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Variations in the Profile and Content of Anthocyanins in Wines Made from Cabernet Sauvignon and Hybrid Grapes

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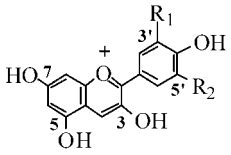
To detect adulteration of wine, it has been proposed that the ratio of acetylated to *p*-coumaroylated conjugates of nine characteristic anthocyanins can be used to determine whether a wine is derived from Cabernet Sauvignon or hybrid grapes. If the ratio is >3, then a wine is classified as being derived from Cabernet Sauvignon grapes. This test has significant commercial implications as it is being used to decide whether Cabernet Sauvignon-labeled wines are genuine and can be imported into Germany. To assess whether this is a valid approach, 24 wines were analyzed, 4 of which were made from hybrids and 20 from Cabernet Sauvignon, with vintages ranging from 1993 to 2000. Only 13 of the Cabernet Sauvignon wines contained all nine of the "characteristic" anthocyanins, and the ratio of acetylated to *p*-coumaroylated derivatives varied from 1.2 to 6.5. It is evident that the use of the anthocyanin ratio method is flawed and that examination of the whole anthocyanin profile and/or investigation of the proportion of monoglucoside and acetylated anthocyanins is a better approach to distinguish between hybrid and Cabernet Sauvignon wines.

KEYWORDS: Anthocyanins; red wine; vinification; adulteration

INTRODUCTION

Anthocyanins are widely dispersed throughout the plant kingdom, being particularly evident in fruit and flower tissue where they are responsible for red, blue, and purple colors. They are also found in leaves, stems, seeds and root tissue (*1*). As well as acting as attractants for pollinating insects, anthocyanins in the epidermis have a protective role acting as a filter reducing the levels of harmful irradiation reaching mesophyll cells (*2*). The term anthocyanidin refers to the aglycon structure (**Figure 1**), and anthocyanins are their conjugated derivatives. The most common anthocyanins are sugar conjugates, typically glucose, but they are also associated with hydroxycinnamates and organic acids such as malic and acetic acids. Although conjugation can take place on carbons 3, 5, 7, 3′, and 5′, it occurs most frequently at the C3 position.

Anthocyanins are responsible for the coloring of black grapes and red wines, but they are lacking in white grapes. They are found principally in the skins of grapes, although they are also present in the flesh of some berries, a characteristic known as teinture. The amount and concentration of anthocyanins in red grapes will vary depending on the variety, maturity, climate, terrior, and fruit yield. The total anthocyanin content of red grapes ranges from about 300 to 7500 μg/g of fresh weight of ripe berries (*3*). The anthocyanins in grape skins are predominately the 3-*O*-glucosides of delphinidin, cyanidin, petunidin, peonidin, and the major compound, malvidin, although coumaric, caffeic, and acetic acid esters have also been detected.



Anthocyanidin	R_1	R_2	Colour
Pelargonidin	H	H	Orange/red
Cyanidin	OH	H	Red
Delphinidin	OH	OH	Pink
Peonidin	OCH_3	H	Bluish purple
Petunidin	OCH_3	OH	Purple
Malvidin	OCH_3	OCH_3	Reddish-purple

Figure 1. Structure and color of the major anthocyanidins in red wine (*2*).

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Anthocyanins are readily extracted from grape skins and provide the vibrant reddish-purple tones of young red wines. They are also known to contribute to the antioxidant and vasodilation activity of red wines (*4*).

Grapevine belongs to the botanical family Vitaceae, comprising 12 or 14 genera, the exact number of which remains under discussion. It is the genus *Vitis* and its various species that are of interest to the wine-maker, particularly *Vitis vinifera*. Although *V. vinifera* produces the best quality wines, other species are found throughout the world. For example, in North America the related species *V. riparia*, *V. rupestris*, and *V. berlandieri* are commonly found and are known to be resistant to low temperature. Likewise, *V. amurensis* is found in the Orient and *V. coignetaiae* in Japan. In viticulture the term hybrid is reserved for vines derived from the crossing of two *Vitis* species (e.g., *V. labrusca* × *V. riparia*), whereas "hybrid wine" describes wines produced from hybrids. A number of hybrid vines (Noah, Herbemont, Jacquez, Clinton, and Isabella) have been prohibited in France since 1953 due to the high methanol content of the resulting wines, although they have been used to fraudulently adulterate better quality Cabernet Sauvignon wines.

Although similar types of anthocyanins are found in different grape varieties, the relative amounts of the individual compounds differ. For example, it has been noted that Pinot Noir grapes contain no acylated anthocyanins (*4*). Features such as these have enabled anthocyanins to be used taxonomically and to detect adulteration in wines. However, the extent of anthocyanin extraction depends on fermentation temperature and duration and the concentration of sulfur dioxide and alcohol (*5*). Analysis of nine anthocyanins and their ratios has been used as a means of validating the identity of the grapes used during vinification to ward against adulteration or fraud. It has been proposed that the ratio of acetylated to *p*-coumaroylated anthocyanins should be >3 if the wine is derived from Cabernet Sauvignon rather than hybrid grapes (*6*), and this test is applied to Cabernet Sauvignon-labeled wines imported into Germany.

The aim of this study was to investigate the nature and profile of anthocyanins in a selection of wines prepared from pure Cabernet Sauvignon and hybrid grape varieties in order to determine whether measuring the ratio of acetylated to *p*-coumaroylated anthocyanins is a reliable means of determining the identity of the vinified grapes.

