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BLUE FLOWER COLOUR DERIVED FROM FLAVONOL–ANTHOCYANIN CO-PIGMENTATION IN *CEANOTHUS PAPILLOSUS*

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Key Word Index—*Ceanothus papillosus*; Rhamnaceae; Californian Lilac; blue flower; acylated anthocyanins; kaempferol 3-*O*-xylosylrhamnoside; mono- and di-coumaroyl delphinidin 3-*O*-rutinoside-7,3′-di-*O*-glucoside.

Abstract—The pigments responsible for the blue flower colour of *Ceanothus papillosus* have been identified. The colour arises from co-pigmentation between the anthocyanins and flavonols. The major anthocyanins have been identified from NMR spectroscopy and degradation studies as the novel acylated delphinidin glycosides, delphinidin 3-*O*-rutinoside 7-*O*-(6-*O*-*p*-coumaroylglucoside) 3′-*O*-glucoside and delphinidin 3-*O*-rutinoside 7,3′-di-*O*-(6-*O*-*p*-coumaroylglucoside), and the major flavonol is kaempferol 3-*O*-xylosyl-(1 → 2)-rhamnoside. The co-pigmentation effect appears to be quite specific, and does not occur to the same extent with other, more common, flavonols. The effect is particularly notable in that an extraordinary, long wavelength visible absorbtion maxima at 680 nm is produced which confers additional blueness. This is proposed to arise from a supramolecular complex of high stoichiometry. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

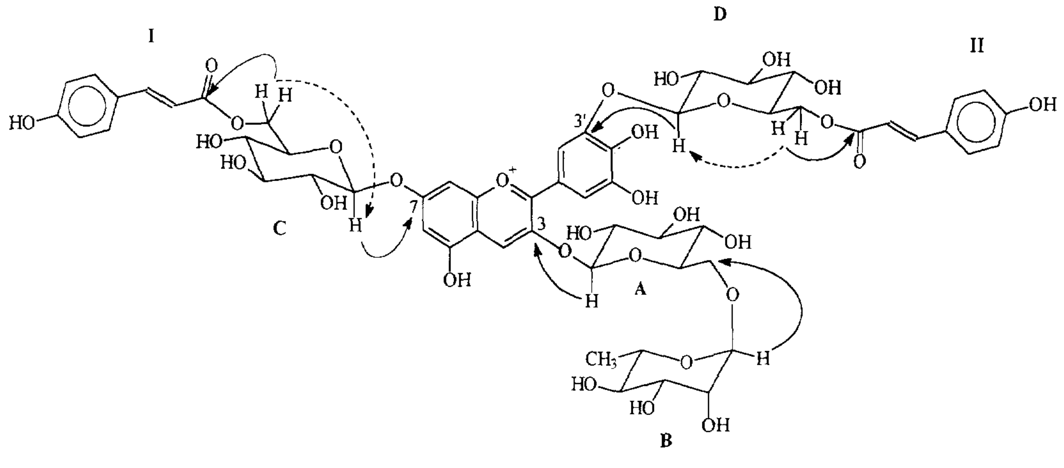
Ceanothus papillosus L. or Californian Lilac [*Ceanothus papillosus* 'Roweanus', (Rhamnaceae)] is a common garden shrub or small tree distinctive for its mass of blue flowers in the springtime. Blue flowers have a particular fascination for the natural product chemist as most blue flowers studied to date have anthocyanin derived pigments with complex acylation and/or derive from supramolocular complexes involving metal ions and/or copigments [1, 2]. This report shows that for *Ceanothus* flowers, the blue colour derives from a specific co-pigmentation effect between the acylated anthocyanin glycosides, delphinidin 3-*O*-rutinoside-7-*O*-(6-*O*-*p*-coumaroylglucoside)-3′-*O*-glucoside, (**1**), delphinidin 3-*O*-rutinoside 7,3′-di-*O*-(6-*O*-*p*-coumaroylglucoside), (**2**), and the kaempferol glycosides present in the petal vacuole.

