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Charge equilibria and p*K* values of 5-carboxypyranomalvidin-3-glucoside (vitisin A) by electrophoresis and absorption spectroscopy

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Abstract—Apparent p*K* values of the wine pigment, 5-carboxypyranomalvidin-3-glucoside (vitisin A), were determined using UV–vis spectroscopy, viz. pK_{a1}=0.98 (±0.10), pK_{H1}=4.51 (±0.03) and pK_{H2}=7.57 (±0.02). An additional ionisation constant at high pH (pK_{a4}=8.84±0.06) was established by high-voltage paper electrophoresis. These data in conjunction with previously published pK_a values determined by high-voltage electrophoresis suggest that in wine (pH 3.2–3.8), 5-carboxypyranomalvidin-3-glucoside exists as a complex mixture of hydrated and non-hydrated, partially ionised species with the predominant species being the quinonoidal base (λ_{max} 498 nm).

1. Introduction

Anthocyanins are an important constituent of young red wine. However, the relative instability of the anthocyanins isolated from grape skins compared with the stability of red wine colour suggests that these anthocyanins participate in reactions during the fermentation and maturation of wine to form stable pigments. Pyranoanthocyanins, including 5-(*p*-hydroxybenzyl)pyranomalvidin-3-glucoside (pigment A),[1] 5-carboxypyranomalvidin-3-glucoside (vitisin A) (**1**),[2,3,4] pyranomalvidin-3-glucoside,[3] pinotin A[5] and 5-(catechin/procyanidin)-pyranomalvidin-3-glucoside[6,7] are members of one group of stable wine pigments. Similar or related pigments have also been reported in strawberries[8] and roses.[9] The structure of these pigments is based on a C-4 substituted anthocyanin with a tetra-cyclic structure. Because of the similarity in structure within this group, it is expected that these compounds behave similarly under aqueous conditions with changing pH. Structural modifications due to differences in charge and hydration can result in changes in colour and perhaps flavour. Pyranoanthocyanins are a significant group of colour compounds in wine and it is important to gain an understanding of their physical chemistry.

Our research has indicated that 5-carboxypyranomalvidin-3-glucoside (**1**) is a common pyranoanthocyanin found in Australian red wines.[10,11] Recently we published three macro-protonation constants using high-voltage paper electrophoresis (HVPE), pK_{a1}=0.95 (±0.10), pK_{a2}=3.56 (±0.06) and pK_{a3}=5.38 (±0.07).[12] In this paper we determined two pK_H values and a fourth pK_a value and examined the possible structural changes 5-carboxypyranomalvidin-3-glucoside (**1**) undergoes with changing pH using visible spectroscopy and ^{13}C NMR.

2. Results and discussion

In addition to the three p*K* values determined at lower pH a fourth ionisation constant (pK_{a4}) was determined by HVPE to be 8.84±0.06. For 5-carboxypyranomalvidin-3-glucoside (**1**) to have four protonation constants, it must undergo structural change to yield four free phenolic OH groups. NMR data presented by Bakker et al.[2] suggest that hydration and ring opening of 5-carboxypyranomalvidin-3-glucoside (**1**) can occur at the C-2 and C-5 positions. Ring opening specifically at the C-5 position (**3a**) has been shown to be induced under hot acidic conditions.[13] If hydration occurs at the C-2 and C-5 positions independently, hydration at the C-2 position gives the colourless C-2 hemiketal species[9] (**2a**), whereas hydration at the C-5 position (**3b**) leaves the chromophore intact and therefore the species will be coloured. However, ring opening of either C-2 or C-5 hydration products will give chromophores with absorbance in the visible region. Ring opening at the C-2 position produces a γ-styrylbenzopyrylium (**2b**), while the C-5 ring open species provides malvidin-3-glucoside-4-(2-ketopropionic acid) (**3b**).