MATERIALS AND METHODS

Wines. Twenty-four samples of French red varietal wines were analyzed (**Table 1**): 20 pure Cabernet Sauvignon wines; two samples of hybrid A (blend of 50% Baco 54−55 and 50% Seybel 1000); and two samples of hybrid B (blend of 30% Clinton, 30% Jacquez, 10% Othello, and 30% Gamay). All of the wine samples from Cabernet Sauvignon or hybrids were from the Languedoc-Roussillon viticultural region of southern France and from a range of vintages. The hybrid wine samples were collected from the 2000 vintage for hybrid A and from the 1998 vintage for hybrid B.

All of the samples came from the wine collection of the Enology Department of the University of Montpellier. All pure Cabernet Sauvignon wines were made with Cabernet Sauvignon grapes from authentic vine batches controlled by ONIVINS official survey. All wine-making was undertaken in experimental department wineries under the control of enologists from the University of Montpellier. Maceration time ranged between 1 and 3 weeks and the maximal temperature from 28 to 35 °C. Sampling and analytical analysis (alcohol content, total acidity, volatile acidity, pH, sugar concentration, and color index) were made directly by the University of Montpellier enologists after malolactic fermentation. No blends of these Cabernet Sauvignon wines were prepared prior to bottling.

Table 1. Details of Wines Analyzed

wine	grape	vintage	total phenol content (mg/L GAE)
1	Cabernet Sauvignon	2000	3676
2	Cabernet Sauvignon	2000	3833
3	Cabernet Sauvignon	1998	4987
4	Cabernet Sauvignon	1994	3897
5	Cabernet Sauvignon	1993	3221
6	Cabernet Sauvignon	1996	1865
7	Cabernet Sauvignon	1999	1554
8	Cabernet Sauvignon	2000	2275
9	Cabernet Sauvignon	2000	3442
10	Cabernet Sauvignon	2000	3293
11	Cabernet Sauvignon	1998	2690
12	Cabernet Sauvignon	1999	2689
13	Cabernet Sauvignon	2000	2811
14	Cabernet Sauvignon	2000	3942
15	Cabernet Sauvignon	1998	2059
16	Cabernet Sauvignon	1998	2360
17	Cabernet Sauvignon	1999	2874
18	Cabernet Sauvignon	2000	2306
19	Cabernet Sauvignon	1999	2820
20	Cabernet Sauvignon	2000	3455
21	hybrid A	2000	3140
22	hybrid A	2000	2870
23	hybrid B	1998	1162
24	hybrid B	1998	1392

In brief, Baco 54−55 is a complex hybrid created by François Baco; it belongs to the group *V. vinifera* × *V. labrusca* × *V. riparia* × *V. rupestris* × *V. aestivalis*. Seybel 1000, known in the United States as Rosette, is a cross of 70 Jaeger and *V. vinifera* and belongs to the group *V. vinifera* × *V. rupestris* × *V. lincecu* M11. Clinton is a natural hybrid belonging to the group *V. labrusca* × *V. riparia*. Jacquez is also a natural hybrid, but from Ohio, and belongs to the group *V. aestivalis* × *V. cinerea* × *V. vinifera*. Othello is a hybrid of Clinton × Black Hambourg and belongs to the group *V. labrusca* × *V. riparia* × *V. vinifera*. Only Gamay belongs to *V. vinifera*.

Hybrid A and B wines were prepared from 50% Baco 54−55 and 50% Seybel and from 30% Clinton, 30% Jacquez, 10% Othello, and 30% Gamay, respectively. In each case the maceration time was 1 week and the maximum temperature 30 °C. These hybrid wines were made in the ENVAT (Conservation of wines collection) at l'Espiguette, France.

Total Phenol Content. Total phenol content was determined using the Folin−Ciocalteu method (*7*). Samples were calibrated against gallic acid, and the results were expressed as gallic acid equivalents (GAE).

LC-MS-MS Analysis. Samples, with no prior treatment, were analyzed on a P4000 liquid chromatograph fitted with an AS 3000 autosampler and with detection by a UV6000 diode array absorbance monitor scanning from 250 to 700 nm (Thermo Finnigan, San Jose, CA). Separation was carried out using a 250 × 4.6 mm i.d. 4 μm Synergy RP-Max column (Phenomenex, Macclesfield, U.K.), maintained at 40 °C and eluted with a 60 min gradient of 5−30% acetonitrile in 1% formic acid at a flow rate of 1 mL/min. After passing through the flow cell of the absorbance monitor, the column eluate was split and 50% directed to a Finnigan LCQ Duo mass spectrometer with an electrospray interface (ESI) operating in positive ion mode. Full-scan MS-MS spectra were obtained from 150 to 2000 U.

HPLC Analysis. Anthocyanins in wine were analyzed quantitatively using a 250 × 4.6 mm i.d. 4 μm Synergy RP-Max column (Phenomenex) eluted with a gradient over 60 min of 5−30% acetonitrile in 5% formic acid at a flow rate of 1 mL/min and maintained at 40 °C. Anthocyanins were monitored at 520 nm and identified on the basis of their elution order, retention time, and analysis of absorbance and mass spectra.

