Pergamon

PII: S0031-9422(97)00595-5

Phytochemistry, Vol. 47, No. 3, pp. 355–361, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

PIGMENT CHEMISTRY AND COLOUR OF *PELARGONIUM* FLOWERS

KEVIN A. MITCHELL, KENNETH R. MARKHAM* and MURRAY R. BOASE†

NZ Institute of Industrial Research and Development, PO Box 31310, Lower Hutt, New Zealand; † NZ Institute for Crop and Food Research Ltd, Private Bag 4005, Levin, New Zealand

(*Received* 1 *April* 1997)

Key Word Index—*Pelargonium*; *Pelargonium* X *domesticum*; Geraniaceae; flower colour; anthocyanins; petunidin-3-*O*-*β*-D-glucopyranoside-5-*O*-*β*-D-[6-*O*-acetylglucopyranoside].

Abstract—The major factors responsible for colour variation in a range of *Pelargonium* species and cultivars were shown to be the types and relative levels of pigments present. Variations in pH and copigment levels were not found to contribute significantly. Flowers with colours ranging from cream and pink through to deep purple, including salmon, orange and red, were studied. While either flavonols or carotenoids were responsible for cream/yellow colouration, all other colours resulted from anthocyanin mixes. The pigments were isolated, quantified and identified by combinations of column and HPL chromatography, absorption spectroscopy and NMR. The major anthocyanins were identified as the 3,5-diglucosides and 3-glucoside-5-(6-acetyl)glucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. The acetates, four of which have not been reported previously, were found predominantly in the regal *Pelargonium* cultivars. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Pelargonium (*Pelargonium* X *domesticum*. L.H. Bail.) flowers (often incorrectly referred to as geraniums) vary in colour from white, through shades of cream ('yellow'), pink, salmon, orange, red and mauve to purple, with many possessing a more intensely coloured 'inner' petal. Notable missing colours include a true yellow and blue. Previous studies of the pigmentation chemistry of *Pelargonium* flowers are limited. Asen and Griesbach [1] demonstrated that most of a range of 20 *Pelargonium* X *hortorum*. L.H. Bail. (zonal pelargoniums) cultivars could be distinguished via HPLC analysis of petal pigments, and in the process identified the anthocyanins as 3,5-diglucosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Accompanying flavonoids were found to be a range of 3-*O*-mono- and 3-*O*-di-glucosides of kaempferol and quercetin and the 7-*O*-glucoside of kaempferol. In a more recent study [2] the anthocyanidin composition of *Pelargonium* X *domesticum*. L.H. Bail. (regal pelargoniums) flowers was determined for 27 cultivars. Again all 6 common anthocyanidins were found, but a distinction was made between inner ('blotched') and outer ('non-blotched') areas of the petal where appropriate. The level of pigmentation in the inner portion was found to be 5–10 times that of the outer portion and to comprise predominantly of delphinidin, petunidin and malvidin.

The object of the present study is to attempt to understand flower colour variation through an investigation of factors such as pigment structure, composition and concentration, pigment:copigment ratio, pH etc. Such an understanding would provide an essential background for any attempt to modify flower colour via genetic manipulation. Preliminary 2D-PC analyses of the flowers used in the current study revealed that flower colour derives predominantly from anthocyanidin and flavonol derivatives. For this reason these components are the prime focus of the investigation. However, since a true yellow is a colour missing from the *Pelargonium* range, the pigmentation in the previously un-examined existing 'yellow' (= cream) horticultural cultivars and original species was also examined.

RESULTS AND DISCUSSION

Cream (*'pale yellow'*) Pelargonium *flowers*

These weakly yellow flowers, noticeably with small petal size in the original non-selected species, were studied primarily in order to determine the source

---

* Author to whom correspondence should be addressed.

Table 1. Pigmentation levels in cream *Pelargonium* flowers

Pelargonium flower	Carotenoids	Flavonols*	Anthocyanin*
'Thompson Morgan' [Thompson and Morgan, Inc. USA]	<0.01	6.0	0.018
'PAC® Botham's Surprise' [Elsner Pac, Germany]	<0.01	6.0	0.011
P. luridum (Andr.) Sweet (sensu lato)	0.11	—	0.015
P. appendiculatum (L.f.) Willd.	0.14	—	0.048
P. alchemilloides (L.) L'Hérit. (sensu lato)	0.14	—	0.044
P. aridum R. A. Dyer	0.20	—	0.011
P. triste (L.) L'Hérit	<0.01	11.0	0.81

(—) Too low to measure.

