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Differences in the Behavior of Anthocyanin Coloration in Wines Made from *Vitis vinifera* and Non-*vinifera* Grapes

Tohru Okuda *, Kyohei Maeda, Itsuki Serizawa, Fumie Watanabe-Saito and Masashi Hisamoto

The Institute of Enology and Viticulture, University of Yamanashi, 1-13-1 Kitashin, Kofu 400-0005, Yamanashi, Japan; fumies@yamanashi.ac.jp (F.W.-S.); hisamoto@yamanashi.ac.jp (M.H.)
* Correspondence: okuda@yamanashi.ac.jp; Tel.: +81-55-220-8608

Abstract: The skins of *Vitis vinifera* species contain 3-glucosyl anthocyanins (3G), but some non-*vinifera* species, such as 'Yama Sauvignon' (YS), contain a large amount of 3,5-diglucosyl anthocyanins (35DG), and the behavior of anthocyanin coloration with respect to pH is quite different. The anthocyanins of YS showed a very weak color at a pH of 3 or higher but a very strong color below a pH of 3. Furthermore, when we investigated the effect of co-pigmentation in commercially available wines, we found that YS red wine contained a large amount of co-pigmented anthocyanins, and even wine aged for about 4 years contained a large amount of co-pigmented anthocyanins. Due to concerns regarding disease resistance, many hybrid varieties of *V. vinifera* and non-*vinifera* species have been bred, but it is important to take these special properties of 35DG into consideration when producing wine.

Keywords: wine; anthocyanin; co-pigmentation; non-*vinifera*; pH

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1. Introduction

Because the color of red wine is directly related to wine price [1], many researchers have studied it, and excellent reviews have been published [2–4]. The color of red wine immediately after production is mainly determined by the anthocyanins extracted from grapes. The majority of red wines produced in many countries are made from *V. vinifera* grapes. However, global warming and the concomitant spread of grape diseases have increased the importance of disease-resistant non-*vinifera* species. Grapes grown in Japan are prone to fungal disease because of the heavy rainfall that occurs during the growing season. Therefore, disease-resistant non-*vinifera* species, such as *V. labrusca*, *V. coignetiae*, *V. amurensis*, and their hybrids, are grown in Japan, and some are used in winemaking. Berries from these species contain 3,5-diglucosidic anthocyanins (35DG), unlike *V. vinifera* berries, which contain 3-glucosidic anthocyanins (3G) [5,6]. Wines containing large amounts of 35DG are produced in Korea, Brazil, and China [7–9], and this has further fueled research on the color of 35DG.

Anthocyanins are compounds that tend to be unstable, and various factors influence their coloration. pH, for instance, is a well-known factor affecting anthocyanin coloration. The effects of global warming on climate can alter the pH of grapes and wines, leading to a decrease in red color intensity. However, there is still insufficient understanding regarding the specific impact of pH on wines containing 35DG, especially under practical winemaking conditions.

Moreover, some anthocyanins decompose during the production and aging of red wine, and others undergo a series of reactions with other compounds to form stable red pigments such as polymeric pigments (PPs) with complex structures and pyranoanthocyanins [3]. The color of red wine is maintained for a long time by PPs produced in a series of complex reactions. In the first step of these complex reactions, anthocyanins extracted from grape berry skins react with other compounds (or themselves), resulting in the stacking of π electrons of anthocyanins; this process is called "co-pigmentation". This

anthocyanin reaction can be divided mainly into two types. In the first type, anthocyanin reacts with other anthocyanin molecules (self-association). In the second type, anthocyanin reacts with other compounds called co-factors. These reactions cause significant changes in color including darkening or a purple shift (hyperchromic effects) [10]. In young red wines made from *V. vinifera*, 30–50% of the red color is believed to be derived from these reactions [2,10,11]. In addition, these reactions are considered to slow down the decomposition of anthocyanins.