RESULTS AND DISCUSSION

Fresh flower tissue was extracted with formic acid–water and the pigment fraction concentrated by passage through Diaion HP-20 resin. HPLC analysis of the concentrated extract and fresh pressed juice from the flowers showed that little decomposition of pigment had taken place during extraction and concentration. The HPLC analysis showed two anthocyanins, **1** and **2**, present in approximately equal amounts, and a range of other flavonoids and cinnamic acid esters. The on-line spectra of **1** and **2** indicated anthocyanins acylated with cinnamic acids. Under mild acid hydrolysis, **2** degraded first to a mixture of compounds which included **1** and eventually gave the aglycone, delphinidin.

The two anthocyanins were purified by polyamide CC followed by preparative HPLC. Base hydrolysis of **1** or **2** produced an anthocyanin, **3**, and *p*-coumaric acid. The relatively short HPLC retention time of the glycoside, **3**, suggested tri- or higher glycosylation. The NMR data for **2** showed one rhamnose and three hexose sugars. Two *p*-coumaroyl groups were also present and the remaining signals could be assigned to the delphinidin nucleus. There was no evidence for other acyl groups. The data for **1** showed signals for only one *p*-coumaroyl group. FAB(+) MS gave molecular ions consistent with this assemblage; **1**, M^+ 1081.3171 *m/z*, $C_{48}H_{59}O_{28}$ requires 1081.3036 and **2**, M^+ 1227.3448 *m/z*, $C_{57}H_{63}O_{30}$ requires 1227.3404 (FAB(-) $[M-2H] = 1226$ *m/z*). Thus **1** and **2** are delphinidin tetraglycosides acylated with one and two *p*-coumaroyl groups, respectively.

An indication of the arrangement of the sugars in **1** and **2** was the presence in the mild acid hydrolysis product mixture from **2**, of delphinidin 3-*O*-rutinoside (identified by HPLC cochromatography and on-line spectral comparison with delphinidin 3-*O*-rutinoside from blackcurrant extract). Also, the ^{1}H NMR signals of the delphinidin B-ring protons in **2** were well separated suggesting substitution at the 3′ position. Fur-



Ceanothus anthocyanin **2** (**1** = 7-O-glucosyl)

Fig. 1. Structure of **1** and **2**. Solid arrows indicate connections evident from long range H—C coupling and dotted arrows H—H coupling.

ther evidence leading to the complete structure of **2** was gained from a series of NMR experiments (HMBC, HMQC and TOCSY). The critical connections deduced from these experiments are summarised in Fig. 1. The long range H—C connections from HMQC are shown as solid arrows while the intraglycosidic H—H linkages from TOCSY are shown as dotted arrows. The fourth sugar is thus shown to be attached at the 7 position of the delphinidin nucleus so the deacylated glycoside, **3**, must be delphinidin-3-*O*-rutinoside-7,3′-di-*O*-glucoside. The three hexose sugars are all *β*-D-glucopyranosides from comparison of their ^{13}C NMR shifts with those for similar compounds and from their ^{1}H NMR anomeric coupling constants. Thus, the structure of **2** is delphinidin 3-*O*-rutinoside-7,3′-di-*O*-(6-*O*-*p*-coumaroyl-*β*-D-glucopyranoside).

The other anthocyanin, **1**, was clearly lacking one of the coumaroyl groups of **2**, since signals for only one *p*-coumaroyl group were seen in the ^{1}H NMR spectrum. The general arrangement of the ^{1}H NMR signals of **1** was somewhat different from that of **2** so the placement of the single acyl group was not immediately obvious. As for **2** a TOCSY experiment linked the H-6 and H-1 protons of the acylated glucose. A subsequent HMBC experiment then linked this anomeric proton to a carbon signal at 146.7 ppm, i.e. the acylated glucose is at the 3′ position of the delphinidin so **1** is delphinidin 3-*O*-rutinoside-7-*O*-*β*-D-glucopyranoside, 3′-*O*-(6-*O*-*p*-coumaroyl-*β*-D-glucopyranoside). Interestingly, the ^{1}H NMR signals for the delphinidin 2′ and 6′ protons of **1** were nearly coincident, suggesting a different conformation for this ring in solution from that in **2**.

