



Anthocyanin Pigments in Redbud (*Cercis* spp) Flowers

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Abstract

Redbud (*Cercis* spp.) is used as a spring flowering ornamental tree and is found wild in much of North America. Typically flowers are light purple although there are selected cultigens that are white, rose, or red-purple. Flowers from cultigens common to the eastern U.S. and from wild Eastern redbud (*C. canadensis*) were collected and tested for color and anthocyanin pigment composition. The anthocyanins cyanidin 3-glucoside, petunidin 3-glucoside, peonidin 3-glucoside, and malvidin 3-glucoside were most abundant in purple, rose, and red-purple redbud flowers and total anthocyanin content was 2263 to 8730 mg.kg DW⁻¹. Small amounts of delphinidin, cyanidin, and petunidin 3, diglucosides were also present. Most of the typical purple-flowered redbuds contained cyanidin 3-glucoside as the dominant pigment, while the red-purple flowered 'Appalachian Red' and 'Crosswicks Red' contained malvidin 3,5-diglucoside as the dominant anthocyanin. An unknown anthocyanin was present in all redbud flowers, and was higher in the red-purple flowered phenotypes. These results show that the color of redbud flowers is from anthocyanins, predominantly cyanidin 3-glucoside and malvidin 3,5-diglucoside, with malvidin 3,5-diglucoside as the primary pigment in red-purple flowers and cyanidin 3-glucoside dominant in purple flowers.

Keywords

Flavonoid, Color, Malvin, Oenin, Nutraceutical, Cyanin, Cyanidin, Malvidin

Introduction

Use of plants as sources of anthocyanins for new uses is an emerging area of research. Certain anthocyanins, such as cyanidins, are thought to be more resistant to oxidation and important in human bioactive defense reactions, while other pigments such as malvidins are valued for imparting stable color to fruits and vegetables, as well as for anti-inflammatory effects [1]. Use of plant-based anthocyanins, regarded as safer alternatives to synthetic colorants, has become increasingly common in the food industry [2]. In addition, anthocyanins extracted from flowers are being explored for use as sensitizer dyes in solar cells [3].

Redbud (*Cercis* spp.), is a small tree of the legume family indigenous to the Americas, Europe, and China. It can be found as a wild spring flowering tree in much of North America [4]. *C. canadensis* var *canadensis*, the Eastern redbud, is found from the eastern and Midwestern US, north to Toronto and south to Oklahoma and north Texas [5]. Botanical varieties "texensis" and "mexicana" are found in Oklahoma/northern Texas, and in south Texas into Mexico, respectively. Various *C. canadensis* phenotypes have been selected from the wild for unusual leaf or flower color or modified tree architecture [6]. 'Appalachian Red'

(*C. canadensis* var *canadensis*) (AR) has flowers with a strong reddish purple color, in contrast to the more common pink-purple color of Eastern redbud (Figure 1). 'Oklahoma' (OK), a cultivar of *C. canadensis* var. *texaniana*, in contrast, has a dark purple-magenta flower color. Weeping cultigens such as 'Traveller' (*C. canadensis* var. *texaniana*) (TRV) and 'Ruby Falls' (RF) have flowers that show typical purple color. An understanding of the pigments in redbud flowers will help establish inheritance of flower color in crosses of redbud cultigens.

Information on the anthocyanin content of redbud (*Cercis* spp.) flowers is lacking. Anti-malarial properties of the leaf and bark of *C. siliquastrum* have been reported and may be from flavonoids or galloyl triterpenes [7].

