Pergamon

PII: S0031-9422(96)00831-X

Phytochemistry, Vol. 45, No. 2, pp. 417–423, 1997

0031-9422/97 $17.00+0.00

MALVIDIN-3-*O*-GLUCOSIDE-5-*O*-(6-ACETYLGLUCOSIDE) AND ITS COLOUR MANIFESTATION IN 'JOHNSON'S BLUE' AND OTHER 'BLUE' GERANIUMS

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

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

(*Received* 14 *October* 1996)

Key Word Index—*Geranium*; Geraniaceae; 'Johnson's Blue'; malvidin-3-*O*-β-D-glucopyranoside-5-*O*-β-D-[6-*O*-acetylglucopyranoside]; flower colour; colour reconstitution; vacuolar pH.

Abstract—A study of the physicochemical factors contributing to flower colour in three 'blue' geraniums, 'Johnson's Blue', *G. pratense* and *G. sanguineum*, has led to an understanding of how such colour variants are produced in geraniums. All three contain the same new anthocyanin, malvidin-3-*O*-β-D-glucopyranoside-5-*O*-β-D-[6-*O*-acetylglucopyranoside], the structure of which was established by 2D-NMR techniques. This anthocyanin is an isomer of the malvidin-3-*O*-β-D-[6-*O*-acetylglucopyranoside]-5-*O*-β-D-glucopyranoside reported recently from *G. sylvaticum*, but it was indistinguishable by HPLC from the pigment sourced from a locally available *G. sylvaticum*. It was accompanied in 'Johnson's Blue' and *G. pratense* with kaempferol and myricetin 3-*O*-glucosides and 3-*O*-sophorosides, but with only low levels of flavonols in *G. sanguineum*. The pH values of pressed juice from these flowers were 5.4, 5.4 and 4.6, respectively. In *in vitro* colour reconstitution experiments, the petal spectra of 'Johnson's Blue' and *G. pratense* could only be produced from the anthocyanin at near vacuolar concentrations, in the presence of molar excesses of kaempferol-3-*O*-sophoroside (the major copigment) and at a pH of 6.6 to 6.8. It is concluded that the pH of epidermal cell vacuoles in these flowers is 1–1.4 units higher than that of the pressed juice. *In vitro* colour reconstitution of this type is proposed as a convenient method for determining the approximate pH of anthocyanin-containing epidermal cell vacuoles. The colours and colour stabilities of all three studied flowers can be adequately accounted for by the pigment structure and concentration, the flavonol type and ratio, and the epidermal pH.

INTRODUCTION

In recent years a number of cultivars in the genus *Geranium* L. have become more popular and commercially available [1,2]. Several cultivars are marketed as blue geraniums. Amongst these are the three studied here, *Geranium* 'Johnson's Blue' (*G. himalayense* Klotzsch. × *G. pratense* L.) (bluish purple), *G. pratense* L. (purplish blue) and *G. sanguineum* L. (bluish magenta). To date no study of the flower colour chemistry of these cultivars has been reported. In 1983 Asen and Griesbach [3] surveyed a wide range of non-blue 'geranium' (actually zonal *Pelargonium*) cultivars for their pigment and copigment components and found that the predominant anthocyanins were 3,5-diglucosides of pelargonidin and peonidin, and that the predominant flavonols were the 3-glucosides and rutinosides of kaempferol. More recently a new anthocyanin, malvidin-3-*O*-(6-*O*-acetylglucoside)-5-*O*-glucoside, has been identified by Andersen *et al.* [4] as the major anthocyanin in *G. sylvaticum* florets. The unacylated compound however has been identified previously at low levels in several other cultivars [3] and in *G. eriostemon* [5]. In the present study, aspects of the chemistry and biochemistry of *G.* 'Johnson's Blue', *G. pratense* (meadow cranesbill) and *G. sanguineum* (bloody cranesbill) are compared and contrasted with similar data for other geranium flowers in an attempt to understand the basis for their novel blue colouration.

