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Anthocyanin Transformation in Cabernet Sauvignon Wine during Aging

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Anthocyanins in Cabernet Sauvignon grapes and wines were elucidated by HPLC-MS/MS. Major anthocyanins in Cabernet Sauvignon grape extract are malvidin 3-*O*-glucoside and malvidin 3-*O*-acetylglucoside. In matured wine, anthocyanins are transformed to anthocyanin-vinyl derivatives, ethyl bridged anthocyanin-flavanol adducts, and anthocyanin-flavanol adducts. The major anthocyanin pigments are malvidin 3-*O*-glucoside-pyruvate, malvidin 3-*O*-acetylglucoside-pyruvate, malvidin 3-*O*-coumaroylglucoside-pyruvate, malvidin 3-*O*-glucoside-4-vinylphenol, malvidin 3-*O*-acetylglucoside-4-vinylphenol, and malvidin 3-*O*-coumaroylglucoside-4-vinylphenol. The presence of syringetin 3-*O*-glucoside and syringetin 3-*O*-acetylglucoside has been established for the first time in grape and wine.

KEYWORDS: Cycloaddition; condensation; Cabernet Sauvignon; wine; pyruvic acid; malvidin 3-*O*-glucoside; malvidin 3-*O*-acetylglucoside; syringetin 3-*O*-glucoside

INTRODUCTION

During red wine aging, anthocyanins undergo condensation reactions to form polymeric pigments. This transformation was thought to be responsible for the color change during wine maturation. Three mechanisms have been postulated to be involved in the condensation reaction (*1*). The first two mechanisms involve direct reaction between proanthocyanidin and anthocyanin, generating colorless compounds or orange xanthylium salts (*2*−*7*). The third mechanism involves acetaldehyde-mediated condensation of anthocyanin and proanthocyanidin, yielding purple pigments with an ethyl bridge (*8*−*13*). Glyoxylic acid, furfural, and HMF were found to react in the same way as acetaldehyde-mediated condensation with anthocyanins or flavanols in the model systems (*14*−*17*). Recent studies further revealed the presence of new pigments other than those previously suggested. These pigments were formed from a new type of reaction between anthocyanin and vinyl derivatives (*18*−*21*). The mechanism was postulated to be a cycloaddition reaction at carbon 4 and the hydroxyl group of carbon 5 of an anthocyanin, with various components possessing a polarizable double bond (*22*). In this reaction, a fourth ring was formed, which was considered to be responsible for their higher stability than the original anthocyanins (*20*). The components that reacted with anthocyanins include 4-vinylphenol, hydroxycinnamic acids, acetone, and several yeast metabolites such as acetaldehyde and pyruvic acid (*20*−*28*).

Direct evidence for the condensation reaction between anthocyanin and flavanol was recently identified in matured Cabernet Sauvignon wine (*7*, *10*, *29*). Evidence of the existence of cycloaddition products was reported in matured port wine (*19*), grape pomace (*21*), and aged Shiraz and Cabernet Sauvignon wines (*23*, *25*, *30*−*31*). Formation of anthocyanin-proanthocyanidin adducts was commonly proposed to explain the loss of astringency during wine maturation, and wine quality was speculated to have something to do with anthocyanin-to-procyanidin ratio due to interaction between anthocyanins and proanthocyanidins during wine maturation (*22*). However, no one has looked at these anthocyanin pigments completely in mature wine. Little is known about mechanisms of anthocyanin transformation and the roles of anthocyanins in wine's reduced astringency during wine maturation.

The objective of this work is to establish anthocyanin profiles and individual anthocyanins in Cabernet Sauvignon wines in selected vintages by LC-MS/MS and to elucidate the mechanisms of anthocyanin transformation. On the basis of anthocyanin profiles established, the involvement of anthocyanins in wine's reduced astringency during maturation is discussed.

MATERIALS AND METHODS

Wines and Grapes. *Vitis vinifera* var. Cabernet Sauvignon grape samples were harvested from different viticultural areas in Central Valley (Fresno-Madera), North Valley (Lodi-Sacramento) and Napa Valley, CA. Young wines were prepared in Research and Development, Canandaigua Wine Company (Madera, CA) following standard wine making procedures from those grapes. Thirty commercial Cabernet Sauvignon wines from different viticultural areas in California at vintage years ranging from 1997 to 2002 were purchased from local supermarkets.