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Received: January 05, 2017; **Accepted:** February 27, 2017; **Published online:** March 02, 2017

Citation: Veazie PP, Ma G, Werner D (2017) Anthocyanin Pigments in Redbud (*Cercis* spp) Flowers. J Hortic Sci Res 1(1):13-18

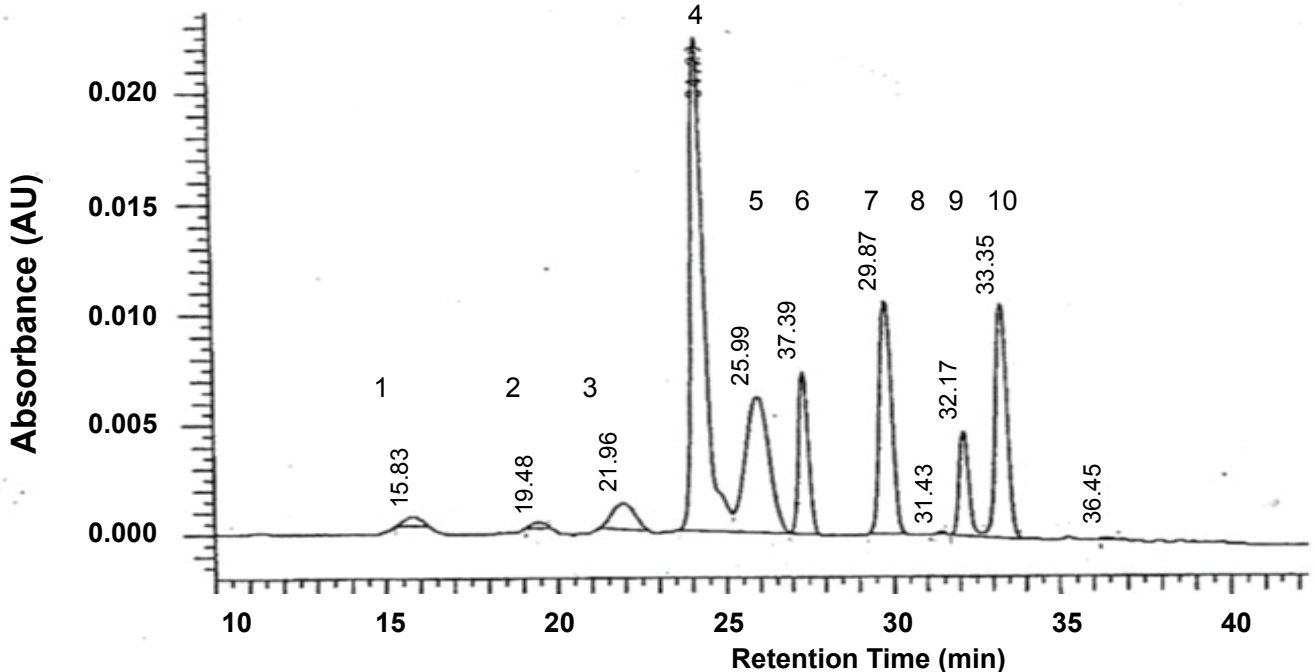


Figure 1: Chromatogram of RF redbud flower extract obtained using high performance liquid chromatography. Peaks identified as follows: 1) DDG; 2) CDG; 3) PTDG; 4) PNDG; 5) MDG; 6) CG; 7) PTG; 8) PNG; 9) UK; 10) MG. RF illustrates the varied peaks detected among redbud cultigens.

Salatino, et al. [8] found only monoglycoside flavonoids of kaempferol, quercetin and myricetin in leaves from multiple species of *Cercis*, while *Cercis chinensis* Bunge flowers are used to treat rheumatic pain [9] and petunidin 3-glucoside, malvidin 3-glucoside and malvidin 3,5-diglucoside were detected in flowers of *C. chinensis* using paper chromatography [10].

This study was done to characterize the amount and type of anthocyanins in redbud flowers among cultigens prevalent in eastern North America.

Materials and Methods

Plant material

Redbud flowers were collected from 12 cultigens in North Carolina in 2015 and 2016. Flowers from Appalachian Red (AR), Crosswicks Red (CR), Alba (AB), Ace of Hearts (AOH), Flame (FL), Big John (BJ), Kays Early Hope (KEH) and Oklahoma (OK) were collected from the JC Raulston Arboretum in Raleigh NC. Flowers from Forest Pansy (FP), Traveller (TRV), Ruby Falls (RF) and a seedling Eastern redbud (ERB) were collected from 3-5 year old trees in Kannapolis NC. Flowers were carefully selected to make sure they were at optimum bloom stage, with full extension of calyx tube but before petals started to flex. Samples of 50 to 100 g were collected each year, with the exception of FL and AOH, where a sample of 30 g was collected only in 2016. All flowers were held in plastic zipper bags on ice until return to the laboratory. Pedicels and sepals were removed and flowers were weighed.

