Adsorption of Anthocyanins by Yeast Cell Walls during the Fermentation of Red Wines

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This paper reports the anthocyanin adsorption profiles of the cell walls of different *Saccharomyces* strains isolated from grapes collected in the Spanish *appellation controlée* regions of La Rioja, Navarra, and Ribera del Duero. These strains are habitually used in red wine-making. The acyl derivatives of anthocyanins (acetyl and *p*-coumaryl compounds) were more strongly adsorbed than nonacyl derivatives. Peonidin-3G was also strongly adsorbed, as were its acyl derivatives. The greater presence of acetyl derivatives in the cell wall adsorbate leads to an increase in yellow color and a reduction in blue color with respect to the corresponding wine.

KEYWORDS: Anthocyanins; *Saccharomyces*; cell walls; color; HPLC-DAD.

INTRODUCTION

The making of red wine involves maceration of the grape skins during alcoholic fermentation. The aim is that pigments in the skins spread throughout the must. However, some of the anthocyanins released are retained by the yeast cell walls and are therefore lost to the wine when the lees is removed.

The cell wall of *Saccharomyces cerevisiae* is made of mannoproteins bound to oligopolysaccharides, which remain exposed on the outside of the cell. These mannoproteins are also bound to glucanose and chitin (*1,2*). The different polarities and the hydrophilic or hydrophobic nature of these wall polymers define the capacity of yeast to retain or adsorb different wine molecules such as volatile compounds (*3*), fatty acids (*4*), or pigments (*5*).

The porosity of the wall also influences adsorption (*6, 7*). An increased surface area provided by interstitial spaces favors adsorption. The surface area of yeast cells in fermenting wine is greater than 10 m²/L of must, and the quantity of anthocyanins adsorbed during fermentation by the different generations of yeast can be very large.

Given the importance of color to red wines, and the quantity of pigments lost when the lees is removed, the aim of this work was to investigate the adsorption of anthocyanins by yeast cell walls during wine-making: (1) to determine whether there are any differences between yeasts strains, and (2) to determine the degree of retention of different anthocyanins.

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MATERIALS AND METHODS

Fermentation. Ten small scale fermentations were established with 5 L of crushed grapes (*Vitis vinifera* L. cv. Cabernet-Sauvignon) to produce wine with an alcohol level of 13.5% v/v and a pH of 3.6. Each must was inoculated with 150 mL of YEPD medium, containing a population of 10^8 cfu/mL of one of the 10 yeast strains (see below). The 10 inocula were synchronized to produce homogeneous populations.

Fermentation was monitored by taking readings of density and temperature and was considered complete when a score of 50 was attained on the Folin-Ciocalteu Index *(8)* (measurement of total polyphenol levels) and no sugar could be detected *(9)*.

Anthocyanins Absorbed by Cell Walls. After fermentation, the skins and seeds were separated from the wines plus lees, leaving 3-L volumes of each of the latter. The lees at the lower part of each deposit was then separated and made up to 225 mL with the corresponding wine. Because this volume of lees corresponded to the 3 L of each fermentation, 75 mL of this suspension corresponded to 1 L of wine. Adsorbed anthocyanins were recovered from 12.5 mL aliquots of these 75 mL suspensions (i.e., from one-sixth of the lees produced per liter of wine). These were washed with 10 mL of distilled water and then centrifuged at 8000 rpm at 4 °C for 5 min. The supernatant was discarded. This was performed twice, to eliminate any remnants of wine. The adsorbed anthocyanins were then extracted by three washes with 10 mL of formic acid/methanol (10:90 v/v) agitating with a Vortex for 30 s. Centrifugation at 8000 rpm followed each wash, and the supernatant was kept. The last 30 mL of solvent, filtered through 0.45 μm filter polyvinylidene fluoride membranes (Millipore, Ireland), as well as samples of the finished wines (also filtered with 0.45 μm membranes) were analyzed spectrophotometrically to determine color and by HPLC-DAD to evaluate their anthocyanin content.

