

PAPER IV

Anthocyanins from flowers of *Hippeastrum* cultivars

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Abstract

The anthocyanins, cyanidin 3-*O*-(6''-*O*- α -rhamnopyranosyl- β -glucopyranoside) (**1**) and pelargonidin 3-*O*-(6''-*O*- α -rhamnopyranosyl- β -glucopyranoside) (**2**), were isolated from the ornamental flowers of a Ugandan *Hippeastrum* cultivar by a combination of chromatographic techniques, and their structures were elucidated mainly by the use of homo- and heteronuclear nuclear magnetic resonance spectroscopy and electrospray mass spectrometry. The same anthocyanins were found in six different *Hippeastrum* cultivars purchased in Norway. However, the absolute amount of the anthocyanins (0.08–1.79 mg/g, fresh weight) and the relative proportions of the individual anthocyanins varied from cultivar to cultivar (13.2–96.5% of **1**). The colours of the fresh petals of the three cultivars ‘Red Lion’, ‘Royal Velvet’ and ‘Liberty’ were described by the CIELab coordinates L^* (lightness), C^* (chroma) and h_{ab} (hue angles). All the cultivars showed hue angles corresponding to scarlet nuances ($h_{ab} = 22\text{--}35^\circ$), with the highest value in ‘Red Lion’. The most reddish petals (in ‘Royal Velvet’) contained the highest relative proportion of **1**. Thus, the *in vivo* colours of these cultivars seem to be correlated with the relative proportions of individual anthocyanin in the petals.

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1. Introduction

The genus *Hippeastrum*, also referred to as *Amaryllis* (Hofmann et al., 2003), belongs to the family Amaryllidaceae. Many of the species in this genus have large and colourful flowers favourable for instance as Christmas and New Year ornamentals (Silberbush et al., 2003). There is a huge diversity of different hybrids of the *Hippeastrum* plant on the market today, and optimization of various effects (temperature, CO₂, saline water, light, phosphorus, nitrogen, growth regulators, and twin scale propagation) on growth and flowering of *Hippeastrum* hybrids have been reported (Bose et al., 1980; Ephrath et al., 2001; Huang et al., 1990a,b; Stancato et al., 1995; Silberbush et al., 2003).

In the family Amaryllidaceae the 3-glucoside and 3-xylosylglucoside of pelargonidin and cyanidin have been identified in *Lycoris* (Arisumi, 1971), and the 3,5-diglucoside of cyanidin, peonidin and pelargonidin, the 3-glucoside of cyanidin and pelargonidin, cyanidin 3-sophoroside and two

partly identified anthocyanins have been detected in *Nerine* (Arisumi and Shioya, 1970). The pigment pelargonidin 3-glucoside has been previously identified in *Hippeastrum* petals as a minor component (Hrazdina, 1988). This tentative identification was based on HPLC of the petal extract hydrolysate and TLC examination of *Hippeastrum* petal extracts, which showed five major components.

In this paper, we report the qualitative and quantitative anthocyanin content, which is responsible for the orange to red colours of seven *Hippeastrum* cultivars. None of the previously reported anthocyanins from the family Amaryllidaceae were identified. The colours of the fresh petals were described by CIELab coordinates for three of the cultivars.

2. Materials and methods

2.1. Plant material

Flowers of *Hippeastrum* × *hybridum* cv. spp. were collected from Makerere University main campus in Kampala (Uganda) in August 2004, and the flowers were kept in a freezer before analysis. The identification of the sample was carried out at the

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Table 1
Calibration equation for determination of anthocyanin concentrations by HPLC

Cy3gal ^a (mM)	Regression equation ^b	r^c	S.D. ^d		S_r^e
			Slope	Intercept	
0.026–4.194	$C_{\text{anthocyanin}} = 8.7 \times 10^{-5} \text{area} - 3.2 \times 10^{-2}$	1.0	7.3×10^{-7}	1.7×10^{-2}	0.035

^a Cyanidin 3-galactoside, isolated from *Aronia melanocarpa*, black chokeberry.

^b Ratios of peak area of cyanidin 3-galactoside vs. the appropriate concentration in mM; seven standards (0.026–4.194 mM).

^c Correlation coefficient.

^d Standard deviation of slope and intercept.

^e Standard error of the estimate.

