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FLAVONOIDS FROM *EUSTOMA GRANDIFLORUM* FLOWER PETALS

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Key Word Index—*Eustoma grandiflorum*; Gentianaceae; prairie gentian; acylated anthocyanins; flavonols; acylated flavonols.

Abstract—The major flavonoids responsible for flower colours of *Eustoma grandiflorum* were characterized by TLC, HPLC, spectral and chemical analyses. Anthocyanins were delphinidin 3-rhamnosylgalactoside-5-glucoside and delphinidin 3-galactoside-5-glucoside, each acylated with *p*-coumaric acid, from the purple cultivar 'Murasaki no Homare' and the pelargonidin analogues, each acylated with either *p*-coumaric or ferulic acids, from the pink cultivar 'Momo no Mine'. The major flavonol copigments were the 3-rhamnosylgalactoside-7-rhamnoside of myricetin, kaempferol and isorhamnetin and the 3-rhamnosylglucoside-7-rhamnoside of kaempferol and isorhamnetin. Flavonols present acylated with *p*-coumaric acid were myricetin 3-rhamnosylgalactoside-7-rhamnoside and robinin in both *cis* and *trans* forms, and isorhamnetin 3-rhamnosylgalactoside-7-rhamnoside. Robinin also was present acylated with caffeic or ferulic acids. Simulated *in vitro* colours obtained from the flavonoids present in this germplasm indicated that good blue colours were not attainable. Good blue colours were formed with delphinidin 3-*p*-coumaroylrhamnosylgalactoside-5-glucoside and *C*-glycosylflavone copigments such as swertisin and isoorientin. These copigments are readily available in other members of the Gentianaceae and this suggests the possibility of genetical engineering endeavours for increasing the colour range of this important new ornamental plant.

INTRODUCTION

Eustoma grandiflorum Griseb., commonly known as Lisianthus or Prairie Gentian, is a new important crop introduction for use as a garden or pot plant and also for cut flowers [1, 2]. The present flower colour range is purple, pink and white. In the present paper the flavonoids responsible for these flower colours were characterized. We determined the possibilities of enhancing the colour range from existing germplasm and also from germplasm available in other members of the Gentianaceae.

RESULTS AND DISCUSSION

Anthocyanins were purified by prep. TLC and two major bands were resolved from each cultivar examined. Spectral properties indicated that they were all acylated 3,5-glycosides [3] and only those from Murasaki no Homare chelated with Al. R_fs before and after base hydrolysis are shown in Table 1. Acylated anthocyanins previously have been reported in the Gentianaceae [4–6].

Base hydrolysis of the two major anthocyanins from Murasaki no Homare each yielded *p*-coumaric acid. Acid hydrolysis of deacylated band 1 yielded delphinidin, galactose, glucose, rhamnose and four intermediate glycosides. They were characterized as the 3-galactoside-5-glucoside, 3-rhamnosylgalactoside, 3-galactoside and the 5-glucoside. Deacylated band 2 yielded delphinidin, galactose, glucose and two intermediate glycosides. They were characterized as the 3-galactoside and the 5-glucoside. For comparable R_fs of the intermediate glycosides formed during acid hydrolysis refer to [3]. The isolated anthocyanins were characterized as delphinidin 3-*p*-coumaroylrhamnosylgalactoside-5-glucoside and delphinidin 3-*p*-coumaroylgalactoside-5-glucoside.

Base hydrolysis of each band from Momo no Mine yielded *p*-coumaric and ferulic acids but HPLC analysis revealed the presence of two-anthocyanins in each of the bands. Resolving the same pelargonidin 3,5-glycoside, acylated with either *p*-coumaric or ferulic acids, was virtually impossible with the prep. TLC solvents used. Raphanusin A (pelargonidin 3-*p*-coumaroylglucosyl-glucoside-5-glucoside) and Raphanusin B (pelargonidin 3-feruloylglucosylglucoside-5-glucoside), reported in *Raphanus sativus* roots, are anthocyanins very similar to those isolated from Momo no Mine flowers. They have identical R_fs in BAW, 1% HCl and HOAc–HCl [3]. There is a slight R_f difference in BuHCl but this solvent was not used to resolve the anthocyanins from Momo no Mine. Acid hydrolysis of deacylated band 1 from Momo no Mine yielded pelargonidin, galactose, glucose, rhamnose and four intermediate glycosides. They were characterized as the 3-galactoside-5-glucoside, 3-rhamnosyl-galactoside, 3-galactoside and the 5-glucoside. Deacylated band 2 yielded pelargonidin, galactose, glucose and two intermediate glycosides characterized as the 3-galactoside and the 5-glucoside. The anthocyanins from Momo no Mine were characterized as pelargonidin 3-*p*-coumaroyl-rhamnosylgalactoside-5-glucoside, pelargonidin 3-feruloylrhamnosylgalactoside-5-glucoside, pelargonidin 3-*p*-coumaroylgalactoside-5-glucoside and pelargonidin 3-feruloylgalactoside-5-glucoside.