RESULTS

The major anthocyanins in 24 wines were initially identified using LC-MS-MS and were subsequently analyzed quantita-

Table 2. Identities, Based on HPLC Retention Times (t_R), λ_{max}, and Tandem MS Fragmentation Data, and Distribution of Major Anthocyanins Found in Red Wines[a]

peak	t_R	λ_{max}	anthocyanin	M^+	fragment 1	fragment 2	fragment 3	wine
A	9.40	518	delphinidin 3,5-diglucoside	627	465 (M^+ – glu)	303 (M^+ – glu – glu)		hybrid A
B	12.25	512	cyanidin 3,5-diglucoside	611	449 (M^+ – glu)	287 (M^+ – glu – glu)		hybrid A
C	13.10	519	petunidin 3,5-diglucoside	641	479 (M^+ – glu)	317 (M^+ – glu – glu)		hybrid A
1	13.10	519	delphinidin 3-glucoside	465	303 (M^+ – glu)			hybrid A; CS
2	nd	nd	cyanidin 3-glucoside	449	287 (M^+ – glu)			CS
D	16.01	525	peonidin 3,5-diglucoside	625	463 (M^+ – glu)	301 (M^+ – glu – glu)		hybrid A
E	16.88	523	malvidin 3,5-diglucoside	655	493 (M^+ – glu)	331 (M^+ – glu – glu		hybrid A
3	19.63	522	petunidin 3-glucoside	479	317 (M^+ – glu)			hybrid A, B; CS
4	22.60	512	peonidin 3-glucoside	463	301 (M^+ – glu)			hybrid A, B; CS
5	23.67	526	malvidin 3-glucoside	493	331 (M^+ – glu)			hybrid A, B; CS
	26.25	519	delphinidin 3-acetylglusoside	507	303 (M^+ – AG)			CS
	30.42	520	delphinidin (*p*-coumaroylglucoside) glucoside	773	611 (M^+ – glu)	303 (M^+ – glu – *p*CG)		hybrid A
	30.95	525	petunidin acetylglucoside	521	317 (M^+ – AG)			hybrid A
	33.67	nd	cyanidin (*p*-coumaroylglucoside) glucoside	757	395 (M^+ – glu)	287 (M^+ – glu – *p*CG)		hybrid A
	33.97	520	petunidin (*p*-coumaroylglucoside) glucoside	787	625 (M^+ – glu)	479 (M^+ – *p*CG)	317 (M^+ – glu – *p*CG)	hybrid A
6	34.25	519	peonidin 3-acetylglucoside	505	301 (M^+ – AG)			hybrid A; CS
7	34.83	529	malvidin 3-acetylglucoside	535	331 (M^+ – AG)			hybrid A, B; CS
	35.93	520	delphinidin 3-(*p*-coumaroyl)glucoside	611	303 (M^+ – *p*CG)			hybrid A
	37.28	528	malvidin (*p*-coumaroylglucoside) glucoside	801	639 (M^+ – glu)	493 (M^+ – *p*CG)	331 (M^+ – glu – *p*CG)	hybrid A
	38.98	513	cyanidin 3-(*p*-coumaroyl)glucoside	595	287 (M^+ – *p*CG)			hybrid A
	39.77	520	petunidin 3-(*p*-coumaroyl)glucoside	625	317 (M^+ – *p*CG)			hybrid A; CS
	40.55	519	malvidin 5-(*p*-coumaroyl)glucoside	639	331 (M^+ – *p*CG)			hybrid A
8	43.02	520	peonidin 3-(*p*-coumaroyl)glucoside	609	301 (M^+ – *p*CG)			hybrid A; CS
9	43.33	529	malvidin 3-(*p*-coumaroyl)glucoside	639	331 (M^+ – *p*CG)			hybrid A, B; CS

[a] t_R, retention tine (min); λ_{max}, maximum wavelength (nm); M^+, molecular ion (U); nd, not detected; glu, glucoside; AG, acetylglucoside; *p*CG, *p*-coumaroylglucoside.

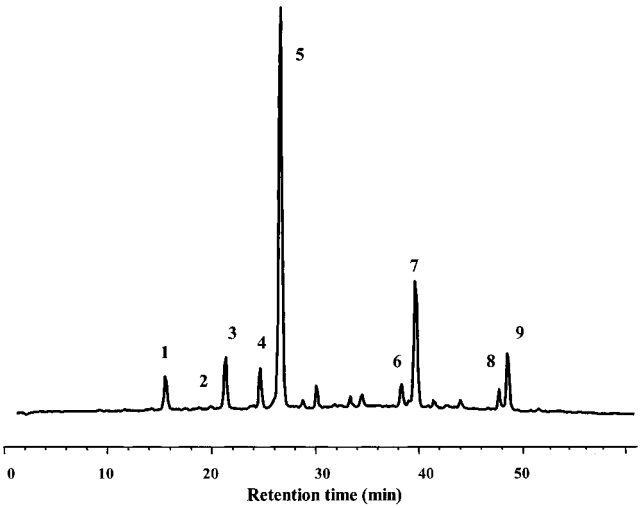

Figure 2. HPLC analysis of nine major anthocyanin peaks in wine 20 (Cabernet Sauvignon, 2000): peak 1, delphinidin 3-glucoside; peak 2, cyanidin 3-glucoside; peak 3, petunidin 3-glucoside; peak 4, peonidin 3-glucoside; peak 5, malvidin 3-glucoside; peak 6, peonidin 3-acetylglucoside; peak 7, malvidin 3-acetylglucoside; peak 8, peonidin 3-(*p*-coumaroyl)glucoside; peak 9, malvidin 3-(*p*-coumaroyl)glucoside.

tively by HPLC with absorbance detection at 520 nm (**Table 2**). Although >20 anthocyanins were identified, many were present in only trace amounts; therefore, for quantitative studies the levels of only the 9 major characteristic anthocyanins were determined in the hybrid and Cabernet Sauvignon wines.

Identification of Anthocyanins for Validation. Five monoglucosides (anthocyanins **1**−**5**) of the anthocyanidins delphinidin, cyanidin, petunidin, peonidin, and malvidin (**Figure 2**) were identified by retention time, elution order, and spectral characteristics and also by using LC-MS-MS to confirm the presence of appropriate M^+ and $M^+ - 162$ U (corresponding to the loss of a glucose moiety). Similarly, the acetylglucose and *p*-coumaroylglucose conjugates of malvidin and peonidin (anthocyanins **6**−**9**) were identified by the loss of 204 U in the case of the acetylglucoside and by the loss of 308 U for *p*-coumaroylglucoside (**Table 2**).