It is empirically known that young red wines made from berries containing 35DG tend to be purple in color, and many of them have very high anthocyanin concentrations and a black-ink-like color. Therefore, co-pigmentation is considered to play an important role in the color of these non-*vinifera* wines. Several reports have been published on the co-pigmentation of 35DG in not only grape berries but also flowers and other plants [12–14]. In addition, although there are only a few examples, there have also been enological studies on the color of wine made from berries containing 35DG [8,15]. For example, muscadine grape berries (*V. rotundifolia*) contain large amounts of 35DG, and their color tends to fade markedly over time; however, it has been shown that a co-factor derived from red clover stabilizes the color tone [16]. It has also been reported that anthocyanins are stabilized by glucose substitution at the 5 position [17]. Lago-Vanzela et al. reported that coumaroylated 35DGs present in large amounts in the hybrid strain 'BRS Violeta' are highly resistant to color degradation [8]. Thus, 35DG may have different properties from 3G in winemaking, although the details are not well understood.

Owing to the importance of co-pigmentation, several studies on red wine co-pigmentation have been conducted [10]. Many experiments on anthocyanins use model systems. In actual wines, however, many compounds and physical phenomena are involved [17], and the structural stability of compounds and the effects of interactions and oxidation should be taken into account. Owing to these complex reactions, the understanding of anthocyanins in wines remains poor. Moreover, most studies on anthocyanins have focused on 3G anthocyanins contained in *V. vinifera* berries.

In this study, we conducted experiments with the aim of elucidating the differences in coloration between wines containing 3G and 35DG and providing insights into considerations when producing wines containing 35DG.

2. Materials and Methods

2.1. Chemicals

Acetaldehyde, ethanol, hydrochloric acid, potassium hydrogen tartrate, and potassium pyrosulfite were obtained from Fujifilm Wako Pure Chemical Co. (Osaka, Japan). Other chemicals used were of analytical grade. Toyopearl HW-40F was a product of TOSOH Co. (Tokyo, Japan). The ODS column was a Strata C18-E 20 g/60 mL Giga tube (Shimadzu, Kyoto, Japan).

2.2. Extraction and Partial Purification of Grape Skin Anthocyanins

'Merlot' (MER, *V. vinifera*) grape skin was separated by hand from grape berries (18.8°Brix) harvested on 15 September, and YS skins were harvested from grape berries (20.7°Brix) harvested on 19 September in 2021 in Yamanashi Prefecture in Japan. MER anthocyanins were extracted from 40 g of skins with 200 mL of 1% HCl via gentle mixing with a magnetic stirrer at 25 °C in the dark. The solution was then filtered through filter paper (No. 2, Advantec Co., Tokyo, Japan) and applied to a Toyopearl HW-40F (100 mL) column [15]. After washing with Milli-Q, anthocyanins were eluted with 0.1% acetic acid in 50% MeOH, and then MeOH was removed via evaporation. Anthocyanins in YS were extracted from 40 g of skins with 200 mL of 1% HCl and applied to a Toyopearl HW-40F column using the same method as MER. Anthocyanins were eluted with 200 mL of Milli-Q and further separated on an ODS column. The ODS column was washed with 200 mL of 0.1% acetic acid in 10% MeOH, anthocyanins were eluted with 0.1% acetic acid in 20% MeOH, and then MeOH was removed via evaporation. The compositions and

structures of anthocyanins were confirmed via HPLC–DAD/MS [15]. Waters Acquity H-class UPLC systems coupled to Waters TQ-XS triple quadrupole mass analyzers (Waters Corporation, Wilmslow, UK) were employed. The chromatographic separation of the analytes was performed on an Acquity UPLC HSS T3 column (1.8 µm, 2.1 × 100 mm; Waters Corporation, Milford, MA, USA). The eluent used for the separation consisted of 0.5% (*v*/*v*) trifluoroacetic acid diluted with ultrapure water (A) and 0.5% (*v*/*v*) trifluoroacetic acid in acetonitrile (B). The flow rate was 0.3 mL/min, and the column temperature was maintained at 40 °C. The autosampler compartment was cooled to 15 °C, and 5 µL was injected. The total run time was 30 min. The gradient system was 0.1–0.5 min, 10% B; 0.5–10 min, 10–18% B; 10–20 min, 18–37% B; 20–23 min, 100% B; 23–30 min, 10% B. The samples were filtered through a 0.45 µm membrane filter. A Xevo TQ-XS mass spectrometer (Waters Corporation, Wilmslow, UK) operating in the positive ESI mode was used for the detection of anthocyanin analytes. The mass spectrometer was operated in full-scan mode (*m*/*z* 100–2000) for the qualification of all analytes. The diode array detector was set to monitor at 280 and 520 nm. MassLynx™ software, version 4.1 (Waters Corporation, Wilmslow, UK) was used for data acquisition and analysis.