To verify that the extraction of anthocyanins was complete, the absorbance of the second wash with formic acid/methanol (10:90 v/v) was measured at 530 nm in a cuvette with a 1-cm path length. This absorbance was 10% less than the initial reading. The UV−visible

Yeast Cell Wall Anthocyanin Adsorbtion

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Table 1. Mean Total Anthocyanin Content and Derivatives in Wines (mg/L), and Cell Wall Adsorbates (mg) in Lees, Corresponding to 1 L of Wine[a]

sample	anthocyanins	3G	PY	6AC	6CAF	6CM	totals
wines	mean	254.15	12.56	140.49	2.38	20.89	430.47
	sd	19.19	2.26	10.80	0.30	3.35	32.33
	%	59.04	2.92	32.64	0.55	4.85	100.00
adsorbates	mean	5.95	0.06	3.58	0.25	3.39	13.23
	sd	1.26	0.02	0.82	0.09	0.72	2.80
	%	44.96	0.42	27.08	1.91	25.64	100.00
	% adsptn	2.34	0.44	2.55	10.59	16.24	3.07

[a] Percentage of adsorption.

spectrum (300−700 nm) of the third wash showed no absorption at 500−550 nm above background.

Yeasts Used in Experimental Fermentations. The strains of yeast used in the experimental fermentations all belonged to *Saccharomyces cerevisiae*, the species habitually employed in red wine-making. They were isolated from grapes collected in the Spanish *apellation controlée* regions of La Rioja (4CV, 5CV, and 9CV), Navarra (7EV, 2EV, and 1EV), and Ribera del Duero (3VA, 1VA, and 7VA). All these strains continue fermentation at above 16% v/v of probable alcohol, have a volatile acidity production of less than 0.3 g/L expressed in acetic acid, produce more than 8 g/L glycerine, are resistant to total SO_2 levels of above 200 mg/L, and produce low levels of SH_2. The commercial yeast S6U (*Saccharomyces uvarum*) (Lallemand Inc., Canada) was also used.

Percentage Color Intensities. Absorbance by the wine at 420, 520, and 620 nm was determined using a DU 70 Beckman Spectrophotometer with a 1 mm path length quartz cell, following the Glories procedure *(10)*. The percentages of red (R), yellow (Y), and blue (B) were recorded.

Analysis of Anthocyanins by Liquid Chromatography. The anthocyanins contained in the 10 wines produced (one from each yeast strain) and in the cell wall adsorbates of the corresponding yeasts were analyzed using a Waters (Milford, MA) HPLC chromatograph equipped with a 600-MS controller, a 717 plus autosampler, and a 996 photodiode-array detector. Gradients of solvent A (water/formic acid, 90:10, v/v) and solvent B (water/methanol/formic acid, 45:45:10, v/v/ v) were used in a reverse-phase Nova-pack C_{18} column (150 × 3.9 mm) as follows: 15−80% B linear (0.8 mL/min) from 0 to 30 min, 80% B isocratic (0.8 mL/min) from 30 to 43 min and washing (100% methanol), plus reequilibration of the column from 43 to 75 min. Detection was performed by scanning from 260 to 600 nm. Quantification was performed against an external standard at 530 nm and expressed as a function of malvidin-3-glucoside chloride concentration. Samples (100 μL) of previously filtered wines and samples (200 μL) of cell wall adsorbates were injected into the HPLC. Determinations were made in duplicate.

The following anthocyanins were identified in both wines and cell wall adsorbates: delphinidin (D), cyanidin (Cy), petunidin (Pt), peonidin (Pn), and malvidin (M), as well as their 3-glucoside (3G), pyruvic (Py), acetyl glucoside (6Ac), cinnamoyl (*p*-coumaryl (6Cm), and caffeyl (6Caf)) glucoside derivates.

The different anthocyanins were identified by their retention time relative to that of the majority anthocyanin in *Vitis Vinifera* L., malvidin-3-O-glucoside, and from their UV−visible absorption spectra.

Correlation analysis was performed to establish relationships between variables. All calculations were made using the PC Statgraphics 5.0 software package (Graphics Software Systems, Rockville, MD).