Keywords: 5-Carboxypyranomalvidin-3-glucoside; Vitisin A; Wine; Anthocyanin; pK_a.
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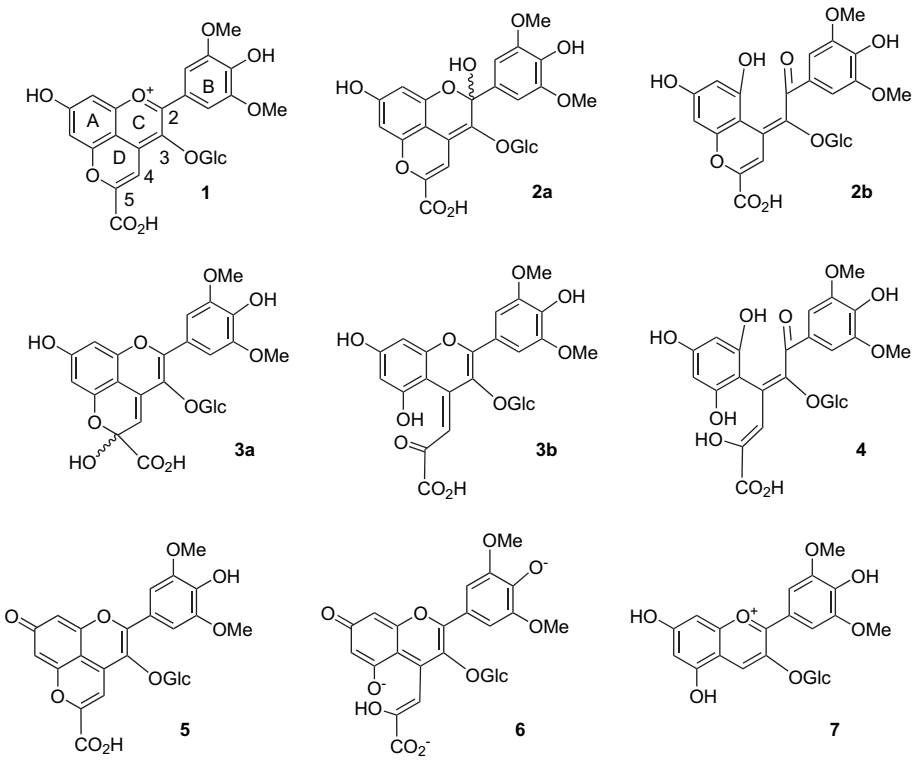

The possibility of ring opening at high pH was investigated by ^{13}C NMR. The data (Table 1) showed that at pH 2.6, 4.4 and 6.8, carbon 5 has a shift at 161.0, 160.7 and 159.6 ppm, respectively, which is consistent with a closed ring pyran. However, at pH 9.6 this carbon has a shift of 183.6 ppm. The doubly (C-2/C-5) opened ring species (**4**) as described by Bakker et al.[2] has a shift of 144 ppm. It is estimated, by comparison with the analogue 2-hydroxychromene-2-carboxylate,[14] that the C-5 carbon of the C-5 hemiketal (**3a**) anion has a shift of approximately 94 ppm. It is therefore suggested that at high pH (pH 9.6) the signal at 183.6 ppm results from the opening of the D-ring.

UV–vis spectroscopic analysis using analytical wavelengths at 510 and 544 nm revealed four plateaus at pH <0.5, 2.7, 6.1 and >9 (Fig. 1). The charge of these plateaus estimated by HVPE is +1, 0, −1.75 and −3, respectively. The first, second and fourth plateaus with integral charge correspond to the flavylium cation (**1**), quinonoidal base (**5**) and the D-ring opened quinonoidal trianion (**6**), respectively. The absorbance maximum, 516 nm, of the flavylium ion (**1**) (Fig. 2) was similar to the flavylium ion of malvidin-3-

Table 1. Shifts for C-5 of 5-carboxypyranomalvidin-3-glucoside compared with literature values in DMSO-d_6 and current data

	Literature	Observed
Flavylium (**1**)	154.5[4]	[a]
Quinonoidal base (**5**)		161.0[b]
Quinonoidal anion		160.7[c]
Quinonoidal dianion		159.6[d]
C-5 hemiketal CO_2^- (**3a**)	(94.3)[f]	
Ring open C-5 hydroxy CO_2^- (**6**)		183.6[e]
Ring open C-5 keto CO_2^- (**3b**)	(198.1)[g]	
Doubly (C-2/C-5) opened ring (**4**)	144.4[2]	

[a] In aqueous solution at pH 0.0, the flavylium ion was insoluble and no ^{13}C NMR data could be obtained.
[b] In aqueous solution pH 2.6.
[c] In aqueous solution pH 4.4.
[d] In aqueous solution pH 6.8.
[e] In aqueous solution pH 9.6.
[f] Derived from structural analogue 2-hydroxychromene-2-carboxylate.[14]
[g] Derived from structural analogue *trans-o*-hydroxybenzylidenepyruvate.[14]