Flower color parameters and pH

Flower color was determined by filling a 20 ml glass cuvette (93-G-10, Starna, Atascadero CA USA) with flowers. The cuvette was fitted with black cloth containing a hole of 8 cm diameter cut in the center. A Konica Minolta CR400 (Konica Minolta, Ramsey, NJ) was calibrated with a white tile, placed so the aperture was centered over the hole and color measured using Hunter coordinates $L^*a^*b^*$, illuminant C, and diffuse illumination/0° viewing angle and 2° observer angle. Hue and chroma were calculated using the formula of McGuire [11]. Each cultigen was measured in duplicate except for FL.

Flower and petal pH were determined by microelectrode in triplicate to determine if pH was related to phenotypic color. Petals were removed from subsamples of redbuds when sufficient sample was available to compare to flowers (calyx, petals, stamens, styles), to determine if petal pH differed from that of the whole flower. Petals and flowers were frozen at -20 °C. Subsamples of frozen flowers or petals were placed in microcentrifuge tubes and crushed with micropestles to release juice. The pH of the juice was determined by placing the tip of a steel pH probe (Hach Co., Loveland CO USA) into the tube.

HPLC analysis

Redbud flowers (5-10 g per sample) were freeze-dried using a VirTis LyoTroll (SP Scientific, Warminster, PA, USA) then were ground with stainless steel balls with

a homogenizer (Geno-Grinder, SPEX, Metuchen, NJ USA). Powders were extracted following the method of Bradish, et al. [12] with slight modification. Briefly, 0.04 g of redbud powder was extracted with 1.5 mL of acidified methanol (formic acid:methanol:deionized water, 3:60:37 v/v/v). Samples were vortexed and centrifuged at 10,600 g for 20 min at 4 °C. All samples were re-extracted and supernatants combined to achieve 98% of total anthocyanin present in samples. Supernatant aliquots of 1 ml were filtered through 0.2 µm PTFE membranes (Fisher Scientific, Pittsburg, PA) into 2 ml amber vials (Agilent), flushed with nitrogen gas, capped, and loaded onto an autosampler coupled to a Hitachi LaChrom HPLC (Hitachi Ltd., Tokoyo), equipped with a UV-VS diode array detector (DAD), controlled temperature auto sampler (4 °C), and column compartment (30 °C). D-2000 software (Hitachi Ltd., Tokoyo) was used as the system run controller and for data processing. Three individual samples were extracted for each cultigen.

Anthocyanin separation was performed using a reversed phase C18 column (Synergi 4 µ Hydro-RP 80 Å, 6 × 250 µm, Phenomenex, Torrance, CA, USA). The mobile phase consisted of 5% formic acid in water (A) and 100% methanol (B) with a flow rate of 1 ml/min using a step gradient of 0 min, 10% B; 5 min, 15% B; 15 min, 20% B; 20 min, 25% B; 25 min, 30% B; 45 min, 60% B; 47 min, 10% B; 60 min, 10% B. Compound concentrations were estimated using standard curves generated by injecting 5 µL of 0.0625-0.5 mg/mL preparations of cyanidin 3-glucoside, cyanidin 3,5-diglucoside, malvidin 3-glucoside, malvidin 3,5-diglucoside as external standards (Chromadex, Irvine, CA; Sigma, St. Louis, MO). Compound identification was performed based on retention time

compared to authentic standards, and previously published reports [13-16]. Each sample was run in duplicate and content reported as mg of cyanidin 3-glucoside equivalents/kg dry weight (DW). Sums of anthocyanins were calculated to obtain total anthocyanin content and each anthocyanin calculated as a percent of the total anthocyanin content.

