Eur Food Res Technol (2005) 220:341–346
DOI 10.1007/s00217-004-1053-8

ORIGINAL PAPER

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Cell wall anthocyanin adsorption by different *Saccharomyces* strains during the fermentation of *Vitis vinifera* L. cv Graciano grapes

Received: 23 July 2004 / Revised: 10 September 2004 / Published online: 11 November 2004

Abstract This work reports the adsorption of anthocyanins by the cell walls of different strains of *Saccharomyces* during the production of red wine from *Vitis vinifera* L. cv Graciano grapes. The anthocyanin derivative contents of the yeast cell walls were substantially different to those of their corresponding wines. Cinnamoyl derivatives (6-*p*-coumaroyl and 6-caffeoyl) were strongly adsorbed while vitisins (adducts of pyruvic acid and acetaldehyde) were weakly adsorbed. The mean total anthocyanin concentration of the wines was 507.64 mg L^{-1} with the following distribution: 3-glucosides (3G), 82.2%; vitisins, 0.97%; 6-acetyl derivatives, 7.44%; 6-caffeoyl derivatives, 1.81%; and 6-*p*-coumaroyl derivatives, 7.54%. A mean of 18.57 mg of anthocyanins were adsorbed by the lees corresponding to 1 L of wine; this quantity was distributed: 3G, 52.60%; vitisins, 0.15%; 6-acetyl derivatives, 4.06%; 6-caffeoyl derivatives, 6.61%; and 6-*p*-coumaroyl derivatives, 36.58%. Large differences were seen between the different yeast strains examined with respect to the quantities of anthocyanins adsorbed. The mean adsorption percentage was 3.67%, but this varied between 1.60% (strain 3VA) and 5.85% (strain 9CV). The adsorption percentage of 6-*p*-coumaroyl derivates for strain 3VA (7.61%) was fourfold less than that of 9CV (28.37%). Strains 2EV and 3VA showed no vitisin adsorption.

Keywords Anthocyanins · Graciano · *Saccharomyces* · Cell walls · Color · High-performance liquid chromatography–diode array detection · Vitisins

Introduction

In red wine production, the composition [1, 2] and porosity [3, 4] of the cell walls of the yeasts used to ferment the must can cause significant losses of aroma and color [5, 6, 7] via the adsorption of volatile compounds, metabolic intermediates and pigment flavonoids (e.g., anthocyanins). This can be a particularly serious problem when grape varieties that show less intense color development are used or in areas where the climate negatively influences the formation of anthocyanins in the fruit [8].

An initial study [9] showed the acylated anthocyanin derivatives (acetyl and *p*-coumaroyl) of Cabernet-Sauvignon grapes to be the most strongly adsorbed by yeast cell walls. These derivatives are very important in red wines since, although some are only present in minor amounts (e.g., *p*-coumaroyl) they are stable [10] and absorb at higher wavelengths (red-bluish pigments) [11]. In addition, differences between the color of the wines and of the cells wall extracts of the lees were found to be owing to the latter accumulating acetyl anthocyanin derivatives. Internationally used grape varieties such as Cabernet-Sauvignon, Cabernet-Franc or Merlot are rich in these compounds [12, 13, 14], which are quantitatively the most important of all the acylated derivatives they contain. The Spanish varieties Graciano and Tempranillo, however, have higher levels of *p*-coumaroyl derivatives [10, 15, 16]. Graciano grapes were used in this study to determine whether a variety with a different quantitative composition in terms of acyl derivatives would show any differences to Cabernet Sauvignon in the adsorption of anthocyanins by the cells walls of the fermenting yeast.

Vitisins A and B (malvidin-3-*O*-glucoside–pyruvate and malvidin-3-*O*-glucoside–vinyl) are very stable anthocyanin derivatives [17, 18, 19] of particular interest in wines that are left to age. Vitisins are not found in grapes;

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rather, they are produced during fermentation [20, 21] through the reaction of malvidin-3-*O*-glucoside and yeast metabolites (pyruvic acid and acetaldehyde). Vitisin A is weakly adsorbed by the cell walls of most yeasts [9]. The use of such strains would be advantageous for maximizing the content of these derivatives, especially in the production of aged wines.

