in <0.2%. Similarly, Crews et al. (2006) reported that the composition of grape seed oils obtained from France, Italy and Spain was 6.6–11.6%, 3.5–3.9%, 14.0–20.9%, 61.3–74.6% and 0.3–1.8% for palmitic, stearic, oleic, linoleic and linolenic acids, respectively. The fatty acid composition of 'Merlot', 'Muscat Bailey A' and Coignetiae seeds at ripe was similar to findings previously reported. Unsaturated fatty acids in grape seed oil were reported to be in the range 85–90% (Ahmadi and Siahshar, 2011; Baydar et al., 2007; Hassanein and Abedel-Razek, 2009). A lower level of unsaturated fatty acids was found only in Ganebu seed oil. Lower levels of linoleic (14.4–19.9%) and higher levels of palmitic (5.5–8.1%) than the other oils account for the scarcity of unsaturated fatty acids in Ganebu seed oil.

Linoleic ($n-6$ fatty acid) and linolenic ($n-3$ fatty acid) acids are essential oils for the human body and must be dietary taken. Linoleic acid ($n-6$) rich vegetable oil had been recommended for human nutrition, because of the greater effect on reducing LDL cholesterol than monoenoic fatty acids (oleic acid). Recently, however, it has been found that an excessive intake of linoleic acid is not good for human health, because it is more susceptible to oxidation than monoenoic fatty acids and hormone-like compounds,

Table 6
Tocopherol and tocotrienol (vitamine E) content in the seed oils of 'Merlot', 'Muscat Bailey A', *Vitis coignetiae* and *Vitis ficifolia* var. *ganebu* at ripe.

| Grapes | Tocopherol and tocotrienol (mg/100 g oil) | | | | | | | | Total |
|--|--|-----------------------------|----------------|----------------|-----------------|------------------|----------------|----------------|--------|
| | α -Toc | α -Toc3 ^z | β -Toc | β -Toc3 | γ -Toc | γ -Toc3 | δ -Toc | δ -Toc3 | |
| Merlot | 10.3 b ^y (18.3) ^x | 17.6 ab (31.0) | 0.3 b (0.4) | 0.5 a (0.8) | 1.1 b (1.9) | 25.2 a (44.6) | 0.8 b (1.4) | 0.9 a (1.6) | 56.6 a |
| Muscat Bailey A | 13.2 b (23.9) | 8.0 b (14.6) | 0.3 b (0.5) | – | 3.4 a (6.2) | 28.6 a (51.9) | 0.8 b (1.5) | 0.8 a (1.4) | 55.0 a |
| <i>V. coignetiae</i> | 11.3 b (16.2) | 23.3 a (33.2) | 0.2 b (0.3) | 0.2 b (0.3) | 0.7 b (1.0) | 32.7 a (46.5) | 0.9 b (1.3) | 0.8 a (1.1) | 70.2 a |
| <i>V. ficifolia</i> var. <i>ganebu</i> | 43.9 a (57.3) | 22.4 a (29.2) | 0.6 a (0.8) | 0.4 a (0.6) | 2.5 ab (3.3) | 4.2 b (5.5) | 2.2 a (2.9) | 0.4 b (0.5) | 76.7 a |

^z β -Tocopherol and tocotrienols are shown as α -tocopherol equivalents.

^y Means ($n=3$) with different letters in each column are significantly different by Fisher's PLSD test at 5%.

^x The values in the parenthesis show the component ratio of Toc and Toc3 in each type of grape seeds.

