Isolation, Identification, and Characterization of New Color-Stable Anthocyanins Occurring in Some Red Wines

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Four new anthocyanin pigments were found in red wines in trace amounts and are thought to be formed in wines during maturation. Their structures were established by FAB-MS and NMR. Two pigments were identified as the 3-glucoside (vitisin A) and the 3-acetylglucoside (acetylvitisin A) of malvidin containing a $C_3H_2O_2$ grouping, linking carbon 4 and the 5-hydroxyl group of its molecule (vitisidin A). The other two anthocyanins were identified as the 3-glucoside (vitisin B) and the 3-acetylglucoside (acetylvitisin B) of malvidin containing a CH═CH moiety linking carbon 4 and the 5-hydroxyl group of its molecule (vitisidin B or decarboxyvitisidin A). Unlike other anthocyanins these novel compounds were found to be wholly or partly resistant to bleaching by sulfur dioxide and express more color up to pH 7 than malvidin 3-glucoside. Detailed spectral measurements from 250 to 770 nm up to pH 7, including the use of CIELAB 76 measurements, indicate the formation of stable quinonoidal bases, confirming that there could be little formation of the colorless carbinol base forms.

Keywords: *Red wine; anthocyanins; vitisin A; vitisin B; vitisidin A; vitisidin B; NMR; FABMS*

INTRODUCTION

Anthocyanins are a widespread source of naturally occurring colorants of foods. Their use as an added color to foods and drinks has been limited hitherto, since they have a number of drawbacks, such as sensitivity to bleaching by sulfur dioxide and limited coloring capability at pH values above 3.5 (Timberlake, 1980). Overcoming such problems would greatly enhance the possibility of using anthocyanins as added food colors, especially since there is a current interest in natural colors with potential added health benefits (Bridle and Timberlake, in press).

Earlier, analyses of red port wine pigments by high-performance liquid chromatography (HPLC) revealed a number of unidentified peaks, eluting soon after malvidin 3-glucoside (Bakker, 1985). Properties of one of these anthocyanins purported by Bakker (1985) included its resistance to color bleaching by sulfur dioxide. This stability in the presence of sulfur dioxide is consistent, by analogy with flavylium salt studies (Timberlake, 1968; Timberlake and Bridle, 1968), with the presence of a 4-substituent on the anthocyanin molecule. During our studies on maturation of red table wines the same anthocyanin, vitisin A (3-formyl-4-D-β-glucopyranosyloxy-8-hydroxy-5-(4-hydroxy-3,5-dimethoxy)phenyl-2-oxo-1,6-dioxa-2,3-dihydrophenalene), was observed, often accompanied by a second unknown anthocyanin. Even smaller concentrations of two other anthocyanins having apparently similar properties were observed in fortified red wines. Recently we have fully identified vitisin A using a combination of FABMS and ^{1}H and ^{13}C NMR (Bakker et al., in press). In this present paper, we report the isolation and structural determination of related novel anthocyanins using FABMS and ^{1}H NMR. On the basis of the full identification of vitisin A, we present the tentative structures of three new anthocyanins. In addition we describe some of the unusual attributes of these four anthocyanins for the first time.

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MATERIALS AND METHODS

Source. Vitisin A has been observed in both red table wines and fortified red wines in our laboratory studies over recent years. However, fortified red wines also showed the presence of further unknown anthocyanins. Initially, ^{1}H NMR spectra and studies of the properties of these anthocyanins were done on small quantities isolated from a fortified dry port wine, prepared from *Vitis vinifera* (var. Touriga Nacional) grapes in 1981 (Bakker and Timberlake, 1985b). These grapes came from the demarcated upper Douro Valley in northern Portugal and were picked at commercial maturity. These grapes were found to give port wines with large concentrations of anthocyanins and containing a high percentage of malvidin 3-glucoside based anthocyanins, including 18% malvidin 3-glucosyl acetate (Bakker and Timberlake, 1986b).

Isolation. Dry fortified red wine was prepared in our laboratory using a standard pilot scale technique (Bakker and Timberlake, 1985b). Approximately 500 mL of this 9 months old wine was concentrated by vacuum rotary evaporation at 40 °C to 250 mL, filtered, and applied to a previously prepared column (50 mm i.d., 600 mm length, prepared by our glassblower) of Sephadex LH-20 in 3% formic acid. The column was eluted with up to 2500 mL of aqueous 3% formic acid. The early eluate contained mainly vitisins, later becoming diluted with other monomeric anthocyanins. No more compounds of interest were eluted after 2500 mL of eluate. The crude vitisin fraction was retained on a ODS minicolumn (Sep-Pak cartridge C18, Waters, Millipore) washed with aqueous 3% formic acid, eluted with methanolic 3% formic acid, and dried at room temperature under reduced pressure in a desiccator. Four different vitisins were obtained in highly purified form by semipreparative HPLC on a 5 μm Spherisorb ODS-2 (Phase Sep) column (8 mm × 100 mm) at 40 °C with a flow rate of 2 mL min^{-1} and detection at 520 nm. The solvent system was a linear gradient using solvent A (1% TFA) and solvent B (methanol) from 20% B to 46% B over 26 min. The compounds were collected and concentrated by vacuum rotary evaporation at 40 °C, and the small liquid samples of about 10 mL were dried at room temperature under reduced pressure in a desiccator. Their purity was tested by HPLC on a reversed phase Spherisorb-hexyl (Phase Sep) column (5 mm × 100 mm) at 35 °C with a flow rate of 1.0 mL min^{-1} and detection at 520, 495, and 280 nm. The following gradient using methanol (solvent B) and water containing 0.6% $HClO_4$ (solvent A) was used: in 6 min from 20% to 23% B, in 8 min from 23% to 30% B, in 5 min from 30% to 40% B, in 8 min

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from 40% to 50% B, and finally to clean the column in 5 min from 50% to 95% B, 5 min at 95% B, in 1 min back to 20% B.