Distribution of Validation Anthocyanins in Wines. Reversed-phase gradient HPLC with absorbance detection at 520 nm was used to quantify the nine major wine anthocyanins, namely, delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, peonidin 3-glucoside, malvidin 3-glucoside, peonidin 3-acetylglucoside, malvidin 3-acetylglucoside, peonidin 3-(*p*-coumaroyl)glucoside, and malvidin 3-(*p*-coumaroyl)glucoside (**Figure 2**).

The relative amounts of individual anthocyanins were expressed as a percentage of the combined levels of the nine major wine anthocyanins (**Table 3**). Although wines 1−20 were all derived from Cabernet Sauvignon grapes and have a similar complement of anthocyanins, the relative amounts of the individual anthocyanins varied markedly. Malvidin 3-glucoside was the major component in all of the wines, but its contribution to the total anthocyanin content ranged from 45.7% (wine 2) to nearly 60% (wine 12). Greater variations are noted with other anthocyanins. Delphinidin 3-glucoside varied 3-fold, from 4.2% in wine 9 to 12.8% in wine 3, whereas the malvidin 3-acetylglucoside content ranged ~3.5-fold from 8.9% in wine 12 to 30.4% in wine 2.

Only 13 of the 24 wines analyzed contained all 9 of the major anthocyanins. All of these wines were made from Cabernet Sauvignon grapes and were from 1998, 1999, or 2000 vintages. In the cases of wines 9 and 12, they contained all of the anthocyanins with the exception of cyanidin 3-glucoside. However, in other wines cyanidin 3-glucoside was only a very minor component compared with the other anthocyanins. Cyanidin 3-glucoside and the acylated and *p*-coumaroylated conjugates of peonidin 3-glucoside were not detected in wine 19 (Cabernet Sauvignon, 1999).

Wine 6 (Cabernet Sauvignon, 1996) contained only malvidin 3-glucoside in detectable quantities, whereas no anthocyanins were detected in wines 4 and 5, which were 1994 and 1993 vintages, respectively (**Figure 3**). With aging, a wine is known to lose its free anthocyanins at the expense of the formation of large, complex condensation products (*8*).

Table 3. Major Anthocyanins in Red Wines Produced from Cabernet Sauvignon and Hybrid Grapes[a]

wine	D-3-G	C-3-G	P-3-G	Pe-3-G	Mv-3-G	Pe-3-AG	Mv-3-AG	Pe-3-CG	Mv-3-CG
1	7.6 ± 0.1	0.6 ± 0.1	6.2 ± 0.1	4.6 ± 0.1	53.4 ± 0.5	2.1 ± 0.1	21.8 ± 0.1	0.5 ± 0.1	3.2 ± 0.1
2	7.0 ± 0.1	0.6 ± 0.0	5.6 ± 0.2	3.1 ± 0.1	45.7 ± 0.5	2.4 ± 0.2	30.4 ± 0.1	0.6 ± 0.1	4.5 ± 0.1
3	12.8 ± 0.0	nd	5.3 ± 0.5	3.2 ± 0.4	51.2 ± 0.4	nd	23.2 ± 0.6	nd	4.2 ± 0.0
4	nd	nd	nd	nd	nd	nd	nd	nd	nd
5	nd	nd	nd	nd	nd	nd	nd	nd	nd
6	nd	nd	nd	nd	100.0 ± 0.0	nd	nd	nd	nd
7	9.4 ± 0.9	1.1 ± 0.1	8.2 ± 0.6	5.9 ± 0.3	54.7 ± 0.9	1.5 ± 0.3	12.3 ± 0.2	1.1 ± 0.2	5.7 ± 0.6
8	6.8 ± 0.4	0.4 ± 0.1	5.7 ± 0.1	3.0 ± 0.1	55.2 ± 0.3	1.7 ± 0.1	22.6 ± 0.3	0.4 ± 0.0	4.2 ± 0.1
9	4.2 ± 0.1	nd	6.5 ± 0.1	5.0 ± 0.2	53.5 ± 0.2	3.1 ± 0.1	18.0 ± 0.0	2.2 ± 0.1	7.5 ± 0.3
10	8.9 ± 0.1	0.8 ± 0.1	6.4 ± 0.1	3.4 ± 0.2	52.0 ± 0.4	1.6 ± 0.1	22.1 ± 0.3	0.5 ± 0.1	4.3 ± 0.5
11	10.2 ± 0.1	1.4 ± 0.2	7.8 ± 0.1	5.8 ± 0.1	50.6 ± 0.5	2.5 ± 0.2	15.9 ± 0.4	1.1 ± 0.1	4.7 ± 0.3
12	8.7 ± 0.2	nd	6.6 ± 0.1	6.6 ± 0.2	59.7 ± 0.5	1.2 ± 0.1	8.9 ± 0.1	1.3 ± 0.2	6.9 ± 0.1
13	5.6 ± 0.2	0.4 ± 0.1	5.1 ± 0.1	2.5 ± 0.1	57.6 ± 0.2	1.5 ± 0.1	22.4 ± 0.2	0.5 ± 0.0	4.3 ± 0.1
14	9.0 ± 0.0	0.6 ± 0.0	6.7 ± 0.0	2.9 ± 0.0	51.4 ± 0.1	1.6 ± 0.0	23.0 ± 0.0	0.7 ± 0.0	4.1 ± 0.1
15	10.9 ± 0.1	0.7 ± 0.2	8.5 ± 0.2	5.4 ± 0.1	54.1 ± 0.9	1.7 ± 0.2	13.8 ± 0.1	0.7 ± 0.1	4.2 ± 0.2
16	10.8 ± 0.5	0.7 ± 0.2	8.9 ± 0.3	6.0 ± 0.1	55.5 ± 1.1	1.3 ± 0.2	9.3 ± 0.2	1.2 ± 0.2	6.2 ± 0.2
17	9.5 ± 0.1	1.3 ± 0.0	7.9 ± 0.2	5.8 ± 0.1	51.7 ± 1.1	2.1 ± 0.3	16.0 ± 0.5	1.3 ± 0.2	4.4 ± 0.2
18	8.2 ± 0.2	0.4 ± 0.1	5.7 ± 0.1	3.4 ± 0.4	53.4 ± 0.1	1.7 ± 0.0	22.6 ± 0.3	0.5 ± 0.1	4.1 ± 0.1
19	6.9 ± 0.8	nd	5.5 ± 0.4	3.9 ± 0.7	51.4 ± 0.2	nd	15.3 ± 0.2	nd	7.0 ± 0.9
20	8.2 ± 0.2	0.5 ± 0.1	6.5 ± 0.1	2.6 ± 0.0	54. 0.3	1.6 ± 0.1	22.0 ± 0.0	0.4 ± 0.1	4.1 ± 0.1
21	35.9 ± 0.1	8.4 ± 0.1	9.0 ± 0.0	2.9 ± 0.0	43.7 ± 0.1	nd	nd	nd	nd
22	36.3 ± 0.5	8.3 ± 0.2	9.1 ± 0.1	3.0 ± 0.1	43.2 ± 0.4	nd	nd	nd	nd
23	2.7 ± 0.1	nd	5.5 ± 0.2	2.5 ± 0.1	84.1 ± 0.2	nd	1.8 ± 0.3	nd	3.3 ± 0.1
24	nd	nd	4.3 ± 0.2	2.3 ± 0.2	93.3 ± 0.3	nd	nd	nd	nd

