The Coloration of *Gladiolus*
I. Survey of Anthocyanins in Petals of *Gladiolus* *

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The six common anthocyanidins found in *Gladiolus* petals occur in four types of glycosilation: 3-glucoside, 3-rhamnoglucoside, 3,5-diglucoside, and 3-rhamnoglucoside-5-glucoside. The six monoglucosides appear in minute quantities, whereas any of the other 18 anthocyanins can serve as the major contributor to the coloration of *Gladiolus* petals. In high performance liquid chromatographic analyses of petal pigment composition of nine cultivars, it was found that the anthocyanins are grouped on the basis of the aglycon substitution. Thus, pelargonidin appears by itself (group I), cyanidin and peonidin constitute group II, and delphinidin, petunidin, and malvidin group III.

Introduction

Flower coloration in gladioli has attracted many workers and stimulated many investigations. These studies required a representative collection of varieties with a complete assortment of colors, and the development of methods for pigment analysis. Robinson and Robinson [1] made one of the early attempts to separate anthocyanins in *G.* × *gandavensis* and described the presence of six anthocyanins without, however, final identification. In later reports on two different gladiolus hybrids, they showed the presence of two anthocyanins [2, 3].

In a short note intended to stimulate a genetic study of gladiolus pigmentation, Macek *et al.* [4] described six anthocyanins. A more detailed study of a random collection of commercial gladiolus varieties was made by Shibata and Nozaka [5] who claimed the identification of seven anthocyanins using paper chromatography. A thorough investigation by Yatomi and Arisumi [6] uncovered the presence of 13 anthocyanins. Five other anthocyanins were mentioned but lack full confirmation. Using a genetic and biochemical rationale, Yatomi and Arisumi predicted also the presence of the mono-glycosides. In a follow-up of this investigation, Arisumi and Kobayashi [7] confirmed the presence of three monoglycosides. These studies [6, 7] claimed full identification of 16 anthocyanins out of the potential 24. Seilleur [8–10] studied quantitatively the pigment relationship between induced mutants of the gladiolus cultivar "Hawaii" and its progenitor. He identified the six aglycons appearing in unidentified anthocyanins.

Genetic studies of gladiolus flower pigmentation are possible only when a simple, sensitive and precise method is available for quantitative and qualitative separation and identification of pigments. Of the potential 24 pigments [7], only 16 were properly identified [6, 7], to the best of our knowledge. One possibility for the inability to recognize the full range of 24 pigments may be due to the lack of sensitivity of the systems which were used.

Recent studies of the application of high performance liquid chromatography (HPLC) for analyses of anthocyanidins [11–13] and anthocyanins [14–17] demonstrated the method's feasibility for pigment identification and screening studies.

The purpose of this investigation was to develop a method capable of rapid and precise analysis of micro-samples with high resolution power which will complete the survey of anthocyanins in gladiolus flowers as an aid to genetic studies.

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Materials and Methods

Plant material

Gladiolus plants were grown during the winter of 1978/79 in an open field at Bet Dagan in the central coastal plain of Israel. Fully expanded petals were harvested in April 1979 and were stored at −25 °C before lyophilizations.

Extraction

Two hundred mg lyophilized petals were extracted in 4 ml methanol, water, conc. HCl (25:75:1, v/v/v) for approx. 15 h.

HPLC

The equipment used has been described previously [17]. Extracts were filtered through a 1-μm M filter (Swinny) before injection onto the chromatographic column. The column (250 × 4 mm) was prepacked with LiChrosorb RP-18 (5 μm) (Merck, Darmstadt). Separation was accomplished by linear gradient elution: from 20 to 100% solvent B in A + B (A, 1.5% *ortho*-phosphoric acid in water; B, 1.5% *ortho*-phosphoric acid, 20% gl. acetic acid, 25% acetonitrile in water), in 60 minutes. Solvent flow-rate was 1 ml/min, and sample size was 25 μl.

Pigment identification

Anthocyanins were identified using the classical methods [18] and those described previously [17]. In all cases, the results obtained from HPLC identifications by means of examining the kinetics of appearance and disappearance of partial hydrolysis products were also identified by the same methods.

Results and Discussion

Four types of glycosylation of the six common anthocyanidins – pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin – with the sugars glucose and rhamnose, were found in petals of commercial tetraploid *Gladiolus* cultivars. The glycosides were found to be 3-glucoside, 3-rhamnoglucoside, 3,5-diglucoside, and the 3-rhamnoglucoside-5-glucoside. Identifications were achieved by co-chromatography (TLC, HPLC) with known compounds isolated from known plant sources [18], sugar analyses, and HPLC of partial hydrolysis products [17].