Quantification and Measurement of Anthocyanin Color

Anthocyanins obtained from MER and YS were quantified using molecular coefficients for malvidin-3-glucoside and malvidin-3,5-diglucoside, respectively, as written in elsewhere [18]. The absorbances at 520 nm (A_{520}) for various anthocyanins concentrations (0.02, 0.4, 0.8, and 1.2 mM) were measured in 5 g/L tartaric acid and 0.2 M NaCl in 12% EtOH, and the pH of the solution was adjusted from 1.0 to 4.2 with 1 M HCl and 1 M NaOH.

2.3. Calculation of Hydration Constant (pKh)

The hydration constants for MER and YS anthocyanins were calculated via the methods used by González-Manzanao et al. [19].

2.4. Wine Samples

Eighty commercial Japanese red wines were used for analysis. Only monovarietal wines were used in this study. The numbers of samples were 24 for 'Muscat Bailey A' (MBA, Bailey × Muscat Humburg, hybrid), 15 for YS, 31 for MER, and 10 for 'Cabernet Sauvignon' (CS, *V. vinifera*). These wines were entered in the Japan Wine Competition between 2017 and 2019. The vintage and the numbers of samples used in this study are shown in Table 1. Samples were stored in the cellar of the Institute of Enology and Viticulture, University of Yamanashi, at approximately 16 °C until use.

Table 1. Number of commercial wines used in this study.

	2017	2016	2015	2012–2014	Total
MBA	2	12	5	5	24
YS	5	6	4	-	15
MER	2	3	15	11	31
CS	1	2	2	5	10
Total	10	23	26	21	80

2.5. Measurement of Co-pigmented Anthocyanins, Free Anthocyanins, and Polymeric Pigments

Various parameters were measured by the method of Darias-Martín et al. [20], which is based on the method proposed by Boulton [21]. The pH values of wine samples were first adjusted to exactly 3.6 with HCl or NaOH. To eliminate the SO_2 effect, 20 µL of 10% acetaldehyde was added to 2 mL of the wine sample, and the mixture was allowed to stand for 45 min. After that, the color of the wine sample was analyzed by measuring the A_{520} (A^{acet}). The color due to PP was analyzed by measuring the A_{520} of the wine sample (A^{SO_2}) after adding 160 µL of 5% SO_2 solution to 2 mL of the wine sample. The

color of the wine sample without the effect of co-pigmented anthocyanins (CA) was analyzed by measuring the A_{520} (A^{20}) of the wine sample diluted with buffer solution in a 1:20 ratio. The buffer solution was prepared by adding 24 mL of pure ethanol into 176 mL of distilled water, into which 0.5 g of potassium bitartrate was added and dissolved. The pH of the solution was adjusted to 3.6 with HCl or NaOH. The absorbance reading of the diluted wine sample was corrected by multiplying it by 20. All absorbance readings were normalized to 10 mm path length. The following were calculated: percentage of color due to CA (CA%) = $[(A^{acet} - A^{20})/A^{acet}] \times 100$; percentage of color due to free anthocyanins (FA) (FA%) = $[(A^{20} - A^{SO_2})/A^{acet}] \times 100$; and percentage of color due to PP (PP%) = $(A^{SO_2}/A^{acet}) \times 100$. The A_{520} of wine sample was also measured using a 2 mm path length cuvette without pH adjustment. The spectrophotometer used was a Hitachi U-2900 (Tokyo, Japan). All measurements were performed in 2019.

2.6. Statistical Analysis

Statistical analysis was performed by the *t*-test with Excel version 2016 (Microsoft, Redmond, WA, USA).