Standards. Malvidin 3, 5-*O*-diglucoside was purchased from Aldrich (Saint Louis, MO), Cyanidin-3-*O*-glucoside, malvidin 3-*O*-galactoside, and syringetin 3-*O*-glucoside were purchased from Indofine (Hillsborough, NJ).

Extraction of Anthocyanins in Cabernet Sauvignon Wines. Cabernet sauvignon wine (5 mL) was passed separately through a C-18

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Table 1. Molecular Ions and Product Ions of Anthocyanins in Cabernet Sauvignon Wine and Grape Skin Extract

peak	retention time	compounds	molecular and product ions
1	5.8	delphinidin 3-*O*-glucoside	465 (M^+), 303
2	8.9	cyanidin 3-*O*-glucoside	449 (M^+), 287
3	12.3	petunidin 3-*O*-glucoside	479 (M^+), 317
4	15.0	malvidin 3-O-galactoside	493 (M^+), 331
5	18.4	Peonidin 3-*O*-glucoside	463 (M^+), 301
6	20.7	malvidin 3-*O*-glucoside	493 (M^+), 331
7	25.5	delphinidin 3-*O*-acetylglucoside	507 (M^+), 303
8	28.8	malvidin 3-*O*-acetylgalactoside	535 (M^+), 331
9	34.4	petunidin 3-*O*-acetylglucoside	521 (M^+), 317
10	39.6	peonidin 3-*O*-acetylglucoside	505 (M^+), 301
11	40.2	malvidin 3-*O*-acetylglucoside	535 (M^+), 331
12	41.3	peonidin 3-*O*-caffeoylglucoside	625 (M^+), 301
13	41.5	malvidin 3-*O*-caffeoylglucoside	655 (M^+), 331
14	42.1	petunidin 3-*O*-coumaroylglucoside	625 (M^+), 317
15	42.3	malvidin 3-*O*-coumaroylgalactoside	639 (M^+), 331
16	43.6	peonidin 3-*O*-coumaroylglucoside	609 (M^+), 301
17	43.8	malvidin 3-*O*-coumaroylglucoside	639 (M^+), 331
18	4.4	malvidin-3-*O*-glucoside-(epi)-catechin	781 (M^+), 619, 467, 373, 344
19	7.1	delphinidin 3-*O*-glucoside-pyruvate	533 (M^+), 371
20	15.1	petunidin 3-*O*-glucoside-pyruvate	547 (M^+), 385
21	24.9	malvidin 3-*O*-glucoside-pyruvate	561 (M^+), 399
22	28.4	malvidin 3-*O*-acetylglucoside-pyruvate	603 (M^+), 399
23	29.4	malvidin 3-*O*-glucoside-acetate	517 (M^+), 355
24	35.6	syringetin 3-O-glucoside	509 (M^+), 347
25	36.7	malvidin 3-*O*-glucoside-acetone	531 (M^+), 369
26	37.0	malvidin 3-*O*-glucoside-ethyl-(epi)catechin	809 (M^+), 519, 357
27	39.1	malvidin 3-*O*-coumaroylglucoside-pyruvate	707 (M^+), 399
28	41.5	malvidin 3-*O*-acetylglucoside-ethylflavanol	851 (M^+), 561, 357
29	42.2	syringetin 3-*O*-acetylglucoside	551 (M^+), 347
30	42.4	malvidin 3-*O*-glucoside-4-vinyl-(epi)catechin	805 (M^+), 643, 491
31	43.6	petunidin 3-*O*-glucoside-4-vinylphenol	595 (M^+), 433
32	45.0	malvidin 3-*O*-glucoside-4-vinylphenol	609 (M^+), 447
33	45.3	malvidin 3-*O*-glucoside-4-vinylcatechol	625 (M^+), 463
34	46.2	malvidin 3-*O*-acetylglucoside-4-vinylphenol	651 (M^+), 447
35	47.2	malvidin 3-*O*-coumaroylglucoside-4-vinylphenol	755 (M^+), 447

Sep-Pak cartridge preconditioned with acidified methanol and water (Waters Corporation). The adsorbed pigments were washed with 5 mL of water and eluted with 2 mL of 0.01% HCl methanol. The eluant was stored at −20 °C prior to HPLC analysis. Wine samples were passed through a 0.45-μM glass microfiber filter (GMF) syringe filter (Whatman Inc, Clifton, NJ) before injection onto HPLC.