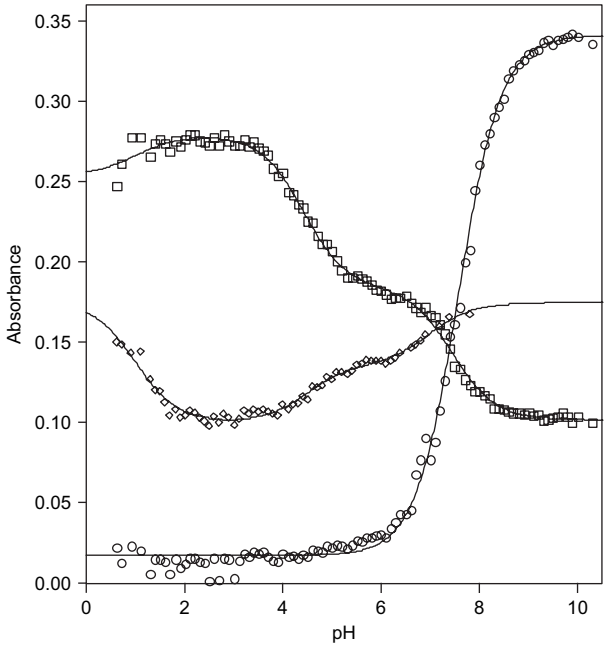

Figure 1. The absorbance of 5-carboxypyranomalvidin-3-glucoside in citrate and sodium pyrophosphate buffers as a function of pH measured at 510 (square), 544 (diamond) and 590 nm (circle).

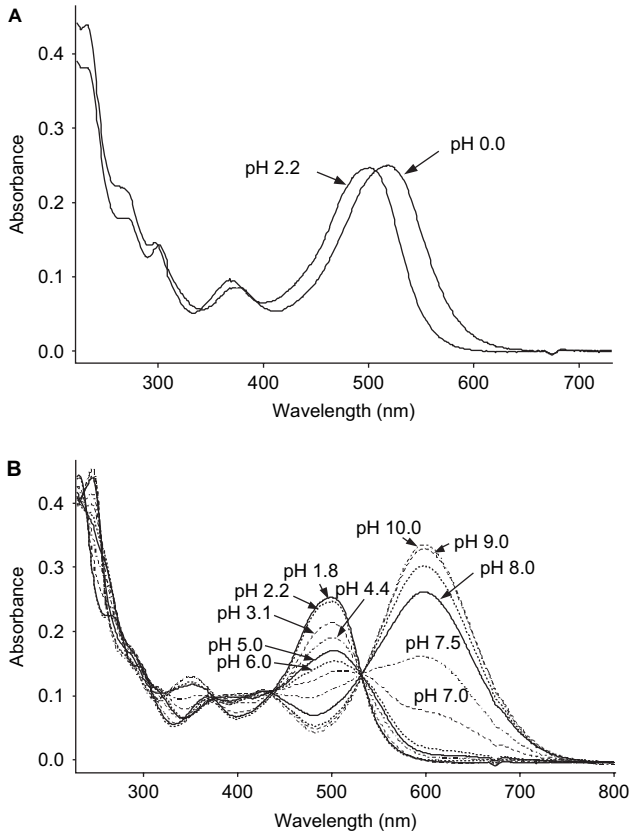

Figure 2. Absorbance spectra for 5-carboxypyranomalvidin-3-glucoside (A) pH 0.0 and 2.2 and (B) from pH 1.8 to 10.

glucoside (**7**) (518 nm),[14] while at high pH (>9) the λ_{max} 599 nm of the D-ring opened quinonoidal trianion was comparable to the quinonoidal dianion of malvidin-3-glucoside (595 nm).[15] These colours were also identical to the red spot at low pH and the blue spot at high pH observed on paper electrophoretograms. At pH 2.7 the spectrum of 5-carboxypyranomalvidin-3-glucoside had a λ_{max} value of 498 nm (Fig. 2), which corresponded to the orange spot detected by HVPE with zero charge that was assigned to the quinonoidal base (**5**).