RESULTS

Petal characteristics

Petals of 'Johnson's Blue' were examined in some detail by stereo-microscopy and it was evident that

*Author to whom correspondence should be addressed.

pigmentation was spread evenly across all cells, each of which appeared the same bluish colour. Examination in cross-section revealed that the pigmentation occurred only in the (papillate) epidermal cells forming the upper and lower surfaces. The double inner layer of parenchymous cells lacked colour. Petal thickness was measured by micrometer at *ca* 100 μm and around 70% of this thickness contained pigmentation.

The fresh petal colours of 'Johnson's Blue', *G. pratense* and *G. sanguineum* were compared by visible absorption spectroscopy. All three gave spectra with major peaks at *ca* 540, 575 and 620 nm representing an equilibrium mix of flavylium and quinonoidal forms of the constituent anthocyanin pigments. Such spectra are typical of purple–blue flowers in which there is no metal ion complexation [6]. From these spectra an estimate of relative colour intensity was made on one batch by comparing the absorbances at λ (max) measured from a 450–650 nm baseline. The figures obtained (Table 1) match what was expected from visual examination, with 'Johnson's Blue' being the most intensely coloured. The degree of blueness determined visually was also estimated from these spectra through a comparison of the relative intensities of the 540 (reddish), 575 (bluer) and 620 (blue) nm absorptions. Thus the 'bluish-magenta' *G. sanguineum* exhibits lower absorption (A) at 620 nm, relative to the 'less blue' absorption bands at 575 and 540 nm, than do 'Johnson's Blue' and *G. pratense*. The spectrum of the bluest flower, *G. pratense* in contrast shows the highest relative ODs at 575 and 620 nm. By way of objective comparison, the colour of 'Johnson's Blue' was assessed as equivalent to violet–blue group 93B on the Royal Horticultural Society's colour chart, while *G. pratense* rated 92A on the violet–blue chart and *G. sanguineum*, 80A on the purple–violet chart.

Petal pH

The petal pH for each of the three flowers was obtained from juice expressed from a number of petals

Table 1. Physical and chemical characteristics of blue *Geranium* petals

Characteristics	*G.* 'Johnson's Blue'	*G. pratense*	*G. sanguineum*
Petal			
Colour of petals: (visual)	Deep bluish purple	Light purplish blue	Bluish magenta
Colour intensity: (A at λ max of petal, *ca* 575 nm)	0.55	0.10	0.23
Petal spectra:			
Relative A at 540 nm	1	1	1
575 nm	1.20	1.47	1.13
620 nm	0.57	0.59	0.32
Chemical			
pH (pressed juice):	5.40	5.43	4.63
Molar concentrations (vacuolar, M L):*			
Anthocyanins	2.8×10^{-3}	nd	nd
Flavonols	5.3×10^{-3}	nd	nd
(dry petals, M/g):†			
Anthocyanins	1.1×10^{-5}	1.1×10^{-5}	1.4×10^{-5}
Flavonols	2.5×10^{-5}	4.1×10^{-5}	0.3×10^{-5}
Molar ratio:			
Flavonols:anthocyanins	2 : 1	4 : 1	1 : 5
2DPC patterns	≡ *G. pratense*	≡ *G.* 'Johnson's Blue'	Very low in flavonols
Pressed juice anthocyanins (%):			
Compound **1**	90.2	74.6	92.2
Malvidin-3,5 diglc **2**	5.2	14.9	4.7
Malvidin-3-glc	1.4	3.0	1.0
Malvidin-5-glc	1.7	2.0	0.5
Flavonol (%):‡			
Kaempferol	64	80	53
Quercetin	9	13	23
Myricetin	27	7	24

*Vacuolar conc. = pressed juice conc. × 100/70; nd = not determined.

†Anthocyanin levels calc. using $\varepsilon = 33\,000$. Flavonol levels calc. as rutin equivalents.

‡Values obtained from acid hydrolysed extracts.

via crushing and centrifugation (pressed juice). The figures obtained suggest that 'Johnson's Blue' and *G. pratense* are very similar with pHs about 5.4, whereas *G. sanguineum* differs with a pH of about 4.6.