3. Results

3.1. Effect of pH on Color and Hydration Constant of Grape Anthocyanins

HPLC chromatograms of anthocyanins that were obtained from MER and YS skins are shown in Figure 1. Anthocyanins in MER skins were 3G. On the other hand, anthocyanins obtained from YS skins were malvidin-3,5-diglucoside and its acetate. These results were the same as those observed by Koyama et al. [6]. We confirmed that more than 95% of the peaks in the solution were anthocyanins when analyzed at 280 nm. The color of these anthocyanins at a concentration of 0.02 mM in a wine-like solution are shown in Figure 2, and their A_{520} nm observations are shown in Figure 3. At a concentration of 0.02 mM, the color of anthocyanins in MER skin decayed with increasing pH, and this effect was more obvious for anthocyanins in YS. The decay of A_{520} for MER anthocyanins linearly changed with increasing pH from a pH of 1 to 4.2. That of YS was higher than MER at an acidic pH of 1 to 2; however, it steeply declined and was very low at a wine pH of 3 to 4.2. The same phenomenon was observed in the anthocyanin concentrations of 0.02, 0.4, 0.8, and 1.2 mM. The hydration constants calculated under these conditions are shown in Table 2. For each anthocyanin concentration, the p*K*h value for MER was 1 value higher than that of YS.

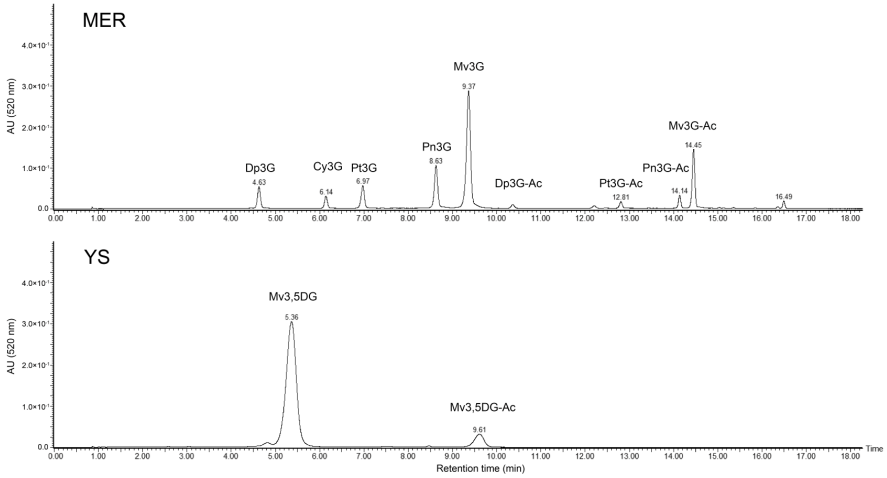

Figure 1. HPLC chromatogram of anthocyanins obtained from MER and YS grape skins. DP3G, delphinidin-3-glucoside; Cy3G, cyaniding-3-glucoside; Pt3G, petunidin-3-glucoside; Pn3G, peonidin-3-glucoside; Mv3G, malvidin-3-glucoside; Dp3G, delphinidin-3-glucoside acetate, Pt3G-Ac, petunidin-3-glucoside acetate; Pn3G-Ac, petunidin-3-glucoside acetate; Pn3G-Ac, peonidin-3-glucoside acetate; Mv3G-Ac, malvidin-3-glucoside acetate; Mv3,5DG, malvidin-3,5-diglucoside; Mv3,5DG-Ac, malvidin-3,5-diglucoside acetate.

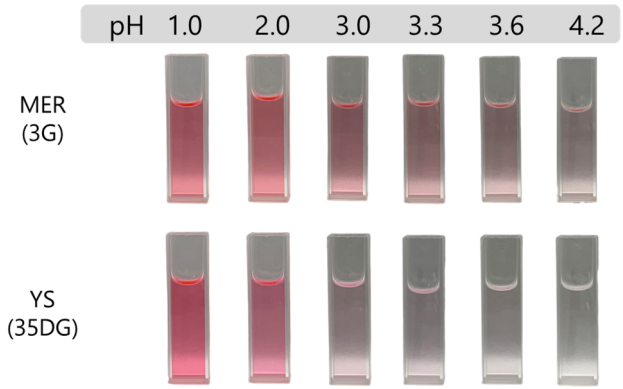

Figure 2. Effect of pH on the coloration of anthocyanins obtained from MER and YS grape skins.