Table I lists 18 of these anthocyanins, each of which can serve as the major contributor to the coloration of *Gladiolus*. This is in agreement with the results and the predictions of Yatomi and Arisumi [6]. The six monoglucosides were omitted from Table I because they always appear in minute quantities. Numbers 1 through 6 designate the aglycons and the letters A, B, and C designate the types of glycosylation. This designation is used in Table II and for peak identification in Fig. 1.

Designation	Compound	t_R	Group[a]
1-A	Pelargonidin 3-rhamnoglucoside-5-glucoside	1333	I
1-B	Pelargonidin 3,5-diglucoside	1519	
1-C	Pelargonidin 3-rhamnoglucoside	2088	
2-A	Cyanidin 3-rhamnoglucoside-5-glucoside	1144	II
2-B	Cyanidin 3,5-diglucoside	1301	
2-C	Cyanidin 3-rhamnoglucoside	1863	
3-A	Peonidin 3-rhamnoglucoside-5-glucoside	1549	
3-B	Peonidin 3,5-diglucoside	1738	
3-C	Peonidin 3-rhamnoglucoside	2339	
4-A	Delphinidin 3-rhamnoglucoside-5-glucoside	924	III
4-B	Delphinidin 3,5-diglucoside	1089	
4-C	Delphinidin 3-rhamnoglucoside	1621	
5-A	Petunidin 3-rhamnoglucoside-5-glucoside	1322	
5-B	Petunidin 3,5-diglucoside	1498	
5-C	Petunidin 3-rhamnoglucoside	2016	
6-A	Malvidin 3-rhamnoglucoside-5-glucoside	1701	
6-B	Malvidin 3,5-diglucoside	1925	
6-C	Malvidin 3-rhamnoglucoside	2565	

[a] The compounds are listed according to their natural distribution.

Table I. The major anthocyanins in petals of *Gladiolus* cultivars and their retention times (t_R in sec) on LiChrosorb RP-18 using a water-acetic acid-acetonitrile gradient.

Table II. Distribution of anthocyanins in petals of *Gladiolus* cultivars.

Cultivar	Visible color[a]	Total absorbance (Dean Dixon = 1)	Compound	Amount, in % of total absorbance	Group[b]
True Love	Scarlet	0.10	1-A	48.6	I
			1-B	19.8	
			1-C	31.6	
Rose Supreme	Venetian-pink (white blotch)	0.12	1-A	44.6	I
			1-B	51.3	
			1-C	4.1	
Intrepid	Vermilion	0.98	1-A	77.1	I
			1-B	14.3	
			1-C	8.6	
NA-17	Rose Bengal	0.34	2-A	38.9	II
			2-B	19.3	
			2-C	4.3	
			3-A	27.3	
			3-B	9.1	
			3-C	1.1	
Rose Delight	Rose-red (yellow-striped)	0.40	2-A	5.1	II
			2-B	24.1	
			2-C	3.9	
			3-A	17.2	
			3-B	48.7	
			3-C	1.1	
Moon Delight	Ruby-red	0.94	2-A	4.3	II
			2-B	23.2	
			2-C	5.4	
			3-A	16.5	
			3-B	49.0	
			3-C	1.7	
Franz Liszt	Cyclamen-purple (yellow-speckled blotching)	0.92	4-A	2.0	III
			4-B	1.1	
			4-C	2.6	
			5-A	14.9	
			5-B	5.0	
			5-C	1.2	
			6-A	55.1	
			6-B	15.6	
			6-C	2.3	
Blue Isle	Spectrum-violet	0.73	4-A	<1.0	III
			4-B	<1.0	
			4-C	1.2	
			5-A	2.6	
			5-B	5.2	
			5-C	<1.0	
			6-A	24.6	
			6-B	64.7	
			6-C	1.7	
Dean Dixon	Violet purple	1.00	4-A	5.1	III
			4-B	23.2	
			4-C	<1.0	
			5-A	10.1	
			5-B	37.5	
			5-C	<1.0	
			6-A	4.5	
			6-B	19.5	
			6-C	<1.0	

[a] According to British Council Dictionary of Color Standards (RHS chart).
[b] Anthocyanins are grouped on the basis of their aglycon substitutions. Group I contains pelargonidin (one substitution in the B-ring); Group II contains anthocyanidins with two substitutions (cyanidin and peonidin); and Group III those with three substitutions (delphinidin, petunidin, and malvidin).