RESULTS AND DISCUSSION

Anthocyanin Profiles in Wines and Yeast Cell Wall Adsorbates. *Adsorption Capacity.* The mean total anthocyanin content of the wines fermented with the 10 yeast strains was 430.47 mg/L. The mean total adsorbate anthocyanin content was 13.23 mg in the lees, corresponding to the fermentation of 1 L of wine (**Table 1**). The distribution of anthocyanins was very

different between the wines and the cells walls. In general, in the wines (**Figure 1a**), anthocyanins glycosylated at position 3 (3G) were the most common (59.04%), followed by acetyl derivatives (6Ac) (32.64%), then cinnamoyl derivatives at 5.40% and pyruvic derivatives at 2.92% (**Table 1**). This is the normal distribution for Cabernet-Sauvignon wines *(11)*. The glycosylated, acetyl, and pyruvic derivatives were slightly less common in the adsorbates than in the wines. However, the quantities of *p*-coumaryl and caffeyl derivatives were very much greater (**Figure 1b**).

Table 1 shows the ratio of each anthocyanin family adsorbed onto the cells' walls/in the wine (expressed as a percentage of adsorption). Glycosylated anthocyanins are the most common both in grapes and the wine. This is logical, because glycosylation is a necessary step for the stability and mobility of anthocyanins. Later, some of the glycosyl derivatives are acylated by acyltransferases *(12)*. However, the percentage adsorption of the acylated derivatives (acetyl, caffeyl, and *p*-coumaryl) is much greater than that of the nonacylated derivates (3G).

The elution order of the reverse phase HPLC column was glycosylated anthocyanins, followed by acetyl derivatives and then cinnamoyl derivatives (caffeyl and *p*-coumaryl derivatives) *(11, 13)* (i.e., in decreasing order of polarity). This is similar to the adsorption profile of the cell wall *p*-coumaryl derivatives, followed by their caffeyl and then acetyl derivatives (i.e., the same order of polarity).

This greater affinity for more apolar acyl derivatives, which are retained longer in the reverse phase column, indicates that, with respect to anthocyanins, the cell wall behaves in a more apolar and hydrophobic fashion than the solvent (wine). This agrees with the results obtained by Lubbers et al. *(3),* who indicate a greater fixing capacity of volatile hydrophobic compounds of yeast cell walls in their study on the adsorption of aromatic compounds. However, it disagrees with the results of Vasserot et al. *(5)*, who, in a study of the five monoglycoside anthocyanins, report a greater percentage adsorption of hydrophilic anthocyanins (delphinidin and petunidin).

The detailed anthocyanin profiles (**Tables 2** and **3**) show that, quantitatively, malvidin-3G and its acylated derivatives are those most adsorbed. Following these compounds at some distance are peonidin-3G, petunidin-3G, and delphinidin-3G (and their derivatives). Cyanidin-3G adsorption was not detected. Within each family, the anthocyanin fixation model shows greater retention of the more apolar anthocyanins (**Figure 2**). The five anthocyanins found in *Vitis vinifera* (delphinidin, cyanidin, petunidin, peonidin, and malvidin − all of which are hydroxylated at the 4′ position) are different in their degree of hydroxylation or methylation of the B ring (positions 3′ and 5′). The most polar is delphinidin (2 hydroxylations), the least polar is malvidin (two methoxylations).

The nonadsorption of cyanidin glycoside might be explained by its low initial concentration in the wine. It is also the starting anthocyanin for the formation of all others through the action of flavonyl-3-hydroxylase and methyl-transferase. Further, because of its highly hydrophilic nature, what is available is more likely to remain in the wine than to enter the yeast cell walls.