Data were subjected to one way ANOVA to determine significance of variables relative to genotype. Where significant, genotype means were separated using HSD or Student's t test, P < 0.05. Correlation of anthocyanin and color parameters was done using Pearson's correlation, P < 0.05.

Results and Discussion

Flower weight, color and pH

Fresh flower weight was highest for FL (0.288 g), followed by KEH, and lowest for AOH (0.012 g) (Table 1). The remaining cultigens were of similar flower weights. Percent dry weight of flowers averaged 15% for all cultigens (data not shown).

Colorimeter data of redbud flowers showed differences primarily in L* values, with AB highest (most white) with L* of 65 and OK lowest with L* of 38. The values of a, b, chroma and hue did not clearly delineate differences in visual color. CR and AR flowers were highest in hue, about 355° (more red purple) while other values (other than those of 'AB') indicated values that were more purple (about 343°). OK, a visually darker purple-magenta flower Figure 2, could not be distinguished from other cultigens using colorimeter values of a, b, or hue. Chroma was lower for the purple flowered redbuds

Table 1: Flower weight and color of redbud genotypes.

Cultivar	Visual color	Flower FW (g ⁻³)	L	a	b	Chroma (°)	Hue (°)	[a/b]*
<i>Cercis canadensis</i> var. <i>canadensis</i>								
Alba (AB)	White	42.92bc ^z	65.67a	-2.24d	10.02a	10.27d	102.56e	0.22d
Appalachian Red (AR)	Red-purple	47.70bc	45.52b	29.44a	-0.85b	28.47a	354.98ab	34.64a
Crosswicks Red (CR)	Red-pink	41.40bc	41.42bc	18.40bc	-1.13bc	18.43bc	356.49a	16.28b
Forest Pansy (FP)	Light purple	45.83bc	43.65bc	19.81bc	-5.7de	20.68bc	344.34bcd	3.48c
Ruby Falls (RF)	Purple	43.88bc	42.06bc	15.72c	-6.47e	17.03bc	337.42d	2.43c
Ace of Hearts (AOH)	Light purple	12.17d	40.54bc	16.28bc	-5.40de	17.15bc	341.67d	3.01c
Eastern Redbud (ERB)	Light purple	46.13bc	44.46bc	16.99bc	-6.79cde	17.24bc	342.57cd	2.50dc
Flame (FL)	Rose pink	288.57a	na ^y	na	na	na	na	na
<i>C. canadensis</i> var. <i>texensis</i>								
Oklahoma (OK)	Dark purple	40.34c	38.11c	17.35bc	-5.21cde	18.11bc	343.29bcd	3.34c
Traveller (TRV)	Light purple	43.21bc	43.01bc	19.46bc	-7.00e	20.61bc	341.56d	2.78c
Hybrids containing <i>C. chinensis</i>								
Big John (BJ)	Light purple	54.9bc	44.58bc	16.40bc	-1.78bcd	16.54c	351.98a	10.33b
Kays Early Hope (KEH)	Light purple	63.13b	41.57bc	21.38b	-8.21e	22.58b	341.39d	2.61c

*[a/b] indicates absolute value of a/b; ^zMeans separated within column by HSD. Different letters indicate significant differences between means, P < 0.05; ^yna = not available.

