Effect of pH, Anthocyanin, and Flavonoid Co-pigments on the Color of Statice Flowers

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Abstract. The anthocyanin isolated from flowers of *Limonium* cvs. Twilight Lavender and Midnight Blue was delphinidin 3,5-diglucoside, and that from 'Blue Bonnet' and 'American Beauty' was delphinidin 3-glucoside. The major flavonoid co-pigments in all 4 cvs. were luteolin and its 6-C-glucoside (iso-orientin). These co-pigments were also present in white 'Iceberg' and yellow 'Gold Coast'. The range of colors from reddish-purple to blue for the various cvs. was directly related to the pH of the tissue.

The increasing commercial importance of annual statice *Limonium* hybrid cvs. has emphasized the need for improvements of their color in both fresh and dried material. We have attempted to define the parameters influencing color in existing cvs. and to provide the geneticist with information for his breeding and selection programs.

Materials and Methods

Solvents. The following solvents were used for chromatographic separation of anthocyanin and other flavonoids or for co-chromatography with known standards:

AFH	Acetone-formic acid-water (5:5:5)
AHH	Acetic acid-HCl-water (15:3:82)
BAW	1-Butanol-acetic acid-water (6:1:2)
BH	1-Butanol-2N HCl (1:1, upper layer)
EPH	Ethyl acetate-pyridine-water (2:1:2)
Forestal	Acetic acid-HCl-water (30:3:10)
H_2O	Water
1% HCl	HCl-H_2O (1:99)
15% HOAc	Acetic acid-water (15:85)
PFH	2-Propanol-formic acid-H_2O (2:5:5)
PhOH	Phenol-water (73:27)

Ratios for all solvents were volume to volume, except PhOH, which was wt to volume.

Isolation, purification, and characterization of anthocyanin. Fresh flowers were dried at 50°C in a forced-draft oven and then ground to pass a 40-mesh screen. The ground tissue was extracted with methanol containing 1% HCl. The anthocyanin was purified by preparative thin-layer chromatography (TLC) on plates with a 2-mm layer of microcrystalline cellulose (Avicel). The solvents used were AHH and PFH. The isolated anthocyanin was finally passed through a column of Sephadex LH-20 (15 x 300 mm) with methanol containing 1% HCl at a flow rate of 1 ml/min and then examined spectrophotometrically foe purity. The purified anthocyanin was co-chromatographed with known standards by TLC on plates containing a 250-μm layer of Avicel. The solvents used were 1% HCL, BAW, BH, and AHH.

The anthocyanin was hydrolyzed by refluxing for 30-45 min (1N NCl). The anthocyanidin and sugar moieties were identified by methods previously described (1). Instead of on filter paper, the products of acid hydrolysis were co-chromatographed with known standards by TLC on plates containing a 250-μm layer of Avicel. Solvents used for the anthocyanidin were Forestal and BH, and those for the sugar were PhOH and EPH.

Isolation, purification, and characterization of flavonoid co-pigments. Flavonoid co-pigments were extracted from the ground tissue with boiling methanol. They were isolated and purified by TLC on plates with 2-mm layers of Avicel. The solvents used were AFH and PFH.

Before acid hydrolysis, each compound was passed through a column of Sephadex LH-20 (15 x 300 mm) with methanol to eliminate contamination from TLC. Each isolated compound was hydrolyzed by refluxing for 1 hr in 1N HCl, using a min of ethanol to effect complete solution. The aglycone was extracted with ethyl acetate, and the sugar moeity in the aqueous phase was examined by methods previously described for the anthocyanin (1).

Flavonoids were characterized by their chromatographic and spectral properties in direct comparison with authentic pigments. Solvents used were BAW, PFH, 15% HOAc, and PhOH. Spectral properties were determined in ethanol. Spectral diagnostic shifts were obtained as previously described (9).

Tissue pH measurement. The pH of epidermal peels, from sepals ca. 10 sq mm, was determined spectrophotometrically (2). Bromcresol green (1.2 x 10^{-3}M) was used to extend the pH range to 5.4.

Intact-tissue spectra. Spectral absorption curves of entire fresh sepals were measured with a spectrophotometer developed in one of our laboratories (4).

Authentic flavonoids. A sample of delphinidin 3,5-diglucoside was provided by Dr. G. Hrazdina. Delphinidin 3-glucoside was isolated from 'Merveille' hydrangea (7), iso-orientin from 'Prof. Blaauw' iris (6), and luteolin from parsley seed (8).