Eleven flavonol copigments were isolated from flower petals (Table 2). The UV spectral properties indicated that they were 3,7-substituted and that six were acylated [7, 8]. All 11 flavonol copigments were present in the two

Table 1. Chromatographic data for the major anthocyanins in *Eustoma grandiflorum* flower petals

	R_f (× 100) in*		
	HOAc–HCl	1% HCl	BAW
Momo no Mine (pink)			
Pelargonidin 3-*p*-coumaroylrhamnosylgalactoside-5-glucoside	34	9	58†
Pelargonidin 3-feruloylrhamnosylgalactoside-5-glucoside	34	9	58†
Pelargonidin 3-rhamnosylgalactoside-5-glucoside‡	70	48	24
Pelargonidin 3-*p*-coumaroylgalactoside-5-glucoside	13	7	60†
Pelargonidin 3-feruloylgalactoside-5-glucoside	13	7	60†
Pelargonidin 3-galactoside-5-glucoside‡	46	21	25
Murasaki no Homare (purple)			
Delphinidin 3-*p*-coumaroylrhamnosylgalactoside-5-glucoside	16	3	20†
Delphinidin 3-rhamnosylgalactoside-5-glucoside‡	54	23	5
Delphinidin 3-*p*-coumaroylgalactoside-5-glucoside	7	1	12†
Delphinidin 3-galactoside-5-glucoside‡	25	7	6

*Measured on microcrystalline cellulose TLC plates; HOAc–HCl = HOAc–HCl–H_2O (15:3:82); 1% HCl (w/v); BAW = *n*-BuOH–HOAc–H_2O (6:1:2).
†Streaked.
‡After base hydrolysis.

Table 2. Chromatographic data for the major flavonol copigments in *Eustoma grandiflorum* flower petals

	R_f (× 100) in*			
Flavonoid copigments	BAW	H_2O	15% HOAc	PhOH
1. Myricetin 3-rhamnosylgalactoside-7-rhamnoside	31	36	63	47
2. Kaempferol 3-rhamnosylgalactoside-7-rhamnoside	52	54	81	83
3. Kaempferol 3-rhamnosylglucoside-7-rhamnoside	51	55	81	72
4. Isorhamnetin 3-rhamnosylgalactoside-7-rhamnoside	46	53	79	89
5. Isorhamnetin 3-rhamnosylglucoside-7-rhamnoside	43	45	75	80
6. Myricetin 3-*p*-coumaroylrhamnosylgalactoside-7-rhamnoside	53	20	59	72
7. Kaempferol 3-caffeoylrhamnosylgalactoside-7-rhamnoside	62	21	65	85
8. Kaempferol 3-*trans*-*p*-coumaroylrhamnosylgalactoside-7-rhamnoside	71	17	67	96
9. Kaempferol 3-feruloylrhamnosylgalactoside-7-rhamnoside	69	16	69	97
10. Isorhamnetin 3-*p*-coumaroylrhamnosylgalactoside-7-rhamnoside	68	8	57	97
11. Kaempferol 3-*cis*-*p*-coumaroylrhamnosylgalactoside-7-rhamnoside	70	17	65	97

*BAW = *n*-BuOH–HOAc–H_2O (6:1:2); PhOH = PhOH–H_2O (73:27 w/w) measured on microcrystalline cellulose TLC plates.

cultivars mentioned above and also in Kiri no Mine, except that myricetin 3-rhamnosylgalactoside-7-rhamnoside was not detected in Momo no Mine. Their HPLC resolution and quantitative distribution are shown in Fig. 1 and Table 3. Those present in the largest concentration in all three cultivars were robinin (kaempferol 3-rhamnosylgalactoside-7-rhamnoside) and robinin acylated with caffeic or ferulic or *trans* or *cis* *p*-coumaric acids. The copigments occurred early in flower development when buds of Murasaki no Homare were *ca* 3 cm long, tight green and showing no purple colour. Their concentration per bud was *ca* that of fully developed flowers.