* Levels are absorption (A) values at λ_{max} for extracts of 50 mg d.w. in 20 ml of solvent (see Experimental).

of their colouration. Sequential extraction of freeze-dried petal material with ether and with acidified aqueous methanol, followed by 2D-PC and absorption spectroscopic analyses, enabled relative levels of the yellow pigments (carotenoids and flavonols) to be determined. As is evident from Table 1, three of the studied taxa, Botham's Surprise, Thompson Morgan and *P. triste*, lack measurable levels of carotenoids but do contain high levels of flavonol glycosides, both 7-*O*-glycosides (quercetin 7-*O*-glucoside and others in Botham's Surprise, Thompson Morgan) and 3-*O*-mono- and di-glycosides (in all three). The other four species exhibit the reverse pigment balance, that is, lacking flavonoids but possessing carotenoids. It is concluded that colouration in these cream flowers results from the presence of flavonols in the former group and from carotenoids in the latter. Anthocyanins were also found. These cause the deep red/black speckling evident in most flowers, but which is significant only in *P. triste*.

Anthocyanin coloured Pelargonium *flowers*

The bulk of *Pelargonium* flowers, i.e. those with colours in shades of pink, orange, salmon, red, mauve and purple, fall into this category. These colours appear to derive entirely from the constituent anthocyanins, since ether extraction of the petals failed to indicate the presence of significant levels of carotenoids. However, because anthocyanin colour manifestation is modified by factors such as pH, copigmentation, concentration and acylation [3, 4], analytical data was recorded for a range of characteristics of potential importance in flower colour (see Table 2). In cases where the 'inner' and 'outer' petal regions exhibited different colours, analyses on both regions were carried out in order to determine what factors are involved in colour differentiation.

The effect of pH. Petal pH levels determined on macerates were confined to a tight range of between 3.0 and 3.9. The higher levels were found only in the red/purple petals, with the outer portion of the petals normally possessing higher pH's than the inner. In general, it seems that petal pH has little effect on colour. For example, N.C. Faiss inner and Dubonnet inner, with very similar ratios, levels etc. and a relatively large difference in pH, both exhibit a very similar colour.

The effect of vacuolar flavonols. Anthocyanin pigments are accompanied by flavonol glycosides in all of these flower petals. The flavonols found in the range of flowers studied were all glycosides of kaempferol, quercetin and myricetin (see Table 2) and included the 3-rhamnosides, 3-glucosides and 3-rutinosides as major components, but with variable distribution. The overall level of flavonoid glycosides is generally exceeded by that of the anthocyanins, i.e. A:F > 1:1 (Table 2). In colour differentiated petals, the relative level of flavonols is higher in the more intensely coloured inner petal than in the outer petal. A consequence of this may be an enhancement of colour intensity through copigmentation between the flavonols and the anthocyanins [3, 4]. Further comment on their possible contribution of the flavonols to colour is presented below.

Anthocyanins and pink colouration. In the cultivars examined, pink colouration results from relatively low levels of anthocyanins in the presence of higher levels of flavonols (Table 2). This higher level of flavonols may be required to stabilise the anthocyanins in this situation by preventing colour loss through hydration [3]. In petals with higher anthocyanin levels, stabilization through self-association tends to occur [4].

The strong pinks, Pink Multibloom and Pink Elite Mix, result from the predominance of peonidin glycosides. The light purple tinge in Pink Multibloom Mix can be accounted for by the replacement of about half of the peonidin with malvidin. In this case the very low total anthocyanin level against the white background of the petal has resulted in a pale colour, which may well be stabilized by the relatively high flavonol level. Pink Multibloom Mix has the highest flavonol to anthocyanin ratio found in this survey.