[a] Quantity of individual anthocyanins expressed as a percentage of the combined levels of all nine anthocyanins: (**1**) delphinidin 3-glucoside (D-3-G); (**2**) cyanidin 3-glucoside (C-3-G); (**3**) petunidin 3-glucoside (P-3-G); (**4**) peonidin 3-glucoside (Pe-3-G); (**5**) malvidin 3-glucoside (Mv-3-G); (**6**) peonidin 3-acetylglucoside (Pe-3-AG); (**7**) malvidin 3-acetylglucoside (Mv-3-AC); (**8**) peonidin 3-(*p*-coumaroyl)glucoside (Pe-3-CG); (**9**) malvidin 3-(*p*-coumaroyl)glucoside (Mv-3-CG). nd, not detected.

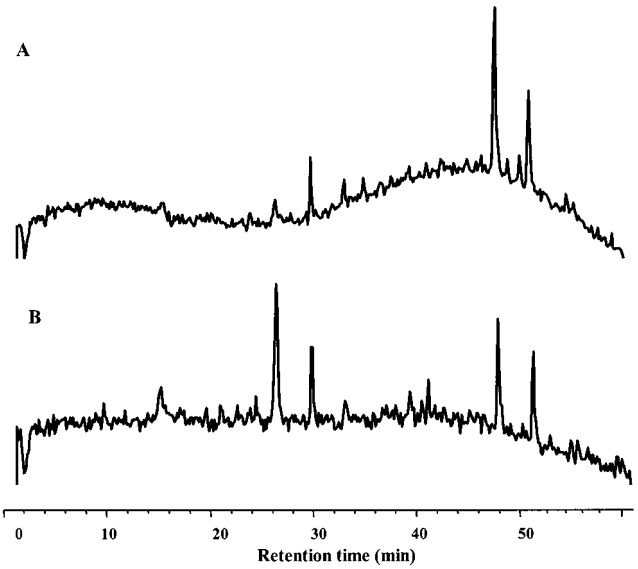

Figure 3. HPLC analysis of the anthocyanins in aged wines: (A) wine 4, Cabernet Sauvignon, 1994; (B) wine 6, Cabernet Sauvignon, 1996.

Table 4 contains information on the overall levels, as a percentage of total anthocyanin content, of anthocyanidin monoglucosides (anthocyanins **1**−**5**), acetylated anthocyanins (anthocyanins **6** and **7**), *p*-coumaroyl conjugates (anthocyanins **8** and **9**), acetylated and *p*-coumaroyl anthocyanidin glucoside conjugates (anthocyanins **6**−**9**), and finally the ratio of the acetylated to *p*-coumaroyl conjugated anthocyanidins. It has been proposed that for a wine to be identified as a genuine Cabernet Sauvignon, the ratio of acetylated to *p*-coumaroylated anthocyanins must be >3, which distinguishes it from wines derived from hybrid grapes (*6*). However, the ratio of acetylated to *p*-coumaroylated anthocyanins in the Cabernet Sauvignon wines analyzed in the current study varied from 1.2 (wine 12) to 6.5 (wine 1), whereas values for the hybrid wines were in the region of 0.4 (**Table 4**).