eicosanoids, formed from linoleic acid, have a negative effect on human health (Matthäus, 2008). Oxidation of LDL cholesterol is one of the causes of atherogenesis in the arterial wall. A high dietary intake of polyunsaturated fatty acids, especially $n-6$ fatty acids, can increase the susceptibility of LDL to oxidation (Kratz et al., 2002). The eicosanoids are formed from not only $n-6$ but also $n-3$ fatty acids, but negative effects, such as blood coagulation, are caused only by the former, while the latter has the opposite effect (Matthäus, 2008). Therefore, reducing the dietary ratio of $n-6$ – $n-3$ fatty acids is important in the risk control of arteriosclerosis and cardiovascular disease. Kris-Etherton et al. (2000) report that the recommended standards of the ratio indicated by national and international organizations range from 2:1–10:1. Grape seed oil is a vegetable oil with high ratio of $n-6$ – $n-3$ fatty acids. Our data for 'Merlot', 'Muscat Bailey A' and Coignetiae seed oils at ripe confirm this: the ratio is 107.8–140.4. It should be noted, however, that for Ganebu seed oil, the ratio of $n-6$ – $n-3$ fatty acids is 50.3, and it has a higher level of monoenoic fatty acids than the other grapes. Although the ratio of $n-6$ – $n-3$ fatty acids of Ganebu seed oil is still considerably higher than the recommendation, the lower $n-6$ – $n-3$ ratio compared with the other oils characterizes its superiority in terms of health properties in grape seed oil.

3.3. Phytosterols: content and composition

The sterol content of grape seed oils, including campesterol, stigmasterol and β -sitosterol, at véraison and at ripe, are shown in Tables 3 and 4, respectively. The total phytosterol content ranged from 287.1 to 346.7 mg per 100 g oil at véraison and from 218.7 to 372.7 mg at ripe. There were no significant differences in the total phytosterol content between grapes, irrespective of the developmental stages. In 'Merlot', the total content of the three phytosterols was slightly lower than that extracted with petroleum ether by Beveridge et al. (2005). The total phytosterol content in this study was close to the lowest levels recorded in ten oil samples from France, Italy and Spain, as reported by Crews et al. (2006). The phytosterol content at ripe was lower than at véraison in oils of 'Merlot', 'Muscat Bailey A' and Coignetiae. On the other hand, the content in Ganebu seed oil was higher at ripe than that at véraison. Significant differences in the content of each of the sterols were found only for stigmasterol between Ganebu and the other grapes; the level in Ganebu at véraison was about 1.6–2.2 \times higher, and that at ripe was about 2.6–2.9 \times higher than in the other grapes.

With regard to the phytosterol composition, β -sitosterol was a predominant sterol in all oil samples, as has been reported by others (Crews et al., 2006; Hassanein and Abedel-Razek, 2009; Pardo et al., 2009). Unlike the phytosterol content, the phytosterol composition showed no obvious differences between the development stages in all grape seed oils (Tables 3 and 4). The constant phytosterol composition from véraison to ripe found in this study was in agreement

with the findings of Rubio et al. (2009). Stigmasterol composition in Ganebu seed oil at véraison and ripe, reflecting the higher content, was 10.1–12.7% and 12.5–14.8% higher, respectively, than that in the other grapes.

Phytosterols have similar chemical structure and biological functions to cholesterol. The most important effect of dietary intake phytosterols in human health is lowering blood cholesterol by inhibiting cholesterol absorption in the intestine (Berger et al., 2004). Although the effect of each sterol in lowering cholesterol still seems to be debatable, Matsuoka et al. (2010) reported that β -sitosterol might have a greater cholesterol-lowering effect compared with the other two sterols, due to its preferential solubility in bile salt solution. Phytosterols may also have beneficial effects on health, such as anticancer (Berger et al., 2004). A case-control study of diet and the risk of ovarian cancer in humans in the USA showed that dietary intake of high levels of stigmasterol may reduce risks of the cancer (McCann et al., 2003). Ganebu seed oil, of which the stigmasterol composition is higher than in the other grape seed oils, may have somewhat different characteristics in terms of human health benefit effects compared with the other grape seed oils.

3.4. Tocopherol and tocotrienol: content and composition

In this study, the tocopherols and tocotrienols, which are collectively known as vitamin E, in grape seed oils ranged from 71 to 112.6 mg per 100 g oil at véraison and from 55.0 to 76.7 mg at ripe. These levels are similar to the data on ten oil samples from France, Italy and Spain (63–1208 mg⁻¹ kg oil) reported by Crews et al. (2006) and those on the mature seeds of seven Turkish cultivars reported by Demirtas et al. (2013). Compared with their levels in 'Merlot' seed oil extracted with petroleum ether (Beveridge et al., 2005), the levels of tocopherols (α - and γ -) and tocotrienols (α - and γ -) in our data, with an exception of the β -tocopherol level (it is less than one-tenth), were almost in agreement with the literature. There were no significant differences in the total level at ripe between grapes.