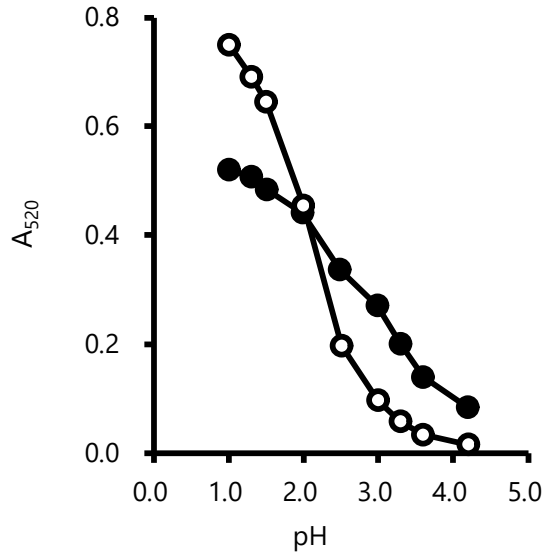

Figure 3. Effect of pH on the coloration of anthocyanins that were obtained from MER (closed circle) and YS (open circle).

Table 2. Calculated hydration constants of anthocyanins obtained from MER and YS.

Anthocyanin Conc. (mM)	MER	YS
0.02	2.92	2.11
0.4	3.26	2.13
0.8	3.46	2.39
1.2	3.76	2.73

3.2. Differences in Average Percentage of Color Due to Three Classes of Pigments in Cultivars in Commercial Wines

The anthocyanin co-pigmentation effect was compared among commercial red wines produced in Japan, namely the following: MER and CS wines, which contain 3G anthocyanins; YS wine, which has large amounts of 35DG; and MBA wine, which has small amounts of 35DG [6]. The average percentages of color due to the three classes of pigments (CA%, FA%, and PP%) in the red wine samples are shown in Tables 3–5, respectively. YS wine showed a much higher CA% than the other wines, although its PP% was much lower. MBA wine also showed a higher CA% and a lower PP% than wines made from *V. vinifera* cultivars MER and CS, although the difference was not statistically significant. We also investigated wines made from other non-*vinifera* cultivars and their hybrids. For example, the CA%, FA%, and PP% of 'Yamabudou' (*V. coignetiae*) wine were 17.2, 40.5, and 42.3; those of 'Gyoujyanomizu' (*V. flexuosa* × 'Merlot') wine were 58.7%, 18.0%, and

23.3%; those of 'Kitanoyume' (*V. flexuosa* × 'Pinot noir') wine were 45.4%, 14.3%, and 40.3%; those of 'Black Queen' ('Bailey' × 'Golden Queen') wine were 8.8%, 44.6%, and 46.6%; and those of 'Kai noir' ('Black Queen' × CS) wine were 0.1%, 47.1%, and 53.1%, respectively. These results suggest that the berries of non-*vinifera* cultivars and their hybrids, except for *V. vinifera* back-crossed varieties such as 'Kai noir' and 'Black Queen', have high CA% and low PP%. However, the number of wine samples was too small (1–2) to draw conclusions for the non-*vinifera* cultivars, except for MBA and YS; thus, these data were excluded from the analysis.

Table 3. Co-pigmented anthocyanins color percentage (CA%) of red wines.

