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Anthocyanins in Petals of Tulip var. President Eisenhower

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The anthocyanins of cultivated tulip flowers were first investigated by Willstätter & Bolton (1916), who identified cyanidin diglucoside in some scarlet-red varieties. Robinson & Robinson (1931) examined 34 varieties of tulips and found that they contained a mixture either of cyanidin and pelargonidin biosides or of cyanidin bioside and delphinidin diglucoside. In these studies most pigments were not fully identified. Shibata (1956) and Shibata & Sakai (1958) isolated crystalline delphinidin 3-rhamnoglucoside and cyanidin 3-rhamnoglucoside. Halevy & Asen (1959) used paper chromatography and spectrographic methods to identify cyanidin 3-glucoside, pelargonidin 3-glucoside and pelargonidin 3-rhamnoglucoside in two tulip varieties in addition to the two pigments isolated by Shibata. Shibata & Ishikura (1960), in a chromatographic examination of 105 tulip varieties, identified also delphinidin 3-glucoside and delphinidin 3,5-diglucoside.

Five anthocyanins have now been separated from the petals of tulip var. President Eisenhower. One of them, pelargonidin 3-glucoside, was not found by Shibata & Ishikura (1960).

EXPERIMENTAL

Separation of crude anthocyanins. Petals (300 g.) were dried at 50° in a forced-draught oven; they were ground and extracted in 500 ml. of methanol containing 1% of HCl for 24 hr. at room temperature. The extract was filtered and the anthocyanins were precipitated with basic lead acetate. The lead salt of the anthocyanins was dried at room temperature and blended with methanol containing 2% (v/v) of concentrated HCl. The precipitated lead chloride was removed by filtration; any residual lead was precipitated by treatment with H₂S. The methanolic solution was evaporated to dryness *in vacuo* at 40° and the residue was dissolved in a minimum volume of methanol. The anthocyanins were precipitated with ether and kept at 3° for 3 days before filtration. The anthocyanins were again dissolved in methanol (fraction A).

The supernatant remaining after precipitation with lead acetate was red-brown, indicating that not all the anthocyanins were precipitated. Chromatography in butan-1-ol-acetic acid-water (6:1:2, by vol.) and 15% (v/v) acetic acid showed that it contained at least two anthocyanins in addition to other flavonoid pigments. The supernatant was shaken several times with ethyl acetate and then extracted in a liquid-liquid extractor for 24 hr. with ethyl acetate, to remove yellow flavonoid pigments. Finally it was

evaporated to dryness and the residue dissolved in methanol (fraction B). The anthocyanins in fractions A and B were combined and further purified by reprecipitating four times by addition of ether. This material (6.5 g.) was dissolved in methanol and applied to cellulose-powder columns (4 cm. $\times$ 50 cm.) prepared as described by Halevy & Asen (1959). The first solvent used was 2N-HCl-butan-1-ol (1:1, v/v; upper phase) when four anthocyanin bands separated. The resolved bands were still contaminated with yellow pigments. Each band was rechromatographed in butan-1-ol-acetic acid-water (6:1:2, by vol.) followed by 15% (v/v) acetic acid. In 15% acetic acid, the last two bands eluted from the first column were each separated into two components. These four were purified further by paper chromatography on Whatman 3MM paper as described by Halevy & Asen (1959). The solvents used were 5% (v/v) acetic acid, propan-2-ol-2N-HCl (1:1, v/v) and ethyl acetate-*tert.*-butanol (commercial)-acetic acid-water (5:4:1:3, by vol.).

Identification of the pigments. Portions (1 ml.) of each of the concentrated anthocyanins were hydrolysed by refluxing with 2N-HCl (1 ml.) for 1 hr. The anthocyanidins were extracted with a small amount of 3-methylbutan-1-ol and chromatographed. The aqueous phase was examined for sugars. Mineral acid was removed from the hydrolysates with di-*n*-octylmethylamine in chloroform (Harborne & Sherratt, 1957a). Sugars were identified by chromatography as described by Halevy & Asen (1959).