Anthocyanins and orange/salmon colouration. Orange Appeal and Salmon Multibloom Mix contain a very similar mix of anthocyanidin types. Both produce almost exclusively pelargonidin derivatives, although Salmon Multibloom Mix also contains a small amount of cyanidin. Orange Appeal has a total anthocyanin level that is twice that of Salmon Multibloom Mix and this, combined with the small difference in anthocyanin type, seems to be responsible for

Table 2. Analytical and colour data for anthocyanin containing *Pelargonium* flowers†

Cultivar (R,Z)		pH	A Level	F Level	A:F Ratio	Flavonols (%) Kaem	Quer	Myr	Anthocyanidins (%) Pel	Cy	Peo	Del	Pet	Mal	RHS Code[1]	Universal colour language colour name[2] or [ISCC-NBS[3] Colour Name[4]]
Pink																
Pink Mbl	Z	3.06	12	45	0.3:1	92	8	0	1	7	92	0	0	0	Fan2, 67C	Deep purplish pink
Pink Mbl Mix	Z	3.12	3.2	16	0.2:1	87	13	0	1	6	43	0	0	50	F2, 68B	Strong purplish pink
Pink Elite Mix	Z	*	16	40	0.4:1	88	12	0	2	7	91	0	0	0	F1, 55A	Deep purplish pink
Orange/Salmon																
Orange Appeal	Z	3.21	63	15	4.2:1	100	0	0	98	0	2	0	0	0	F1, 32A	Vivid reddish orange
Salmon Mbl Mix	Z	*	31	9.1	3.4:1	97	3	0	94	5	1	0	0	0	F1, 41C	Moderate reddish orange
Red																
Red/Pink Mbl	Z	3.16	38	35	1.1:1	92	8	0	64	2	34	0	0	0	F1, 52A	Vivid red
Red Mbl	Z	3.03	102	23	4.4:1	95	6	0	74	2	21	0	0	3	F1, 40A	Vivid reddish orange
Red Elite Mix	Z	3.13	81	18	4.5:1	95	5	0	76	2	22	0	0	0	F1, 40B	Vivid reddish orange
Red/Purple																
Karl Hagele	Z	3.3	30	82	0.4:1	84	15	1	0	9	43	5	3	40	F2, 66B	Vivid purplish red
NC Faiss	R	*	67	34	2.0:1	29	20	51	0	0	1	51	17	31		
Outer		3.85	16	0.4	40:1	92	8	0	0	0	1	19	8	72	F2, 67B	Vivid purplish red
Inner		3.88	112	34	3.3:1	20	22	58	0	0	0	54	18	28	F4, 187A	Dark red
Jessica May	R	3.14	44	8.2	5.4:1	78	8	13	57	7	10	9	4	13		
Outer		*	38	3.2	12:1	95	5	0	81	5	11	0	0	3	F1, 44A	Vivid red
Inner		*	73	19	3.8:1	64	18	18	37	7	7	20	9	20	F2, 59B	Deep purplish red
Hazel Cherry	R	3.29	74	25	2.9:1	56	14	30	42	7	5	26	8	12		
Outer		3.59	46	1.7	27:1	72	19	9	74	2	8	3	1	12	F1, 50A	Strong red
Inner		3.22	91	40	2.3:1	52	13	35	33	7	5	27	10	18	F4, 187B	Dark red
Dubonnet Cl	R	*	148	22	6.7:1	19	19	62	15	8	6	33	14	24		
Outer		3.72	150	4.6	33:1	27	20	53	21	5	6	30	11	27	F2, 60A	Deep red
Inner		3.19	148	43	3.5:1	13	20	67	4	3	2	54	14	23	F4, 187A	Dark red

† Abbreviations: Mbl = Multibloom; Cultivar R = regal and Z = zonal; A level = Anthocyanin level (× 10^{-6} mole g^{-1} d.w.); F level = Flavonol level in Rutin equivalents (× 10^{-6} mole g^{-1} d.w.); A:F Ratio = Anthocyanin to flavonol mole ratio; Kaem = Kaempferol; Quer = Quercetin; Myr = Myricetin; Pel = Pelargonidin; Cy = Cyanidin; Peo = Peonidin; Del = Delphinidin; Pet = Petunidin; Mal = Malvidin.

* pH not established.

[1] Royal Horticultural Society (1986). Royal Horticultural Society colour chart. Royal Hort. Soc., London, and Flower Council of Holland, Leiden.

[2] Huse, R. D. and Kelly, K. L. (1984): A contribution toward standardization of colour names in horticulture. The American Rhododendron Society, U.S.A.