The intense blue colour of *C. papillosus* petals is particularly striking and although a visible spectrum of the petal could not be recorded with a conventional spectrophotometer due to the miniature size of the petals, the pressed juice obtained from centrifugation of crushed petal tissue was the same intense blue as the petals themselves. The visible spectrum of the juice is shown in Fig. 2(A). At the pH of this pressed juice, 5.4, anthocyanins with trihydroxylated B-rings typically display three visible absorbance maxima at *ca* 520, 570 and 610 nm (see for example refs [3, 4]) and appear purple. The spectrum of the *C. papillosus* pressed juice is unusual in that a fourth strong absorbance maximum occurs at 680 nm. This extra absorption maximum explains the strong blue colouration of the petals. Upon dilution of the pressed juice with pH 5.4 buffer the 680 nm absorption reduced in intensity until, at a dilution factor of approximately five times, the visible absorption spectrum resembled a typical three maxima anthocyanin spectrum.

Intramolecular co-pigmentation

The structures of **1** and **2** are similar to other pigments isolated from violet and blue flowers such as gentiodelphin and the ternatins [5, 6]. These compounds are believed to be stabilised by an intramolecular co-pigmentation effect where the cinnamic acids provide the pyrilium ring with protection from hydration resulting in increased stability at typical petal vacuolar pH [5]. Although there is good evidence from NOE effects that one of the cinnamoyl groups in gentiodelphin is stacked to the pyrilium ring at low pH, the colour stability at higher pH is strongly suggestive of the second cinnamoyl group shielding the other side of the pyrilium ring. Anthocyanin **2** would be expected to show similar behaviour and indeed gives quite stable colour intensity in solution

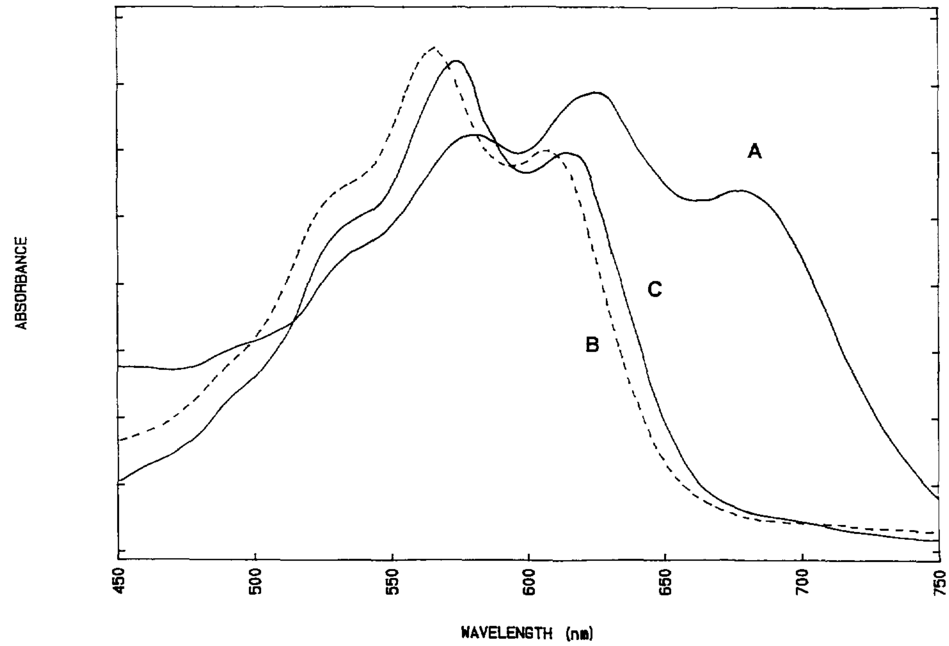


Fig. 2. Visible spectra of *C. papillosus* flower pressed juice (A), anthocyanin **1** (B) and anthocyanin **2** (C) in pH 5.5 buffer.