The aims of the present work were (a) to study the adsorption of anthocyanins by yeast cells walls during the fermentation of *V. vinifera* L. cv Graciano grapes, (b) to verify the existence of yeast strains that adsorb anthocyanins weakly and to detect any differences in the adsorption of stable anthocyanin derivatives (acetyl, cinnamoyl (*p*-coumaroyl plus caffeoil) and vitisins glucosides) by different yeast strains, and (c) to determine the differences in the color variables of yeast cell wall extracts and their corresponding wines.

Materials and methods

Fermentations. Fifteen small scale fermentations were undertaken (three for each of the different yeast strains studied) using 5 L of crushed, de-stemmed *Vitis vinifera* L. *cv.* Graciano grapes (must pH 3.5, potential alcohol content 13.5%, v/v). Musts were inoculated with 150 mL of YEPD medium [22] containing 10^8 cfu mL^{-1} of yeast. The inocula were synchronized to homogenize the populations. Fermentation was monitored by taking readings of density and temperature, and was considered complete when a Folin-Ciocalteau index of 50 was obtained [23] (measurement of total polyphenol levels) and reducing sugar levels were <3 g L^{-1} [24].

Anthocyanins adsorbed by cell walls. The anthocyanins adsorbed by the yeast cell walls were extracted as was described in a previous work [9]. Briefly: adsorbed anthocyanins were recovered from 12.5 mL wine-lees suspensions. These were washed with 10 mL of distilled water and then centrifuged at 8,000 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 formic acid:methanol (10:90), agitating with a Vortex for 30 s. Centrifugation at 8,000 rpm followed each wash and the supernatant was kept. The last 30 mL of solvent were filtered through a 0.45 μm filter polyvinylidene fluoride membrane (Millipore, Ireland), as were samples of the finished wines, and analyzed spectrophotometrically to determine color. High-performance liquid chromatography–diode array detection (HPLC-DAD) was used to evaluate their anthocyanin content.

Yeast strains used. The strains of yeast used in the experimental fermentations all belonged to *Saccharomyces cerevisiae*. These were isolated from grapes collected in the Spanish *appellation contrôlée* regions of La Rioja (9CV), Navarra (2EV) and Ribera del Duero (3VA and 7VA). The commercial yeast S6U (*Saccharomyces uvarum*) (Lallemand, Canada) was also used.

Color percentage intensity. The absorbance of the wines produced was measured at 420, 520 and 620 nm using a Jasco 530 spectrophotometer with a quartz cell of 1 mm path length, according to the Glories procedure [25, 26]. The variables calculated were: intensity, tonality, and percentages of red (%R), yellow (%Y) and blue (%B).

Anthocyanin analysis by liquid chromatography. The anthocyanins contained in all the fermentations and in the cell wall adsorbates were analyzed using a Waters (Milford, MA) HPLC-DAD in a reverse-phase Nova-pack C_{18} column [20]. Nineteen anthocyanins were identified in the fermentations and grouped according to the different anthocyanin derivatives found: anthocyanidin-3-glucosides (3G), acetylated derivatives (6Ac), caffeoyl derivatives (6Caf), coumaroyl derivatives (6Cm), and vitisins A and B (vitisins). The different anthocyanins were identified by their relative retention times compared to the majority anthocyanin of *Vitis vinifera* L., malvidin-3-*O*-glucoside, and by their UV-visible absorption spectra.

Statistics. Means, standard deviations, ANOVAs and LSD were calculated using the PC Statgraphics 5.0 software package (Graphics Software, Rockville, MD, USA).

Results and discussion

Adsorption of *V. Vinífera* L. cv Graciano grape anthocyanins by the cell walls of *Saccharomyces*

The mean total anthocyanin content of all the wines was 507.64 (range 469.18–548.35) (Table 1), while the mean total anthocyanin content of the cell walls was 18.57 mg (range 8.78–30.91). To further analyze the results they were grouped according to the different anthocyanin derivatives found, such as 3G, 6Ac, 6Caf, 6Cm, vitisins,

Table 1 Mean and standard deviation of total anthocyanin content and derivatives in wines (milligrams per liter) and cell wall adsorbates (mg) in lees corresponding to 1 L of wine. Data represent mean value for three replicates ± standard deviation. 3G Anthocyanidin-3-glucosides, 6Ac acetylated derivatives, 6Caf caffeoyl derivatives, 6Cm coumaroyl derivatives, vitisins vitisins A and B