Model *in vitro* absorption spectra were simulated from the major anthocyanin and flavonol copigments isolated from Murasaki no Homare to determine the colour range obtainable from the existing germplasm (Fig. 2). Absorption at the λ_{max} for intact flowers was *ca* one-half that shown in Fig. 2A and was adjusted only for comparing the shape of the curve with those of the model systems. The estimated path length of upper and lower epidermal cells, where the colour of the flowers occurred, was *ca* 0.1 that of the model systems.

The absorption spectrum and colour obtained with 10^{-3} M delphinidin 3-*p*-coumaroylrhamnosylgalactoside-5-glucoside and 2×10^{-3} M robinin, at *ca* the pH of the vacular content of epidermal cells (6.5), closely resembled that of Murasaki no Homare flowers (Fig. 2B). The λ_{max} was 570 nm with shoulders at 538 and 622 nm. Substituting robinin acylated with either *cis* or *trans* *p*-coumaric acid for robinin, or slight changes in pH, had little effect on the colour or absorption spectra. A combination of robinin and either acylated form in ratios of 1:3 or 3:1 (final concentration 2×10^{-3} M) also

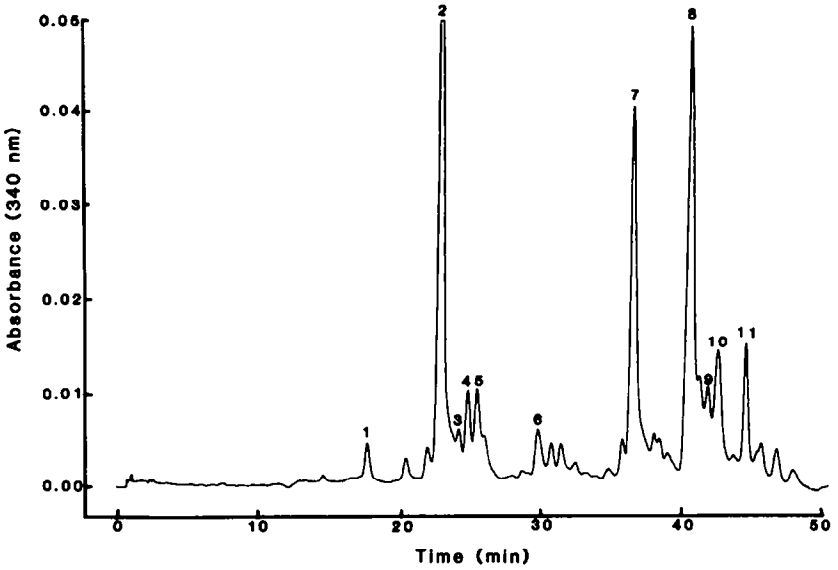

Fig. 1. Analytical HPLC separation of the major flavonoids from flower petals of *Eustomia grandiflorum* cultivar 'Kiri no Mine'. Column = Water's radial pak (C_{18}) with radial compression separating system; solvents = 1% Et_3N buffered to pH 3 with H_3PO_4 (pump A), MeCN (pump B); elution programme = 8% B 9 min, 8–12% B linear gradient 0.1 min, 12–31% B linear gradient 50 min; flow rate = 3.5 ml/min (*ca* 1600 psi); detection = adsorption at 340 nm. For key to compounds separated, see Table 2.

resulted in little change in colour or absorption spectra. The absorption spectrum and colour obtained with 10^{-3} M delphinidin 3-*p*-coumaroylrhamnosylgalactoside-5-glucoside and 2×10^{-3} M swertisin (6-*C*-glucosylgenkwanin) at *ca* the pH of the vacular content of epidermal cells, was different and much bluer than those of intact flowers (Fig. 2C). The λ_{max} was 583 nm with shoulders at 538 and 630 nm. The bluer colour was due to the bathochromic shift of the λ_{max} and increased adsorption at 630 nm and longer wavelengths. The same effect was evident when isoorientin (6-*C*-glucosylluteolin) was substituted for swertisin.