[3] Inter-Society Colour Council—National Bureau of Standards;

[4] Kelly, K. L. and Judd, D. B. (1976): Colour: *Universal Language* and *Dictionary of Names*, National Bureau of Standards Special Publication 440. Washington: US Government Printing Office.

the difference in colour. Mirroring the anthocyanins, Orange Appeal produces only kaempferol as its flavonol type while Salmon Multibloom Mix also produces a small amount of quercetin.

Anthocyanins and red colouration. The three red cultivars are all very similar in pigment and flavonol composition. The dominant pelargonidin is accompanied by peonidin in all three and the major flavonol is kaempferol. In terms of colour, no copigmentation effect is evident in that the higher relative flavonol levels in Red/Pink Multibloom are not reflected in altered colouration. The significantly higher levels of anthocyanins, and in particular pelargonidin, distinguish these flowers from the pink flowers (above), and accordingly would account for the red colouration.

Anthocyanins and red/purple colouration. Flowers in this category generally exhibited colour differentiation between inner and outer portions of the petal. In the cultivars in which the outer petal is noticeably more red than the inner, e.g. Jessica May and Hazel Cherry, the anthocyanidin mix in the outer portion is significantly richer in pelargonidin than it is in the inner. This is mirrored in the flavonols by a predominance of kaempferol. The visibly more purple inner portions of the petals are characterized by much higher levels (*ca* 50% of total) of 3′,4′,5′-trioxygenated anthocyanins and reduced pelargonidin levels. This change is reflected to a lesser extent in the flavonol composition. Both the presence of the 3′,4′,5′-trioxygenated anthocyanins and the higher flavonol to anthocyanin ratio would account for the increased blueness of the inner petal relative to the outer. Consistent with this the inner petal portions of the purplish N.C. Faiss, and to a lesser extent those of Karl Hagele and Dubonnet, contain relatively high levels of 3′,4′,5′-trioxygenated anthocyanins. Karl Hagele is unusual amongst the red/purple cultivars studied, in that a high level (*ca* 50%) of 3′,4′-dioxygenated anthocyanins accompanies the 3′,4′,5′-trioxygenated anthocyanins. In this case it appears that the reddish tinge is produced by the 3′,4′-dioxygenated anthocyanins whereas in cultivars such as, Jessica May and Hazel Cherry, it is produced by the high pelargonidin levels.

Anthocyanin structures

The anthocyanidins contributing to the different flower colours discussed above, are all present in the flowers in glycosidic form (anthocyanins). 2D-PCs of all cultivars revealed the presence of up to three distinct groups of anthocyanins with the more highly pigmented cultivars tending to possess all three. In the TBA–15% HOAc solvents used, the minor group, I, which was of sporadic occurrence, ran as do mono-*O*-glycosides. Group II types which were ubiquitous, and group III types which were found predominantly in the Red/Purple cultivars (Table 2), possessed PC mobilities broadly consistent with those of 3,5-diglucosides [5]. HPLC supported these observations. The relationships between these groups were established through acid hydrolysis and co-chromatography. Thus group I glycosides were demonstrated to be 3-*O*-monoglucosides from their HPLC on-line absorption spectra and by HPLC comparison with authentic 3-*O*-glucosides (e.g. from *Rosa*). Likewise a number of group II anthocyanins were shown to be chromatographically identical with authentic 3,5-di-*O*-glucosides from *Rosa* and *Geranium* flowers. Mild acid hydrolysis of the group III anthocyanins converted