CA%	Average	2017	2016	2015	2012–2014
MBA	3.4 ± 3.5^{A}	9.7 ± 3.2^{Ac}	2.9 ± 2.7^{Ab}	3.5 ± 4.0^{Ac}	0.2 ± 0.4^{Aa}
YS	24.0 ± 10.1^{B}	33.6 ± 9.7^{Bb}	21.2 ± 4.2^{Ba}	16.1 ± 8.3^{Ba}	
MER	2.5 ± 3.2^{A}	9.2 ± 3.4^{Ac}	4.1 ± 3.2^{Abc}	2.3 ± 2.9^{Aab}	0.7 ± 0.8^{Aa}
CS	2.1 ± 3.0^{A}	10.0	1.6 ± 1.3^{Aa}	0 ± 0^{Aa}	1.3 ± 1.6^{Aa}

Percentages of each fraction are expressed as mean ± SD. Different superscript letters indicate significant differences ($p < 0.05$). Large letters show statistical differences between cultivars, and small letters show statistical differences between vintages of wines in a cultivar.

Table 4. Free anthocyanin color percentage (FA%) of red wines.

FA%	Average	2017	2016	2015	2012–2014
MBA	42.7 ± 6.5^{B}	50.6 ± 8.7^{Aa}	44.7 ± 7.1^{Aa}	41.1 ± 5.9^{Ba}	41.0 ± 3.4^{Ba}
YS	48.7 ± 7.9^{C}	41.8 ± 6.5^{Aa}	54.1 ± 6.5^{Bb}	49.1 ± 5.4^{Bab}	
MER	33.6 ± 8.0^{A}	42.5 ± 5.6^{Ab}	50.9 ± 9.9^{ABb}	30.6 ± 5.5^{Aa}	31.6 ± 3.0^{Aa}
CS	38.4 ± 10.6^{BC}	50.0	39.8 ± 0.4^{ABa}	49.3 ± 1.1^{Ba}	31.2 ± 9.7^{ABa}

Percentages of each fraction are expressed as mean ± SD. Different superscript letters indicate significant differences ($p < 0.05$). Large letters show statistical differences between cultivars, and small letters show statistical differences between vintages of wines in a cultivar.

Table 5. Polymeric pigment color percentage (PP%) of red wines.

PP%	Average	2017	2016	2015	2012–2014
MBA	54.0 ± 7.2^{B}	39.7 ± 5.0^{Ba}	52.4 ± 6.9^{Bb}	55.3 ± 6.1^{Bb}	58.8 ± 3.5^{Ab}
YS	16.1 ± 13.4^{A}	24.6 ± 4.9^{Aa}	24.7 ± 6.4^{Aab}	34.7 ± 7.1^{Ab}	
MER	63.9 ± 9.0^{C}	48.3 ± 9.0^{Ba}	45.0 ± 10.3^{Ba}	67.1 ± 4.5^{Cb}	67.7 ± 2.6^{Cb}
CS	59.5 ± 11.3^{BC}	40.0	58.6 ± 1.8^{Ba}	50.7 ± 1.1^{Ba}	67.7 ± 8.4^{BCa}

Percentages of each fraction are expressed as mean ± SD. Different superscript letters indicate significant differences ($p < 0.05$). Large letters show statistical differences between cultivars, and small letters show statistical differences between vintages of wines in a cultivar.

Compared with wines made from the non-*vinifera* cultivars and their hybrids, wines from *V. vinifera* cultivars MER and CS had low CA% and high PP%. These results are significant in terms of understanding the characteristics of commercial wines. Anthocyanins are gradually oxidized and polymerized during wine aging, and co-pigmentation is considered to affect oxidation and polymerization reactions, leading to a gradual decrease in CA% [10]. Therefore, it was concluded that an analysis that takes aging period into account is necessary.

3.3. Changes in the Percentage of Color Due to Three Classes of Pigments during Aging

Tables 2–4 also show how CA%, FA%, and PP% change with vintage. In MBA, MER, and CS, CA% was approximately 10% after aging for two years (2017) but decreased with further aging, reaching nearly 0% after aging for five years. Because only one sample was investigated for CS in 2017, no standard deviation is shown in the tables, and the value was excluded from statistical analysis. On the other hand, in YS, CA% was 33.6% two years after production, and although it decreased with aging, it remained at 16.1% even after aging for four years (2015).

The FA% for all cultivars was relatively high, ranging from approximately 30 to 50%. In addition, some samples showed no significant differences in FA%, although FA% seemed to decrease after aging for approximately five years. On the other hand, PP%, which is due to PPs formed by polymerization, was 25–48% two years after production and gradually increased with aging.