Extraction of Anthocyanins in Cabernet Sauvignon Grape Skin. Cabernet Sauvignon grape skins (50 g) were extracted twice with 100 mL of methanol. Extract was combined and evaporated under reduced pressure to remove methanol. Residue was reconstituted to 20 mL with water. Water solution (5 mL) was passed through a preconditioned C-18 Sep-Pak cartridge (Waters Corporation). The adsorbed pigments were washed with 5 mL of water and eluted with 2 mL of 0.01% HCl methanol. The eluant was stored at −20 °C prior to HPLC analysis. Cabernet Sauvignon grape skins were also extracted with 75% acetone with 0.2% acetic acid to compare anthocyanin profiles in different extraction procedures.

Preparation of Model Solution. Malvidin 3, 5-diglucoside (10 000 mg/L) and catechin (4400 mg/L) in the presence of acetaldehyde (0.1%, v/v) were prepared in a medium that contained 5% tartaric acid (w/v) and 10% ethanol (v/v) and were adjusted to pH 3.2 with NaOH. The mixtures were stored at 20 °C in sealed dark glass vials. Samples were taken periodically and diluted 1→5 with model wine solution before injection onto HPLC.

HPLC/DAD/ESI-MS/MS Analyses. LC/ESI-MS/MS experiments were performed on an Agilent 1100 LC/MSD Trap-SL mass spectrometer (Palo Alto, CA) equipped with an electrospray ionization (ESI) interface, 1100 HPLC, a DAD detector, and Chemstation software (Rev.A.09.01). Anthocyanins were monitored at 520 nm. The column used was a 150-cm × 2.0-mm i.d., 4-μm Synergi hydro-RP 80 Å (Phenomenex, Torrance, CA). Solvents were (A) 10% acetic acid/0.1% TFA/5% acetonitrile/84.8%water (v/v/v/v) and (B) acetonitrile. Solvent gradient was 0−30 min, 0−10% B; 30−40 min, 10−30% B; 40−50 min, 30−40% B. Flow rate was 0.2 mL/min, injection volume was 3 μL, and column temperature was 25 °C. The ESI parameters were as folows: nebulizer, 30 psi; dry gas (N_2), 12 L/min; dry temp, 350 °C; trap drive, 50; skim 1, 40 v; skim 2, −5.0 v; octopole RF amplitude, 150 vpp; capillary exit, 103.4 v. The ion trap mass spectrometer was operated in positive ion mode scanning from *m*/*z* 150 to *m*/*z* 2000 at a scan resolution of 13 000 amu/s. Trap ICC was 20 000 units and maximal accumulation time was 200 ms. MS-MS was operated at a fragmentation amplitude of 1.2V and threshold ABS was 3 000 000 units.

RESULTS AND DISCUSSION

Anthocyanin profiles in Cabernet Sauvignon wine and grape skin extract were established and elucidated by LC-MS/MS analysis. Molecular ions and product ions of anthocyanins were included in **Table 1**. After comparing anthocyanin profiles of grape skin extracts using a different extraction solvent system from that of grape juice, several artificial peaks were found to be generated using 75% acetone with 0.2% acetic acid as extracting solvent. These artificial peaks with trace quantity might be due to interaction between acetone and anthocyanins at acidic media as previously reported (*24*). Therefore, 100% methanol was selected as extracting solvent. Anthocyanin profiles of grape skin extract with 100% methanol from Central Valley, North Valley, and Napa Valley were identical, even though the ratio of individual anthocyanins in these three grape skin extracts was different. Grape skin extract with 100% methanol from Napa Valley was selected as the reference to illustrate anthocyanin transformation during wine making and wine maturation. Anthocyanins in Cabernet Sauvignon grape skin extract were in agreement with previous reports (*32*−*33*).