NMR Analysis. ^{1}H NMR (200 MHz) spectra of all four anthocyanins were obtained in CD_3OD containing 0.1% DCl with TMS internal standard; spectra for the two main anthocyanins were also prepared in DMSO-d_6/TFA (9:1, v/v), a solvent system used to avoid deuteration of the vitisins.

FAB-MS Analysis. A small sample of anthocyanin was dissolved in M HCl, placed on a clean copper tipped probe, dried under vacuum, and mixed with glycerol on the probe before insertion in the FAB source. Spectra were obtained using a resolution of 1000, with the gun producing a 5−7 kV beam of xenon atoms.

Colorimetric Measurements. The color expression as a function of pH was measured in aqueous solutions containing 0.1 M citric acid using two methods: samples. (i) A scanning spectrophotometer was used to measure the absorbance from 250 to 770 nm, using a cell path length of 10 mm. The pH values were adjusted with sodium bicarbonate. (ii) Tristimulus measurements (CIELAB 76) were collected by scanning the acidic aqueous solutions by spectrophotometer from 380 to 770 nm, equipped with a computer software program to calculate the L^*, a^*, and b^* values, from which the hue angle (degrees) was calculated (Bakker et al., 1986). L^* denotes lightness (100) to darkness (0), while a hue angle decreasing from 90 to 0° indicates a change from a brownish-redness to bluish-redness.

Bleaching by sulfur dioxide was determined by adding small amounts of a stock solution of 1000 mg L^{-1} sulfur dioxide to an aqueous solution of anthocyanin in 0.1 M citric acid. The A_{max} was determined in a 10 mm cell using the spectrophotometer, and the data were corrected for the dilution. After the last measurement with 250 mg L^{-1} sulfur dioxide, the cells were covered, kept at room temperature for 48 h, and remeasured.

The effect of Al^{3+} on the λ_{max} was done by addition of one drop of a 5% solution of $AlCl_3$ to a solution of the anthocyanin in methanol containing 0.01% HCl. The absorbance was measured from 250 to 770 nm, using a cell path length of 10 mm.

RESULTS AND DISCUSSION

General. This is the first study to unequivocally identify pigments occurring in *V. vinifera* wines. The existence of colored compounds other than the monomeric anthocyanins occurring in grapes was for the first time alluded to by Somers (1966). The polymeric red pigments first investigated by Somers (1966) are likely to have included the vitisins. However, this is the first report of these compounds with properties quite different from those of anthocyanins. The vitisins were isolated from wine and identified using FABMS, ^{1}H NMR, and ^{13}C NMR in addition to standard colorimetric measurements to complement and enhance the studies on their properties.

Occurrence. The anthocyanins, vitisin A, and vitisin B (4-D-β-glucopyranosyloxy-8-hydroxy-5-(4-hydroxy-3,5-dimethoxy)phenyl-2-oxo-1,6-dioxaphenalene) were first encountered in small quantities in fortified red wines; in addition, small quantities of two anthocyanins with similar properties, vitisin A-X and vitisin B-X, were also observed. Our laboratory studies have revealed the presence of vitisin A also in maturing red table wines. Recently, we have found a small amount of vitisin A in the skins of some deep frozen stored grapes, but it does not occur in the any of the skins of fresh grapes we have examined (unpublished data). Vitisins B and B-X have mainly been observed in maturing fortified red wines (Bakker, 1985). An important difference between the maturation of red table wines and fortified wines is the higher concentration of acetaldehyde in the latter, derived from the short fermentation time. At approximately halfway through the fermentation, the latter is stopped before completion by the addition of fortifying grape spirit in order to retain some of the natural sweetness (Reader and Dominguez, 1994). The grape spirit also forms an important contribution to the acetaldehyde concentration (Bakker, 1986). The acetaldehyde plays an important role in the maturation processes whereby the anthocyanins polymerize with other phenolic compounds naturally present during maturation (Bakker, 1986; Bakker et al., 1993). Most evidence indicates that vitisin B is an intermediate in the maturation processes. For example, small amounts of this anthocyanin were formed in model wine solutions containing acetaldehyde and anthocyanins extracted from grape skins. Further evidence indicating the involvement of malvidin 3-glucoside is the observation that vitisin B was also formed in model wines prepared from extracts of white grapes with added malvidin 3-glucoside and acetaldehyde (Bakker, 1985). Smaller quantities of a related compound, vitisin B-X, were also observed.

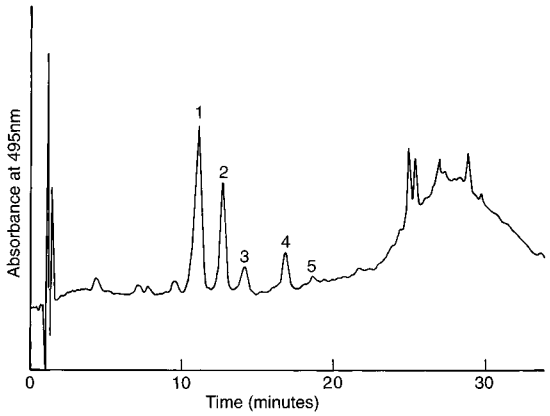

Figure 1. HPLC trace recorded at 495 nm of 9 month old port wine, showing (1) malvidin 3-glucoside, (2) vitisin A, (3) vitisin B, (4) acetylvitisin A, and (5) acetylvitisin B.

HPLC Analysis. Using high-performance liquid chromatography these four compounds eluted shortly after malvidin 3-glucoside (Figure 1). In all the fortified wine samples examined the concentrations of these compounds were small, no more than 10 mg L^{-1}; however, as the wines matured, the vitisin compounds formed a larger percentage of the of the total anthocyanins determined. Vitisin B showed an initial increase in concentration followed by a decrease and was prevalent in port wines which contained between 60 and 150 mg L^{-1} acetaldehyde. Vitisin A was also present only in low concentrations, but it is very resistant against aging, and in a port wine which had been allowed to age for about 1 year, it was often the only monomeric anthocyanin left. For example, Figure 1 shows that in a 9 month old port wine the total monomeric anthocyanin concentration as determined by HPLC was 40.5 mg L^{-1}, with 37.5% malvidin 3-glucoside, 20.3% vitisin A, 4.9% vitisin B, 7.4% vitisin A-X, and 1.7% vitisin B-X.