Petal chemistry

A 2D-PC screen of the acidic extracts from all three flowers revealed that 'Johnson's Blue' and *G. pratense* contained closely similar arrays of anthocyanins and flavonol glycosides. *G. sanguineum* differed in that although the anthocyanin components appeared to be the same, the flavonol glycosides were almost completely missing. Approximate flavonol–anthocyanin molar ratios determined on these extracts, both from their absorption at λ (max) 352 and 530 nm, and by HPLC, confirmed that *G. sanguineum* contained the lowest ratio (Table 1). Thus whereas *G. pratense* gave a ratio of 4 : 1 and G. 'Johnson's Blue' a ratio of 2 : 1, the low flavonol level in *G. sanguineum* resulted in a ratio of only 1 : 5.

Acid hydrolysis of the crude extracts followed by HPLC comparison with authentic anthocyanidin standards revealed that the anthocyanins in all three flowers were based predominantly on malvidin, accompanied by low levels of the other five common anthocyanidins. Further, it was established by HPLC analysis of the pressed juice, that the malvidin occurred in the vacuole as one major glycoside. In all three flowers, one anthocyanin (**1**, RT 22.6 min) was predominant to the extent of 75–92% with the second largest peak (**2**, RT 14.6 min) contributing 5–15% and exhibiting an identical absorption spectrum with maxima at 274.5, 300 sh, 343.5 and 527.5 nm. Mild acid treatment converted **1** readily into **2** indicating that the former is an acid labile acyl derivative of the latter. The absorption spectrum and chromatographic mobility of **2** was supportive of a malvidin-3,5-diglycoside formulation, and indeed authentic malvidin-3,5-diglucoside ex *Pelargonium* × *domesticum* 'Dubonnet' cochromatographed with it. Furthermore, the partial hydrolysis products from malvidin-3,5-diglucoside, malvidin-3-glucoside and malvidin-5-glucoside, cochromatographed with the partial hydrolysis products from **2**. From these data it is concluded that the major anthocyanin in all three flowers is an acylated malvidin-3,5-diglucoside which, together with its deacylated product (which is possibly an artefact) comprises in excess of 95% of the total anthocyanins (Table 1). The vacuolar concentration of this anthocyanin in the pigmented cells was determined from pressed juice by absorption spectroscopy to be 2.8×10^{-3} M l^{-1}.

Compound **1** was isolated in sufficient quantity for NMR studies by repeated column chromatography of the 0.1% aqueous TFA extract. The ^{1}H-NMR spectrum (Table 2) confirmed the above structural features and in addition revealed an acetyl methyl signal at δ 2.03 which integrated for three protons, together with downfield shifted glucose 6-CH_2 multiplets centred at δ 4.45 and 4.25. The ^{13}CNMR spectrum provided support for these observations (Table 2) with acetyl carbon signals at δ 20.7 and 172.7 and glucose C-6 signals at δ 64.6 (acylated) and 62.6 (non-acylated). Accordingly **1** is defined as malvidin-3,5-diglucoside mono-acetylated at the 6-position of one of the glucosyl residues. The acetyl function was shown to reside on the 5-linked glucosyl residue by a 2D-^{1}H, ^{1}H-TOCSY experiment. This proved that the glucose residue with its H-1 signal at δ 5.16 also gives rise to the H-6 signals at δ 4.28/4.45. It is this glucose, therefore, that carries the 6-linked acetyl group. The signal at δ 5.16 is long-range coupled (HMBC) to the C-5 signal at 156.6 ppm and is, therefore, assigned to the H-1 of the 5-linked glucose. Conversely, the sugar H-1 signal at δ 5.34 is long-range coupled to C-3 at 146.8 ppm, so confirming its assignment to the 3-linked glucose. Compound **1** is therefore assigned the structure malvidin-3-*O*-β-D-glucopyranoside-5-*O*-β-D-[6-*O*-acetylglucopyranoside], which is a new anthocyanin. The major anthocyanin from *G. sylvaticum* has recently been assigned a similar structure [4], but with the acetyl group positioned at the 6-hydroxyl of the 3-linked glucose on the basis of a 2D-^{1}H, ^{1}H-COSY study. In our hands, the major anthocyanin from a locally sourced *G. sylvaticum* was indistinguishable from **1** by HPLC.