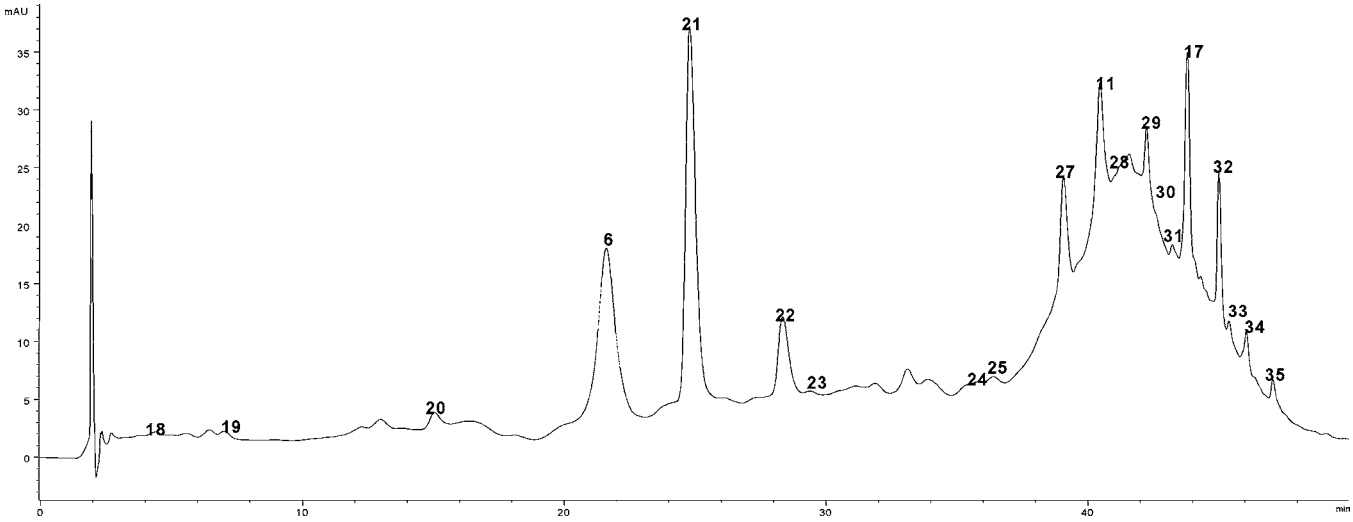

Figure 1. HPLC chromatogram of Cabernet Sauvignon wine from Napa valley at 520 nm.

However, peaks 4, 8, and 15 with trace quantities were not included in the previous reports. These three peaks had the same molecular and product ions as malvidin 3-*O*-glucoside (peak 6), malvidin 3-*O*-acetylglucoside (peak 11), and malvidin 3-*O*-coumarylglucoside (peak 17), respectively. Also, peaks 4, 8, and 15 were eluted earlier than their glucosidic counterparts. UV−vis spectra also could not distinguish differences among peaks 4, 8, and 15 from peaks 6, 11, and 17. These results suggested that the differences between peaks 4, 8, and 15 and peaks 6, 11, and 17 were a glycosidic pattern. Malvidin 3-*O*-galactoside along with malvidin-3-*O*-glucoside was identified in black bilberry and blueberry extracts, and anthocyanin 3-*O*-galactosides were eluted earlier on reverse-phase HPLC than their glucosidic counterparts (*34*−*35*). Peaks 4, 8, and 15 could well be malvidin 3-*O*-galactoside, malvidin 3-*O*-acetylgalactoside, and malvidin 3-*O*-coumaroylgalactoside, respectively. Peak 4 was further confirmed by comparison of malvidin 3-*O*-galactoside chloride standard based on their retention times and mass spectral.