Results

The anthocyanin present in 'Blue Bonnet' and 'American Beauty' statice was chromatographically indistinguishable from delphinidin 3-glucoside (Table 1). The visible λ_{max} in methanol containing 0.01% HCl was 535 nm and the $\frac{E440}{E535}$ was 0.18, typical of a 3-substitution. No absorption band was evident in the 300-330 nm region, confirming that the compound was not acylated. Acid hydrolysis yielded delphinidin and glucose in a ratio of ca. 1:1.

The anthocyanin present in 'Twilight Lavender' and 'Midnight Blue' statice was chromatographically indistinguishable from delphinidin 3,5-diglucoside (Table 1). The visible λ_{max} was similar to that of delphinidin 3-glucoside,

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but the $\frac{E440}{E535}$ was only 0.10, typical of a 3,5-substitution. There was no spectral evidence that the compound was acylated. Acid hydrolysis yielded delphinidin and glucose in a ratio of ca. 1:2.

The 2 major flavones present in all of the 6 cvs. were luteolin and iso-orientin (Tables 1 and 2). Both flavones showed the following spectral characteristics: a free 7-hydroxyl, by the 12 nm shift of band II in alcoholic sodium acetate; a free ortho-dihydroxyl group, by the 28-29 nm shift of band I in the presence of H_3BO_3 and sodium acetate; and a free 5-hydroxyl, by the spectral shifts of band I with $AlCl_3$. Acid hydrolysis yielded no hydrolyzable sugar and did not alter the R_f values of either compound. Acid hydrolysis of iso-orientin yielded a minor spot, which migrated behind the parent compound in all solvents. This minor spot was orientin formed by the 6-glycosyl-8-glycosyl isomerization taking place via pyran ring opening in the acid solution (12).

Four flavonols were present at low concn in all 6 cvs. They were characterized as quercetin, quercetin glycosides, and a myricetin glycoside.

The pH of epidermal peels from sepals was as follows: 'American Beauty' 3.88; 'Iceberg' 3.95; 'Gold Coast' 4.10; 'Twilight Lavender' 4.15; 'Midnight Blue' 4.26; and 'Blue Bonnet' 4.70.

The in-vivo absorption spectra of each of the 6 cvs. revealed an absorption band in the 350 nm region (Fig. 1). This absorption band was attributed to the flavonoid co-pigments. A slightly longer λ_{max} in this region for 'Gold Coast' was probably due to the influence of the aurones in this tissue (3). The visible pigment present in 'Midnight Blue', 'Blue Bonnet', and 'Twilight Lavender' all showed absorption bands in the 500, 530, 570, and 620 nm regions. The absorption bands were more definitive red, and blue colors in higher plants. Because anthocyanins, at pH ca. 4 and above, are virtually colorless and there are relatively few known anthocyanidins, the extensive range of flower colors requires further explanation (10). Co-pigmentation, the complexing of anthocyanins with flavonoids and related compounds was suggested as early as 1931 (11). This phenomenon has now been shown to offer a suitable explanation for the color of many flowers (5). Co-pigmentation occurs with glycosides of all 6 common anthocyanidins and has the effect of greatly increasing their absorbance as well as changing their λ_{max} to longer wavelengths.

The co-pigmentation of a delphinidin glycoside (delphinidin 3-p-coumaroylrutinoside-5-glucoside) with iso-orientin has been demonstrated (6). In this study the colors of the statice cvs. were due to the co-pigmentation of delphinidin 3-glucoside or delphinidin 3,5-diglucoside with luteolin and iso-orientin. The major co-pigments in these cvs. were the same, but the glucoside pattern of the anthocyanin varied. Delphinidin 3,5-diglucoside was present in 'Twilight Lavender' and 'Midnight Blue', and delphinidin 3-glucoside was present in 'American Beauty' and 'Blue Bonnet'. The effect of various glycosides of the same anthocyanidin on co-pigmentation has not been fully established, but preliminary work indicates that the effect is minimal. The importance of the type of co-pigment, concn of anthocyanin, and the molar ratio of anthocyanin to co-pigment in the cvs. with the higher pH. 'American Beauty', which had the lowest pH, had a broad absorption band in the 560 nm region, with a shoulder in the 620 nm region.

Discussion

Anthocyanins are considered to be responsible for most pink,

Table 1. Chromatographic properties of the anthocyanin and the major flavonoid co-pigments isolated from statice flowers.