Horvath et al. (2006) observed changes in the levels of tocopherol/tocotrienol and tocotrienol biosynthesis activity during seed development of 'Albert Lavallée' (*V. vinifera*). They found a gradual decrease in tocopherol levels during seed development and an accumulation of tocotrienol, especially in the γ form, from the beginning of the lag phase of berry development (25 days before véraison) through 10 days after véraison. Although in our results, total tocopherol/tocotrienol levels in grape seed oils, with an exception of Ganebu, were lower at ripe than that at véraison, the γ -tocotrienol level decreased, rather than increased, from véraison to the ripe stage. We have only limited information about tocopherol and tocotrienol synthesis and metabolism in grape seeds. In order to explain this discrepancy, it appears necessary to elucidate

the species specificity and the factors affecting the physiological processes.

Our data reveal that in terms of the tocopherol/tocotrienol composition of 'Merlot,' γ -tocotrienol was most abundant, followed in order by α -tocotrienol and α -tocopherol. This composition is in good agreement with that reported by Hassanein and Abedel-Razek (2009), and Demirtas et al. (2013). High levels of α - and γ -tocotrienol is a well-known feature of grape seed oils. Coignetiae seed oil at ripe showed this feature but Ganebu seed oil was unique (Tables 5 and 6). Ganebu seed oil at ripe showed only about one-eighth of the γ -tocotrienol composition of 'Merlot' seed oil. On the other hand, the α -tocopherol content in Ganebu seed oil was significantly higher than that of 'Merlot' seed oil and the composition was about three times higher than that of 'Merlot.'

This unique tocopherol/tocotrienol composition in Ganebu seed oil may be reflected in its functional properties. Although all four tocopherols have vitamin E activity in the human body, α -tocopherol reveals the highest activity of all because the β -, γ - and δ -forms are not converted to the α -form in humans, and the affinity of β -, γ - and δ -forms for α -tocopherol transfer protein in the liver is lower (Schneider, 2005). They also have antioxidant activity in cells, although their effect on the oxidative stability of oils is weak (Matthäus, 2008). When the antioxidant activity of α -tocopherol *in vitro* is 100%, the values for β -, γ - and δ -tocopherol are reported to be 71, 68 and 28%, respectively (Schneider, 2005). On one hand, tocotrienols have been suggested to be superior in terms of physiological activities compared with α -tocopherol; they have, for example, hypocholesterolemic effects, antithrombotic effects and antitumor effects (Therriault et al., 1999). Overall, our results suggest that Ganebu seed oil can be a better source of vitamin E than the other grape seed oils examined in this study, although, in humans, it might be inferior in terms of some functional properties.

4. Conclusions

Different, species-specific, characteristics of seed oil were found in *Vitis coignetiae* (Coignetiae) and *V. ficifolia* var. *ganebu* (Ganebu) grapes grown under the same cultivation conditions such as soil, climate and agricultural practices. Coignetiae seed oil, irrespective of the developmental stage of the seed, showed common characteristics in the composition of fatty acids, and the content and composition of phytosterols and tocopherols/tocotrienols with those in the other species compared in this study and in the literatures. Coignetiae seeds may be utilized as an oil source; its oil has similar characteristic to the grape seed oil currently used.

On the other hand, the seed oil of Ganebu revealed unique characteristics of fatty acids, phytosterols and tocopherols/tocotrienols. In the seed oil of Ganebu, the composition of palmitic acid was higher, and that of linoleic acid was lower, than in the other seed oils. In addition, the oil had abundant stigmasterol and α -tocopherol. With regard to the health benefits of grape seed oil, this fatty acid composition may reduce the unfavorable characteristics, while the high levels of stigmasterol and α -tocopherol may reinforce the beneficial characteristics. Thus, the Ganebu seed can be a useful source for grape seed oil, although the oil content is slightly lower than that of the other grapes.

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