The flavonoid 'copigments' accompanying the above anthocyanin in the vacuole of 'Johnson's Blue' are kaempferol glycosides together with lower levels of quercetin and myricetin glycosides (Table 1). On a 2D-PC, four significant flavonol glycosides were evident, two of which gave kaempferol and glucose on hydrolysis, and two of which gave myricetin and glucose. The lower running compounds (15% HOAc) were spectrophotometrically and chromatographically (2D-PC) identical with the 3-*O*-glucosides of kaempferol and myricetin, while the major higher running compound was spectrophotometrically identical to, and cochromatographed (2D-PC, HPLC) with kaempferol 3-*O*-sophoroside. By analogy, the fourth significant glycoside is considered to be myricetin 3-*O*-sophoroside. Compounds from the ill-defined, low level 2D-PC spots between the kaempferol and myricetin mono- and di-glucosides and which gave quercetin on hydrolysis, possess the chromatographic and spectrophotometric properties expected for the quercetin equivalents of the kaempferol and myricetin compounds. Thus, the major flavonoid 'copigments' are kaempferol 3-*O*-glucoside and sophoroside, accompanied by lower levels of the myricetin equivalents and traces of the quercetin equivalents. The same flavonols were present in *G. pratense*.

In vitro *reconstitution of petal colour*

Compound **1** and the major flavonol glycoside (kaempferol 3-*O*-sophoroside) constituent of 'Johnson's Blue' and *G. pratense* were combined, in the appropriate ratios in a citrate buffer, in an attempt to

Table 2. NMR data for malvidin-3-*O*-glucoside-5-*O*-[6-*O*-acetylglucoside], **1** (500 MHz; CD_3OD–CF_3CO_2D, 19 : 1)

Position	δC (ppm)*	δH*
Malvidin		
2	164.3	
3	146.8	
4	136.0	9.06*s*
5	156.6	
6	106.1	6.96*d* (J = 2 Hz)
7	169.7	
8	97.5	7.09*d* (J = 2 Hz)
9	157.2	
10	113.5	
1′	119.6	
2′	111.0	7.93*s*
3′	149.8	
4′	147.2	
5′	149.8	
6′	111.0	7.93*s*
5-Glucose		
1	102.3	5.16*d* (J = 8 Hz)
2	74.4	3.3–3.8
3	77.6	3.3–3.8
4	71.4[a]	3.3–3.8
5	75.9	3.77 *m*
6	64.6	4.28 *dd* (12, J = 7 Hz) 4.45 *dd* (J = 12, 2 Hz)
3-Glucose		
1	104.1	5.34 *d* (7.5 Hz)
2	74.9	3.3–3.8
3	78.5	3.3–3.8
4	71.5[a]	3.3–3.8
5	79.1	3.3–3.8
6	62.6	3.72 *m*, 3.95 *dd* (J = 12, 2 Hz)
Acetate		
CO	172.7	
CH_3	20.7	2.03 *s*
Methoxyl	57.3	3.97 *s*

*Assigned by ^1H,^1H-COSY, HMBC and ^1H,^1H-TOCSY; assignments bearing the same superscript may be reversed.

recreate the petal spectra and thereby to establish that a pH of *ca* 5.4 and the flavonol–anthocyanin ratios are the key factors in determining petal colour. Marked differences in colour stability (or colour intensity at a fixed time after mixing) were apparent. For example, in the absence of flavonol at an anthocyanin concentration of 3.5×10^{-4} M l^{-1}, colour stability/intensity was poor (see Fig. 1). Colour stability/intensity was considerably improved in the presence of a 4 : 1 excess of flavonol, and even more so in the presence of an 8 : 1 excess (Fig. 1). Maximum colour stability/intensity was observed with near vacuolar concentrations of *ca* 1.8×10^{-3} M l^{-1} (Fig. 1).

At the pH values used for these experiments (5.5–5.6) the spectrum of **1** alone did not match those of the 'Johnson's Blue' and *G. pratense* petals. In particular, the three major absorption bands appeared at *ca* 525, 562 and 612 nm instead of *ca* 540, 575 and 620, respectively. The addition of a four molar excess of flavonol, however, produced the required blue shifts to *ca* 540, 575 and 620 nm with the relative intensities of the longer wavelength bands increasing with higher levels of flavonol. Even at a flavonol : anthocyanin ratio of 8 : 1, however, the absorbance ratios of these bands still did not equate to those measured for the petals (Table 1). It was subsequently shown that by altering the pH, via a stepwise addition of small volumes of 1 M NH_4OH, the required ratios could be achieved if the pH was adjusted to between 6.6 and 6.8. A similar attempt to match the petal spectrum of *G. sanguineum* with the appropriate mix of **1** and flavonol revealed that a pH of about 5.6 was required (although the middle absorption band remained at *ca* 565 nm).