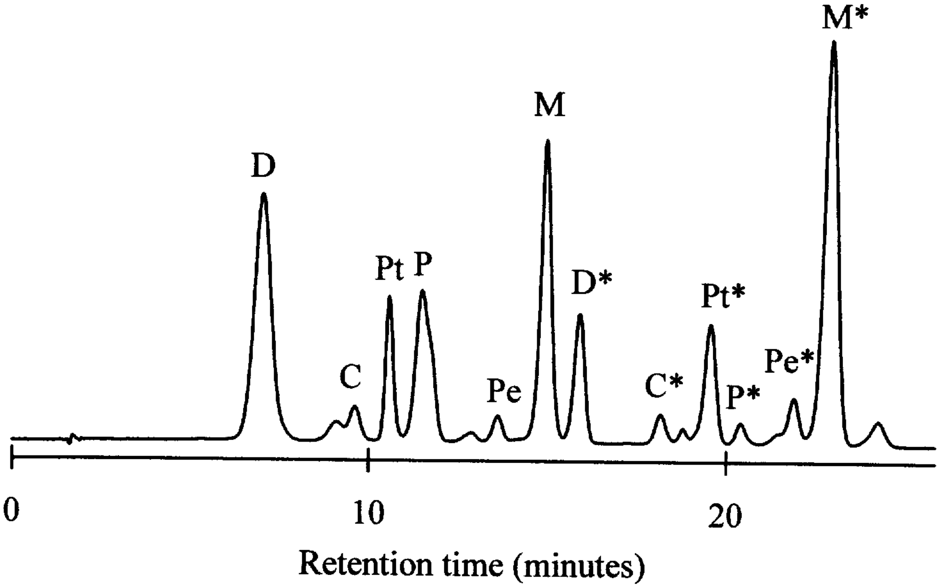

Fig. 1. HPLC of anthocyanins from Dubonnet *Pelargonium* cultivar. Identified compounds (retention time): 3,5-diglucosides; D = delphinidin (7.14), C = cyanidin (9.73), Pt = petunidin (10.69), P = pelargonidin (11.61), Pe = peonidin (13.74), M = malvidin (15.08); 3-*O*-glucosides-5-*O*-[6-acetyl]glucosides; symbols as above but asterisked, D* (16.03), C* (18.28), Pt* (19.68), P* (20.53), Pe* (22.03), M* (23.04).

them to the group II equivalents with no sign of intermediate products. This suggests that group III anthocyanins are mono-acylated derivatives of anthocyanidin 3,5-di-*O*-glucosides.

In order to establish the structures of the acylated anthocyanins, individual compounds were isolated by repeated column chromatography of the crude extracts on polyamide, followed by a final clean-up on LH-20 and/or preparative HPLC, with HPLC monitoring. The HPLC relationship between these compounds is well exemplified by the chromatogram presented in Fig. 1 which represents the range of anthocyanins found in the Dubonnet cultivar. The heavily pigmented Dubonnet cultivar, which accumulates the 3,5-di-*O*-glucosides and acylated derivatives of all six common anthocyanidins, was used as a source for all acylated pigments studied here.

Purified samples of compounds **3**, **5** and **6** on FAB mass spectrometry produced MH^+ ions of *m/z* 683, 667 and 697, respectively. The differences between these molecular weights are accounted for by the different anthocyanidins produced from each on hydrolysis, i.e. petunidin (MW 316) from **3**, peonidin (MW 300) from **5** and malvidin (W 330) from **6**. The remaining portion of all three can therefore be ascribed to the 3- and 5-linked glucoses and a single acetyl group. The presence of the single acetyl group was confirmed for all anthocyanins **1**–**6** by the ^1H NMR spectra (Table 3), each of which revealed a three proton singlet at *ca* δ 2.05. In addition, the appearance of downfield shifted glucose H-6 signals centred at *ca* δ 4.45 and 4.27 (Table 3) requires that the acetyl group be attached to the 6-hydroxyl of one of the glucoses [6]. ^{13}C NMR data available for compounds **3**–**6** confirmed this in that one glucose C-6 signal appeared at *ca* 64.5 ppm, i.e. *ca* 2 ppm downfield from the other. The attachment of the acetyl to the 6-position of the 5-*O*-glycosyl residue was established for each of the six anthocyanins via 2D-^1H, ^1H-TOCSY and HMBC experiments. In this way the H-1 of the 5-linked glucose, with its signal centred at *ca* δ 5.15 (established by HMBC), was demonstrated to reside on the same sugar as the H-6 protons represented by signals shifted downfield by acetylation. In a like manner the 3-linked sugar with H-1 at *ca* δ 5.3 was shown to contain H-6 protons with signals in the usual range for glucosides [6].

On the basis of the above evidence, the tabulated NMR data and the analysis of hydrolysis products, it is concluded that the anthocyanins in Dubonnet are the 3-*O*-β-D-glucopyranoside-5-*O*-β-D-glucopyranosides and the 3-*O*-β-D-glucopyranoside-5-*O*-β-D-[6-*O*-acetyl] glucopyranosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. HPLC comparisons confirmed that the major anthocyanins in all cultivars are combinations of some or all of these compounds with the acetylated compounds occurring predominantly in the regal pelargoniums and Karl Hagele (Table 2). Most of these acetylated anthocyanins are described here for the first time. Very few 3,5-diglucosides of anthocyanidins with acetyl groups attached to the sugar residues have been reported previously [7, 8, 9]. Of these only two are 3-glucosyl-5-acetylglucosides, with pelargonidin and malvidin being the aglycones in the pigments from *Verbena hydrida* [7, 8] and *Geranium* 'Johnsons Blue' [9] flowers, respectively.