The acyl derivatives of all anthocyanins were those most strongly adsorbed, especially the *p*-coumaryl and acetyl derivatives of peonidin and malvidin. However, it is important to point out that there is, in fact, a much greater concentration of
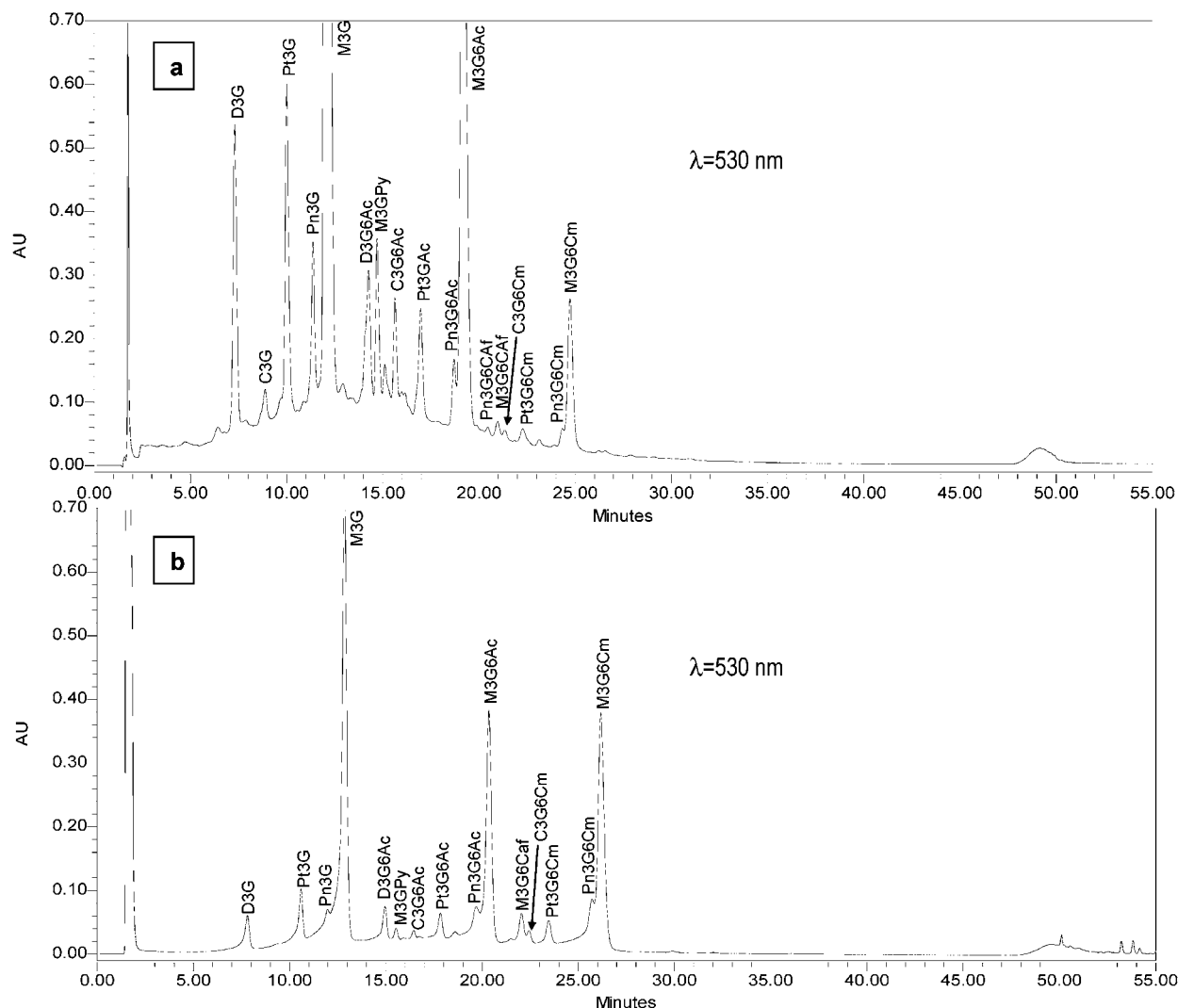
Figure 1. HPLC-DAD chromatograms for strain 7VA. (a) Anthocyanins in wine. (b) Anthocyanins adsorbed by the cell wall. λ 530 nm.

Table 2. Anthocyanin Content in Wines Fermented by the Different Yeast Strains (mg/L)