The UV−vis spectrum of vitisin A recorded in 0.6% $HClO_4$ in H_2O and methanol (65:35), using a diode array scanning HPLC detector, has been shown to be quite different from the main anthocyanin in wines, malvidin 3-glucoside (Bakker et al., in press). The λ_{max} of for malvidin 3-glucoside was 529 nm, whereas vitisin A had a λ_{max} of 514 nm. The solvent in which the anthocyanins are dissolved has a small effect on the λ_{max}, as can be

Table 1. ^{1}H NMR Spectral Data of Vitisins A and B in TFA/DMSO-d_6 (9:1) and of Vitisins A and B and Acetylvitisins A and B in CD_3OD Containing 0.1% DCl Using 200 MHz Analysis[a]

	TFA/DMSO-d_6 (9:1)				CD_3OD/0.1% DCl							
	vitisin A		vitisin B		vitisin A		vitisin B		acetylvitisin A		acetylvitisin B	
H	∂	*J*	∂	*J*	∂	*J*	∂	*J*	∂	*J*	∂	*J*
					Aglycon							
α	8.02	s			8.00	s			8.04	s		
α_1			8.60	d, 5.37			8.35	s			8.38	s
α_2			7.43	d, 5.37								
2′6′	7.88	s	7.83	s	7.78	s	7.73	s	7.81	s	7.76	s
8	7.49	d, 2.00	7.43	d, 1.95	7.35	d, 1.95	7.29	d, 1.96	7.37	d, 2.20	7.32	d, 1.95
6	7.27	d, 2.00	7.21	d, 1.95	7.18	d, 1.95	7.12	d, 1.71	7.20	d, 1.70	7.13	d, 1.96
OME(3′5′)	3.99	s	3.95	s	4.00	s	3.99	s	4.00	s	3.99	s
					Sugar							
anomeric H	4.77	d, 7.30	4.74	d, 7.57	4.72	d, 7.60	4.70	d, 7.60	4.72	d, 8.00	4.73	d, 7.80
acetate									1.79	s	1.80	s

[a] *J*: binding constant. s: singlet. d: doublet.

Table 2. Absorbance Maxima (λ_{max}) in Aqueous Solution Containing 0.1 M Citric Acid and Effect of the Addition of $AlCl_3$ on λ_{max} (nm) in Methanol Containing 0.01% HCl

	H_2O with 0.1 M citric acid, pH 2−4		methanol with 0.01% HCl		methanol with 0.01% HCl + Al^{3+}	
anthocyanin	λ_{max} (nm)	$\Delta\lambda_{vismax}$ (nm)	λ_{max} (nm)	$\Delta\lambda_{vismax}$ (nm)	λ_{max} (nm)	$\Delta\lambda_{vismax}$ (nm)
vitisin A	500, 365	−18[a]	518, 370, 274	−19[a]	535	+17[b]
vitisin B	482, 352	−36[a]	498, 355, 270	−39[a]	498	0[b]
acetylvitisin A			523, 370, 270	−13[c]	535	+12[b]
acetylvitisin B			503, 355, 270	−33[c]	503	0[b]
malvidin 3-glucoside	518		537, 280			
malvidin 3-acetyl-glucoside	518		536, 280			

[a] From malvidin 3-glucoside. [b] Shift as a result of Al^{3+} addition to methanol containing 0.01% HCl. [c] From malvidin 3-acetylglucoside.

seen by comparing this value for vitisin A with data from Table 2.

Structural Identification of Vitisin A. The full structural elucidation of vitisin A has been done using a combination of physical methods, i.e., fast atom bombardment mass spectrometry (FABMS) and ^{1}H and ^{13}C NMR. The compound was shown to be malvidin 3-glucoside with a $C_3H_2O_2$ link between positions 4 and the 5-hydroxyl of the molecule with a β glucoside at the three position (Bakker et al., in press). The full structure (**1**) of the flavylium form of vitisin A is shown in Figure 2. We present here the tentative structures of the other three anthocyanins, based on data obtained from FABMS and ^{1}H NMR, interpreting these by analogy with vitisin A.

FABMS. Analysis of vitisin A by FABMS produced a molecular ion (M^+) of 561 mass units, accompanied by a fragment ion of 399 (162 mass units less, corresponding to the loss of glucose), compared to a M^+ of 493 and an aglycon of 331 for malvidin 3-glucoside. Thus vitisin A is a monoglucoside with an aglycon (vitisidin A) 68 mass units (corresponding to C_3O_2) heavier than malvidin; this additional mass is accounted for by the $C_3H_2O_2$ link between positions 4 and the 5-hydroxyl of the molecule (see Figure 2). Analysis of vitisin B by FABMS gave a molecular ion (M^+) of 517 mass units, accompanied by a fragment ion of 355 attributed to the aglycon (vitisidin B) after the loss of glucose. Hence this aglycon was 44 mass units lighter than the vitisidin A and 24 units heavier than malvidin. These differences may indicate CO_2 loss from vitisidin A, leaving a malvidin structure with two additional C's substituted at carbon 4 and oxygen 5 with a HC═CH moiety.

The masses of vitisin A-X and B-X were 603 and 559, respectively, with aglycons of 399 and 355. Thus the aglycons have the same mass as vitisidin A and vitisidin B after the loss of 204 mass units. The loss of 204 indicates the loss of acetylglucoside, indicating that the sugar moiety may be acylated with acetic acid (Bakker and Timberlake, 1985a). Since the fortified wines in which these anthocyanins were observed contained more than 60% malvidin-based anthocyanins, with major contributions from malvidin 3-acetylglucoside (up to 18%) (Bakker and Timberlake, 1985b), it is possible that both the nonacylated and the acylated novel anthocyanins were formed from malvidin 3-glucoside and malvidin 3-acetylglucoside as their precursors.