EXPERIMENTAL

Plant material. The Goldsmith F1 hybrid zonal cultivars [pink, orange/salmon and red groups—Table 2] were obtained as seed from Watkins New Zealand Ltd., PO Box 468, New Plymouth. The regal cultivars and the zonal Karl Hagele [red–purple group—Table 2] were obtained from St Martins Geranium Nursery Ltd., 13A St Martins Road, Christchurch 2, New Zealand. The 5 (cream) species [Table 1] except *P. triste* were obtained by seed from The South African Pelargonium and Geranium Society, PO Box 55342, Northlands, 2116 Johannesburg, South Africa. *Pelargonium triste* was obtained from Mara Nurseries, Meremere Road., RD12, Hawera, New Zealand. Voucher specimens have been deposited in the international registered herbarium at Massey University, Palmerston North (MPN), with numbers as follows: Table 1 specimens (in sequence): MPN 24555, 24556,

Table 3. Proton NMR data (CD_3OD–CF_3CO_2D, 19:1) for acylated *Pelargonium* anthocyanins*

Acylated 3,5-di	Nuclear protons							5-Glucose		3-Glucose		Other	
glucoside	H-4	H-6	H-8	H-2′	H-3′	H-5′	H-6′	H-1	H-6	H-1	H-6	$COCH_3$	OCH_3
Pelargonidin (**4**)	9.19*s*	7.12*d* (1.7)	7.01*d* (2)	8.66*d* (9)	7.60*d* (9)	7.60*d* (9)	8.66*d* (9)	5.17*d* (7.8)	4.27/4.48*dd* (12,7)/(12,2)	5.27*d* (7.8)	NC	2.06*s*	—
Cyanidin (**2**)	9.13*s*	7.09*d* (NC)	7.00*d* (NC)	8.08*d* (2.5)	—	7.04*d* (9)	8.36*dd* (9,2.5)	5.16*d* (8)	4.27*m*/4.48*dd* (12,2)	5.28*d* (8)	3.75*m*/3.96*dd* (12,2)	2.06*s*	—
Peonidin(**5**)	9.13*s*	7.10*d* (1.5)	7.00*d* (2)	8.23*d* (2)	—	7.04*d* (9)	8.31*dd* (9,2.5)	5.14*d* (8)	4.24/4.41*dd* (12,7)/(12,2)	5.27*d* (8)	3.65*m*/3.92*dd* (12,2)	2.03*s*	3.99 *s*
Delphinidin (**1**)	9.07*s*	6.98*d* (1.7)	7.06*br*	7.82*s*	—	—	7.82*s*	5.17*d* (8)	4.25*m*/4.44*m*	5.32*d* (7.5)	NC	2.05*s*	—
Petunidin (**3**)	9.09*s*	6.98*d* (1.7)	7.08*d* (1.2)	8.01*d* (2)	—	—	7.81*d* (2)	5.16*d* (8)	4.27/4.44*dd* (12,7)/(12,2)	5.32*d* (7.5)	3.7*m*/3.95*dd* (12,2)	2.06*s*	3.99*s*
Malvidin (**6**)	9.06*s*	6.96*d* (2)	7.09*d* (2)	7.93*s*	—	—	7.93*s*	5.16*d* (8)	4.28/4.45*dd* (12,7)/(12,2)	5.34*d* (7.5)	3.72*m*/3.95*dd* (12,2)	2.03*s*	3.97*s*

* Assigned by ^1H, ^1H-COSY, HMBC and ^1H, ^1H-TOCSY; signal couplings in Hz are in parentheses; NC = not clear.

24553, 24551, 24550, 24552, 24554. Table 2 specimens (in sequence): MPN 24537–24549.