Table 4. Relative Amounts of the Main Groups of Anthocyanins in Red Wine Produced from Cabernet Sauvignon and Hybrid Grapes[a]

wine	anthocyanin monoglucosides	acetylated anthocyanins	*p*-coumaroyl anthocyanins	acetylated and *p*-coumaroyl anthocyanins	ratio *p*-coumaroylated to acetylated
1	72.4 ± 0.3	23.9 ± 0.2	3.7 ± 0.2	27.6 ± 0.5	6.5
2	62.0 ± 0.5	32.9 ± 0.3	5.1 ± 0.2	38.0 ± 0.5	6.4
3	72.5 ± 0.6	23.2 ± 0.6	4.2 ± 0.0	27.5 ± 0.6	5.5
4	nd	nd	nd	nd	
5	nd	nd	nd	nd	
6	100.00	nd	nd	nd	
7	79.4 ± 0.7	13.8 ± 0.5	6.8 ± 0.8	20.6 ± 1.3	2.0
8	71.1 ± 0.4	24.4 ± 0.4	4.5 ± 0.1	28.9 ± 0.5	5.4
9	69.3 ± 0.3	21.1 ± 0.1	9.6 ± 0.4	30.7 ± 0.5	2.2
10	71.5 ± 0.6	23.7 ± 0.4	4.7 ± 0.6	28.5 ± 1.1	5.0
11	75.9 ± 0.2	18.4 ± 0.6	5.7 ± 0.4	24.1 ± 1.0	3.2
12	81.7 ± 0.2	10.1 ± 0.2	8.2 ± 0.2	18.3 ± 0.5	1.2
13	71.3 ± 0.3	23.9 ± 0.3	4.8 ± 0.2	28.7 ± 0.5	5.0
14	70.6 ± 0.1	24.6 ± 0.0	4.8 ± 0.1	29.4 ± 0.1	5.1
15	79.6 ± 0.3	15.6 ± 0.3	4.9 ± 0.3	20.4 ± 0.6	3.2
16	72.4 ± 0.5	10.5 ± 0.3	6.2 ± 0.1	16.7 ± 0.5	1.7
17	76.2 ± 1.2	18.1 ± 0.8	5.7 ± 0.4	23.8 ± 1.2	3.1
18	71.1 ± 0.3	24.3 ± 0.3	4.6 ± 0.2	28.9 ± 0.5	5.3
19	77.7 ± 1.1	15.3 ± 0.2	7.0 ± 0.9	22.3 ± 1.1	2.2
20	71.9 ± 0.3	23.6 ± 01	4.5 ±0.2	28.1 ± 0.3	5.3
21	94.5 ±0.1	1.6 ± 0.0	3.9 ± 0.0	5.5 ± 0.1	0.4
22	94.8 ± 0.3	1.6 ± 0.1	3.6 ± 0.2	5.2 ± 0.3	0.4
23	94.9 ± 0.2	1.8 ± 0.3	3.3 ± 0.1	5.1 ± 0.4	0.6
24	100	nd	nd	nd	nd

[a] No anthocyanidins were detected in wines 4 and 5. Anthocyanin monoglucosides: delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, peonidin 3-glucoside, malvidin 3-glucoside. Acylated anthocyanins: peonidin 3-acetylglucoside, malvidin 3-acetylglucoside. *p*-Coumaroylated anthocyanins: peonidin 3-(*p*-coumaroyl)glucoside, malvidin 3-(*p*-coumaroyl)glucoside. nd, not detected.

LC-MS-MS Identification of Anthocyanins in Hybrid Wines. Although only the relative levels of the 9 major anthocyanins were used for Cabernet Sauvignon validation, many more anthocyanins were identified in the 24 wines analyzed, particularly hybrid A (**Table 2**). In contrast, hybrid B contained relatively few anthocyanins (**Figure 4**).

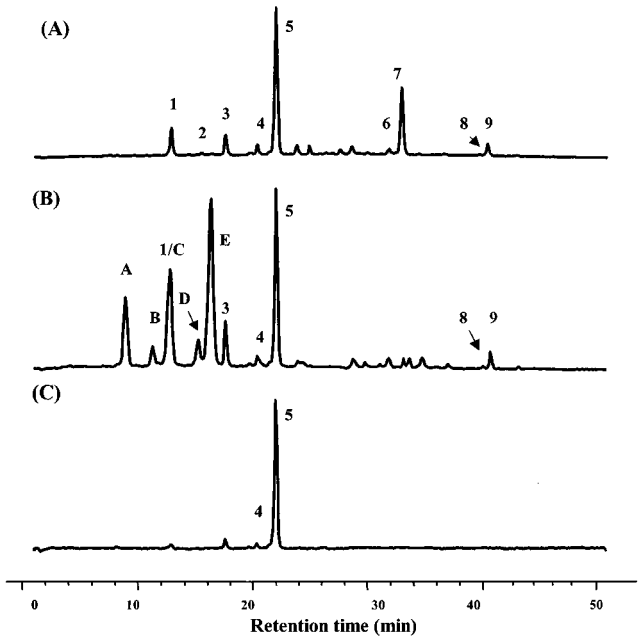

Figure 4. HPLC analysis of the anthocyanins in aliquots of (A) Cabernet Sauvignon wine 20, (B) hybrid wine A, and (C) hybrid wine B. Peaks are labeled according to **Table 2**.

Diglucosides. The most striking feature of hybrid A wines (**Figure 4**) is the presence of delphinidin 3,5-diglucoside, cyanidin 3,5-diglucoside, petunidin 3,5-diglucoside, peonidin 3,5-diglucoside, and malvidin 3,5-diglucoside (peaks A−E). These compounds were identified on the basis of their MS-MS fragmentation pattern, their absorbance spectra, and HPLC elution order. In each case the M^+ fragmented sequentially to give ions corresponding to the monoglucoside and the aglycon (**Table 2**). This pattern of fragmentation suggests that the two glucosides are attached to the anthocyanidin moiety at different positions. If the sugars had been attached sequentially at the same position, it is unlikely that they would have ionized to produce a fragment corresponding to a monoglucoside intermediate. The compounds were tentatively identified as 3,5-diglucosides on the basis of a review of the literature (*3*), which indicates that this is the most common configuration for anthocyanin diglucosides in red wines.