^{1}H NMR. Table 1 shows ^{1}H NMR shifts using DMSO-d_6/TFA (9:1) for vitisins A and B. By comparison with the data for vitisin A (Bakker et al., in press) the presence of H-6 and H-8 in the A ring and H-2′6′ and two methoxy groups in the B ring can also be established for vitisin B. However, vitisin A does not have the low-field H-4 (8.6−9.1 ppm) singlet of characteristic for flavylium salts and anthocyanins (Nilsson, 1973), instead it has a more upfield singlet at 8.02 ppm assigned to H-α on the D ring. Vitisin B has a doublet at 8.60 ppm with a coupling constant (J = 5.37) and a second doublet at 7.43 ppm with the same coupling constant. This indicates that there are two H's attached to adjacent carbons, separated by a double bond. Hence vitisin B aglycon is also based on the malvidin aglycon structure, with two additional carbons, as indicated by FABMS, and with an H attached to each carbon, separated by a double bond. These the two extra carbons are most likely C-α-1 and C-α-2. The tentative structure (**2**) based on this evidence is shown in Figure 2.

The anomeric proton signals of the sugars in these anthocyanins are also rather more upfield than is typical for anthocyanins with a 3-glucoside (e.g., 5.4 for malvidin 3-glucoside) (Bakker et al., in press). The large coupling constants for the anomeric protons of the

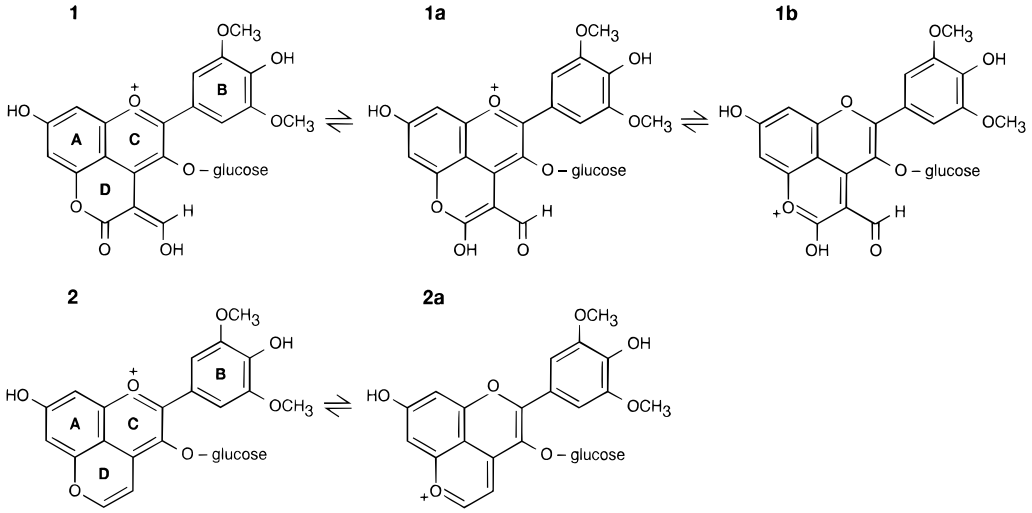

Figure 2. Structures of vitisins A (**1**) and B (**2**), also showing the isomeric structures of the flavylium forms.

glucose indicate a β glucoside linkage. The glucoside is attached at the 3 position in all these anthocyanins.

The shifts for all four anthocyanins obtained in CD_3OD with 0.1% DCl are shown in Table 1. There are only relatively small differences in shifts as a result of solvent. One important difference is that the two doublets obtained for vitisin B DMSO-d_6/TFA and assigned to C-α-1 and C-α-2 are missing in the spectra obtained using CD_3OD with 0.1% DCl. In the latter solvent there is only one signal at 8.35 ppm, without any apparent coupling (Table 1). Thus one of the two H's has deuterated in this solvent. This is likely to be the proton (α-1) nearer the C ring, and the small upfield shift for α-2 would be expected. We have previously compared the spectra of 7-hydroxyflavylium chloride substituted with a methyl group at position 6, at position 8, or at position 4 (Timberlake et al., 1986). In CD_3OD−DCl, the H-4 signal of 4-CH_3-7-(OH)-flavylium chloride was absent as expected. However, the CH_3 signal was also absent, although it occurred in the spectra of 6-CH_3 and 8-CH_3-7-(OH)-flavylium chlorides. Thus it appeared that there was rapid exchange between the protons of the 4-CH_3 group and the solvent. Changing the solvent to DMSO-d_6/TFA (90:10) overcame this restriction, and the CH_3 signal appeared in the appropriate place.

The other two anthocyanins, vitisins A-X and B-X, gave similar spectral results using CD_3OD with DCl (Table 1), showing that both compounds are malvidin 3-glucoside based, by analogy with the interpretation of the data for vitisins A and B and the FABMS data. Deuteration again lost the two doublets attributed to C-α-1 and C-α-2; instead there is a singlet at 8.38 ppm in vitisin B-X. However, in addition a large single shift was observed for each compound at 1.79 and 1.80 ppm. Under these conditions malvidin 3-acetylglucoside has a single peak at 2.02 ppm attributed to the three protons of the acetate group, which was acylated at the 6 position of the glucose (Bakker and Timberlake, 1985a). Hence the observed chemical shifts for these anthocyanins can probably be attributed to the three protons of the acetate group. Thus the most likely tentative structures based on ^{1}H NMR and FABMS data are acetylvitisin A and acetylvitisin B, with the sugar being acylated at the 6 position.

Properties of the Vitisins. All four anthocyanins have their 4 positions substituted, and it is anticipated that this substitution affects the properties of these anthocyanins in solutions. Substitution at the 4 position gives greater resistance to color loss with sulfur dioxide, greater color expression at higher pH values, and increased stability. A number of experiments showed the different behavior of these unusual anthocyanins, which are not only 4 substituted but also have their 5 position substituted. Most experiments are reported just for vitisins A and B; the acylated vitisins would be expected to behave similarly.