The first pK (0.98±0.10), determined using the 544 nm analytical wavelength, denotes a shift in wavelengths from λ_{max} 516 to 498 nm (Fig. 2A) and was similar in magnitude to pK_{a1} estimated by HVPE.[12] A hypsochromic shift of the absorbance maximum with increasing pH was also observed for other pyranoanthocyanins, 5-carboxypyranopelargonidin,[8] pinotin A[5] and 5-carboxypyranocyanidin-*O*-glycosides.[16]

An isosbestic point at 532 nm (Fig. 2B) was indicative of an equilibrium between the major species at pH 2.2 (**5**) (charge 0, λ_{max} 498 nm) and at pH 10 (**6**) (charge −3, λ_{max} 599 nm). However, between these two pH values a plateau can be observed at pH 6.1 (Fig. 1), and the shift from the species (**5**) at pH 2.2 to the species (**6**) at pH 10 was actually made up of two equilibrium constants, pK_{α} (4.51±0.03) and pK_{β} (7.57±0.02). pK_{α} was associated with a loss of colour, suggesting that this was the hydration constant pK_{H1}, with hydration occurring at the C-2 position. However, if hydration occurs at the C-2 position, it is still possible for carboxyl group to ionise. Using the method of Perrin et al.[17] it is predicted that pK_a of the C-2 hemiketal (**2a**) carboxyl is approximately 4.2. Expected spectroscopic differences between the hemiketal and the hemiketal anion are minimal. This hydration constant pK_{H1} is larger than that for malvidin-3-glucose (pK_{H1} 2.6)[18] demonstrating that the D-ring helps to stabilise the anthocyanin chromophore.

pK_{β} was associated with an increase in colour, a shift in absorbance to 599 nm and dehydration at the C-2 position. This pK is analogous to pK_{H2} of malvidin-3-glucoside (5.79)[15] and the magnitude of difference between pK_{H1} and pK_{β} (3.06) is similar to pK_{H1} and pK_{H2} of malvidin-3-glucoside (3.19). However, pK_{β} is between pK_{a3} and pK_{a4} as determined by HVPE. ^{13}C NMR demonstrated that ring opening occurs at the C-5 position at high pH (pH 9.6). It is therefore probable that pK_{β} represents the change from the C-2 hemiketal (**2a**) anion to the malvidin-3-glucoside-4-(2-ketopropionic acid) trianion (**6**). At pH 4.5, where according to the HVPE the maximum concentration of the quinonoidal anion was expected, the UV–vis spectrum showed a λ_{max} of 504 nm. This was not significantly different from the quinonoidal base and agrees with the colour revealed by HVPE (Table 2). At pH 7.1 a shoulder at 548 nm corresponds to the maximum concentration of the quinonoidal dianion (Fig. 2B), which agrees with the purple spot observed by HVPE at this pH.

In dilute aqueous solution, it appears that hydration at the C-2 and C-5 positions are competing reactions and that hydration in one ring inhibits hydration in the second ring. Hydration at the C-2 position is favoured at low pH, while at high pH hydration at the C-5 position is favoured. However, while the hydration at the C-2 position at low pH (3–6) is only a minor component occurring in equilibrium with the neutral and/or charged quinonoidal species, at high pH

Table 2. Macro-protonation and hydration constants of 5-carboxypyranomalvidin-3-glucoside according to HVPE and absorbance spectroscopy

Assignment	Ionisation Charge	HVPE				Spectroscopic			
		Plateau pH	Spot colour	pK	Value	Plateau pH	λ_{max} (nm)	pK	Value
Flavylium cation (**1**)	+1	<1.0	Red	pK_{a1}	0.95	<0.5	516	pKa_1	0.98
Quinonoidal base (**5**)	0	2.4	Orange	pK_{a2}	3.56		498		
Quinonoidal Monoanion	−1	4.5	Orange	pK_{a3}	5.38		504		
Quinonoidal dianion	−2	7.0	Purple	pK_{a4}	8.84		547		
C-2 hemiketal (**2a**)	0					2.7		pK_{H1}	4.51
C-2 hemiketal anion	−1					6.1		pK_{H2}	7.57
Malvidin-3-glucoside-4-(2-ketopropionic acid) trianion (**5**)	−3	10	Blue			>9.0	599		