The above observations necessitated that the experiments carried out at pH 5.5–5.6 be repeated at higher pH. As before it was found that at pH 6.6 the colour

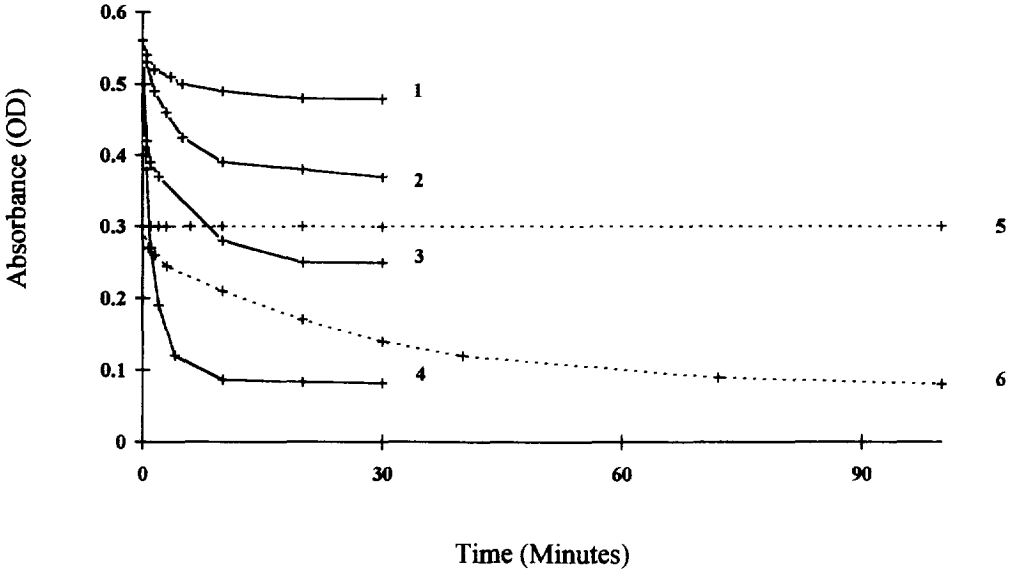

Fig. 1. Colour stability/intensity of anthocyanin **1** under various conditions. (1) Anthocyanin **1**:kaempferol 3-sophoroside (1 : 4), final anthocyanin concentration 1.8×10^{-3} M l^{-1} (pH 5.5–5.6). (2) Anthocyanin **1**:kaempferol 3-sophoroside (1 : 8), final anthocyanin concentration 3.5×10^{-4} M l^{-1} (pH 5.5–5.6). (3) Anthocyanin **1**:kaempferol 3-sophoroside (1 : 4), final anthocyanin concentration 3.5×10^{-4} M l^{-1} (pH 5.5–5.6). (4) Anthocyanin **1** concentration 3.5×10^{-4} M l^{-1} (pH 5.5–5.6). (5) Anthocyanin **1**:kaempferol 3-sophoroside (1 : 2), final anthocyanin concentration 1.76×10^{-3} M l^{-1} (pH 6.6). (6) Anthocyanin **1**, concentration 1.76×10^{-3} M l^{-1} (pH 6.6).

stability was poor in the absence of flavonol copigments. However in the presence of even a two molar excess of flavonols, the intensity of the initial bluish purple colour remained unchanged for the full 4.5 hr monitoring period (see Fig. 1). As at pH 5.5–5.6, the flavonols brought about blue shifts in all three absorption bands. It was observed that as the pH approached the upper end of the pH 6.3–6.8 range, a spectrum more akin to that of *G. pratense* petals was obtained, that is, with a more pronounced 575 nm peak (see Table 1).