	Yeast strain	3G	Vitisins	6Ac	6Caf	6Cm	Totals
Wines	9CV	433.91±5.21	7.37±0.42	39.09±0.65	9.68±0.19	38.86±0.44	528.91±4.75
	2EV	432.21±3.96	2.82±0.15	37.47±0.02	8.69±0.12	37.31±0.35	518.50±4.27
	3VA	450.02±2.06	3.65±0.17	39.63±0.20	10.27±0.07	44.78±0.20	548.35±1.95
	7VA	385.78±0.51	5.92±0.19	37.04±0.25	8.73±0.03	35.77±0.04	473.25±0.07
	S6U	385.38±2.84	4.92±0.21	35.65±0.36	8.61±0.04	34.61±0.24	469.18±2.74
	Mean	417.46±27.84	4.94±1.68	37.77±1.52	9.20±0.69	38.27±3.69	507.64±32.48
	%	82.24	0.97	7.44	1.81	7.54	100.00
Adsorbates	9CV	16.22±1.40	0.06±0.03	1.49±0.23	2.12±0.25	11.02±0.96	30.91±2.83
	2EV	10.88±0.65	0.00±0.00	0.64±0.05	1.35±0.13	6.78±0.33	19.64±1.05
	3VA	4.63±1.33	0.00±0.00	0.23±0.09	0.51±0.29	3.41±1.00	8.78±2.69
	7VA	10.84±1.45	0.07±0.01	0.96±0.19	1.47±0.19	8.18±1.15	21.53±2.95
	S6U	6.26±1.07	0.01±0.01	0.45±0.15	0.69±0.25	4.57±0.64	11.98±1.97
	Mean	9.77±4.34	0.03±0.03	0.75±0.47	1.23±0.63	6.79±2.88	18.57±8.29
	%	52.60	0.15	4.06	6.61	36.58	100.00

Table 2 Percentage of adsorption (AP) of each anthocyanin derivative calculated as the relationship between the anthocyanins in the lees corresponding to 1 L of wine and the concentration in the wine at the end of fermentation. Data represent mean value for three replicates±standard deviation. 3G Anthocyanidin-3-glucosides, 6Ac acetylated derivatives, 6Caf caffeoyl derivatives, 6Cm coumaroyl derivatives, vitisins vitisins A and B

Yeast strain	3G	Vitisins	6Ac	6Caf	6Cm	Totals
9CV	3.74±0.33	0.80±0.44	3.80±0.54	21.91±2.71	28.37±2.51	5.85±0.54
2EV	2.52±0.13	0.00±0.00	1.71±0.12	15.48±1.27	18.16±0.76	3.79±0.18
3VA	1.03±0.30	0.00±0.00	0.59±0.24	4.93±2.84	7.61±2.27	1.60±0.50
7VA	2.81±0.37	1.19±0.07	2.59±0.54	16.84±2.07	22.88±3.23	4.55±0.62
S6U	1.62±0.28	0.28±0.25	1.25±0.41	8.03±2.82	13.20±1.80	2.55±0.41
Mean	2.34±1.01	0.45±0.52	1.99±1.21	13.44±6.69	18.04±7.74	3.67±1.59

and finally total anthocyanins (the sum of these groups). Figure 1a shows the chromatogram for the wine produced with strain 3VA (a weak adsorber of anthocyanins) at 525 nm. Figure 1b shows the same for the cell wall extract of the yeasts. A clear reduction in the area of the peaks can be seen, more so in those corresponding to 3-glucosides, 3-(6-acetyl) glucosides and vitisins. Figure 1c shows the chromatogram of the cell wall extract of strain 7VA at 525 nm. This strain showed greater anthocyanin adsorption than 3VA.