Color Characteristics. Both major new pigments exhibit hypsochromic spectral shifts from malvidin 3-glucoside: vitisin A, 18−19 nm, and vitisin B, 36−39 nm, depending on the solvent (Figures 3 and 5, and Table 2). Unlike usual anthocyanins, absorption peaks also occur in the near-UV (352−370 nm); this feature appears to be characteristic of 4-substituted anthocyanins since purpurinidin glycoside, also 4-substituted, also exhibits a pronounced peak near 350 nm (Timberlake and Bridle, 1980). Similar, but slightly less marked, hypsochromic shifts from malvidin 3-acetylglucoside occurred with acetylvitisin A and acetylvitisin B (Table 2). A possible explanation for these hypsochromic effects may be that the + charge on the flavylium C ring may be delocalized by resonance and partly reside on the oxygen atom of the newly formed D ring. Such a rearrangement of charge within the main vitisin B structure (**2**) would give structure **2a** and account for its orange color (Figure 2). Although the lactonic carbonyl group does not usually form its enolic isomer, it is possible that small amounts of the latter are formed and so allowing the possibility of forming structure **1b** by delocalizing of charge as seen in Figure 2. A small hypsochromic shift, such as the one observed, might then be expected. The NMR spectrum indicated the presence of only one isomer flavylium cation isomer (Bakker et al., in press). A possible explanation is a very fast equilibrium rate between these two possible forms, leading to an average NMR spectrum .

Effect of Aluminum Chloride. The addition of one drop of a 5% solution of $AlCl_3$ to a solution of vitisin A in methanol containing 0.01% HCl produced a small bathochromic shift of the λ_{max} from 518 to 535 nm , as shown in Table 2. Acetylvitisin A showed a slightly smaller shift (+12 nm). This phenomenon with anthocyanins is usually a diagnostic of the presence of two adjacent hydroxyl groups in the B ring (e.g., shift of 44 nm for cyanidin 3-glucoside). It does not occur with malvidin 3-glucoside, which has only one free hydroxyl

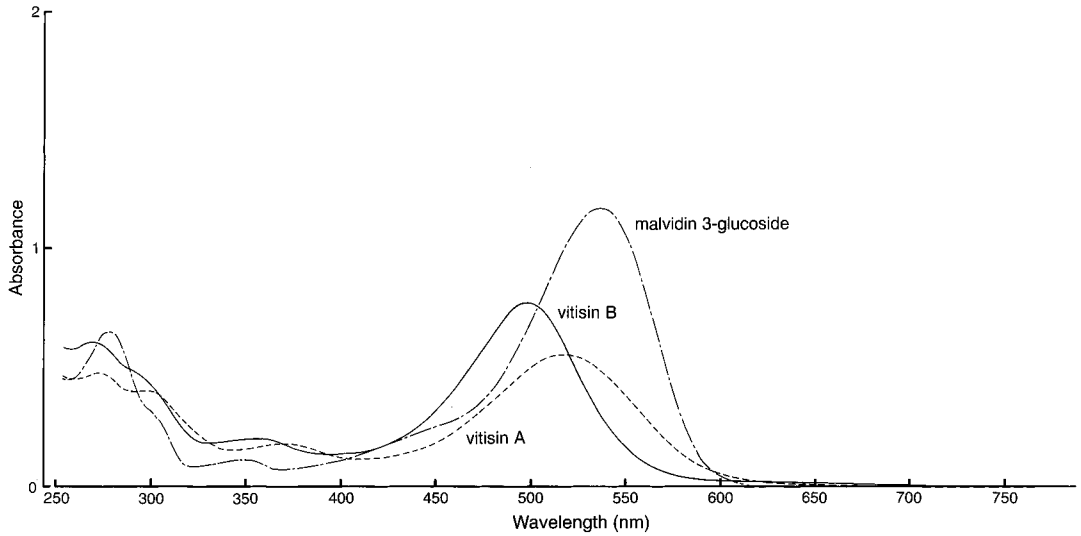

Figure 3. UV−vis spectra collected from 250 to 770 nm of vitisin A, vitisin B, and malvidin 3-glucoside dissolved in methanol containing 0.01% HCl.

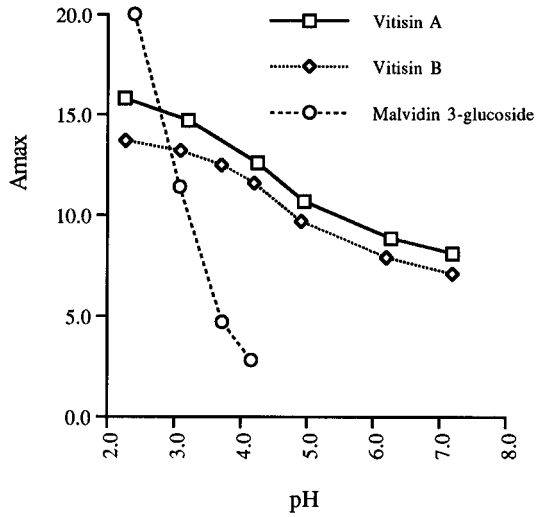

Figure 4. Effect of changes in pH on the maximum absorbance values (A_{max}) of malvidin 3-glucoside and vitisins A and B in aqueous solutions containing 0.1 M citric acid.

group on the B ring. But aluminum chelation also occurs for instance between the 3-hydroxyl or the 5-hydroxyl and the 4-carbonyl groups of flavones. The aluminum shift with vitisin A and acetylvitisin A can be attributed to similar chelation between $C_{\alpha 2}$=O and C_{α}−OH of these compounds. For the vitisin B compounds no such shift was observed upon the addition of $AlCl_3$. Hence this provides further evidence for their tentative structures, which do not contain adjacent hydroxyl or carbonyl groups. It is worth noting that the acylation of the glucose with acetic acid produces a shift of 5 nm for both acetylvitisin A and acetylvitisin B.