Botany Department (Makerere University), and a voucher specimen was deposited in the herbarium (Byamukama No. 21). The flowers of the other six *Hippeastrum hybridum* cultivars, 'Red Lion', 'Royal Velvet', 'La Paz', 'Jungle Star', 'Magic Green' and 'Liberty' (dark red) were purchased in Bergen (Norway) in November 2004.

2.2. Isolation and identification of anthocyanins

The Ugandan *Hippeastrum* sample (100 g) was extracted with 1% trifluoroacetic acid (TFA) in methanol. The filtered extract was concentrated under reduced pressure, purified by partition (several times) against ethyl acetate and applied to an Amberlite XAD-7 column. The anthocyanins adsorbed to the column were washed with water, and eluted from the column with methanol containing 1% TFA. The concentrated anthocyanin extract was purified by Sephadex LH-20 chromatography using 50% aqueous methanol containing 1% TFA as eluent. The individual anthocyanins were separated using preparative HPLC (Gilson 305/306 pump equipped with a UV 6000LP detector) equipped with an ODS Hypersil column (25 cm × 2.2 cm; i.d.; 5 μm). Two solvents were used for elution: A = formic acid–water (1:9, v/v) and B = formic acid–water–methanol (1:4:5, v/v). The elution profile consisted of a linear gradient from 10% to 100% B for 30 min, isocratic elution (100% B) for the next 12 min, followed by a linear gradient from 100% to 10% B for 2 min. The flow rate was 14 mL/min for 44 min, and aliquots of 500 μL were injected.

The NMR experiments were obtained at 400.13 and 100.62 MHz for ¹H and ¹³C, respectively, on a Bruker DMX-400 instrument at 25 °C. The deuteriomethyl ¹³C signal and the residual ¹H signal of the solvent, CF₃COOD–CD₃OD (95:5, v/v), were used as secondary references (49.0 and 3.4 ppm from tetramethylsilane for ¹H and ¹³C, respectively).

The 1D ¹H NMR and the 2D HMBC, HSQC and COSY experiments were obtained with the 5 mm TB1 probe. Mass spectral data were achieved by a LC–MS system (Waters 2690 HPLC-system connected to Micromass LCZ mass spectrometer) with electrospray ionisation in positive mode (ESP+). The following ion optics was used: Capillary 3 kV, cone 30 and 60 V, and extractor 7 V. The source block temperature was 120 °C and the desolvation temperature was 150 °C. The electrospray probe-flow was adjusted to 100 μL/min. Continuous mass spectra were recorded over the range *m/z* 150–800 with scan time 1 s and interscan delay 0.1 s.

2.3. Co-chromatography (TLC, on-line HPLC)

Co-chromatography included TLC and on-line HPLC. TLC was carried out on microcrystalline cellulose (F 5556, Merck) with the solvent FHW (HCO₂H–conc HCl–H₂O, 1:1:2, v/v). The HPLC instrument (HP-1050 module, system, Hewlett-Packard) was equipped with an ODS Hypersil column (25 cm × 0.46 cm, 5 μm). Two solvents: A, water (0.5% trifluoroacetic acid) and B, acetonitrile (0.5% trifluoroacetic acid) were used for elution. The elution profile for HPLC consisted of initial conditions with 90% A and 10% B followed by linear elution for 10 min (14% B), isocratic elution 10–14 min, and the subsequent linear conditions; 18 min (16% B), 22 min (18% B), 26 min (23% B), 31 min (28% B) and 32 min (40% B), isocratic elution 32–40 min, and final linear elution 40–41 min (10% B). Aliquots of 15 μL were injected and the flow rate was 1 mL/min. Prior to injection, all samples were filtered through a 0.45 μm Millipore membrane filter. All the UV–vis absorption spectra were recorded on-line during HPLC analysis, and the spectral measurements were made over the wavelength range 200–600 nm in steps of 2 nm.

Table 2
Chromatographic and spectral data of the anthocyanins in *Hippeastrum* spp.

Compound	R_f (TLC), FHW	t_R (HPLC) (min)	Absorption maxima (nm)	$A_{440}/A_{\text{vis-max}}$ (%)	ES-MS (M^+ , A^+) (<i>m/e</i>)
1	0.57	23.7	510	35	595, 287
2	0.73	27.1	502	48	579, 271
1^a	0.55	23.9	510	35	
2^b	0.46	27.1	502	48	

See Fig. 1 for structures.

^a Cyanidin 3-rutinoside from blackcurrant (*Ribes nigrum*) (Frøylog et al., 1998).