In the wines, the majority anthocyanins were non-acylated 3G (Table 1) (mean concentration 417.46 mg L^{-1}, some 82.2% of the total amount). The most common acylated derivatives were the 6Cm (mean concentration 38.27 mg L^{-1}; 7.5%), followed by the 6Ac (37.77 mg L^{-1}; 7.4%). The 6Caf concentration was 9.20 mg L^{-1} (1.81%) and that of the vitisins was 4.94 mg L^{-1} (0.97%). This distribution of the different derivatives is normal for wines made with traditional Spanish grape varieties such as Graciano or Tempranillo. The cinnamoyl derivatives (6Cm and 6Caf) were more common than the 6Ac [11], which is different to what is seen with internationally used varieties such as Cabernet-Sauvignon or Merlot, in which the 6Ac derivatives predominate [13, 14, 27]. However, the anthocyanin distribution of the cell wall extracts was different, with the 3G derivatives being the most common (Table 1). The mean content for the strains studied was 9.77 mg, although they only represented 52.6% of the total amount (82.2% in wines). The most important acylated derivatives were the 6Cm (6.7 mg; 36.6% of the total amount). The coumaroyl derivatives are therefore strongly adsorbed by the yeast cell walls, as seen in the fermentation of Cabernet-Sauvignon musts [9]. The mean value for the adsorbed 6Ac derivatives (Table 1) was 0.75 mg (4.1% of the total). The mean content of adsorbed 6Caf derivatives was 1.23 mg (6.6%), greater than that for the 6Ac, despite there being four times as much 6Ac as 6Caf derivatives in the wine. This shows the important adsorption of the cinnamoyl anthocyanin derivatives (6Cm and 6Caf) by *Saccharomyces* cell walls. The vitisins were only adsorbed in a small way; the mean cell wall content was 0.03 mg (0.15%). These extracts were not found at all in the cell wall extracts of strains 3VA and 2EV (Table 1).

Proportions in which the different anthocyanin-derived pigments were adsorbed

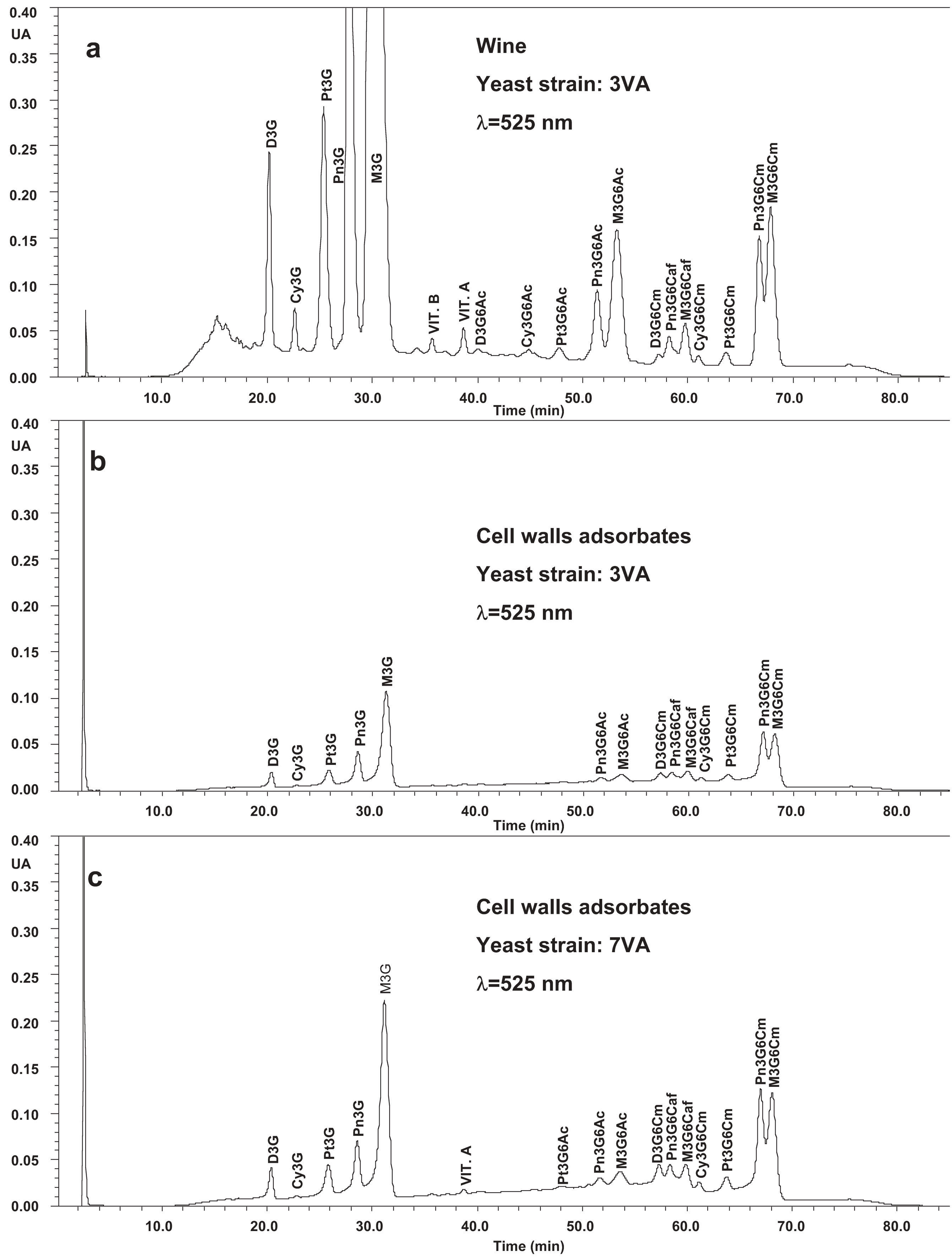
To establish a balance between the anthocyanin concentration of the wines produced by the different yeast strains and the quantities present in the yeast cell walls themselves, the relationships between each anthocyanin or anthocyanin group (3G, vitisins, 6Ac, 6Caf, 6Cm and total anthocyanins) in the cell walls of yeasts that fermented 1 L of wine (expressed in milligrams) and the wines themselves (expressed in milligrams per liter) were calculated. These values were termed adsorption percentages (AP). The APs obtained for the mean component values associated with the studied strains were (Table 2) 3G, 2.34%; vitisins, 0.45%; 6Ac, 1.99%; 6Caf, 13.44%; 6Cm, 18.04%; and total anthocyanins, 3.67%.