at pH 5–6 [Fig. 3(A)]. As might be expected, **1** is less stable [Fig. 3(B)], consistent with a stabilisation mechanism where the two *p*-coumaroyl groups in **2** are able to form a sandwich which protects the central anthocyanin ring from hydration. The single *p*-coumaroyl group in **1** is only able to protect one side of this ring. Perhaps supportive of this concept is the fact that the three visible absorption maxima of **2** in pH 5.5 buffer show *ca* 8 nm bathochromic shifts, relative to **1**, typical of co-pigmentation. Also, the relatively upfield ^{1}H NMR chemical shifts of the delphinidin H-4 and coumaroyl protons in **1** and **2** are similar to those seen in other anthocyanins stabilised by intramolecular stacking involving *p*-coumaroyl groups [6]. These shifts are due to a ring current anisotropic effect generated by the proximity of the coumaroyl and delphinidin groups and it appears both *p*-coumaroyl groups in **2** show this effect supporting a sandwich type stacking structure for **2** even at low pH.

Intermolecular co-pigmentation

Intramolecular co-pigmentation is only part of the rationale for the blue colour of petals. The four maxima visible spectrum of the *C. papillosus* pressed juice is not produced by **2** (or **1**) alone which exhibit maxima only at around 536 (529), 576 (565) and 615 (607) nm. Thus another effect must be responsible for the additional peak at 680 nm.

Intermolecular co-pigmentation, especially that between anthocyanins and flavonols or flavones, is the most common colour enhancement effect seen in flowers studied to date. The flavonoid copigments in *C. papillosus* petals were examined and found to be mainly kaempferol glycosides. A group of several glycosides were present and all showed similar chromatographic properties (HPLC, TLC). Only the major glycoside, **4**, could be purified sufficiently for structure identification and was identified as kaempferol 3-*O*-xylosyl-(1 → 2)-rhamonoside by comparison of ^{13}C and ^{1}H NMR data with literature values [7]. The 1-2 linkage was confirmed by the downfield shift position of the rhamnose H-2 proton signal (identified from H—H coupling with the anomeric proton) at 4.01 ppm. At least three other glycosides, also thought to be glycosylated kaempferol-3-*O*-rhamnosides (on the basis of on-line spectra from HPLC analysis and the ^{1}H NMR spectra of the mixtures), were also present. Such glycosides are uncommon and have been reported only recently from natural sources. The ratio of total flavonols to anthocyanins in the pressed juice was approximately 16:1.

Co-pigmentation experiments with an increasing ratio of the major flavonol, **4**, to the diacylated anthocyanin, **2**, showed a remarkable change in the visible spectrum with the appearance of the fourth absorbance at maxima at 680 nm, which is seen in the petal pressed juice (Fig. 4). The relative intensity of this peak increases proportionately with the increase in flavonol level until at a ratio of about 8:1 flavonol–anthocyanin, the pressed juice spectrum is recreated. The colour change from violet-blue to blue is clearly visible. A similar effect was observed with a crude mixture of flavonol glycosides from the petal extract. The positions of the three lower wavelength visible maxima are essentially unchanged on combination with the flavonols indicating the intramolecular co-pigmentation is unaffected. The same experiment with other more common flavonol glycosides such as rutin, kaempferol 3-sophoroside and kaempferol 3-rham-