During winemaking and wine maturation, new anthocyanin pigments were developed, and original anthocyanins were decreased. In 30 commercial Cabernet Sauvignon wines, anthocyanin profiles were very similar. However, matured wines had a higher percentage of newly formed anthocyanin-derived pigments than young wines (data not shown). Therefore, Cabernet Sauvignon wine from Napa Valley 1997 and two-month old Cabernet Sauvignon wine made from Napa Valley grapes in Research and Development, Canandaigua Wine Company, Inc., were selected as representatives of matured and young wine, respectively. During winemaking, new anthocyanin pigments (peaks 18−35) were formed. With the progress of wine maturation, these anthocyanin-derived pigments became dominant (Peaks 21, 22, 27, 32, and 34) (**Figure 1**). Peak 21, with molecular ion at *m*/*z* 561 and product ion at *m*/*z* 399, was assigned to be malvidin 3-*O*-glucoside-pyruvate (Vitisin A) (*20*−*21*). Similarly, peaks 19 and 20 were delphinidin 3-*O*-glucoside-pyruvate and petunidin 3-*O*-glucoside-pyruvate, respectively. Peaks 22 and 27 were malvidin 3-*O*-acetylglucoside-pyruvate and malvidin 3-*O*-coumaroylglucoside-pyruvate, respectively. Peak 23 was identified as malvidin 3-*O*-glucoside-acetate after comparison with reference data (*20*). Peaks 32, 34, and 35 had product ions at $m/z = 447$ and were elucidated as malvidin 3-*O*-glucoside-4-vinylphenol, malvidin 3-*O*-acetylglucoside-4-vinylphenol, and malvidin 3-*O*-coumaroylglucoside-4-vinylphenol, respectively (25). Peak 33 had molecular and product ions at *m*/*z* 625 and 463 and was elucidated as malvidin 3-*O*-glucoside 4-vinylcatechol, which agreed with previous reports (*25*, *28*). However, it is important to point out that the concentration of malvidin 3-*O*-glucoside-vinylcatechol in wine was much lower than that of malvidin 3-*O*-glucoside-4-vinylphenol, even though a significant amount of caffeic acid was monitored in the wine and grape extract (data not shown). The explanation can be that *p*-coumaric acid, but not caffeic acid, was decarboxylated during fermentation (36), and reaction between malvidin 3-glucoside and decarboxylated product of *p*-coumaric acid, 4-vinylphenol, went to completion rather fast (*18*, *28*). The reaction of caffeic acid and malvidin 3-O-glucoside took place directly during wine storage, and reaction proceeded very slowly (*28*).

Peak 18 at RT 4.4 min had molecular ion at *m*/*z* 781 and product ions at *m*/*z* 619 and *m*/*z* 467. Product ion at *m*/*z* 619 was a fragment of *m*/*z* 781 by loss of a glucose, and *m*/*z* 467 was a fragment of *m*/*z* 619 resulting from retro-Diels−Alder cleavage (−152 amu) in (+)-catechin or (−)-epicatechin (**Figure 2**) (*26*). Because MS data itself could not distinguish the isomers of a compound, peak 18 was assigned to be malvidin 3-*O*-glucoside-(+)-catechin or malvidin 3-*O*-glucoside-(−)-epicatechin. Peak 30 at RT 42.4 with molecular ion at *m*/*z* 805 and product ions at *m*/*z* 643 and 491was assigned as either malvidin 3-*O*-glucoside-vinyl-(+)-catechin or malvidin 3-*O*-glucoside-vinyl-(−)-epicatechin based on report recently in the literature (*26*). Peak 26 had molecular ion at *m*/*z* 809 and product ions at *m*/*z* 519 and *m*/*z* 357. Product ion at *m*/*z* 519 was a fragment from *m*/*z* 809 by loss of either (+)-catechin or (−)-epicatechin. Product ion at *m*/*z* 519 could be further fragmented to yield a product ion at *m*/*z* 357 by loss of a glucose moiety (**Figure 3**). Peak 26 was suggested to be malvidin 3-*O*-glucoside-ethyl-(+)-catechin or malvidin 3-*O*-glucoside-(−)-epicatechin (*29*, *37*). In the model solution containing catechin, malvidin 3, 5-diglucoside, and acetaldehyde, two major compounds were produced. Two enantiomers had identical molecular and product ions at *m*/*z* 971, 681, 519, and 357, which corresponded to a structure in which malvidin 3, 5-diglucoside, and catechin were linked by an ethyl bridge. Enantiomers might be due to presence of an asymmetric carbon in the ethyl bridge (*29*). The fragmentation pattern of compounds produced in the model system was the same as that of peak 26. In both cases, the C−C bond connected ethyl group to catechin moiety was cleaved before sugar moiety was cleaved. This fragmentation pattern was quite unique because in most cases, only sugar moiety was cleaved