These values agree with those obtained in an earlier study of anthocyanin adsorption during the fermentation of Cabernet Sauvignon grapes [9] (3G, 2.34%; vitisins, 0.44%; 6Ac, 2.55%; 6Caf, 10.59%; 6Cm, 16.24% and total anthocyanins 3.07%). The small difference in adsorption (0.6%) between this initial study and the present work, in terms of total anthocyanin content, is probably owing to the greater anthocyanin content of the Graciano-origin wines (507.64 mg L^{-1} compared to 430.47 mg L^{-1}; Table 1).

The differences in the adsorption of the 6Ac and cinnamoyl (6Cm and 6 Caf) derivatives are to be expected if it is taken into account that the 6Ac are the most common acylated derivatives in Cabernet Sauvignon wines (30% of the total content) [9]. In the present Graciano-derived wines, the majority of acylated anthocyanins were cinnamoyl derivatives (6Cm and 6Caf), with the 6Ac making up only 7.4% of the total (Table 1).

The differences in the adsorption of anthocyanin derivatives are owing to the different polarity of each derivative according to the hydroxylation/methoxylation grade in B ring and the acyl moiety [9].

The vitisins were only weakly adsorbed in Graciano fermentations (0.45%) (Table 2), as in Cabernet Sauvignon wine production. The formation of these molecules [20] is greatest during the middle (VIT A) and final (VIT B) stages of fermentation. Therefore, since these compounds are not present in the initial phases of fermentation, the yeast cell walls might become saturated with other majority anthocyanins, which are present in the grapes. They may then be unable to adsorb the vitisins


a
Wine
Yeast strain: 3VA
λ=525 nm
b
Cell walls adsorbates
Yeast strain: 3VA
λ=525 nm
c
Cell walls adsorbates
Yeast strain: 7VA
λ=525 nm
UA
Time (min)
D3G
Cy3G
Pt3G
Pn3G
M3G
VIT. B
VIT. A
D3G6Ac
Cy3G6Ac
Pt3G6Ac
Pn3G6Ac
M3G6Ac
D3G6Cm
Pn3G6Caf
M3G6Caf
Cy3G6Cm
Pt3G6Cm
Pn3G6Cm
M3G6Cm

when they eventually form. This is important since, although the vitisins are minority pigments in wines, they are of great importance during aging since they are more stable [28, 29] than the other anthocyanins.