4. Discussion

The effect of pH on coloration differed significantly, as illustrated in Figures 2 and 3. Since the color of anthocyanins is influenced by the co-pigmentation effect, pH experiments were conducted even at low concentrations (0.02 mM) where co-pigmentation effects may be minimal, and 0.2 M NaCl was added to prevent ionic interactions between molecules.

The hydration constants for MER and YS anthocyanins differed by approximately one unit. Since this value is exponential, it suggests that YS anthocyanins may be ten times more susceptible to hydration compared to MER anthocyanins, meaning only one-tenth of the anthocyanins may exhibit coloration under the same pH conditions. It is important to note that since the anthocyanins used were a mixture, the hydration constant values obtained in this experiment are not considered to have any particular significance and may vary depending on the composition of anthocyanins.

The anthocyanin co-pigmentation effect in the commercial wines made from different cultivars was apparent from the differences in CA% and the effect of aging on CA%. Heras-Roger et al. found significant differences in CA% and aging period among wines made from different cultivars [22,23]. We also found a statistically significant difference in CA% between wines made from different cultivars. CA% in YS wine was considerably higher than those in MBA, MER, and CS wines, suggesting that wine made from this cultivar differs from those made from MBA, MER, and CS. However, it is not easy to account for the high CA% in YS wine because of the many factors affecting co-pigmentation [10,14,17].

It is known that anthocyanin concentration is crucial for the induction of co-pigmentation. Compared with the average CA% of approximately 16 in MER wines measured by Heras-Roger et al. [22], the average CA% in MER wines obtained in this study was considerably low at 2.5%. Although we did not measure the color intensity ($A_{420} + A_{520} + A_{620}$) of the wine samples in this study, the average color intensity (or color density) of Japanese MER wines was determined to be approximately six in our previous study [24]. On the other hand, the color intensity reported by Heras-Roger et al. was as high as twelve, suggesting that the concentration of anthocyanins indeed affects CA%. In addition, wines obtained through the co-fermentation of YS, which has a high CA%, and MER did not enhance the red color in our preliminary experiments. The concentration of flavonol co-factors in the pericarp of YS is lower than that in *V. vinifera* cultivars MER and CS, although it has been reported that the concentration of anthocyanins is much higher in YS than in *V. vinifera* species [6]. These findings suggest that the high CA% in YS wines is derived at least in part from the high concentration of anthocyanins.

YS wine contains large amounts of 35DG (Figure 1). The higher CA% in YS wine than in the other wines can be considered to be due to the structural differences between anthocyanins. It has been reported that the binding of glucose to the 5 position in anthocyanins enhances the co-pigmentation effect, resulting in stronger hyperchromic effects [25,26]. This seems to be related to the strong purple color often seen in wines with high 35DG contents, such as those made from YS and *V. coignetiae* ('Yamabudou').

MBA wine also contains 35DG, but its amount is much smaller than that of 3G [5,6]. Owing to this difference in anthocyanin composition, MBA wine showed similar characteristics to *V. vinifera* wines, namely, low CA%. Heras-Roger et al. investigated the correlation between CA% and various compounds in 250 red wines and found that many compounds including flavonols, hydroxycinnamic acid, ethanol, and gallic acid affect CA% in red wines [17]. They examined *V. vinifera* wines and concluded that the results are attributable to 3G. On the other hand, Zhao et al. investigated the effects of gallic acid, (−)-epicatechin, and quercetin-3-*O*-glucoside as co-factors on 3G and 35DG and found that the effects of

these co-factors were greater on 35DG than 3G in a model system [27]. It is possible that the high CA% in YS wine is due to the presence of compounds other than anthocyanins. Few studies have been conducted on compounds that affect the CA% of 35DG, and further research is warranted.