	D3G	C3G	Pt3G	Pn3G	M3G	Pn3GPy	D3G6Ac	M3GPy	Pt3G6Ac	Pn3G6Ac	M3G6Ac	Pn3G6Caf	M3G6Caf	Pn3G6Cm	M3G6Cm
4CV	22.37	2.78	26.77	9.77	176.87	0.92	13.95	11.99	10.43	5.38	100.03	0.45	2.10	1.48	16.13
5CV	29.05	2.89	30.62	10.83	198.16	1.04	15.18	12.43	11.80	5.77	113.39	0.47	1.99	2.00	19.73
9CV	21.85	2.79	26.90	9.31	178.03	1.03	14.08	12.36	10.55	5.53	102.14	0.46	2.17	1.44	16.53
7EV	29.30	2.84	31.14	11.45	206.64	1.12	16.07	13.56	12.87	6.17	120.59	0.52	1.92	1.90	22.62
2EV	22.81	2.55	25.67	10.44	185.90	0.80	13.06	10.89	10.26	5.78	104.81	0.40	1.89	1.48	16.63
1EV	24.70	2.03	26.78	9.31	189.34	0.62	14.60	11.38	10.10	8.13	106.17	0.47	1.30	2.26	18.25
3VA	30.12	2.47	30.39	11.38	199.00	0.61	15.16	7.20	11.85	6.80	119.17	0.72	1.86	2.46	23.69
1VA	27.58	2.66	30.86	11.13	197.36	0.85	16.40	9.70	11.96	7.87	117.25	0.74	1.95	2.22	22.91
7VA	24.00	3.34	27.64	10.47	182.08	1.01	15.41	14.99	10.92	5.48	103.85	0.49	1.48	1.75	18.17
S6U	20.19	2.49	23.71	9.32	165.39	0.91	12.87	12.16	9.80	5.34	97.96	0.56	1.90	1.77	15.49
Mean	25.20	2.68	28.05	10.34	187.88	0.89	14.68	11.67	11.05	6.23	108.54	0.53	1.86	1.88	19.02
% CV	14.0	12.7	9.1	8.3	6.7	19.5	8.1	18.2	9.1	16.6	7.7	21.7	14.3	19.0	16.1

malvidin derivatives in wine, meaning that a smaller percentage of them are actually adsorbed, compared to those of peonidin (**Figure 3**).

There was very little adsorption of the caffeyl and pyruvic derivatives of peonidin, possibly because of their low content in wine (which might make their quantitative detection in the cell walls impossible). Further, pyruvic derivatives are mostly formed from the pyruvate made during alcoholic fermentation; their presence increases after removal of the lees.

The coefficient of variation (CV) for each anthocyanin (**Tables 2** and **3**) differed depending upon the yeast strain used

for fermentation, meaning that the structure of their cell wall differs. In the wines (**Table 2**), strain identity had more influence on the final concentration of the monoglycosides of the most hydroxylated anthocyanin (i.e., delphinidin) and less influence on the most methoxylated (i.e., malvidin). No such differences are seen with respect to acyl derivatives, although the CV of cinnamoyl derivatives is slightly higher. The peonidin derivatives show the widest ranging CVs.

Among the anthocyanins adsorbed by the cell walls (**Table 3**), the same can be said for the CVs of monoglycosylated derivatives, although the least hydroxylated anthocyanins show

Table 3. Anthocyanin (mg) Adsorbed by Cell Walls of Yeast that Fermented 1 L of Wine