Acylated Anthocyanins. The presence of acetylated and *p*-coumaroylated conjugates of malvidin and peonidin has been widely reported, and these compounds were among the nine major anthocyanins quantified for validation purposes (*6*). However, hybrid A was also found to contain a range of acylated mono- and diglucosides. Although acylation of a monoglucoside occurs preferentially on the glucose moiety at the C3 position, hybrid A also appears to contain an anthocyanin acylated on the C5 glucose and other diglucosides where only one sugar has been acylated (**Table 2**).

Three *p*-coumaroylated diglucosides were detected: delphinidin (*p*-coumaroylglucoside) glucoside (t_R = 30.42 min), petunidin (*p*-coumaroylglucoside) glucoside (t_R = 33.97 min), and malvidin (*p*-coumaroylglucoside) glucoside (t_R = 37.28 min). These compounds presented a complex fragmentation pattern. The cyanidin and petunidin conjugates each produced an M^+ ion that fragmented to produce ions corresponding to the loss of glucose and *p*-coumaroylglucose. For example, petunidin (*p*-coumaroylglucoside) glucoside (**Table 2**) has an M^+ at 787 U, which yields fragment ions at 625, 479, and 317 U. These correspond to a loss of a glucose (M − 162), a *p*-coumaroylglucose group (M − 308), and both glucose and *p*-coumaroylglucose (M − 470). Delphinidin and cyanidin (*p*-coumaroylglucoside) glucosides are present at much lower concentrations than petunidin *p*-coumaroylated diglucoside, and the delphinidin and cyanidin glucose intermediates were not detected. These compounds have been previously described in Concord grape juice (*9*).

Two malvidin conjugates were detected (40.55 and 43.33 min), which share the same ionization pattern (**Table 2**). As the compound eluting at 43.33 min is known to be malvidin 3-*p*-coumaroylglucoside, the earlier eluting peak is tentatively identified as malvidin 5-*p*-coumaroylglucoside, but further work is necessary to confirm this assignment.

Distribution of Anthocyanins in Hybrid Wines. The anthocyanin profiles of both of the hybrid wines were very different from each other and also from that of Cabernet Sauvignon (**Figure 4**). The 3-glucosides of delphinidin, petunidin, peonidin, and malvidin (anthocyanins **1** and **3**−**5**) were detected in both example wines produced from hybrid grapes (**Figure 4**). Compared with Cabernet Sauvignon wines, hybrid A had a proportionally higher anthocyanin monoglucoside content. It also contained a number of earlier eluting peaks (anthocyanins **A**−**E**, **Figure 4B**), which were identified as diglucosides (**Table 2**). None of these compounds were present in detectable quantities in either the Cabernet Sauvignon or hybrid B wines (**Figure 4A**,**C**). Likewise, the acetylated and *p*-coumaroylated anthocyanins were not present in the hybrid B wines at quantifiable amounts, although trace levels of some of these derivatives were detected by LC-MS-MS (**Table 2**).

DISCUSSION

Of 20 Cabernet Sauvignon wines analyzed, only 12 contained an acetylated to *p*-coumaroylated anthocyanin ratio of >3, which has been proposed to be the defining feature of Cabernet Sauvignon wines (*6*). The acetylated to *p*-coumaroylated anthocyanin ratio ranged from 1.2 in wine 12 to 6.5 in wine 1 (**Table 4**). Of the wines known to be produced from Cabernet Sauvignon grapes, five had a ratio of <3 (wines 7, 9, 12, 16, and 19). These wines would, therefore, have failed the Cabernet Sauvignon validation criteria of Marx et al. (*6*) and been rejected as adulterated or misrepresented. This casts serious doubt on the validity and rigorousness of the ratio method.

There are a number of reasons why the Cabernet Sauvignon-derived wines may have failed the validation test. The aim of the test was to confirm that the anthocyanin profile of a wine is closely related to that of the purported grape of origin. However, vinification, the process of producing wines from grapes, involves extracting anthocyanins and other phenolics from the grapes and subjecting them to changes in acidity, temperature, and alcohol content. Each of these processes would be expected to influence the anthocyanin profile and content of the resulting must and alter it from that of the original grape.

Indeed, although grape skins contain significant levels of petunidin and delphinidin and lesser amounts of cyanidin and peonidin, in addition to malvidin, 7 months into vinification the malvidin-derived anthocyanins contribute 85% of total anthocyanins in wine (*3*). The major anthocyanin derivatives are malvidin 3-*O*-glucoside, malvidin 3-*O*-(6-*O*-acetyl)glucoside, and malvidin 3-*O*-(6-*O*-*p*-coumaroyl)glucoside (*3*). Anthocyanins are rapidly extracted into the wine from grape skins, reaching a maximum by day 3 and decreasing thereafter (*10*). The falling levels of anthocyanins are due to the formation of complexes with other phenolics (*8*) as opposed to their degradation or low extraction efficiency.