Analytical procedures (*data for Table* 1). Ground, freeze-dried petal material (25 mg) was extracted first with Et_2O (20 ml) for 2 hr with stirring. After settling, the absorbance was measured at λ_{max} in the range 410–452 nm. The Et_2O was then decanted and the plant material washed with fresh Et_2O, dried, and extracted with HOAc–H_2O–MeOH, 2:5:13 (20 ml). The A of the clear supernatant was measured (after dilution) at λ_{max} *ca* 350 nm. Next, to 0.5 ml of the undiluted extract, was added 50 μl of 3 M HCl and A measured at λ_{max} *ca* 540 nm. The values obtained were adjusted for dilutions and expressed in terms of A for extracts of 50 mg d.w. in 20 ml of solvent (Table 1). 2D-PC [10] followed by the isolation of key flavonoid components from the chromatogram, and absorption spectroscopy, permitted the determination of flavonoid type [10]. The major yellow fluorescent (in 366 nm) spot on the chromatogram of Botham's Surprise was shown to be due to quercetin-7-*O*-glucoside (absorption spectroscopy, β-glucosidase hydrolysis, product analysis by TLC [10]).

Analytical procedures (*data for Table* 2). Ground, freeze-dried petal material (50 mg) was extracted 3 × (5.0, 2.5 and 2.5 ml) with MeOH–H_2O–HOAc, 70:23:7 and the extracts combined. Anthocyanin levels were determined from the A at λ_{max} *ca* 500–540 nm in extracts diluted with aq. HCl (pH 1), using an ε value of 33 000. Flavonol levels were determined by analytical HPLC (see below). Integrals of all peaks showing flavonol type spectra were added, and the total converted to rutin equivalents using a graph of rutin concn vs peak area determined using an authentic sample. Flavonol aglycone and anthocyanidin types represented were determined on hydrolysed extracts. The extract (1 ml) was heated in a test-tube with 3 M HCl (1 ml) at 100° for 30 min and the cooled product extracted thoroughly with amyl alcohol (or occasionally, sequentially with EtOAc and amyl alcohol when HPLC did not adequately separate the anthocyanidins from the flavonols). The extract(s) was evapd and the residue redissolved in 0.1 M methanolic HCl for HPLC analysis. Aglycones were identified by comparison of their R_ts and absorption spectra with those of authentic standards. Ratios between types were determined from peak integrals, which for the anthocyanidins were adjusted to compensate for differences in λ_{max} relative to the 530 nm detection wavelength. Normalizing multiplication factors, calculated from A at λ_{max} divided by A at 530 nm, were as followed: delphinidin 1.00, malvidin 1.00, petunidin 1.01, peonidin 1.02, cyanidin 1.04, pelargonidin 1.26. Petal pH values were determined by crushing fresh petal material to a pulp in an Eppendorf tube, and measuring the pH of the macerate using a Hanna HI 2031B spear-head micro-electrode.

Extraction and isolation procedures. Frozen 'Dubonnet', used as a source of anthocyanins for structure studies, was ground and extracted × 2 with 0.1% aq. TFA. The combined extracts were applied to a short polyamide column (Macheray–Nagel-CC6) and eluted with 0.1% methanolic TFA. The eluate was evapd, freeze dried, redissolved in a small vol. of 0.1% aq. TFA and chromatographed on polyamide (MN-CC6), eluting with 0.1% aq. TFA containing increasing proportions of 0.1% methanolic TFA. Frs determined to be of interest by HPLC were reduced in vol., freeze dried, redissolved in a small vol. of 0.1% aq TFA and chromatographed on LH-20 using 0.1% TFA in H_2O–MeOH (4:1) for elution. Frs were monitored by HPLC and those containing single components were used for structure studies. Others were further sepd by prep. HPLC.

Analytical HPLC. Analytical HPLC was performed using a Waters 600E solvent delivery system, Waters 996 diode array detector, and a Jasco 851-AS intelligent sampler, results being analysed using Waters Millenium 2010 software. An injection vol. of 10 μl was used with a Merck LiChrospher 100RP-18 endcapped column (5 μm, 4 × 119 nm). Elution (0.8 ml min^{-1}, 30°) was performed using a solvent system comprising solvent A (1.5% H_3PO_4) and solvent B (HOAc–CH_3CN–H_3PO_4–H_2O, 20:24:1.5:54.5) mixed using a linear gradient starting with 80% A, decreasing to 33% A at 30 min, 10% A at 33 min, and 0% A at 39.3 min. Anthocyanins were detected at 530 nm and flavonols at 352 nm.