DISCUSSION

The colours of the three *Geranium* flowers investigated here represent a colour gradient from bluish magenta (*G. sanguineum*) through bluish purple (*G.* 'Johnson's Blue') to purplish blue (*G. pratense*). These colours are all produced in petal vacuoles with essentially the same anthocyanin, **1**. Perhaps the most important difference of relevance to relative colour is the pH level. It is recognized that higher pH levels favour the quinonoidal structural forms of anthocyanins [7]. Thus, in the present study the flowers with the higher pH would be expected to be bluer due to the enhanced levels of anthocyanin quinonoidal forms relative to the magenta flavylium ion form with which they are in equilibrium [7]. The noticeably bluer shading in 'Johnson's Blue' and *G. pratense*, therefore, can be rationalized on this basis. The small differences between the petal spectra of these two flowers may also be rationalized on this basis if the pH of the epidermal cells of the latter are slightly less acidic than in 'Johnson's Blue' (see above).

Additionally it was demonstrated in the *in vitro* colour reconstitution experiments, that the presence of excess flavonol also produces blue shifts in the absorption bands together with a blueing effect due to a relative increase in the level of absorption at 575 and 620 nm. This latter effect was shown to be greater with higher levels of flavonol, which would contribute to the increased blueness of *G. pratense* relative to 'Johnson's Blue'. Flavonols have been implicated previously in copigmentation phenomena resulting in blue shifts and intensity changes in the absorption bands [6, 7]. This is probably brought about through preferential stabilization of the quinonoidal base form as has been observed previously with malvidin-3,5-diglucoside when copigmented with caffeine [9]. The colour stability/intensity in these *Geranium* flowers appears to be due to a combination of copigmentation with flavonol and self-association (cf. refs. [10] and [11]) resulting from the high vacuolar concentration of pigment (Fig. 1).

The petal spectra of all three species are not reproducible *in vitro* in the absence of flavonol or at the pH of the pressed juice. In the presence of flavonol it is only at a pH of *ca* 6.6–6.8 (for *G.* 'Johnson's Blue' and *G. pratense*), and *ca* 5.6 (for *G. sanguineum*) that petal spectra are produced. It is concluded therefore that the pH of the pigment containing epidermal cells is 1 to 1.4 pH units higher than measured for the pressed juice. It follows that the pH of the parenchymal cells must be significantly lower than that

of the epidermal cells in these petals. Such pH differences are greatly in excess of the 0.2–0.3 pH units reported for *Azalea* [12], *Salvia* [13] and *Poinsettia* [13], but are in accord with recently published data on morning glory (*Ipomoea tricolour*) petals [14], in which the epidermal cell pH was measured directly to be *ca* 7.7, while the parenchymal cells measured only 6.0. In the absence of the sophisticated pH microelectrode equipment needed for measurement of cellular pH, the *in vitro* reconstitution approach outlined above, in which the anthocyanin is effectively used as an indicator, offers a convenient alternative. This approach however is only valid if the petal colour is produced solely by the components included in the reconstitution, and if all epidermal cells possess the same colour.

In summary, for the species studied, it has been established that the impressive 'blue' colouration in 'Johnson's Blue' and *G. pratense* can be accounted for by the basic colour of anthocyanin **1**, modified by copigmentation with flavonol glycosides and by epidermal cell pH levels markedly higher than those of the pressed juices.

EXPERIMENTAL

Blue geranium plants were purchased from commercial garden centres in Christchurch, New Zealand and were grown at the New Zealand Institute for Crop and Food Research Ltd, Levin, in polythene planting bags, containing a steam-sterilized bark and pumice (50 : 50) potting mix with added fertiliser (NPK, 3 : 1 : 2). Plants were located in a temperature controlled green-house and upon flowering during September to February, petal material was collected, and if not analysed promptly, was frozen at −80°. Plant identities were checked by M. R. Boase using the keys, descriptions and illustrations in books on hardy geraniums [1,2] and by consulting M. F. Shearer, geranium specialist, Christchurch. Voucher specimens are held by M. R. Boase, at Crop and Food Research.