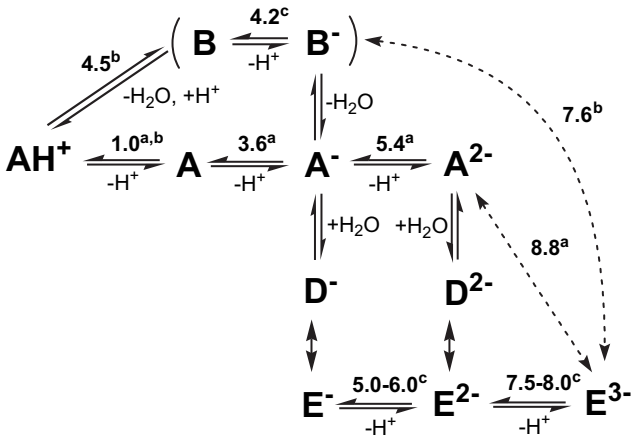

Figure 3. Observed ionisation and hydration of 5-carboxymalvidin-3-glucoside with values as determined by (a) HVPE, (b) UV–vis spectroscopy and (c) prediction using the methods outlined by Perrin[17] where **A** represents the flavylium/quinonoidal species, **B** the C-2 hemiketal species, **D** the C-4 hemiketal and **E** the 4-(2-ketopropionic acid) species.

(>9) hydration at the C-5 position forms the major species. As with malvidin-3-glucoside it appears that the C-2 hemiketal (**2a**) is favoured over the formation of the chalcone (**2b**).[15] In contrast, C-5 hemiketal (**3a**) is unstable and ring opening occurs. At wine pH (3.2–3.8) 5-carboxypyranomalvidin-3-glucoside is in a complex mixture of neutral and anionic, hydrated and non-hydrated states with the principal species being the orange quinonoidal base (Fig. 3).

3. Methods

3.1. Isolation of 5-carboxypyranomalvidin-3-glucoside

The method used for isolation was described in Asenstorfer et al.;[7] the purity (determined by HPLC) was 96.6%. Found: ES-MS (+ve) *m/z* of 561.3 (M^+), 399.2 aglycone (M^+-162); ES-MS (−ve) *m/z* 559.4 (M^+), 515.6 (M^+-44), 353.4, 339.4, 325.4, 311.2; ε_{520} (pH 0.0) 25 000.

3.2. Synthesis

^{13}C 5-labelled 5-carboxypyranomalvidin-3-glucoside was synthesised using labelled sodium pyruvate-2-^{13}C (Cambridge Isotope laboratories, MA, USA) according to Fulcrand et al.[4] This was purified by C18 cartridge adsorption (Sep-Pak classic, Waters). The synthetic product had an identical spectrum and HPLC retention time as the natural product.

3.3. NMR

One dimensional ^{13}C NMR was performed in 20% D_2O using a 600 MHz Varian Inova spectrometer using a spectral width of 35 kHz, a recycle time of 3.5 s and 4000 transients.

3.4. Ionspray mass spectrometry

The ionspray mass spectra of the compounds were obtained by an API-300 mass spectrometer coupled with an ionspray interface (PE Sciex, Thornhill, Ontario, Canada). The ionspray and orifice potentials were 5.5 kV and 30 V for the positive ion mode and −4.5 kV and −30 V for the negative ion mode, respectively. The curtain (nitrogen) and nebuliser (air) gases were set at 8 and 10 units, respectively. The isolated compound in methanol/water was introduced into the mass spectrometer by a flow injector (8125, Rheodyne, Cotati, CA) with a 5 μL sample loop connected to the ionsprayer. The injected solution was delivered by 50% acetonitrile acidified with 2.5% acetic acid at a rate of 5 μL min^{-1}, using a syringe pump (Cole–Parmer, Niles, IL, USA). All data of mass spectra were processed using Bio-Multiview software 1.2ß3 (PE Sciex).

3.5. Proton dissociation and hydration constants using HVPE and UV–vis spectroscopy

pK_a values were determined by HVPE as described in Tate[19] and Asenstorfer et al.[15] Buffers used were 0.05 mol L^{-1} citrate (pH 2.6–8.0), 0.1 mol L^{-1} oxalate (pH 1.2–5.0) and 0.05 mol L^{-1} phosphate/oxalate (pH 7.0–10.4). Determination of pK values and assignment of spectra of the major tautomeric species by UV–vis spectrometry used the methods described in Asenstorfer et al.[12,15] Buffer systems used were 0.2 mol L^{-1} citrate (pH 0.7–7.8) and 0.1 mol L^{-1} pyrophosphate (pH 2.2–10.3). Concentration of 5-carboxypyranomalvidin-3-glucoside solutions was 9.6 μmol L^{-1}

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