It is well known that CA% is approximately 30–50% in young red wines, and CA% is decreased and PP% is increased in aged wines. Gutiérrez et al. reported that CA% was 32–43% immediately after alcoholic fermentation but decreased to 20–34% after aging for three months, with FA% decreasing simultaneously. After aging for nine months, CA% was decreased to ND—5% [28]. They also showed that PP% was approximately 20% immediately after alcoholic fermentation but increased to approximately 30–44% after aging for nine months. These results were observed in wines from *V. vinifera* cultivars CS, Cencibel, and Syrah [28]. On the other hand, Darias-Martín et al. analyzed aged red wines made from Listán negro (*V. vinifera*) and found that CA% in these wines remained at 22.3 and 18.5% and FA% was 37.0 and 32.5% after aging for one and two years, respectively [20]. Heras-Roger et al. showed that CA% decreased and PP% increased with aging, and the differences were statistically significant [22]. The decrease in CA% and the increase in PP% with aging are common phenomena seen at least in *V. vinifera* wines. However, the degree of change in CA% and FA% during aging varied markedly among cultivars, suggesting that the stability of anthocyanins also varied markedly among cultivars. In *V. vinifera* wines, it is known that some anthocyanins are rapidly converted into pyranoanthocyanins in the early stage of aging [14,29]. Pyranoanthocyanins are considered to be PP% in our experimental protocol because they are not susceptible to decolorization by SO_2. Pyranoanthocyanins may also be involved in co-pigmentation, but the details are not well understood as of yet [14]. Moreover, pyranoanthocyanins are not generated from 35DG because of the absence of a free hydroxyl group at the 5 position of the A-ring in anthocyanins [27]. It is necessary to further investigate the effects of the absence of pyranoanthocyanin formation during the aging process of 35DG. It has also been reported that anthocyanins form covalent bonds with other compounds through the formation of ethyl bridges to produce complexes during the aging process [30]. The formation of ethyl bridges is mediated by acetaldehyde produced by the reaction of ethanol in wine with reactive oxygen species. In other words, the polymerization reaction of anthocyanins via ethyl bridges requires an oxidation reaction with reactive oxygen species. Phenols including anthocyanins have high antioxidant activity for scavenging reactive oxygen species, and the high antioxidant activity of malvidin-3-*O*-(6-*O*-*p*-coumaroyl glucoside)-5-glucoside has been reported [31]. From these reports, we consider that the presence of antioxidants in wine, including anthocyanins, markedly influences the reactions of anthocyanins during aging.

5. Conclusions

The coloration of anthocyanins is a highly complex phenomenon influenced by various factors, including the types of anthocyanins, pH, self-association, and cofactors. In this study, our focus was on understanding the color development of red wine under practical winemaking conditions. To investigate the effect of pH on anthocyanin coloration, we used a mixture of anthocyanins extracted from grape skins. As shown in these experiments, the coloration of 3G and 35DG are quite different under environmental pH conditions. When making wine that contains 35DG, pH control is important, and it is necessary to take into account that a small change in pH can significantly change the color of the wine. The same concept can be considered when blending wines containing 35DG.

YS wine containing large amounts of 35DG had high CA%, and the color change due to aging was slow when CA% was high. Although the reason for this high CA% in YS is currently unknown, we suggest that the concentration of anthocyanins, the presence of compounds that affect co-pigmentation such as co-factors, and antioxidant activity affect CA%. Moreover, a high ratio of hydrated anthocyanins to flavylium types in YS anthocyanins may also affect the high CA%.

In at least the grape varieties used in this study, our findings indicate that wines containing more 35DG have higher CA% and are expected to behave differently in color development during wine aging when compared to wines made from *V. vinifera* grapes. We still have to determine the reasons for these findings. Because these findings were observed in commercial wines, it is important to develop an enological method that takes into consideration color changes due to the aging of anthocyanins in order to produce high-quality non-*vinifera* wines.

Many non-*vinifera* hybrids have been bred in Japan, and most of them have high concentrations of 35DG. Therefore, the metabolic pathway for the biosynthesis of 35DG may be more genetically dominant than the 3G biosynthetic pathway. It is accepted that many non-*vinifera* hybrids will be developed in the future from the perspective of disease resistance, but when the berries of such hybrids are used in winemaking, consideration must also be given to the composition of anthocyanins.

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