Extraction and isolation procedures. Pressed juice was obtained from petal material by crushing and centrifugation, and was used subsequently for pH analysis, HPLC and spectrophotometric studies. For most analyses, and for the isolation of **1** from *G* 'Johnson's Blue', plant material was crushed and extracted with 0.1% aq. TFA. The isolation of **1** was achieved by (a) applying this extract to polyamide (Macherey–Nagel polyamide CC6); (b) eluting the flavonoids with 0.1% methanolic TFA; (c) redissolving the evapd eluate in 0.1% aq. TFA; and (d) chromatographing it on polyamide (CC-6, MN) in 0.1% aq. TFA containing increasing proportions of 0.1% methanolic TFA up to *ca* 30%. Compound **1** was the only significant red-coloured band. Any de-acylated material preceded this band. Final clean-up was achieved on LH-20 in 0.1% TFA (in H_2O–MeOH, 80 : 20). The freeze-dried product was dissolved in MeOH-d_4–TFA (19 : 1) for NMR studies. Flavonol glycosides were analysed by 2D-PC of the crude extract in TBA (*t*-BuOH–HOAc–H_2O, 3 : 1 : 1) and 15% HOAc. R_f values for the four clearly visible compounds in *G.* 'Johnson's Blue' and *G. pratense* (TBA/15% HOAc) : 0.74/0.43 (K-3glc); 0.48/0.26 (Myr-3 glc); 0.63/0.65 (K-3-soph); 0.40/0.58 (Myr-3-soph). The 2D-PC of *G. sanguineum* exhibited an indistinct flavonol glycoside(s?) spot in the region 0.30/0.55. Structures were established by absorption spectroscopy, hydrolysis and analysis of products, and by cochromatography with authentic samples (see ref. [15] for methods).

HPLC conditions. Analytical HPLC was routinely carried out at 35° on a Merck Lichrospher 100RP-18 end-capped column (5 μM, 11.9 × 4 cm) at an elution rate of 0.8 ml min^{-1} using a solvent system comprising solvent A (1.5% aq. H_3PO_4) and [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 mins, 10% A at 33 mins and 0% A at 39.3 mins. Typical retention times: malvidin-3,5-diglucoside (14.78 min), malvidin-3-glucoside (18.34 min), malvidin-5-glucoside (19.38 min), compound **1** (22.64 min). All three blue geraniums contained these components which were identified by cochromatography with authentic samples obtained from malvidin-3,5-diglucoside (1N HCl/100°/30 min).

Petal/vacuolar characters. Petal spectra were measured in a spectrophotometer by pressing petals onto transparent adhesive tape and fixing this to the cuvette carriage. Vacuolar concentrations of anthocyanins were determined using pressed juice diluted 60 × with 1N HCl. Using the Beer–Lambert equation and $\varepsilon = 33\,000$ a concentration of 1.96×10^{-3} M l^{-1} was obtained for *G.* 'Johnson's Blue'. A microscopic study of a petal cross-section indicated that only 70% of the petal thickness was pigmented. The conc. adjusted for this was calculated as 2.8×10^{-3} M l^{-1}. Flavonol levels were estimated approximately from the same curves, but end absorption introduced inaccuracies. Ratios of flavonols to anthocyanins were determined from the M/g values of each, measured on freeze-dried petals. Anthocyanins were quantified as described for pressed juice and flavonols were quantified by HPLC. The sum of the integrals of all flavonol HPLC peaks was used to calc. flavonol levels in rutin equivalents using a standard curve to relate peak area to moles of rutin (see Table 1).

In vitro *reconstitution of flower colour and copigmentation experiments* (*pH* 5.5–5.6). Data for plots 2, 3 and 4 in Fig. 1 were obtained as follows. Standard solns of **1** in 0.1 M citrate buffer, pH 5.4 (2.54×10^{-3} M l^{-1}) and kaempferol-3-sophoroside in 0.1 M trisodium citrate (2.6 mg/ml) were prepd. Compound **1** (82.7 μl) was evapd to dryness *in vacuo* in a small vial and then redissolved in 133 μl of 0.1 M citric acid (with warming). This was added to a 1 cm pathlength cuvette containing (for curve 3) 200 μl of the kaempferol-3-sophoroside solution plus 262 μl of 0.1 M trisodium citrate, and the absorption spectra measured between 400 and 750 nm at *ca.* 0, 1, 3, 5, 10, 20