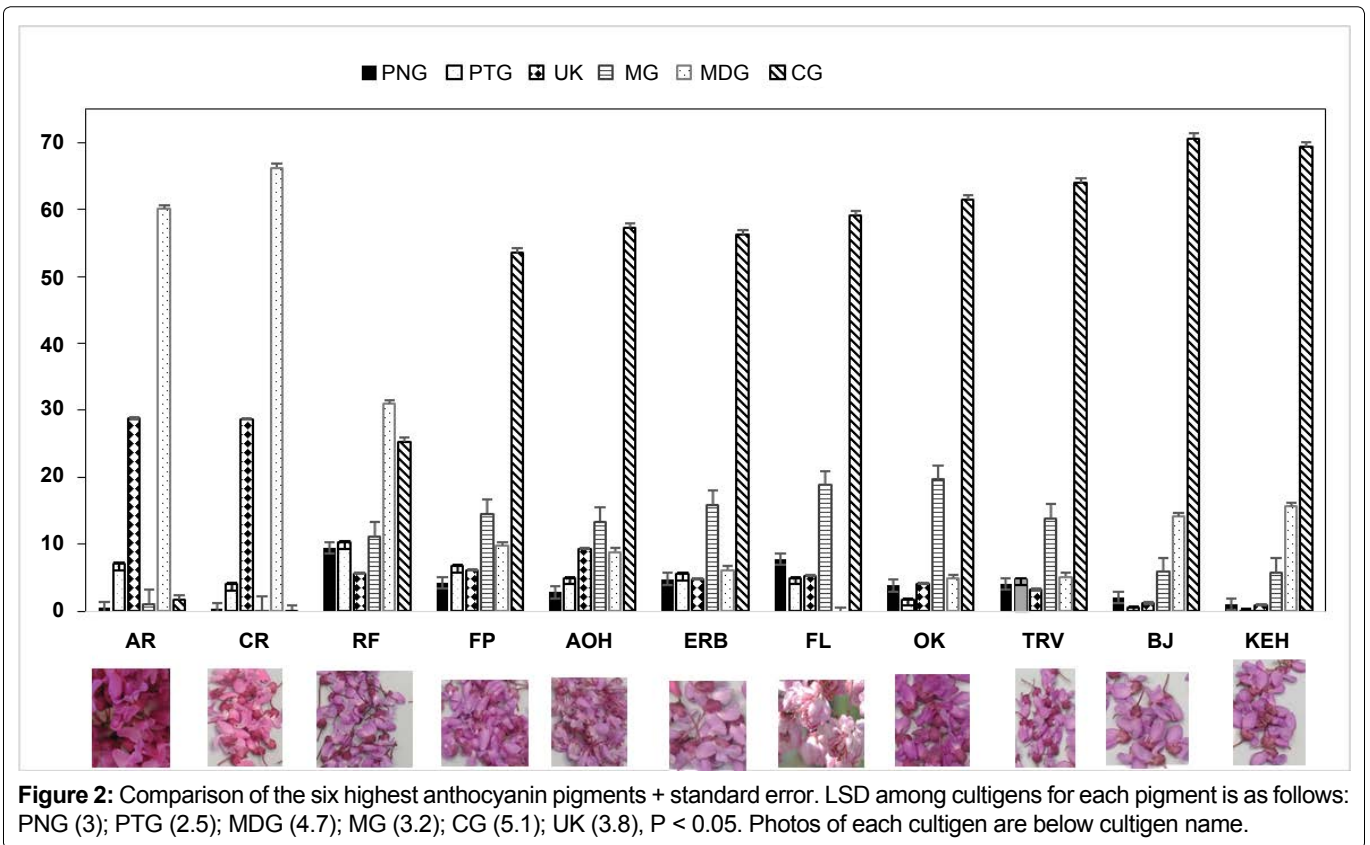


Table 2: Flower and petal pH of redbud genotypes.

Genotype	Flower*	Petal
AB	4.88a	4.48a
AR	4.75ab	4.51a
CR	4.60bc	4.28b
RF	4.58bc	4.21bc
FP	4.58bc	4.21bc
AOH	4.89a	4.55a
ERB	4.58bc	4.26bc
FL	4.23d	Na
OK	4.58bc	4.33b
TRV	4.45c	4.13c
BJ	4.55bc	4.21bc
KEH	4.64bc	4.39b

*Means separated within column by HSD. Different letters indicate significant differences between means, $P < 0.05$.

than for the white or red-purple flowers, indicating the reduced reflectance and reduced brightness of the purple colors. Using an absolute value of a/b, differences among red, white and purple flowered types could be found but this value could not differentiate the light purple from dark purple cultigens.