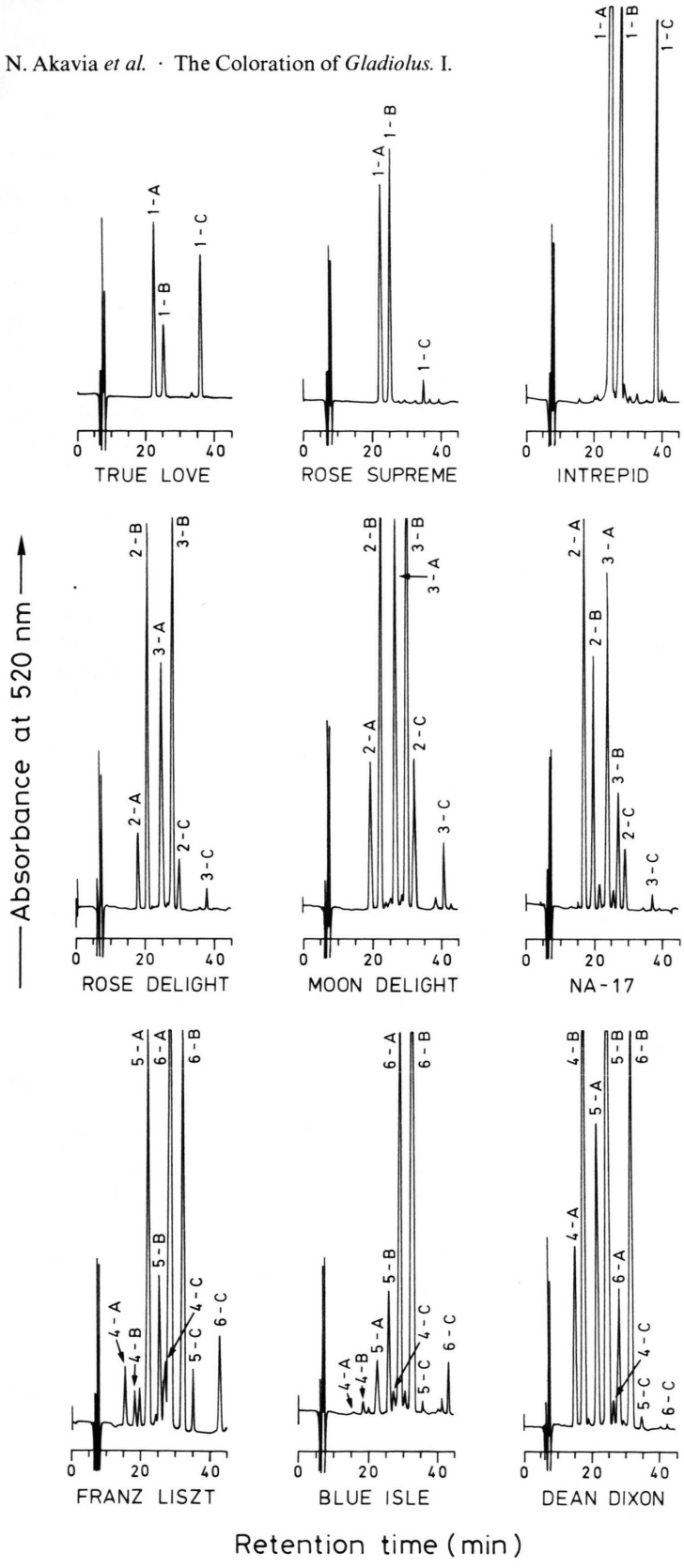

Fig. 1. HPLC analyses of anthocyanins in petals of nine *Gladiolus* cultivars. For peak identification, see Table I.

Delphinidin 3-rhamnoglucoside-5-glucoside is the first compound to be eluted: 15.4 min, and malvidin 3-rhamnoglucoside the last, with a retention time of 42.75 min. Fig. 1 shows HPLC analyses of nine *Gladiolus* cultivars, which represent a full range of petal colors except white and yellow, in which anthocyanins are not responsible for the visible color. Table II lists the identified peaks and the quantitative data from these analyses. It is obvious that in *Gladiolus* cultivars anthocyanins are grouped on the basis of the aglycon substitution. Thus, pelargonidin appeared by itself (group I), cyanidin and peonidin constituted group II, and delphinidin, petunidin and malvidin-group III. An intermixing of these groups is possible. Among the progeny of parents belonging to different groups, we found individual plants which contained all three groups. This is in agreement with the findings of Seilleur [9] who worked with *cv.* "Hawai". In a previous communication [13] we reported the occurrence of all six aglycons in *cv.* "Judith".

Arisumi and Kobayashi [7] reported the existence of 3-monoglucosides of pelargonidin, malvidin, and petunidin in considerable amounts. We detected all six monoglucosides, but in minute quantities only. The small peak in the chromatogram of *cv.* "True Love" appearing between 1-B and 1-C, has been identified as pelargonidin 3-glucoside (Fig. 1). *Cv.* "Moon Delight" shows peonidin 3-glucoside between 2-C and 3-C, and *cv.* "Blue Isle" – malvidin 3-glucoside between 5-C and 6-C.

The results presented in the present study and the methods using HPLC as described, are prerequisites for further studies of *Gladiolus* coloration, dealing with pigment genetics and regulation of glycoside biogenesis. HPLC enables accurate qualitative pigment analyses within minutes, using extracts from a few milligrams of petal tissue.

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