^b Pelargonidin 3-glucoside from strawberries (*Fragaria ananassa*) (Andersen et al., 2004), M^+ = molecular ion, A^+ = fragment.

2.4. Quantitative determinations

The quantitative determination of the anthocyanin content in the six *Hippeastrum* cultivars, purchased in Norway, was related to a standard curve based on pure amounts of cyanidin 3-galactoside, isolated from *Aronia melanocarpa*, black chokeberry (Chandra et al., 2001). Integration data recorded during on-line HPLC analysis detected at 520 ± 20 nm were directly correlated with a regression equation based on seven different concentrations of cyanidin 3-galactoside recorded by on-line HPLC under similar conditions (Table 1).

2.5. Colour measurements

The surface colour of fresh flowers of the three *Hippeastrum* cultivars 'Red Lion', 'Royal Velvet' and 'Liberty' were analysed with a Hunter colorimeter (HunterLab D25 DP-90000) at CIE D65/10° illumination/viewer conditions. The colours were expressed as CIE $L^*C^*h_{ab}$ values, and the average of six measurements was used. The CIE $L^*C^*h_{ab}$ system is a one colour system that takes into account all aspects to describe colour. The L^* describes the lightness of the colour, going from black ($L^* = 0$) to white ($L^* = 100$). The C^* describes the chroma (saturation) of the colour, a measure of how far from the grey tone the colour is. The higher the C^* value the more saturated the colour is. The last parameter, h_{ab} , describes the hue of the colour, i.e. colour tonalities (red, green, yellow, etc.). This is based on the CIE $L^*a^*b^*$ system, which is again based on the tristimulus values X , Y and Z (Gonnet, 1998; Gonnet and Hieu, 1992). A red colour has h_{ab} around 0° while yellow is described by a h_{ab} around 90°. In this way, it is easy to predict that for instance h_{ab} 45° corresponds to orange.

3. Results

3.1. Anthocyanin identification

The HPLC chromatogram of the crude extract of the Ugandan *Hippeastrum* sample detected at 520 ± 20 nm showed two major peaks. The anthocyanins in the extract (**1** and **2**) were purified by partition against ethyl acetate followed by Amberlite XAD-7 column chromatography, and thereafter separated by Sephadex LH-20 chromatography and preparative HPLC.

The anthocyanins from *Hippeastrum* × *hybridum* cv. spp. were co-chromatographed (TLC and online HPLC) with anthocyanins from black currant (*Ribes nigrum*) (Frøytlog et al., 1998) and strawberries (*Fragaria ananassa*) (Andersen et al., 2004) (Table 2).

The ^1H NMR spectrum of **2** showed a 4H AA'XX' system at δ 8.69 and 7.14. These signals were assigned to H-2',6' and H-3',5', respectively, due to the $^3J_{\text{CH}}$ couplings giving cross peaks at δ 8.69/166.54 (H-2',6'/C-4') and δ 7.14/121.14 (H-2',6'/C-1') in the HMBC spectrum. The other proton signals of the aglycone were at δ 9.10 (H-4), δ 7.02 (H-8) and δ 6.77 (H-6) (Table 3). From these signals, the individual carbons of the anthocyanidin were assigned based on the cross peaks in the HMBC spectrum, in accordance with pelargonidin (Table 3). Starting from the

Table 3

^1H and ^{13}C NMR shift values (ppm) and proton–proton coupling constants (Hz) for cyanidin 3-*O*-(6''-*O*- α -rhamnopyranosyl- β -glucopyranoside) (**1**) and pelargonidin 3-*O*-(6''-*O*- α -rhamnopyranosyl- β -glucopyranoside) (**2**) in $\text{CD}_3\text{OD}:\text{CF}_3\text{COOD}$ (95:5, v/v) at 25 °C