Petal pH has been implicated in various flowers as important to maintain pigment color in red or blue forms [17]. The pH of all redbud flowers and petals measured was acidic, under a pH of 5.0 table 2 and was slightly higher than that of petals. Among the redbud flowers, the pH was highest for AOH, AB, and AR and lowest for FL. Petal pH ranged from 4.13 in TRV to 4.48 in AB.

Anthocyanin content and profile

A total of nine anthocyanins were identified in redbud flowers using HPLC (Table 3). These included the monoglucosides petunidin 3-glucoside (PTG), peonidin 3-glucoside (PNG), cyanidin 3-glucoside (CG), malvidin 3-glucoside (MG) and the diglucosides delphinidin 3,5-diglucoside (DDG), petunidin 3,5-diglucoside (PTDG), peonidin 3,5-diglucoside (PNDG), malvidin 3,5-diglucoside (MDG) (Figure 1). An additional unknown peak was present in all cultigens. Delphinidin 3-glucoside (DG) and pelargonidin pigments were not detected in any of the redbud cultigens, indicating that synthesis pathways for cyanidin and delphinidin are dominant in *Cercis*, and that peonidin synthesis from cyanidin and petunidin, and malvidin synthesis from delphinidin are active [18]. PTDG was present only in trace amounts in the redbuds tested. Only the mono and diglucoside pigments PTG, MG, and MDG were detected in *C. chinensis* flowers using paper chromatography [10].

The relative amounts of pigments varied widely among redbud cultigens (Table 3 and Figure 2). In AB, only trace amounts of total anthocyanin (CG, UK) were found (Table 3). Total anthocyanin content was highest for RF, followed by OK and AR. TRV, ERB, FP, and AOH were similar in total anthocyanin content. BJ, KEH and FL were lowest in total anthocyanin content. The cultigens KEH and BJ had proportionally more CG and MDG and less MG than the other purple-red flowered

Table 3: Anthocyanins identified in redbud flowers, mg·kg DW⁻¹.

Cultigen	Delphinidin 3,5-diglucoside (DDG) [*]	Cyanidin 3,5-diglucoside (CDG)	Petunidin 3,5-diglucoside (PTDG)	Peonidin 3,5-diglucoside (PNDG)	Malvidin 3,5-diglucoside (MDG)	Cyanidin 3-glucoside (CG)	Petunidin 3-glucoside (PTG)	Peonidin 3-glucoside (PNG)	Malvidin 3-glucoside (MG)	Unknown (UK)	Total
AB	0.0b	0.00e	0.00c	0.0e	0.0f	1.8f	0.0e	0.0e	0.0c	4.1g	5.9f
AR	0.0b	38.2b	0.0b	0.0e	3117.5a	88.8f	365.7b	24.9e	57.5c	1492.3a	5184.8b
CR	0.0b	10.9cde	0.8b	0.0e	2426.7c	1.4f	150.6cde	10.7e	5.4c	1051.0b	3657.6c
FP	0.0b	2.8cde	0.0b	15.4e	244.3de	1335.2e	157.4cd	103.5cd	342.0b	146.7e	3598.4cd
RF	206.1a	90.3a	0.0b	317.1a	2707.0b	2210.6bc	898.3a	825.3a	979.7a	495.4c	8729.7a
AOH	3.2b	13.0cd	0.0b	12.0e	320.5de	2062.1bcd	179.1c	100.1cd	481.2b	333.1d	3598.4cd
ERB	1.4b	5.0cde	0.0b	31.7de	162.6ef	1460.2de	151.5cde	123.6c	410.3b	126.5f	2596.6cde
FL	2.1b	6.6cde	0.0b	88.6c	0.0f	1474.2de	125.9cde	194.3b	470.6b	131.8ef	2494.1e
OK	1.8b	4.30cde	0.2b	27.0de	250.0de	3133.2a	94.9cde	192.8b	1006.4a	214.5e	5098.4b
TRV	0.6b	1.8de	0.0b	41.6de	190.7ef	2339.6b	179.8c	148.7bc	504.6b	119.0f	3656.9c
BJ	4.0b	14.1c	0.0b	65.8cd	315.0de	1598.3cde	14.5de	46.9de	135.0c	27.6g	2263.4e
KEH	11.6b	12.4cd	5.7a	147.6b	403.2d	1781.9bcde	6.9de	26.9e	150.3c	22.0g	2568.4de

*Means separated within column by HSD. Different letters indicate significant differences between means, P < 0.05.

cultigens (Figure 2). RF flowers were consistently highest of all cultigens in all diglucosides except MDG and in all monoglucoside anthocyanins except CG (Table 3).