|	D3G	C3G	Pt3G	Pn3G	M3G	Pn3GPy	D3G6Ac	M3GPy	Pt3G6Ac	Pn3G6Ac	M3G6Ac	Pn3G6Caf	M3G6Caf	Pn3G6Cm	M3G6Cm
4CV	0.32	nd	0.65	0.61	4.45	nd	0.36	0.10	0.37	0.55	2.80	nd	0.34	0.65	2.75
5CV	0.35	nd	0.60	0.53	4.08	nd	0.32	0.07	0.32	0.51	2.53	nd	0.29	0.71	2.49
9CV	0.28	nd	0.55	0.52	3.77	nd	0.25	0.07	0.30	0.48	2.37	nd	0.09	0.53	2.39
7EV	0.29	nd	0.64	0.73	4.49	nd	0.18	0.05	0.43	0.62	2.70	nd	0.24	1.09	2.86
2EV	0.52	nd	0.73	0.83	6.73	nd	0.36	0.04	0.31	0.48	3.72	nd	0.42	0.65	4.13
1EV	0.28	nd	0.67	0.68	4.85	nd	0.33	0.07	0.37	0.56	2.87	nd	0.27	0.80	3.15
3VA	0.15	nd	0.53	0.63	3.87	nd	0.19	0.03	0.28	0.42	1.97	nd	0.13	0.58	1.99
1VA	0.27	nd	0.62	0.63	4.27	nd	0.29	0.04	0.26	0.46	2.32	nd	0.23	0.99	2.30
7VA	0.33	nd	0.66	0.66	4.78	nd	0.34	0.08	0.23	0.58	2.56	nd	0.29	0.64	2.92
S6U	0.16	nd	0.36	0.41	2.98	nd	0.16	0.03	0.12	0.17	1.38	nd	0.24	0.66	1.68
Mean	**0.30**		**0.60**	**0.62**	**4.43**		**0.28**	**0.06**	**0.30**	**0.48**	**2.52**		**0.25**	**0.73**	**2.66**
% CV	34.50		16.82	18.72	22.08		28.22	44.49	29.07	25.81	24.12		37.41	24.47	25.52
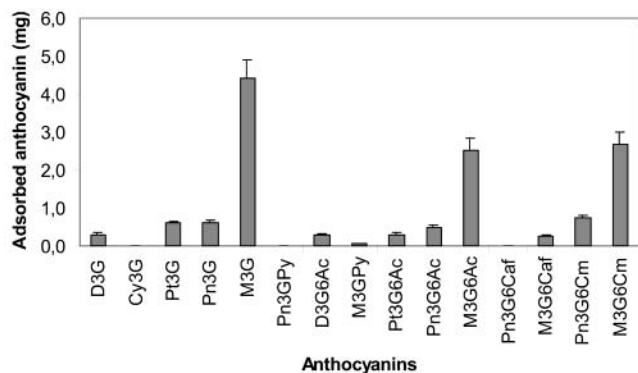
Figure 2. Mean anthocyanin content of cell walls (cells that have fermented 1 L of wine).
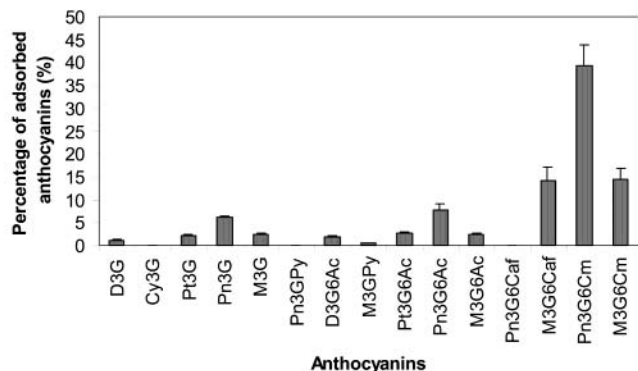

Figure 3. Percentage of adsorbed anthocyanins. Relationship between anthocyanins adsorbed by the lees that fermented 1 L of wine and wine anthocyanin concentration.

higher CVs than in the wine. With regard to acetyl derivatives, cell wall values were similar (though slightly higher) than those for wine, independent of their degree of hydroxylation. The different CVs for the cinnamoyl derivatives appear to confirm that yeast cell walls have different structures, especially when the values for malvidin acylated with caffeic acid (CV 37.5) are compared with those of malvidin acylated with *p*-coumarylic acid (CV 25.5). The greater hydroxylation of caffeic acid may be compensated by the formation of a hydrogen bond between the two hydroxy groups in ortho (positions 3′ and 4′).

The pyruvic derivatives in the wines and the cell walls show higher CV values; although in the latter, no adsorption of peonidins was detected.

Figure 2 shows the mean anthocyanin content (mg) absorbed by cell walls corresponding to the fermentation of 1 L of wine. The most adsorbed anthocyanin is malvidin and its derivatives,

Table 4. Mean Intensity, Tonality, and Percentage Color in Wines and Cell Wall Adsorbates for the Ten Yeast Strains

		intensity	tonality	%Y	%R	%B
wines	mean	2.48	0.51	30.23	59.71	10.06
	%CV	6.1	3.5	1.9	1.5	3.5
adsorbates	mean	0.98	0.60	34.95	58.22	6.83
	%CV	25.7	9.8	4.5	5.8	41.3

followed by the remaining anthocyanins in increasing order of polarity.