Compounds	R_f (x100) in[z] 1% HCl	AHH	BH	BAW	PFH	15% HOAc	PhOH	Products of acid hydrolysis
Statice flavonoids								
1[y]	3	18	10	16	--	--	--	Delphinidin + glucose
2[x]	10	26	4	8	--	--	--	Delphinidin + glucose
3[w]	--	--	--	76	32	3	64	Luteolin
4[w]	--	--	--	36	64	31	50	Iso-orientin + orientin
Authentic flavonoids								
Delphinidin 3-glucoside	4	17	11	16	--	--	--	Delphinidin + glucose
Delphinidin 3,5-diglucoside	10	26	4	9	--	--	--	Delphinidin + glucose
Luteolin	--	--	--	76	33	4	65	Luteolin
Iso-orientin	--	--	--	38	65	32	51	Iso-orientin + orientin

[z]See materials and methods section.
[y]Present only in 'Blue Bonnet' and 'American Beauty'.
[x]Present only in 'Twilight Lavender' and 'Midnight Blue'.
[w]Present in 'Blue Bonnet', 'American Beauty', 'Twilight Lavender', 'Midnight Blue', 'Iceberg', and 'Gold Coast'.

Table 2. Spectral properties of the major flavonoid co-pigments isolated from statice flowers.

Compounds	λ_{max} (nm) in EtOH Band I Alone	+NaOEt	NaOAc +H_3BO_3	+$AlCl_3$	Band II Alone	+NaOAc
Statice flavonoids						
3 or 4[z]	350	408	378	405	255 268[y]	267
Authentic flavonoids						
Luteolin or iso-orientin	350	407	379	405	255 269[y]	267

[z]Present in cvs. Blue Bonnet, American Beauty, Twilight Lavender, Midnight Blue, Iceberg, and Gold Coast.
[y]Indicates a shoulder.

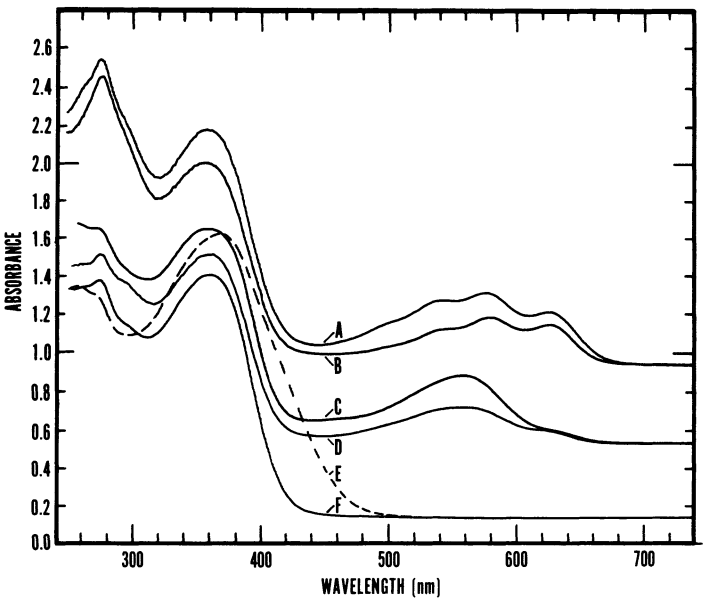

Fig. 1. In-vivo absorption spectra of sepals from statice flowers and pH of epidermal peels (absorbance offset 0.8 for A and B and 0.4 for C and D)

A. 'Midnight Blue'	pH 4.26
B. 'Blue Bonnet'	pH 4.70
C. 'American Beauty'	pH 3.88
D. 'Twilight Lavender'	pH 4.15
E. 'Gold Coast'	pH 4.10
F. 'Iceberg'	pH 3.95

on color have been reported (5). In-vivo absorption spectra (Fig. 1) of the 4 colored cvs. showed no great change in the ratios of anthocyanin to co-pigment, so these ratios cannot explain the range in color from red-violet to blue. The pH of the tissues varied considerably. 'American Beauty' was red-violet and had the lowest pH (3.88). 'Blue Bonnet' was the most blue and had the highest pH (4.70). The pH and color of 'Twilight Lavender' and 'Midnight Blue' were intermediate to 'American Beauty' and 'Blue Bonnet'. Therefore, the color of each of these cvs. was primarily influenced by the pH of the tissue.

Within the relatively constant ratio of co-pigment to anthocyanin found in commercial statice cvs., the control of pH was apparently the underlying factor in selection of the various colors. Improvement of the blue shades would require finding germplasm producing higher pH and co-pigment to anthocyanin ratios. Redder shades would require lower pH, a combination of lower pH with more co-pigment, or increased anthocyanin concn. The low pH and high co-pigment concn of the white cv. Iceberg suggest it would be a promising parent when breeding for redder shades. A white cv. with high pH and high co-pigment concn would be more likely to produce progeny with improved blue shades. The yellow cv. Gold Coast has a high concn of co-pigment, but its usefulness in breeding would probably be limited except for the production of mahogany colors, because of the visible absorption of the aurones present in its tissue.

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