The pigments were identified by chromatographic and spectral comparison with authentic pigments. Authentic pigments were obtained from *Euphorbia pulcherima* var.

Ruth Eecke (Asen, 1958) and tulip varieties Queen of the Night (Shibata, 1956) and Pride of Haarlem (Halevy & Asen, 1959).

The R_F values were determined by descending chromatography. The solvents used for identification of anthocyanidins were: acetic acid-12N-HCl-water (30:3:10, by vol.); formic acid-12N-HCl-water (5:2:3, by vol.); butan-1-ol-2N-HCl (1:1, v/v, upper phase); 3-methylbutan-1-ol-12N-HCl-water (5:1:1, by vol.). The solvents used for identification of anthocyanins are listed in Tables 1 and 2. The absorption spectra of the anthocyanidins and anthocyanins were determined in ethanol containing HCl (0.01N), with a Beckman Model DU spectrophotometer.

RESULTS

The R_F values and absorption-spectra determination of the aglycones disclosed that pigments 1 and 2 were delphinidins, and that pigments 3-6 were pelargonidins. The R_F values of the delphinidin derivatives are presented in Table 1 and those of the pelargonidin derivatives in Table 2.

Pigment 1 was delphinidin 3-glucoside and pigment 2 was delphinidin 3-rhamnoglucoside. Further confirmation was obtained by identification of the sugar produced by hydrolysis.

Hydrolysis of pigments 1 and 4 gave only glucose, but pigments 2, 3 and 5 yielded both glucose and rhamnose. The R_F values of pigment 4 in all 11

Table 1. Values of R_F and $\lambda_{\max}$ for delphinidins of tulip var. *President Eisenhower*

The solvents used were: *a*, butan-1-ol-acetic acid-water (4:1:5, by vol.; upper phase, 16 hr.); *b*, butan-1-ol-2N-hydrochloric acid (1:1, v/v, upper phase, 16 hr.); *c*, 1% (w/v) hydrochloric acid (8 hr.); *d*, water-acetic acid-12N-hydrochloric acid (82:15:3, by vol., 16 hr.); *e*, *m*-cresol-acetic acid-water (25:1:24, by vol., upper phase, 24 hr.); *f*, phenol-water (73:27, w/w, 16 hr.); *g*, ethyl acetate-*tert.*-butanol (commercial)-acetic acid-water (5:4:1:3, by vol., 16 hr.); *h*, 15% (v/v) acetic acid (8 hr.).

Compound	R_F in indicated solvent								$\lambda_{\max}$ (m μ)
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>	<i>g</i>	<i>h</i>	
Tulip anthocyanins									
Pigment 1	0.25	0.11	0.04	0.19	0.19	0.45	0.22	0.27	552
Pigment 2	0.29	0.14	0.11	0.38	0.39	0.44	0.23	0.44	553
Authentic pigments									
Delphinidin 3-glucoside	0.24	0.12	0.05	0.18	0.17	0.44	0.23	0.27	552
Delphinidin 3-rhamnoglucoside	0.28	0.14	0.12	0.36	0.36	0.44	0.23	0.43	552

Table 2. Values of R_F and $\lambda_{\max}$ for pelargonidins of tulip var. *President Eisenhower*

The solvents used were: *a-h*, as in Table 1; *i*, 5% (v/v) acetic acid (8 hr.); *j*, propan-2-ol-2N-hydrochloric acid (1:1, v/v, 16 hr.); *k*, water.