Effect of pH. Color expression of anthocyanins is dependent on the pH value of the medium (Brouillard, 1982), and for malvidin 3-glucoside, the pigment occurs under acidic conditions (pH 0−6) as an equilibrium mixture mainly of the colored flavylium cation and the colorless carbinol base forms. At low pH (<2.5) the flavylium structure dominates the carbinol, whereas toward neutrality, the opposite is true. Hence malvidin 3-glucoside itself does not confer much color to a solution in the pH range 4−6. In contrast both vitisin A and vitisin B show a large amount of color expression up to pH values near neutrality. Figure 4 shows the A_{max} values of vitisins A and B in comparison with that for malvidin 3-glucoside. Although the amount of color expressed by both these novel anthocyanins shows a clear pH dependence, their color sensitivity to pH is much less than that for malvidin 3-glucoside. More detailed studies on the equilibrium distribution of anthocyanins and flavylium salts (Brouillard et al., 1982; Mazza and Brouillard, 1987) showed that a 4-substituent enhanced the pH range in which the flavylium structure dominates to approximately pH 5, while the other more dominant form of the mixture was the quinonoidal base, which itself is slightly blueish red in color. The evidence presented here on vitisins A and B would indicate that the distribution of structures with pH is more akin to an aglycon structure with a 4 substituent than malvidin 3-glucoside. However, further detailed studies need to be done to determine the distribution of the anthocyanin structures as a function of pH.

Examination of the scans collected for vitisins A and B (Figure 5) gives further evidence of the preponderance of the quinonoidal base structures. The scans show a reduction in A_{max} values of both anthocyanins, as expected from a reduction in concentration of the flavylium structure. However, above pH 6, an increase in absorbance at around 600 nm indicates contributions in color from the more blueish quinonoidal base. Above this pH value, the λ_{max} for vitisin A shifts from 500 to 510 nm and for vitisin B from 488 to 500 nm. Both anthocyanins also show a clear λ_{max} at 365 and 352 nm, respectively, which appears to be present between pH 2 and 4. Above pH 4 this λ_{max} disappears into a shoulder at near 380 nm. Published spectra of malvidin 3-glucoside show no such a pronounced λ_{max} in this region and also no change into a shoulder with increasing pH values.

The above spectra were also investigated using tristimulus CIELAB 1976 measurements (Bakker et al., 1986). With increasing pH, malvidin 3-glucoside rapidly lost color (increasing L^* values) whereas both vitisin A and vitisin B largely retained their color intensity between pH 2 and 5, whereafter they actually increased in darkness (decreasing L^* values) (Figure 6a). Likewise the hue angles of the vitisin solutions were constant between pH values 2 and 4 and then became bluer as the pH increased to pH 7 (Figure 6b), so indicating the formation of stable quinonoidal bases and

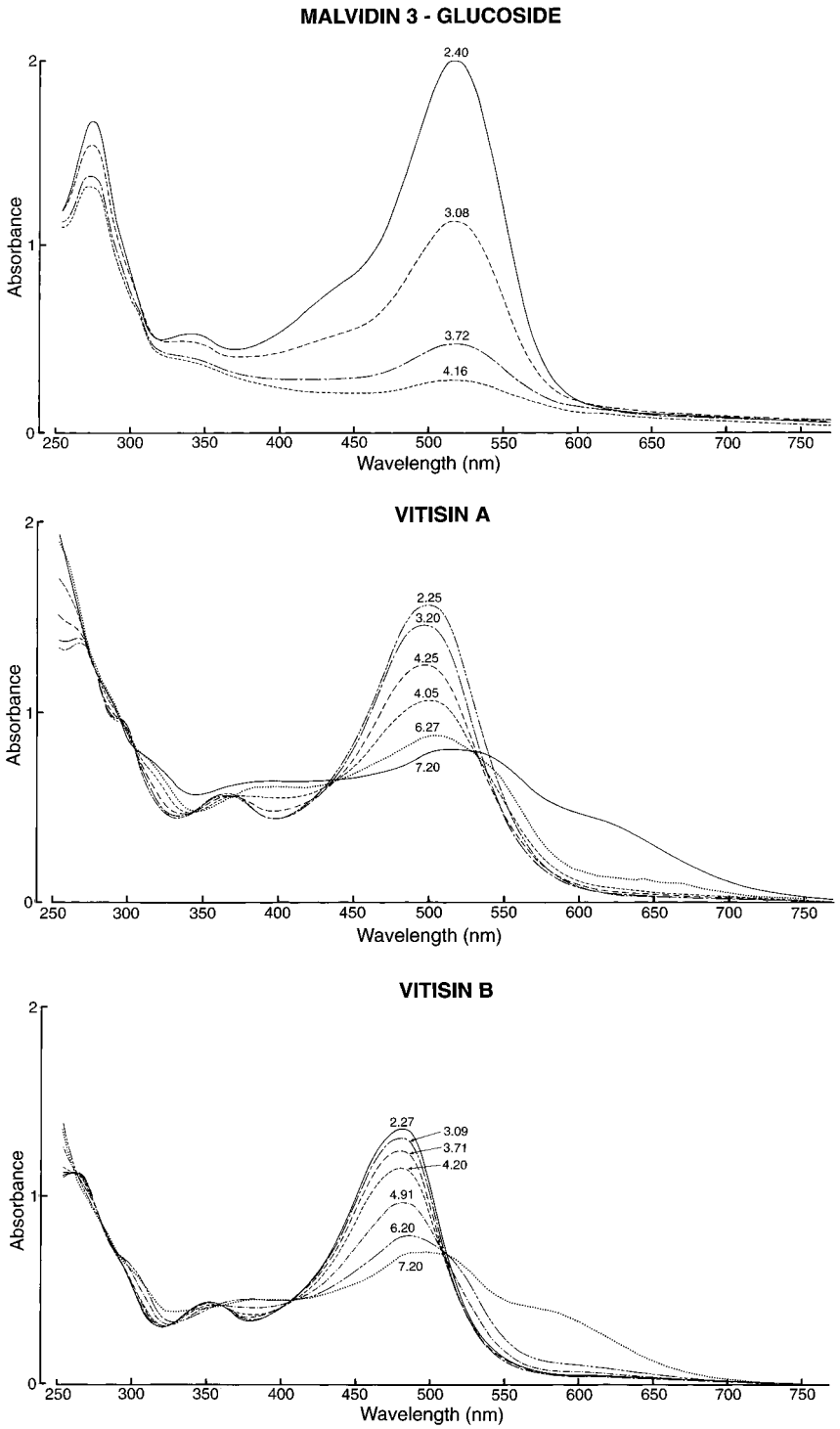

Figure 5. Effect of changes in pH on the UV−vis spectra collected from 250 to 770 nm of malvidin 3-glucoside and vitisins A and B in aqueous solutions containing 0.1 M citric acid.

confirming that there could be little formation of the colorless carbinol base forms.