Although the concentration of anthocyanin to copigments in model systems was not the same as those present in epidermal cells the effect on colour was quite evident. With existing germplasm the colour range of *Eustoma grandiflorum* appears to be limited to various shade of pink and purple. The *C*-glycosylflavones swertisin and isoorientin, which are not present in *Eustoma grandiflorum*, produced much bluer colours than the natural flavonol copigments. These *C*-glycosylflavones are abundant in the Gentianaceae [9, 10]. If these types of copigments could be introduced into *Eustoma grandiflorum* germplasm then the colour range could be increased to include blue flowers.

EXPERIMENTAL

Plant material. Seeds of cultivar 'Marasaki no Homare' (purple flower) were obtained from the Fukukaen Nursery and Bulb Co., Ltd. 9-26, 2-chome, Matsubara, Naka-ku, Nagoya, Japan. Seeds of cultivars 'Momo no Mine' (pink flower) and 'Kiri no Mine' (white flower) were obtained from T. Sakata and Co., C.P.O. Box Yokohama No. 11, Yokohama, Japan 220-91. Plants were grown at Beltsville, Maryland under cultural practices previously described [1]. Petals of flowers, after anthesis, were dried in a forced-air oven at 40° and then ground to pass a 40 mesh screen.

Isolation and characterization. The dried tissue was extracted with petrol and then the anthocyanins were extracted with 0.5% HCl–MeOH. The extracts were reduced to almost dryness at 40° under red. pres., taken up in a minimum vol. of MeOH and filtered. All solvents used for CC of anthocyanins contained 10 ml 2 N HCl/l. The concd extract was placed on a column of Sephadex LH-20 made with 70% MeOH and eluted with the same solvent. The eluate, reduced to a minimum vol., was then placed on a column of MN-polyamide SC-6 made with H_2O, washed with 30% MeOH and eluted with 50% MeOH. Final purification was by prep. TLC on micro-crystalline cellulose with BAW, *n*-BuOH–HOAc–H_2O (6:1:2) and 15% HOAc.

Flavonol copigments were extracted with hot MeOH. The extracts were reduced to almost dryness at 40° under red. pres., taken up in 50% MeOH, filtered and then placed on a polyvinylpyrrolidone (PVP) column made with H_2O. The flavonol copigments were banded into eight fractions by gradient elution with H_2O–MeOH (0–100% MeOH). Any remaining compounds were eluted with 10% HOAc–MeOH. Flavonols in each band were resolved by HPLC procedures similar to those previously described [11]. To eliminate any HPLC contamination, all resolved compounds were placed on a column of MN-polyamide SC-6 made in H_2O and eluted with MeOH.

Acylated compounds were base hydrolysed in 1% KOH for 30 min at room temp. under N_2, acidified and the acyl moiety was extracted with Et_2O. The aq. residue was placed on a column of MN-polyamide SC-6 made in H_2O, eluted with H_2O to remove the salt, and then the deacylated compound was eluted with 70% MeOH. Controlled acid hydrolysis and sugar analysis were by procedures previously described [11, 12].

Isolated compounds were characterized, along with known standards, by HPLC, R_fs, UV and Vis. spectral analyses and the products of controlled acid or base hydrolysis. *Cis* and *trans* *p*-coumaric acids were determined by HPLC and GC/MS. The free acids obtained from base hydrolyses were used for HPLC

Table 3. Concentration of the major flavonoid copigments in *Eustoma grandiflorum* flower petals (μg/100 mg dry wt)*

Flavonoid copigments	Cultivar		
	Kiri no Mine (white)	Momo no Mine (pink)	Murasaki no Homare (purple)
1. Myricetin 3-rhamnosylgalactoside-7-rhamnoside	201	N.D.†	14
2. Kaempferol 3-rhamnosylgalactoside-7-rhamnoside	2360	3050	3346
3. Kaempferol 3-rhamnosylglucoside-7-rhamnoside	232	310	310
4. Isorhamnetin 3-rhamnosylgalactoside-7-rhamnoside	410	428	540
5. Isorhamnetin 3-rhamnosylglucoside-7-rhamnoside	417	435	546
6. Myricetin 3-*p*-coumaroylrhamnosylgalactoside-7-rhamnoside	230	208	18
7. Kaempferol 3-caffeoylrhamnosylgalactoside-7-rhamnoside	1584	696	450
8. Kaempferol 3-*trans*-*p*-coumaroylrhamnosylgalactoside-7-rhamnoside	1906	3953	4157
9. Kaempferol 3-feruloylrhamnosylgalactoside-7-rhamnoside	450	1125	540
10. Isorhamnetin 3-*p*-coumaroylrhamnosylgalactoside-7-rhamnoside	589	733	786
11. Kaempferol 3-*cis*-*p*-coumaroylrhamnosylgalactoside-7-rhamnoside	436	1137	1025
Total	8815	12075	11732

*Dry tissue extracted with petrol.
†N.D. = not detected.