Compound	R_F in indicated solvent											$\lambda_{\max}$ (m μ)
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>	<i>g</i>	<i>h</i>	<i>i</i>	<i>j</i>	<i>k</i>	
Tulip anthocyanins												
Pigment 3	0.41	0.36	0.29	0.54	0.63	0.77	0.38	0.70	0.57	0.59	0.40	518
Pigment 4	0.45	0.40	0.15	0.39	0.67	0.78	0.48	0.56	0.40	0.40	0.22	519
Pigment 5	0.38	0.32	0.23	0.48	0.65	0.77	0.40	0.64	0.49	0.52	0.30	518
Pigment 6	0.68	0.64	—	—	0.66	0.97	0.66	0.78	—	—	—	519
Authentic pigments												
Pelargonidin 3-glucoside	0.44	0.39	0.15	0.38	0.66	0.78	0.47	0.56	0.40	0.40	0.21	518
Pelargonidin 3-rhamnoglucoside	0.38	0.32	0.24	0.47	0.65	0.77	0.39	0.62	0.48	0.52	0.29	518

solvents corresponded to those of pelargonidin 3-glucoside, and those of pigment 5 to pelargonidin 3-rhamnoglucoside.

Paper chromatography showed that pigment 3 was neither pelargonidin 3-rhamnoglucoside, nor any of the pelargonidin pigments reported by Harborne (1958). The possibility of an organic acid being bound to the pigment was tested by treatment with sodium hydroxide according to Shibata & Ishikura (1960), but none could be detected. The pigment remained unchanged and is presumably not acylated.

A controlled hydrolysis of pigments 3 and 5 was conducted according to Harborne (1960). The resulting fractions were identified by chromatographic comparison with authentic pigments in solvents *a-d* of Table 1. Both pigments gave only pelargonidin 3-glucoside in addition to the unhydrolysed pigment and the aglycone. Therefore pigment 3 appears to be a new anthocyanin and an isomer of the already known pelargonidin 3-rhamnoglucoside found in several plants (Asen, 1958; Halevy & Asen, 1959; Harborne, 1958).

Pigment 6 was not obtained pure because it decomposed during paper chromatography. It was contaminated with traces of a yellow pigment and compounds which fluoresced in ultraviolet light. Decomposition also occurred when the pigment solution was stored for a few weeks at -3° . The R_F values of pigment 6 were higher than those reported for pelargonidins (Harborne, 1958). Pigment 6 showed two characteristic features of an acylated pigment (Harborne, 1958): it gave two spots when chromatographed in solvent *b*, and alkaline treatment produced a substance with lower R_F .

DISCUSSION

Two isomeric 3-glucosides of pelargonidin were reported by Harborne & Sherratt (1957*b*). A second instance of the occurrence of isomeric anthocyanins now has been found in the pelargonidin 3-rhamnoglucosides of the tulip.

The two delphinidins identified in President Eisenhower tulip were found by Shibata & Ishikura (1960) in many other tulip varieties, but in the 105 varieties (including Pride of Haarlem but not President Eisenhower) they tested only one pelargonidin derivative, the 3-rhamnoglucoside, was detected. In var. Pride of Haarlem, Halevy &

Asen (1959) identified not only pelargonidin 3-rhamnoglucoside but also pelargonidin 3-glucoside. The pigments have similar R_F values in solvent *b*, which was the only one used by Shibata & Ishikura (1960), although they can be easily separated in several other solvents (Table 2).

Robinson & Robinson (1931) divided the tulip varieties they tested into two groups according to their anthocyanin content. One group contained pelargonidin and cyanidin pigments, and the other delphinidin derivatives, sometimes with cyanidins free from pelargonidins. The variety Pride of Haarlem apparently demonstrates a third group containing cyanidin, delphinidin and pelargonidin pigments (Halevy & Asen, 1959). Now a variety has been found in which only delphinidin and pelargonidin derivatives occur.

SUMMARY

1. Six anthocyanins were isolated from petals of tulip var. President Eisenhower.
2. Five of them were identified by chromatographic and spectrophotometric methods as: delphinidin 3-glucoside, delphinidin 3-rhamnoglucoside, pelargonidin 3-glucoside, pelargonidin 3-rhamnoglucoside, and a new isomer of pelargonidin 3-rhamnoglucoside.
3. The sixth pigment was not identified.

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