Differences in anthocyanin adsorption by the different yeast strains

The differences between the anthocyanin adsorption profiles of the studied yeasts was very notable. The mean AP for total anthocyanins for all the strains together was 3.67% (Table 2), ranging from 1.60% for 3VA and 5.8% for 9CV. Some strains therefore adsorb three times as much as others. The mean percentage adsorption of the 6Cm for all the studied strains together was 18.04% (Table 2): an appreciable reduction of the wine content of these compounds. Once again, however, there was great variation: for 3VA this value was only 7.6%, rising to 28.4% with 9CV (a fourfold difference).

The 6Cm are the acylated derivatives that absorb at higher wavelengths than other anthocyanins, making them of special interest. Currently, bluish hues are highly valued in the production of wine considered to be of good chromatic quality. It is therefore undesirable that these derivatives be adsorbed by the cell walls of some yeast strains at percentages of around 30% (the AP for the 3Gs is only 2.3%; range 1.03%–3.74%). This increases the importance of selecting a yeast with a low adsorption profile, such as 3VA. The situation is similar for the 6Caf derivatives. The mean AP for these compounds was 13.44% (much higher than that of the 3G derivatives), with a range from 4.93% (3VA) to 21.91% (9CV) (a fourfold difference).

The 6Ac were adsorbed in much smaller quantities than the cinnamoyl derivatives. The mean AP for all the studied strains was 1.99%, similar to that of the 3G compounds. Even so, the range (0.59% for 3VA to 3.80% for 9CV) differed by a factor of 6.

To determine whether the yeast strains showed any significant differences with respect to the APs for the different anthocyanins, the results were examined by ANOVA; significance was set at $P \leq 0.05$. A multiple rank test was also performed. The differences between 3VA and 7VA, 3VA and 9CV, 7VA and S6U, and 9CV and S6U were significant for all anthocyanins (3G, vitisins, 6Ac, 6Caf, 6Cm and total anthocyanins). Strains 3VA and S6U had the lowest APs and only showed significant differences with respect to the values for 6Cm, 3G and total anthocyanins. Strains 7VA and 9CV, and 2EV and 3VA, showed significant differences in the adsorption of all anthocyanins except for the vitisins. Strains 2EV and 7VA showed significant differences for all except 3G and 6Caf. Finally, strains 2EV and S6U showed significant differences for all the anthocyanins studied except for the vitisins and 6Ac. Except for the vitisins, the most important differences were always seen between strains 3VA and S6U (with low APs) and strains 7VA and 9CV (with somewhat higher APs). Strain 2EV showed intermediate APs.

These differences in the anthocyanin adsorption by the strains are due to the dissimilar compositions of their cell walls, probably because of the different contents of polar groups exposed on cell wall surfaces. These groups can increase or decrease anthocyanin adsorption on cell surfaces.

Fig. 1a–c. High-performance liquid chromatography–diode array detection chromatograms at 525 nm. **a** Chromatogram for wine fermented by strain 3VA. **b** Chromatogram for 3VA cell wall extract. **c** Chromatogram for 7VA cell wall extract

Effects on color

The mean color intensity of the wines was 1.589 (with a 9.04% coefficient of variation [CV]) and mean tonality was 0.452 (with a CV of 3.54%). The mean color intensity of the cell wall extracts was 0.872; variability between the yeast strains, however, was high (CV=37.47%). The weakly adsorbing strains (3VA and S6U) showed low color intensities (0.451 and 0.614 respectively) while the strongly adsorbing strains, 9CV and 7VA, showed values of 1.227 and 1.081 respectively. The weak adsorption of anthocyanins by 3VA was reflected in the highest wine color intensity (1.802); wine made with 9CV (the strongest anthocyanin adsorber) had a color intensity below the mean (1.695). The tonality of the cell wall extracts (0.856) was greater than that of the wines (0.452).

In summary, these results show that different yeast strains adsorb anthocyanin derivatives to different degrees, and that appropriate strains should be selected to improve the color of wines.

Acknowledgements The authors wish to acknowledge the financial support of the *Ministerio de Ciencia y Tecnología* (MCyT) (Project AGL-2003–07394-C02–01 and –02), the assistance provided by J. A. Sanchez and I. Izquierdo, and the help of Mr. Julián Suberviola of EVENA (*Estación de Viticultura y Enología de Navarra*).

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