Sulfur Dioxide Bleaching. It is well-known that anthocyanins are readily bleached by sulfur dioxide with reversible formation of colorless addition products. Considering that sulfur dioxide is widely used in food products, it is surprising that only two groups have attempted to quantify such a significant interaction. Timberlake and Bridle (1967) calculated apparent formation constants of the sulfur dioxide complexes of a range of flavylium salts and anthocyanins. The reactions of the flavylium cation with OH^- (hydration) and with HSO_3^- were shown to be of similar nature (nucleophilic addition) (Timberlake and Bridle, 1966). Later, Brouillard and El Hage Chahine (1980) showed that the addition of the bisulfite anion to the flavylium cation results in a Meisenheimer-type adduct S and they calculated the stability constant of S for cyanin. It had been shown earlier that flavylium cations containing a phenyl or methyl group at position 4 are practically unaffected by sulfur dioxide (Timberlake and Bridle, 1968). The simplistic implication is that bisulfite adds at position 4 of the flavylium cation and that there is some steric hindrance to its addition at position 2, which is the preferred site for nucleophilic addition of OH^-. An alternative explanation is that substitution at position 4 of the flavylium cation affects the distribution of charge throughout the molecule so that positions 2 and

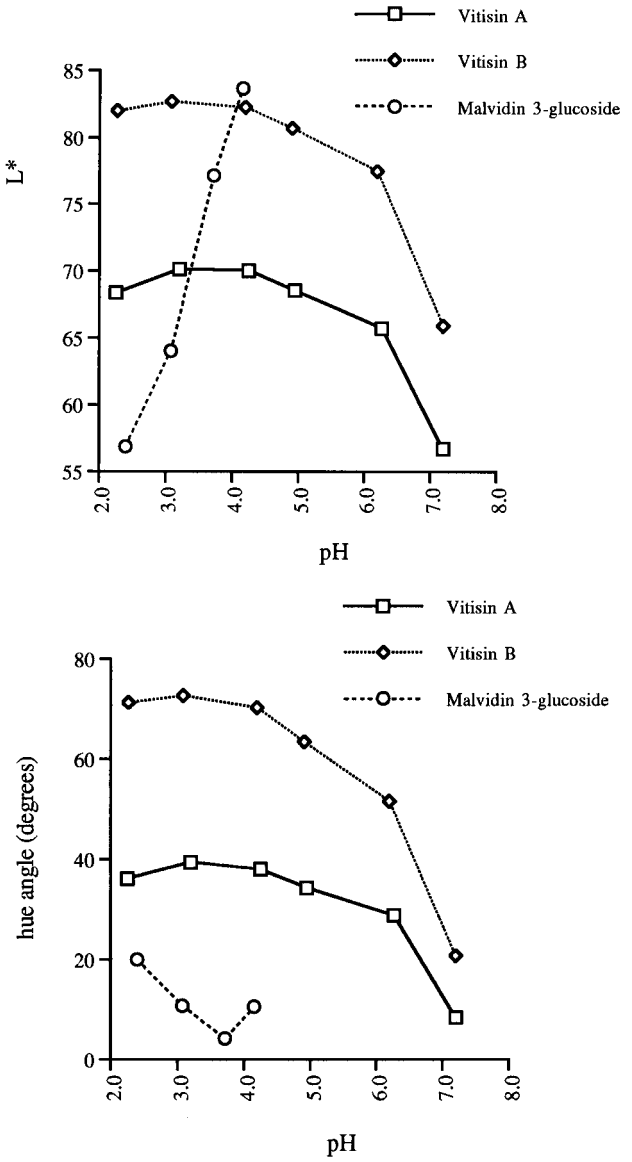

Figure 6. Tristimulus CIELAB 76 color measurements, showing variation of L^* (lightness) and hue angle (degrees) with pH for vitisins A and B and malvidin 3-glucoside.

4 become less reactive to nucleophilic attack. Thus, as has been mentioned, Mazza and Brouillard (1987) and Brouillard et al. (1982) have shown that, unlike anthocyanins (cyanin), 4-methyl- and 4-carboxyl-substituted flavylium salts form very little color base forms in acid solution, rather the quinonoidal bases predominate. Formation of colorless bisulfite adducts would be expected to parallel that of the colorless carbinol bases, resulting in very little bleaching by sulfur dioxide.

Vitisin A is entirely resistant to bleaching by sulfur dioxide. An aqueous solution of vitisin A at pH 3.85 was not bleached by the addition of up to 250 mg L^{-1} sulfur dioxide; even 48 h after the addition the maximum absorbance (A_{max}) remained unchanged (Figure 7). Vitisin B was partly bleached by sulfur dioxide but was much more resistant than malvidin 3-glucoside, which was almost completely decolorized under similar conditions (Figure 7).