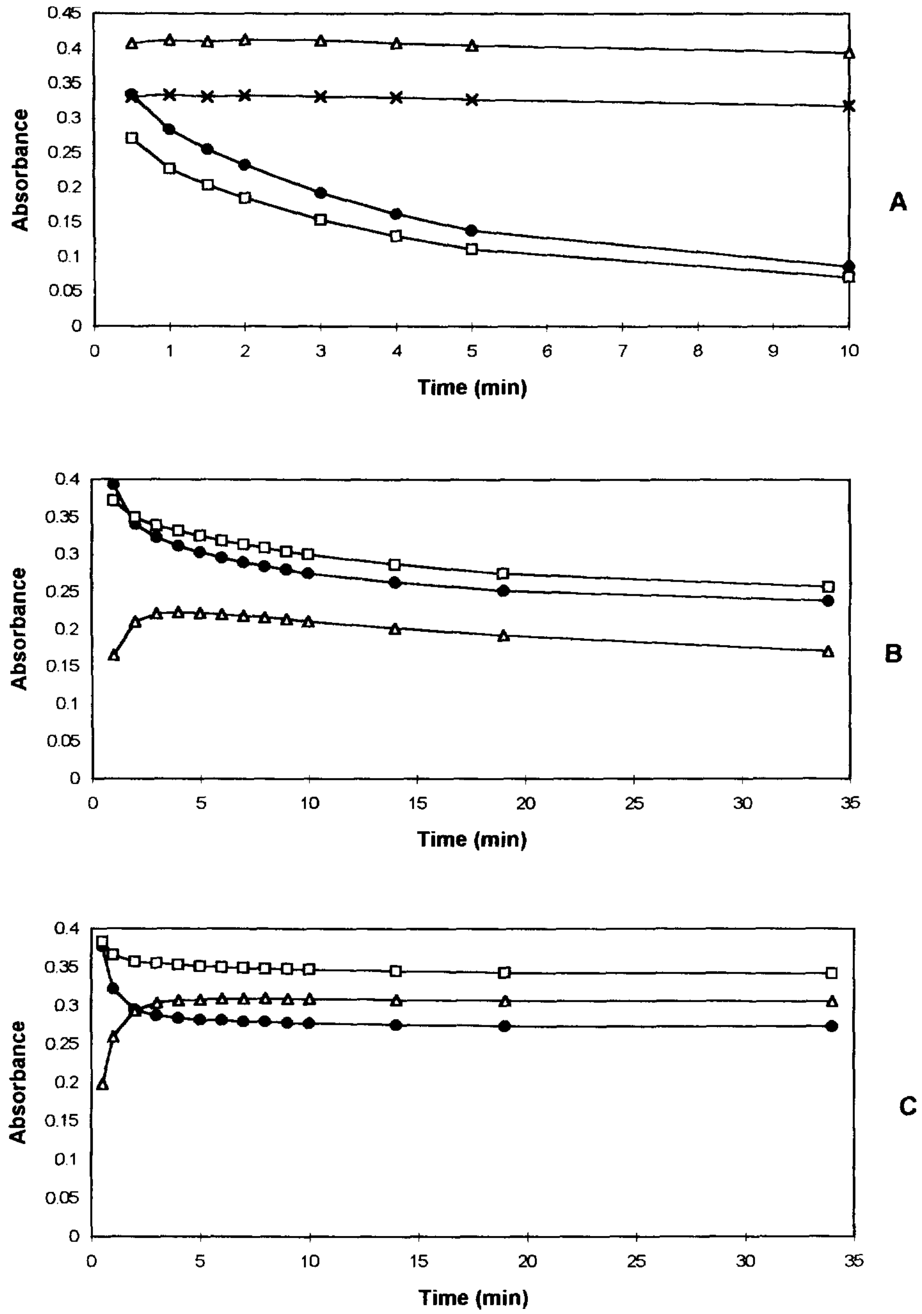


Fig. 3. Stability of anthocyanins and anthocyanin-copigment complexes in pH 5.5 buffer. (A) **1** (●—● 570 nm, □—□ 612 nm) and **2** (△—△ 570 nm, x—x 612 nm); (B) **1**+8 equivalents **4** (●—● 570 nm, □—□ 612 nm, △—△ 680 nm); (C) **2**+8 equivalents **4** (●—● 570 nm, □—□ 612 nm, △—△ 680 nm).

noside, showed little change from the visible spectrum of **2** alone, with either no peak at 680 m (kaempferol-3-sophoroside) or only a low intensity shoulder (rutin, kaempferol-3-rhamnoside). Thus, the blueing effect is apparently due to a specific interaction between the anthocyanins and the relatively unusual kaempferol glycosides present in *C. papillosus* petals.

There are some revealing differences between anthocyanins **1** and **2**. Unlike **2**, when **1** is combined with **4** the positions of the three lower wavelength visible maxima are shifted to positions similar to those seen for **2** alone, consistent with a typical intermolecular co-pigmentation effect between **1** and **4**. Also, as discussed above, the pressed juice spectrum is recreated by combination of **2** and **4** at a ratio of around 1:8 but for **1** the ratio required is higher at about 1:14. Furthermore, the colour stability of **1** in combination with the flavonol is less than that for **2** [Fig. 3(C)] indicating that **2** probably retains the sandwich type intramolecular co-pigmentation in the presence of the flavonols.