	Pigment 1		Pigment 2	
	^{13}C (ppm)	^1H (ppm), J (Hz)	^{13}C (ppm)	^1H (ppm), J (Hz)
Aglycone				
2	163.65		165.02	
3	145.33		146.25	
4	136.25	9.09 s	136.52	9.10 s
5	158.68		159.55	
6	104.01	6.78 s	103.21	7.02 d, 2.9
7	170.46		172.12	
8	95.21	7.02 s (br)	96.50	6.77 s (br)
9	157.40		157.58	
10	113.30		112.26	
1'	121.34		121.14	
2'	118.02	8.17 d, 2.1	115.05	8.69 d, 9.2
3'	146.67		118.50	7.14 d, 9.2
4'	155.95		166.54	
5'	117.48	7.14 d, 9.2	118.50	7.14 d, 9.2
6'	128.52	8.70 dd, 9.2, 2.1	135.05	8.69 d, 9.2
β -Glucopyranoside				
1''	102.92	5.36 d, 7.7	103.75	5.37 d, 7.8
2''	74.51	3.77 dd, 9.1, 7.7	74.45	3.75 dd, 9.0, 7.8
3''	77.78	3.62 m	77.89	3.61 m
4''	70.89	3.50 t, 9.8	70.85	3.50 m
5''	77.22	3.81 m	77.04	3.81 m
6A''	67.50	4.15 dd, 11.2, 1.7	67.69	4.16 dd, 11.1, 1.5
6B''	67.50	3.68 m	67.69	3.68 m
α -Rhamnopyranosyl				
1'''	102.05	4.74 d, 1.6	102.03	4.74 d, 1.6
2'''	71.25	3.90 dd, 3.5, 1.6	71.65	3.90 dd, 3.5, 1.6
3'''	72.33	3.72 m	72.38	3.71 m
4'''	73.70	3.42 m	73.72	3.41 m
5'''	69.55	3.65 m	69.66	3.65 m
6'''	17.58	1.26 d, 6.2	17.69	1.27 d, 6.2

doublet at δ 5.37 ($J = 7.7$ Hz, H-1''), the observed cross peak (δ 5.37/3.75) in the DQF-COSY spectrum permitted the assignment of H-2''. The chain of coupled protons H-2'', H-3'', H-4'', H-5'' and H-6A'' and 6B'' were thereafter assigned similarly (Table 3). The chemical shifts and the coupling constants (Table 3) were in accordance with a β -glucopyranosyl. A cross peak at δ 5.37/146.25 in the HMBC spectrum between H-1'' and C-3 of the aglycone showed that this sugar unit was connected to the 3-position of the aglycone. By using the doublet at δ 4.74 ($J = 1.6$ Hz) as starting point in the DQF-COSY spectrum, it was likewise possible to assign all the chemical shifts for a second monosaccharide moiety, rhamnopyranosyl (Table 3). The linkage point between the two sugars was established to be at the glucosyl 6''-position by the cross peak at δ 4.74/67.11 (H-1'''/C-6'') in the HMBC spectrum. A molecular ion at m/z 579 in the ES-MS spectrum of **2**, and the fragment ion at m/z 271 confirmed the structure to be pelargonidin 3-*O*-(6''-*O*- α -rhamnopyranosyl- β -glucopyranoside) (Fig. 1).

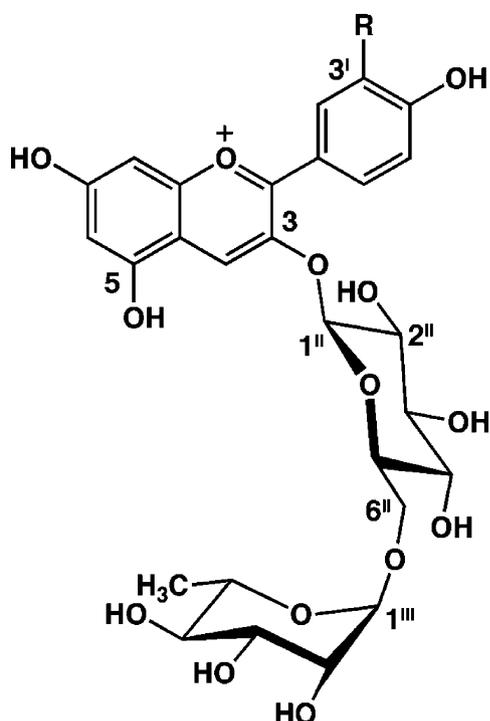


Fig. 1. The structure of the anthocyanins identified in *Hippeastrum* spp. R=OH: cyanidin 3-*O*-(6''-*O*- α -rhamnopyranosyl- β -glucopyranoside) (**1**), and R=H: pelargonidin 3-*O*-(6''-*O*- α -rhamnopyranosyl- β -glucopyranoside) (**2**).

Based on assignments (Table 3) from homo- and hetero-nuclear NMR experiments it was possible in a similar manner as described above for **2**, to identify pigment **1** to be cyanidin 3-*O*-(6''-*O*- α -rhamnopyranosyl- β -glucopyranoside) (Fig. 1). A molecular ion at m/z 595 in the ES-MS spectrum of **1**, and the fragment ion at m/z 287 (cyanidin), confirmed this structure.