and 30 min after mixing. Following each run the pH was measured and confirmed to be in the range 5.5–5.6. Absorbance was measured for the *ca* 575 nm peak from a baseline extrapolated back from 750 nm. Data for plot 1 in Fig. 1 was obtained in a similar manner, but using a soln of **1** in 0.1 M citric acid (2.3×10^{-3} M l^{-1}) and a soln of kaempferol-3-sophoroside in MeOH (7.4×10^{-3} M l^{-1}). The flavonol solution (190 μl) was evapd to dryness in a small vial together with 0.1 M trisodium citrate (400 μl). The residue was redissolved in 0.1 M trisodium citrate (47 μl), combined with **1** (153 μl) in a 1 mm pathlength cuvette and the spectra determined as above. The measured pH was 5.5. This pH was adjusted by the stepwise addition of 5–10 μl aliquots of 1M NH_4OH until the form of the absorption spectrum best matched the petal spectrum of *G.* 'Johnson's Blue'. At this stage the pH was remeasured at *ca* 6.6. A similar experiment, but with a flavonol : anthocyanin ratio of 1 : 5 gave an unstable colour which declined rapidly in intensity. Adjustment of the pH as above, to match the spectrum with that of *G. sanguineum*, indicated that a pH of *ca* 5.6 was required. The low intensity and high noise level in this spectrum however made the match difficult.

In vitro *reconstitution of flower colour and copigmentation experiments* (*pH* 6.3–6.8). Repeat experiments at pH 6.3–6.8 were carried out using solns of **1** (5.2×10^{-3} M l^{-1}) in 0.1 M citrate buffer pH 5.4, and kaempferol 3-sophoroside (7.4×10^{-3} M l^{-1}) in MeOH. A dilute aq. NaOH soln was also prepd, and the volume of 0.1 M citric acid required to produce a pH of 6.6 on admixture with 145 μl determined. Two samples of **1** (100 μl) and one of kaempferol 3-sophoroside (140 μl) were dried in vials under vacuum. For the two pH 6.6 runs, the kaempferol 3-sophoroside in dilute NaOH (145 μl), or the NaOH alone, was added to **1** dissolved in 0.1 M citric acid (150 μl) and the absorption spectrum monitored at *ca* 575 nm (see Fig. 1). The pH was then measured. The volume of NaOH was varied to produce the pH required for other runs.

Acknowledgement—The authors wish to thank Dr Herbert Wong of IRL for running the NMR spectra, and Dr Stephen J. Bloor of IRL for helpful suggestions during the course of this work.

REFERENCES

1. Yeo, P. F., *Hardy Geraniums*. Timber Press, Portland, OR, 1992.
2. Bath, T. and Jones, J., *The Gardeners Guide to Growing Hardy Geraniums*. David and Charles, England, 1994.
3. Asen, S. and Griesbach R., *Journal of the American Society of Horticultural Science*, 1983, **108**(5), 845.
4. Andersen, O. M., Viksund, R. I. and Pedersen, A. T., *Phytochemistry*, 1995, **38**(6), 1513.
5. Ueno, N., Takemura, E. and Hayashi, K., *Botanical Magazine (Tokyo)*, 1969, **82**, 155.
6. Markham, K. R. and Ofman, D. J., *Phytochemistry*, 1993, **34**(3), 679.
7. Brouillard, R. and Dangles, O., in *The Flavonoids-Advances in Research since* 1986, ed. J. B. Harborne. Chapman and Hall, London, 1994, p. 565.
8. Ishikura, N. and Yoshitama, K., *Journal of Plant Physiology*, 1984, **115**, 171.
9. Brouillard, R., Wigand, M. C., Dangles, O. and Cheminat, A., *Journal of the Chemistry Society, Perkin Transactions*, 1991, **II**, 1235.
10. Hoshino, T., Matsumoto, U. and Goto, T., *Phytochemistry*, 1981, **20**, 1971.
11. Hoshino, T., *Phytochemistry*, 1991, **30**, 2049.
12. De Loose, R., *Scientia Horticulturae*, 1978, **9**, 285.
13. Stewart, R. N., Norris, K. H. and Asen, S., *Phytochemistry*, 1975, **14**, 937.
14. Yoshida, K., Kondo, T., Okazaki, Y. and Katou, K., *Nature*, 1995, **373**, 291.
15. Markham, K. R., *Techniques of Flavonoid Identification*. Academic Press, London, 1982. p. 36.