The appearance of the fourth absorbance maximum

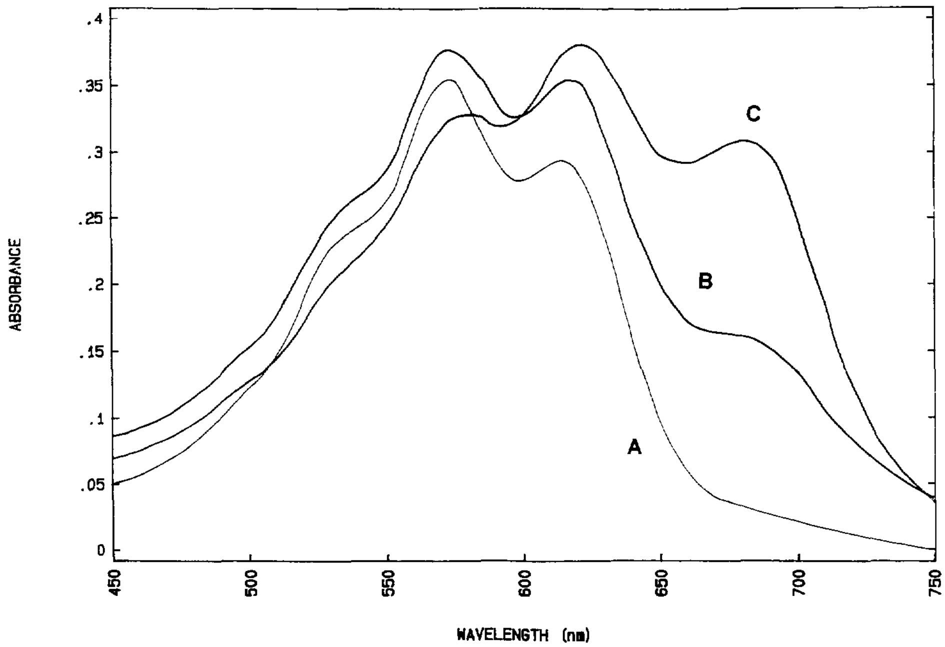


Fig. 4. Visible spectra showing effect of addition of **4** to **2**. (A) **2** in pH 5.5 buffer, (B) **2**+2 equivalents of flavonol glycoside **4**, (C) **2**+8 equivalents **4**.

is not typical of co-pigmentation, which usually produces only small shifts in the existing anthocyanin visible absorption maxima. It is quite possible that the new peak at 680 nm is due to a second effect such as an association of a number of anthocyanin and flavonol glycoside molecules to form a larger complex. There is some supporting evidence for the 680 nm peak arising from such a complex: (i) the peak is lost when the pressed juice is diluted, or on addition of small amounts of DMSO, which is behaviour typical of anthocyanin self association [8, 9]; (ii) the absorption at 680 nm increases over several minutes after combination of flavonol and anthocyanin with a concurrent decrease in the other maxima especially the 580 nm peak [Fig. 3(B), (C)], suggesting slow formation of a complex derived from at least one of the other species present; (iii) such long wavelength absorptions with anthocyanins have only been reported previously in cases where large supramolecular complexes have been shown to be formed, e.g. protocyanin, a metal-anthocyanin-flavone complex, has a peak at 676 nm [10]. The involvement of a metal ion is unlikely in *C. papillosus* given the ability to recreate the pressed juice spectrum from purified anthocyanin and flavonol alone.

The intense blue colouration of *C. papillosus* flowers is due to the co-existence of a number of coloured chemical species involving the two anthocyanins and the several kaempferol glycosides present in the petal vacuole. The diacylated anthocyanin, **2**, is not only stabilised by intramolecular co-pigmentation but appears to form a larger supramolecular complex involving flavonol glycosides. The other anthocyanin, **1**, is stabilised by both intramolecular co-pigmentation (with the single *p*-coumaroyl group) and intermolecular co-pigmentation with the flavonol glycosides. Compound **1** is also able to form the same larger complex proposed for **2**, but at higher levels of co-pigment. The co-pigmentation in **1** is less robust than that in **2**, consistent with the higher level of intramolecular interactions in **2**. The larger complexes are major contributors to the petal blueness as solutions of **2** or **1** alone are blue-violet.