4. Discussion

4.1. Anthocyanin content in petals of *Hippeastrum* cultivars

The 3-*O*-(6''-*O*- α -rhamnopyranosyl- β -glucopyranosides) of cyanidin and pelargonidin have as far as we know previously not been reported to occur in any species in the genus *Hippeastrum* nor the family Amaryllidaceae. To examine the anthocyanin content of *Hippeastrum* cultivars more systematically, six

Table 4
Anthocyanin content in the petals of various *Hippeastrum* cultivars

<i>Hippeastrum</i> cultivars	1 (%)	2 (%)	mg anthocyanins/g fresh petal
'Red Lion'	13.2	86.8	1.34
'Liberty'	55.5	44.3	1.74
'Jungle Star'	63.9	35.2	0.23
'Royal Velvet'	94.2	4.4	1.79
'Magic Green'	95.6	4.4	0.08
'La Paz'	96.5	2.02	0.72
<i>Hippeastrum</i> spp.	11.4	89.5	–

1 = cyanidin 3-*O*-(6''-*O*- α -rhamnopyranosyl- β -glucopyranoside), and **2** = pelargonidin 3-*O*-(6''-*O*- α -rhamnopyranosyl- β -glucopyranoside).

Table 5

CIElab parameters for petals of three *Hippeastrum* cultivars with uniform petal colour

<i>Hippeastrum</i> cultivar	L^*	C^*	h_{ab}
'Royal Velvet'	34.6 \pm 0.3	40.1 \pm 2.4	22.3 \pm 0.9
'Liberty'	33.7 \pm 1.3	48.9 \pm 3.7	26.8 \pm 1.5
'Red Lion'	38.6 \pm 0.8	63.9 \pm 4.8	35.2 \pm 2.2

L^* = lightness of the colour (black; L^* = 0, white; L^* = 100), C^* = chroma (saturation) of the colour and h_{ab} = hue of the colour, i.e. colour tonalities (red, green, yellow, etc.).

cultivars with various petal colours and colour patterns were purchased. The anthocyanin content of these cultivars was determined by their UV-vis spectra and co-chromatography (HPLC and TLC) with authentic anthocyanins (**1** and **2**) (Table 2). All the seven *Hippeastrum* cultivars were found to contain the same two anthocyanins, **1** and **2**, in varying amounts (Table 4). Thus, the 3-*O*-(6''-*O*- α -rhamnopyranosyl- β -glucopyranoside) of cyanidin and pelargonidin were found to characterise the genus *Hippeastrum*/*Amaryllis*. The absolute amount of anthocyanins within the petals of the various cultivars varied between 0.08 and 1.79 mg/g fresh weight (Table 4).

4.2. *In vivo* petal colour versus anthocyanin content

The colours of the fresh petals of the three *Hippeastrum* cultivars 'Red Lion', 'Royal Velvet' and 'Liberty' were described by the CIElab coordinates L^* (lightness), C^* (chroma) and h_{ab} (hue angles) (Table 5). The other samples 'Magic Green', 'Jungle Star' and 'La Paz' have not been analysed by these coordinates due to lack of uniform petal colours.

All the three samples were described by having hue angles corresponding to scarlet nuances ($h_{ab} = 22\text{--}35^\circ$) with the highest value in 'Red Lion'. This latter cultivar also revealed the highest L^* and C^* values (Table 5). The hybrids 'Liberty' and 'Royal Velvet' showed similar quantitative anthocyanin content (Table 4) and similar L^* values (Table 5), while 'Red Lion' had the lowest anthocyanin content and highest L^* value. With respect to a correlation between the CIElab parameters and the qualitative anthocyanin content in the three hybrids, both the C^* and h_{ab} values increased with increasing proportions of **2** at the expense of **1**. The most red petal colour, which was expressed by 'Royal Velvet', contained the highest relative proportion of the anthocyanin (**1**) with the highest λ_{max} -value (Table 2). Thus, the *in vivo* colour of these hybrids seems to be correlated with the type of anthocyanidin in the petals. Flowers containing pelargonidin (**2**) as the major anthocyanidin revealed a more orange colour than those having the corresponding cyanidin-derivative (**1**) as the major anthocyanin (Tables 4 and 5).

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