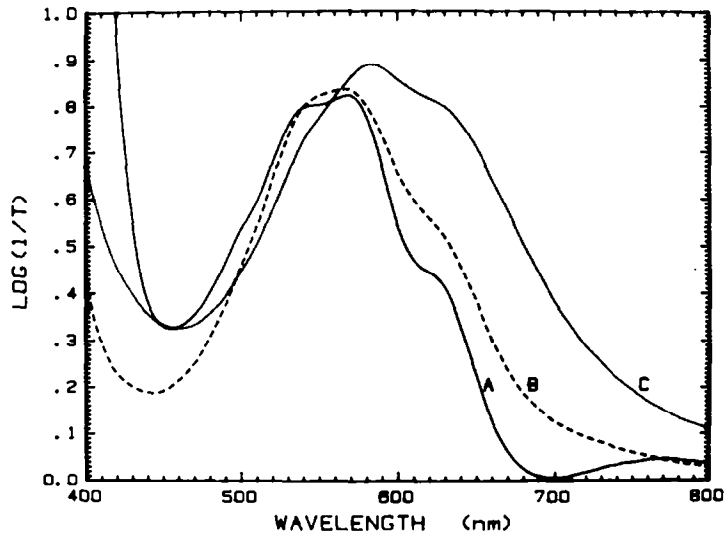

Fig. 2. Simulated *in vitro* absorption spectra of the major anthocyanin and flavonol copigment isolated from *Eustoma grandiflorum* cultivar 'Murasaki no Homare' and the major anthocyanin and swertisin. A, Intact flower petal, pH 6.40. B, Delphinidin 3-*p*-coumaroylrhamnosylgalactoside-5-glucoside, 10^{3} M + robinin, 2×10^{-3} M, 1 mm path length, pH 6.50. C, Delphinidin 3-*p*-coumaroylrhamnosylgalactoside-5-glucoside, 10^{3} M + swertisin 2×10^{-3} M, pH 6.50.

analyses and the trimethylsilyl derivatives were used for GC/MS analyses.

Flavonoid and cinnamic acid HPLC resolution. Each sample consisted of 100 mg of petrol extracted dry petals. Anthocyanins were extracted by blending for 30 sec in 50 ml 0.5% HCl–MeOH. Flavonol copigments were extracted with MeOH. Anthocyanins were resolved by HPLC procedures previously described [13]. HPLC parameters for the resolution of flavonol copigments were the same as those used with the 1st solvent system to resolve hydroxycinnamic acid and flavonols from geranium florets [14].

Caffeic, ferulic, sinapic and *p*-coumaric acids were resolved by HPLC on a 7.8 × 300 mm column of Bondapak C_{18} with 16% MeCN in a 2% HOAc. The flow rate was 3.0 ml/min (*ca* 1500 psi) and detection was by UV absorption at 340 nm. The resolution of *cis* and *trans* *p*-coumaric acid was accomplished with a radial-pak A cartridge (reverse phase permanently bonded octadecylsilane, particle size 10 μm) and a radial compression separating system. The solvent was 6% MeCN in 2% HOAc at a flow rate of 2.0 ml/min (*ca* 1500 psi) and detection was by UV absorption at 340 nm.

In vitro *simulated anthocyanin-copigment complexes.* Stock solns (0.5 ml) of the anthocyanin or flavonol copigments, containing twice the required normality, were prepared in citrate–phosphate buffer pH 2.40 and 0.28 N NaOH, respectively. Each stock soln was immersed in hot H_2O for a few sec and then an equal vol. of each copigment was thoroughly mixed with an equal vol. of the anthocyanin. HPLC revealed that less than 1% of the acylated robinin compounds were base hydrolysed by this procedure. The pH of each complex was measured with a combination pH microelectrode. Absorption spectra were measured with a spectrophotometer designed for analyses of dense light-scattering samples [15] in a 1 mm cell *ca* 5 min after mixing.

The pH of epidermal cells. The pH of epidermal cells was determined microspectrophotometrically using indicator dyes [16].

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