EXPERIMENTAL

NMR experiments were run at 500 MHz or at 300 MHz (75 MHz for ^{13}C). Anthocyanin samples were dissolved in 1% CF_3COOD in CD_3OD. Flavonols were run in DMSO-d_6. HPLC analyses were performed using a Waters 600 solvent delivery system coupled to a Waters 994 PDA detector. Samples of model flavonols used in co-pigmentation experiments are from a collection of flavonoids previously isolated from natural sources.

Plant material. Flowers were collected from a single shrub at the Gracefield Science Centre, Wellington, NZ and processed immediately.

Isolation of anthocyanins. The fresh flowers (170 g) were extracted by blending with 5% aq. formic acid and standing at room temp. for 4 hr. The extract was absorbed onto a Diaion HP-20 column and washed with H_2O. The pigments were eluted with EtOH–HOAc–H_2O (10:1:9), concd on a rotary evaporator and the aq. pigment mixt. freeze-dried. The pigment fr. was applied to a polyamide column (MN-SC.6). **2** was eluted with formic acid–H_2O (1:19) and **1** with

EtOH–formic acid–H_2O (4:1:17). **1** and **2** were further purified by prep. HPLC run on a Phenomenex C-18 (5 μm, 10 × 250 mm) column at 30° with a 1.5 ml min^{-1} flow rate using an isocratic solvent system; HOAc–CH_3CN–TFA–H_2O (100:115:5:780). Frs were evapd by rotary evaporation and freeze drying.

Isolation of flavonols. Fractions rich in flavonol glycosides obtained from the polyamide column above were further purified by silica gel CC using $CHCl_3$–MeOH gradients and Sephadex LH-20 (MeOH). Individual column frs were analysed for purity by HPLC.

Delphinidin 3-O-*rutinoside*-,7-O-*β*-D-*glucopyranoside*,3′-O-(6-O-*p*-*coumaroyl*-*β*-D-*glucopyranoside*) **1**. UV $\lambda_{\max}^{0.1\,\mathrm{N\,HCl}}$ nm: 286, 309, 528 (ratio 309:528 = 1.2:1); ^{1}H NMR δ 8.49 (1H, *s*, H-4 of del), 7.85 (2H, *d*, H-2′,6′ of del), 7.11 (1H, *d*, J = 16 Hz, H-α of II), 7.02 (1H, *br s*, H-8 of del), 6.78 (2H, *d*, J = 8 Hz, H-2, 6 of II), 6.79 (1H, *br s*, H-6 of del), 6.52 (2H, *d*, J = 8 Hz, H-3,5 of II), 5.85 (1H, *d*, J = 16 Hz, H-β of II), 5.33 (1H, *d*, J = 7.5 Hz, H-1 of C), 5.28 (1H, *d*, J = 7.5 Hz, H-1 of D), 4.94 (1H, *d*, J = 10.5 Hz, H-6a of C), 4.86 (1H *d*, J = 7.5 Hz, H-1 of A), 4.77 (1H, *s*, H-1 of B), 1.25 (3H, *d*, J = 6 Hz, H-6 of B); ^{13}C NMR data, see Table 1.