Figure 3 shows the ratio between the quantity of adsorbed anthocyanins by the lees corresponding to the fermentation of 1 L of wine with respect to wine anthocyanin content (expressed as a percentage). Peonidin and its acetyl, and especially its coumaroyl, derivatives are those most adsorbed. This is very noticeable if compared with the same derivatives of malvidin, which paradoxically, are present in greater amounts in the wine and are more apolar, and should therefore be more adsorbed. However, the lower stearic impediment of the B ring of peonidin, owed to a single methoxylation at position 3′, might favor its better interaction with the cell wall, noticeably increasing its fixation.

The Influence of Anthocyanin Adsorption by Cell Walls on Wine Color. The color (*10, 14*) of the cell wall adsorbates is much less intense than that of the wines themselves, although the former do have greater tonality (**Table 4**). This reduced color intensity is a consequence of the lower total adsorbed anthocyanin content of the walls (**Table 1**). The greater tonality is due to an increase in yellow tones and an important reduction in blue (**Table 4**). The adsorbates are less red than the wines, but not greatly so.

To verify the relationship between the modification of the color percentages and the anthocyanin profiles of the wines and adsorbates, Pearson's correlation coefficient was used. A weak negative correlation was found between the percentage of yellow and the concentration of *p*-coumaryl derivatives in wines ($R = -0.6714$; $p < 0.05$). However, in the adsorbates, a strong negative correlation was detected between the percentage of yellow and all acetyl derivatives ($R = -0.8615$; $p < 0.001$). This correlation was significant for acetyl derivatives of malvidin ($R = -0.7881$; $p < 0.01$) and petunidin ($R = -0.8096$; $p < 0.01$) and especially so for peonidin ($R = 0.9278$; $p < 0.001$).

Influence of Yeast Strain on Anthocyanin Adsorption. Because not all anthocyanins are adsorbed in the same proportion by the different strains, differences might be expected in the structure of their cell walls. Some strains, such as S6U and 3VA showed low anthocyanin adsorption (**Figure 4**), especially of acyl derivatives, while others such as 2EV and 1EV showed
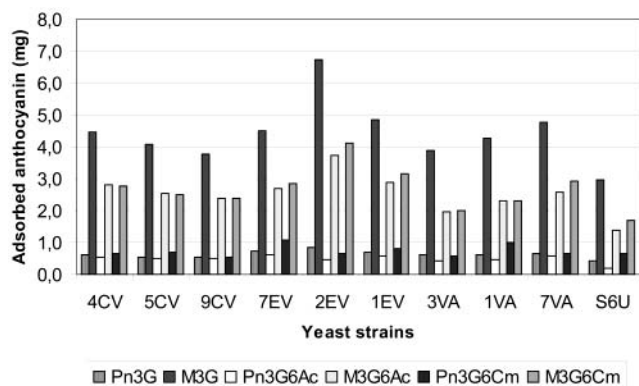
Figure 4. Adsorption by yeasts (that had fermented 1 L of wine) of peonidin and malvidin glycosides and their acetyl and *p*-coumaryl derivatives.

high adsorption (1.68 mg of malvidin-3-(6-*p*-coumaryl)-glucoside was adsorbed by S6U and 4.13 mg by 2EV).

CONCLUSIONS

In the alcoholic fermentation of the must of *Vitis vinifera* L. cultivar Cabernet-Sauvignon, differential anthocyanin adsorption was seen in the cell walls of different strains of *Saccharomyces spp*. Acyl derivatives (*p*-coumaryl and acetyl) were more strongly adsorbed than nonacyl derivatives. Also, anthocyanins with a greater degree of methoxylation (malvidin and peonidin) were more adsorbed than those most hydroxylated (delphinidin and petunidin). This suggests that adsorption involves a hydrophobic interaction. The different CV values for the mean concentrations of each anthocyanin in the wine and adsorbate indicate differences in the structure and composition of the cells walls of the different strains.

The adsorption of peonidin and its derivatives is slightly greater than that of malvidin and its derivatives, even though the former is slightly less apolar. This might be explained in the stearic differences of these molecules which provide some adsorption advantage to peonidin.

The cell wall adsorbates showed a greater percentage of yellow and a fall in blue color. This correlated statistically with high acetyl derivative contents, especially of the most apolar (petunidin, peonidin, and malvidin).

Differences were seen in anthocyanin adsorption capacity between the different yeast strains, some adsorbing more than twice as much as others.

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