Formation and Maturation. The color changes well-known in red wines are due to condensation reactions between anthocyanins and other phenolic compounds naturally occurring in wines. Three mechanisms have been proposed and summarized by Ribéreau-Gayon (1982). The first mechanism involves the reaction

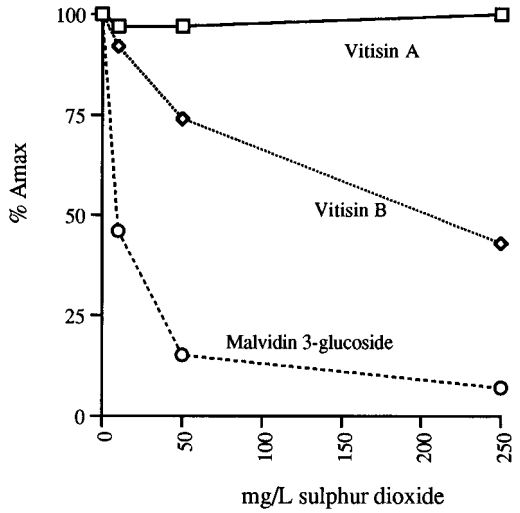

Figure 7. Percentage A_{max} for vitisin A (pH 3.85, A_{max} 13.05), vitisin B (pH 3.80, A_{max} 11.80), and malvidin 3-glucoside (pH 3.45, A_{max} 13.50) with increasing sulfur dioxide concentrations.

of a 4-carbonium ion of procyanidin with anthocyanin (carbinol base form) at position 6 or 8, thus forming a dimer. The second mechanism is the reverse of the first one, with an anthocyanin 4-carbonium ion reacting with procyanidin at the 6 or 8 position. This dimer can undergo further condensation reactions. Evidence for the occurrence of mechanism 2 is the formation of xanthylium salts in stored grape juices and wines (Timberlake and Bridle, 1976). The third mechanism involves the condensation of anthocyanin and procyanidin by the intermediary of acetaldehyde, a reaction first proposed by Timberlake and Bridle (1976). Further evidence for this mechanism has since been reported (Bakker et al., 1993). The work we present here on vitisins provides additional support for mechanism 2. Our results provide a further indication that anthocyanins in red wine can polymerize through their 4 positions with phenolics, since vitisin A appears to be unaffected by the aging reactions undergone by other monomeric anthocyanins. After 9 months of maturation of a port wine, vitisin A is the anthocyanin present in the highest amount; most of the color is due to polymerized pigments.

From our observations it would seem that these compounds can be formed during storage of wines (Bakker et al., in press). Vitisin B in model wines was formed in the presence of malvidin 3-glucoside, acetaldehyde, and an extract of white grapes. Since under these conditions no vitisin A was observed, there may be different pathways to form these compounds.

Relevance to Wine Color. The color properties of the vitisins are expected to influence the red wine color and may explain some of the observations made by many enologists regarding the color changes in red wines during maturation. The hypsochromic spectral shifts of both visitins from malvidin 3-glucoside (Table 2) indicate a shift to orange-brown. Further evidence of the browner color of the vitisins is shown in Figure 6b, showing that at typical wine pH values the vitisins are relatively browner than malvidin 3-glucoside. It is reported that maturation of red wines is accompanied by changes in the color from a bright red to a more brownish red (Somers, 1966, 1968). The vitisins will make a color contribution at a lower wavelength, i.e., toward the orange-brown region, than malvidin 3-glucoside, the dominant anthocyanin in most young wines, which has a much redder absorbance maximum than

the vitisins. Little is known about the color properties of the polymerized anthocyanins, regarding both the quantity and quality of color on a molecular basis, so the relative contribution of the vitisins to a wine also containing polymeric compounds is difficult to assess.

The greater color expression in aqueous solutions of the vitisins than malvidin 3-glucoside at pH values more typical for red wine (about 3.8) may also be significant in wine (Figure 4). Only a small proportion of the anthocyanins is expected to be in the colored form at wine pH, since the equilibrium distribution of malvidin 3-glucoside at this pH value is favoring the noncolored forms, rather than the strongly colored flavylium salt (Timberlake, 1980). Hence despite the low concentrations of the vitisins encountered in the wines analyzed to date, their contribution to the color is expected to be relatively greater than the contribution of malvidin 3-glucoside, including the acylated forms.

Their greater resistance to bleaching by sulfur dioxide (Figure 7) may be significant for the color of a red wine containing anthocyanins. In wines low in acetaldehyde bleaching of the anthocyanins can occur, but vitisin A, and to a lesser extent vitisin B, would maintain its color. Polymeric pigment fractions estimated by bleaching of the monomeric anthocyanins by sulfur dioxide would almost certainly also have included part of the vitisins as polymeric compounds.

Conclusions. It has been shown elsewhere that vitisin A consists of malvidin 3-glucoside containing a $C_3H_2O_2$ link between carbon 4 and the 5-hydroxyl of the molecule (Bakker et al., in press). A tentative structure is now proposed for vitisin B, which is decarboxyvitisin A or malvidin 3-glucoside with a CH=CH structure linking carbon 4 and the 5-hydroxyl group. Vitisin A is less red than malvidin 3-glucoside, and vitisin B is orange in color; possible explanations for these spectral shifts are suggested. Both vitisins A and B also occur in acylated forms, having the 6 position of the sugar acylated with acetic acid. As expected, these novel structures possess properties which are unusual for anthocyanins. Vitisin A shows a shift with Al^{3+}, attributed to $C_{\alpha 2}$=O and the C_{α}−OH groups, and vitisin B does not show this shift. Vitisin A is entirely protected from bleaching by sulfur dioxide, while vitisin B shows greater resistance than malvidin 3-glucoside. Both vitisin A and vitisin B have a greater color expression at higher pH values in comparison with malvidin 3-glucoside. There is evidence of differences in the equilibrium concentrations of the various structures, with the dominant mixture containing the flavylium structure and the quinonoidal base.

Further investigations to study the possible formation of these compounds will allow us to shed more light on one of the pathways of port wine maturation, during which the monomeric anthocyanins rapidly disappear, accompanied by a color change from brick red to a more tawny hue. Since these anthocyanins have some properties desirable for their use as food colors, formation pathways may also give the possibility of their use as food colors. A number of properties, such as light and temperature stability as well as their resistance to reactions with ascorbic acid still remain to be investigated.

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