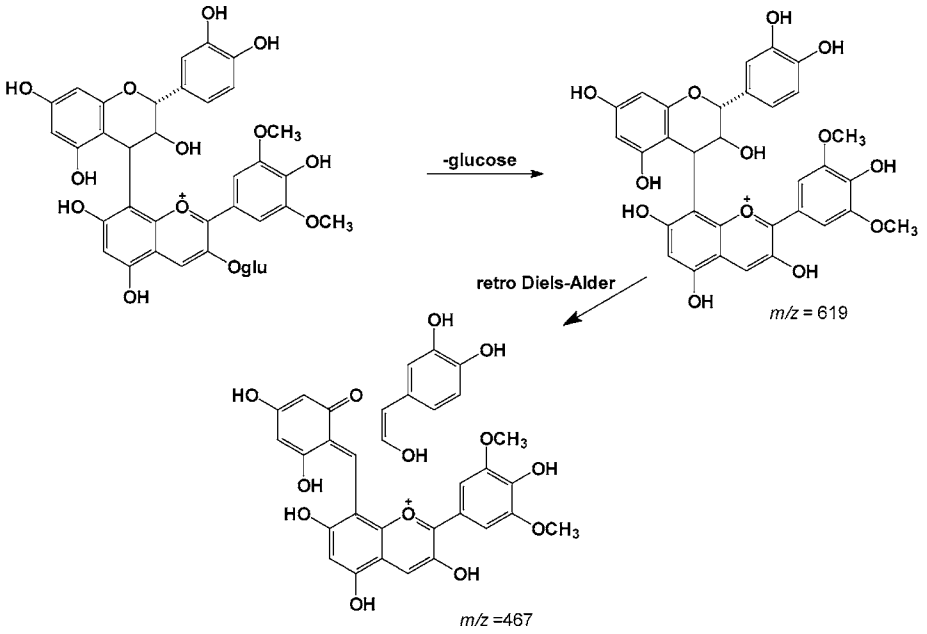

Figure 2. Fragmentation pattern of peak 18.

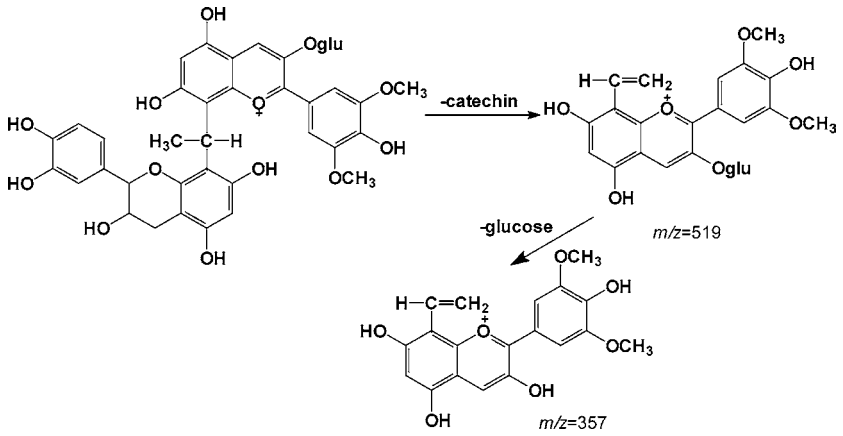

Figure 3. Fragmentation pattern of peak 26.

under this ESI condition (*38*−*39*). Similarly, peak 28 had molecular ion at *m*/*z* 851 and product ions at *m*/*z* 561 and 357, which was assigned to be malvidin 3-*O*-acetylglucoside-ethyl-(+)-catechin or malvidin 3-acetylglucoside-ethyl-(−)-epicatechin. To date, this compound has not been reported in the literature.

Peak 24 at RT 35.6 min had molecular ion at *m*/*z* 509 and product ion at *m*/*z* 347. Product ion at *m*/*z* 347 was a fragment from molecular ion at *m*/*z* 509 by loss of a glucose moiety. This compound did not have maximal absorbance around 520 nm, but it showed strong signal on MS detector. Molecular ion at *m*/*z* 509 was 16 amu higher than malvidin 3-*O*-glucoside, which was suggested to be a hydroxyl group attached to C-4 of malvidin 3-*O*-glucoside or an equilibrium form of malvidin 3-*O*-glucoside (*25*). However, UV spectrum showed this compound had maximal absorbance at 360 nm instead of 520 nm, suggesting a flavonol component instead of an anthocyanin. Peak 24 was then elucidated to be syringetin 3-*O*-glucoside after further compared with standard. Similarly, peak 27 had molecular and product ions at *m*/*z* 551 and *m*/*z* 347. The molecular and product ions of peak 29 were 42 amu higher than those of peak 24, which suggested an acetylglucoside moiety in the compound. Peak 29 was assigned to be syringetin 3-*O*-acetylglucoside. This was the first time the existence of syringetin 3-*O*-glucoside and syringetin 3-*O*-acetylglucoside in grape and wine was confirmed.