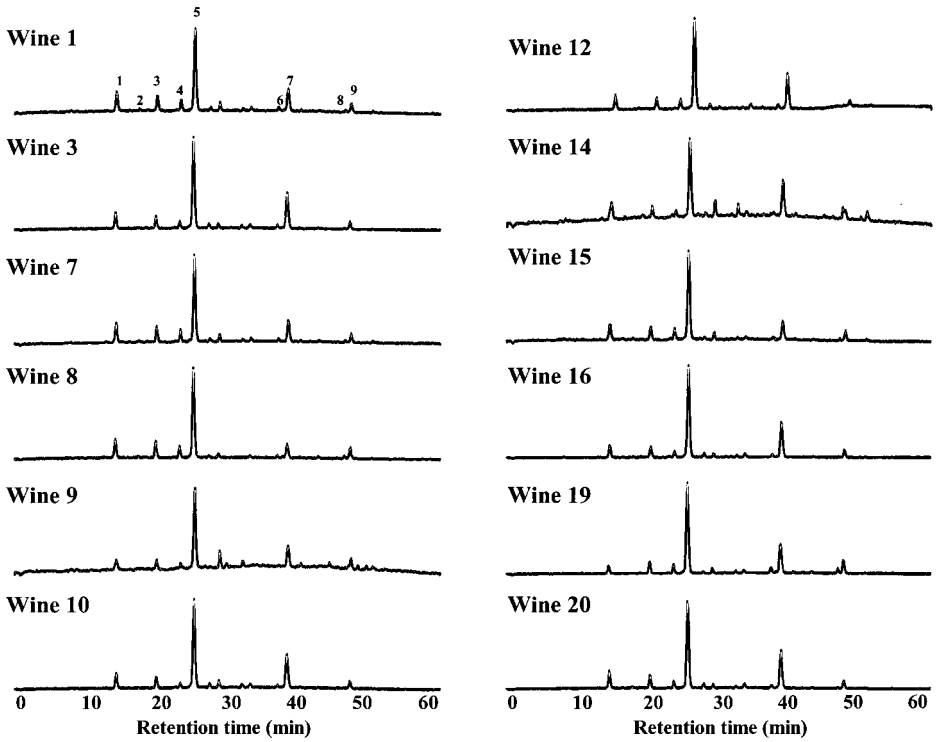

Figure 5. HPLC analysis of the anthocyanins in a selection of 12 Cabernet Sauvignon wines. Peaks are labeled according to **Table 2**.

The temperature maintained during vinification has been shown to affect the final concentration of anthocyanins in a wine (*11*). For example, thermovinification makes use of a particularly high temperature for a short period prior to fermentation. It is reported to increase the extraction of tannins and stabilize wine color. The concentrations of anthocyanins in wine have been shown to increase after thermovinification (*12*), but this is not sustained and the levels subsequently decrease (*13*).

Those Cabernet Sauvignon wines with a ratio of acetylated to *p*-coumaroylated anthocyanins of <3 may have undergone treatments during vinification that retarded the extraction of anthocyanins. Similarly, the age of a wine has a huge influence on the phenolic profile. Whereas a young wine contains many anthocyanins (**Figure 2**), as it ages these smaller units condense with other phenolics and form large polymeric pigments (*8*). These produce a characteristic, broad underlying peak that greatly increases the background noise of a gradient HPLC trace (**Figure 3**). The polymers absorb around 420 nm, accounting for the maturing of the color of a wine from a vivid purple/red to orange/brown. The identity and chemical nature of many of these complex pigments remain unknown, although malvidin−catechin dimers have been reported in model wine solution and also wine (*14*). These complexes may form from acylated anthocyanins, resulting in a decline in the level of the monomeric parent compounds and, in due course, a fall in the ratio of acetylated to *p*-coumaroylated anthocyanins in some Cabernet Sauvignon wines to values of <3.

It has been shown here that validation of wines using the ratio approach is severely flawed, as the level of phenolics, including anthocyanins, in wines is dependent on so many factors. Comparison of the chromatograms of wines 4 and 6 (**Figure 3**), which were a 1994 and a 1996 vintage, respectively, with a young wine, wine 20 (**Figure 2**), highlights the effect of aging on the anthocyanin profile of red wine. Consequently, any approach to validation making use of anthocyanins would have to be carried out on a new or very young wine. However, ratios of <3 were also found in wines from 1998, 1999, and 2000 vintages. These wines would have had little chance to undergo condensation reactions between anthocyanins and other phenolics, suggesting that the low levels of acylated anthocyanins may be due to other influences during viticulture or vinification.

The anthocyanin profile of Cabernet Sauvignon wines is well-defined (**Figure 5**) and is generally dominated by monoglucosides (**Table 4**). In contrast, the acetylated and, particularly, the *p*-coumaroylated anthocyanins quantified in the ratio validation method are minor components. The major difference observed between the anthocyanin profiles of Cabernet Sauvignon, hybrid A and B wines is the presence and absence of individual anthocyanins. Hybrid B wines contained only malvidin 3-glucoside, whereas hybrid A wines were characterized by the presence of significant levels of diglucosides (anthocyanins **A**−**E**, **Figure 4**). In a bid to determine a more robust approach to validating the identity of wine, it was noted that hybrid wines had an anthocyanin monoglucoside content of >90%, whereas in Cabernet Sauvignon wines it was <90%. Likewise, the proportion of acetylated anthocyanins in Cabernet Sauvignon wines was >5%, and it was <2% for hybrid wines. It is proposed that examination of these two values is a more realistic quantitative assessment than the ratio approach. If a wine fails this test, then a full audit of its anthocyanin profile is required prior to any decision on its grape origin and validity of labeling.

The failure of the ratio method of Marx et al. (*6*) to confirm the identity of pure Cabernet Sauvignon wine casts considerable doubt on the continued use of this approach in such a lucrative industry. Examination of the entire anthocyanin profile of a wine in parallel with a quantitative assessment of the proportion of monoglucosides and acetylated anthocyanins appears to be a more thorough and reliable approach. However, this study highlights the need for further extensive analysis of the anthocyanin profile of Cabernet Sauvignon, and other grape varieties, from different geographical and enological origins.

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