Delphinidin 3-O-*rutinoside*-7,3′-*di*-O-(6-O-*p*-*coumaroyl*-*β*-D-*glucopyranoside*), **2**. UV $\lambda_{\max}^{0.1\,\mathrm{N\,HCl}}$ nm: 290, 305, 542 (ratio 305:542 = 1.5:1); ^{1}H NMR δ 8.31 (1H, *s*, H-4 of del), 7.94 (1H, *s*, H-2′ of del), 7.56 (1H, *br s*, H-6′ of del), 7.35 (1H, *d*, J = 16 Hz, H-β of I), 7.02 (1H, *d*, J = 16 H-β of II), 6.80 (1H, *br s*, H-8 of del), 6.78 (2H, *d*, J = 8 Hz, H-2,6 of I), 6.77 (1H, *d*, J = 1.5 Hz, H-6 of del), 6.66 (2H, *d*, J = 8 Hz, H-2,6 of II), 6.53 (2H, *d*, J = 8 Hz, H-3,5 of II), 6.44 (2H, *d*, J = 8 Hz, H-3,5 of I), 6.15 (1H, *d*, J = 16 Hz, H-α of I), 5.87 (1H, *d*, J = 16 Hz, H-α of II), 5.39 (1H, *d*, J = 7.5 Hz, H-1 of C), 5.24 (1H, *d*, J = 7.5 Hz, H-1 of D), 5.05 (1H *d*, J = 10.5 Hz, H-6a of D), 4.9 obsc (H-1 of A), 4.85 obsc (H-6a of C), 4.73 (1H, *s*, H-1 of B), 4.22 (1H, *t*, J = 10 Hz, H-6b of C), 4.14 (1H, *d*, J = 10.5, H-6a of A), 4.08 (1H *t*, J = 11.5 Hz, H-6b of D), 4.01 (1H, *dt*, J = 9.5,2.5 Hz, H-5 of C), 3.9 (1H, *dt*, J = 9.5,2 Hz, H-5 of D), 1.25 (3H, *d*, J = 6 Hz, H-6 of B); ^{13}C NMR data, see Table 1.

Kaempferol-3-O-*xylosyl*-(1 → 2)-*rhamnoside*, **4**. ^{1}H NMR and ^{13}C NMR data consistent with lit. [7].

Co-pigmentation and other experiments. All experiments were in pH 5.5 McIlvaine buffer. The concn of flavonols was calcd as rutin equivalents from HPLC peak integration data at 352 nm. Anthocyanin concns were determined as cyanidin-3,5-diglucoside equivalents (ε = 35 000) from visible absorption maxima in 0.1 N HCl. The concn of anthocyanin in the pressed juice was approx. 1.1 mM. For co-pigmentation experiments the flavonol was dissolved in 200 μl buffer which was then added to the freeze-dried anthocyanin, mixed and immediately transferred to a 1 mm pathlength cuvette for spectroscopic analysis. Spectra presented in the figures were measured about 20 min after mixing. The concentration of anthocyanin used in these experiments was 0.14 mM.

Table 1. ^{13}C NMR spectral data for *C. papillosus* anthocyanins, **1** and **2**, CD_3OD–CF_3COOD

		1	**2**
Delphinidin	2	163.5	163.4
	3	146.8	146.9
	4	133.9	133.2
	5	157.9	157.9
	6	104.4	104.7
	7	167.5	167.2
	8	96.0	95.2
	9	155.8	155.6
	10	114.8	114.0
	1′	119.6	119.7
	2′	116.0	116.5
	3′	146.7	147.4
	4′	146.3	146.5
	5′	147.7	147.8
	6′	110.2	110.4
Glucose (A)	1	103.2	103.3
	2	74.5	74.6
	3	77.7	77.7
	4	71.3	71.1
	5	77.9	77.8
	6	67.9	67.7
Rhamnose (B)	1	102.2	102.0
	2	71.8	71.8
	3	72.6	72.4
	4	74.0	73.8
	5	69.9	69.7
	6	18.0	17.8
Glucose (C)	1	101.5	100.7
	2	74.4	74.4
	3	77.7	77.6
	4	73.0	71.4
	5	75.3	75.9
	6	65.3	62.6
Glucose (D)	1	101.4	101.9
	2	74.5	74.4
	3	77.2	77.2
	4	72.9	72.7
	5	76.1	75.9
	6	65.0	64.6
p-Coumaroyl (I)	C=O		168.7
	α		114.2
	β		147.2
	1		126.3
	2,6		130.7
	3,5		116.9
	4		161.2
p-Coumaroyl (II)	C=O	168.2	168.2
	α	114.0	114.7
	β	145.5	145.6
	1	126.2	126.1
	2,6	130.5	130.7
	3,5	116.7	117.0
	4	160.6	161.0

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