Our data on the anthocyanin analyses of Cabernet Sauvignon wines confirmed that the mechanisms of anthocyanin transformation during wine maturation include cycloaddition (peaks 19−23, 27, 30−35), and condensation (peaks 18, 26, and 28). In the aged Cabernet Sauvignon wines we selected, malvidin 3-*O*-glucoside-pyruvate, malvidin 3-*O*-acetylglucoside-pyruvate, malvidin 3-*O*-glucoside-4-vinylphenol, and malvidin 3-*O*-acetylglucoside-4-vinylphenol were dominant pigments. Recently, researchers further found that the formation of malvidin 3-*O*-glucoside-4-vinylcatechol derivatives resulted from direct interaction between anthocyanins and caffeic acid (*28*). Because hydroxycinnamic acids, such as caffeic acid, might contribute to bitterness and harshness in wine from sensory evaluation (*40*), the reaction between anthocyanins and hydroxycinnamic acids might contribute to the reduction of harshness of wine during wine maturation.

The amount of identified anthocyanin-vinylflavanol, anthocyanin-flavanol, and anthocyanin-ethyl-flavanol adducts in wine was not significant. The formation of anthocyanin-vinylflavanol and anthocyanin -ethyl-flavanol adducts was speculated to follow the acetaldehyde-induced polymerization reaction first proposed by Timberlake et al. to give an unstable ethanol adduct

(*9*). The ethanol adduct could then be protonated to form ethyl-flavanol cation or produce vinyl-flavanol by loss of a water molecular. Vinyl-flavanol reacted with malvidin 3-*O*-glucoside to form malvidin-3-*O*-glucoside-vinylcatechin following the same mechanism as the formation of anthocyanin-4-vinylphenol. Ethyl-flavanol could further react with malvidin 3-*O*-glucoside to form malvidin 3-*O*-glucoside-ethyl-(+)-catechin. Because acetaldehyde was suggested to be produced by the oxidation of ethanol with air in the presence of phenolic compounds during wine maturation (*41*), a condensation reaction would have occurred only when oxygen was present. Es−Safi et al. (*42*) further discovered that in the mixture containing malvidin 3-*O*-glucoside, epicatechin and acetaldehyde, two reactions competed for the formation between epicatechin-ethyl-epicatechin and malvidin 3-*O*-glucoside-ethyl-epicatechin. Epicatechin-ethyl-epicatechin derivatives were formed first and subsequently transformed to colored derivatives, which seem to be more stable. The formation of the trimeric and tetrameric colored derivatives was also monitored. From a sensory point of view, the formation of anthocyanin-ethyl-flavanol adducts was commonly proposed to explain loss of astringency during wine aging. If ethyl-linked flavanol could be converted to ethyl-flavanol or vinyl-flavanol cation, the reduced astringency of procyanidin could be achieved without oxygen present. In 30 Cabernet wines analyzed, only a trivial amount of individual anthocyanin and proanthocyanidin derivatives were found in young and matured wines. However, besides peaks identified, a broad band at retention time 37−48 min underneath peaks 27−35 remained to be identified. This broad band had absorbance at 520 nm, indicating that it contained the flavylium units. This band could be oligomeric or polymeric anthocyanin and flavanol derivatives. In a total ion chromatogram, no signals were monitored in that region, which might be due to complexity of these compounds and low concentration of each compound. In addition to monomer flavanols, the acetaldehyde-induced condensation occurred between anthocyanin and proanthocyanidin oligomers or polymers (*42*); this further increased the complexity of the band. Moreover, in several wines analyzed, the only pigments left were a hump at retention time 37−48 min. Wines with only hump left did not tend to be smoother than others with a significant amount of anthocyanin-pyruvate and anthocyanin-4-vinylphenol derivatives left from the evaluation of our taste panel (data not shown). This can be explained that after certain stage, free anthocyanin may not be further available to reduce the bitterness and astringency of proanthocyanidins. The only reaction is degradation of anthocyanin derivatives. Other factors, such as direct interaction between anthocyanins and hydroxycinnamic acids, may also play a role in reduced astringency in matured wine. Acetaldehyde-induced polymerization between proanthocyanidins may be another mechanism participating in loss of astringency during wine maturation (*43*). It will be interesting to further examine the sensory properties of components from this broad band to further address how anthocyanins are involved in reducing astringency during wine maturation.

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