Preparative HPLC. Prep. HPLC was performed using a Jasco LG-980-02 ternary gradient unit, a Jasco PU-980 intelligent HPLC pump, a Gilson 234 sampling injector, a SciTech column heater, a Waters 994 programmable photodiode array detector and an Isco foxy-200 x-y fr. collector. An injection volume of 100 μl was used with an Alltech econosil C18 column (10 μm, 10 × 250 mm). Solvent system as above. Frs of interest were diluted *ca* × 10 with H_2O and applied to prewashed (0.1% aq. TFA) reversed phase C18 followed by elution of anthocyanins with 0.1% methanolic TFA.

NMR measurements. ^{1}H NMR spectra of anthocyanins were measured at 500 MHz using CD_3OD–CF_3CO_2D, 19:1 as solvent and are presented in Table 3. ^{13}C NMR spectra were measured at 75 MHz using the same solvent. The spectra of the acylated anthocyanins differed only in the signals of the anthocyanidin. A typical example, petunidin-3-*O*-β-D-glucopyranoside-5-*O*-β-D-[6-*O*-acetylglucopyranoside] exhibited signals at: anthocyanidin: 164.9 (C-2), 146.9^a (C-3), 135.7 (C-4), 156.7 (C-5), 105.9 (C-6), 169.4 (C-7), 97.3 (C-8), 157.2 (C-9), 113.5 (C-10), 119.8 (C-1′), 109.8 (C-2′), 149.9 (C-3′), 146.2^a (C-4′), 156.7 (C-5′), 114.2 (C-6′); 5-glucose: 102.3 (C-1″), 74.4 (C-2″), 77.6 (C-3″), 71.5^b (C-4″), 75.9 (C-5″), 64.5 (C-6″); 3-glucose: 103.9 (C-1‴), 74.8 (C-2‴), 78.4 (C-3‴), 71.4^b (C-4‴), 79.0 (C-5‴), 62.6 (C-6‴); other: 172.6, 20.7 (acetyl), 57.2 (OMe) ppm. Key HMBC correlations supporting

---

[a,b] Assignments with the same superscript may be reversed.

both ^{13}C and ^{1}H signal assignments include: H-4/C-2, 3, 5, 9 and 10; H-6/C-5, 7, 8 and 10; H-8/C-6, 7, 9 and 10; H-2′/C-2, 3′, 4′ and 6′; H-6′/C-2, 2′ and 4′; H-1″/C-5; H-1‴/C-3; H-6″a, 6″b/acetyl CO; OMe/C-3′.

Acknowledgements—The authors wish to thank Susan Read of NZ Institute for Industrial Research and Development (IRL) for technical assistance, Dr Herbert Wong of IRL for running the NMR spectra and Rosemary Webby of IRL for preliminary studies of some flavonol glycosides.

REFERENCES

1. Asen, S. and Griesbach, R., *Journal of the American Society of Horticultural Science*, 1983, **108**, 845.
2. Fujioka, M., Kato, M., Kakihara, F. and Tokumasu, S., *Journal of the Japanese Society of Horticultural Science*, 1991, **59**, 823.
3. Brouillard, R. and Dangles, O., in *The Flavonoids—Advances in Research since* 1986, ed. J. B. Harborne. Chapman and Hall, London, 1994, p. 565.
4. Goto, T. and Kondo, T., *Angewandte Chemie—International Edition in English*, 1991, **30**, 17.
5. Harborne, J. B., *Phytochemical Methods*, Chapman and Hall, London, 1973, p. 63.
6. Markham, K. R. and Geiger, H., in *The Flavonoids—Advances in Research since* 1986, ed. J. B. Harborne. Chapman and Hall, London, 1994, p. 441.
7. Toki, K., Saito, N., Kuwano, H., Terahara, N. and Honda, T., *Phytochemistry*, 1995, **38**, 515.
8. Toki, K., Saito, N., Terahara, N. and Honda, T., *Phytochemistry*, 1995, **40**, 939.
9. Markham, K. R., Mitchell, K. A. and Boase, M. R., *Phytochemistry*, 1997 **45**, 417.
10. Markham, K. R., *Techniques of Flavonoid Identification*. Academic Press. London, 1982, p. 15.