AR and CR had distinctly reddish-purple flowers, and high amounts of MDG and UK compared to other cultigens (Figure 2 and Table 3). The visual phenotype resembles that of cyclamen flowers with MDG as the dominant pigment [19]. The unknown pigment may further enhance the reddish color of AR and CR. In contrast, the higher amounts of CG combined with MG in may explain the purple-red cultigen color of the other redbuds. Although DDG can confer a blue or purple color to plant tissues [18], the low amount of DDG among redbud cultigens table 3 may have had little effect on color phenotype.

Cultigens exhibiting red-purple blooms were dominant in MDG (60% of total pigment) and in UK (30% of total pigment) (Figure 2). Redbud flowers of the typical purple-red color were higher in CG (54-71%) and MG (6 to 20% of total anthocyanin). RF, although similar in color phenotype to other purple-red flowers, had an anthocyanin profile that was between the red-purple CR and AR and the purple-red cultigens (Figure 2 and Table 3). MG and CG as percent total anthocyanin in RF were about half that of OK, but much higher than that of AR (Figure 2). In contrast, the percent of MDG in RF was three times higher than OK yet only half that of AR. The more purple color of OK compared to RF may be the result of a high total anthocyanin content with proportionally less MDG and more CG than RF.

Use of reflectance colorimetry sometimes correlates well to total anthocyanin or the major anthocyanins present. Correlations of color values with anthocyanins showed that as total anthocyanin increased, L (darkness) decreased and UK increased (Table 4). Hue was not significantly correlated to any of the anthocyanins. Chroma, or distance from gray tone, was positively but weakly correlated to MDG and UK. Colorimeter a* values were positively correlated with UK and MDG while b* values were negatively correlated with CG and MG and positively correlated to UK. The color parameter a/b, sometimes used for distinguishing color changes with ripeness (red to green/yellow to blue), had the highest correlations with UK and MDG table 4, as the cultigens highest in UK and MDG also were most red and least blue.

The more acidic pH of redbud would indicate that CG and MG would be expressed as a red color [18]. Copigmentation of anthocyanins with flavonols commonly occurs in flowers, and was found in Rhododendron, a flower with cyanidin and malvidin mono and diglucosides [20]. In the present study, only *Cercis*, anthocya-

Table 4: Correlation of redbud flower color parameters with anthocyanins.

Anthocyanin	Color parameter					
	L	a	b	Chroma	Hue	a/b
CG	-0.43**	-0.12	-0.56**	-0.22	0.17	-0.34*
MG	-0.32	-0.14	0.45**	-0.30	-0.21	-0.23
MDG	-0.10	0.36*	0.24	0.34*	0.21	0.43**
UK	-0.10	0.48**	0.35**	0.48**	0.24	0.54**
Total anthocyanin	-0.39**	0.22	-0.22	0.13	0.31	-0.16

*,** indicate significance at P < 0.05.

nins were analyzed and only with HPLC; use of liquid chromatography-mass spectrometry will be needed to identify flavanols contributing to redbud pigmentation.

Conclusions

The pigment profile of anthocyanins in redbud is presented for several cultigens commonly found in North America. Relative total anthocyanin ranged from almost none in AB to 9.3 g/kg DW⁻¹ among pigmented flowers. Three cultigens with the purple flower phenotype were found to have an anthocyanin profile containing predominantly CG and MG while the reddish-purple phenotype of AR and the pink-red phenotype of CR was found to contain predominantly MDG and an unknown pigment. To our knowledge, this is the first detailed information on anthocyanin pigments in *Cercis* cultigens found in North America.

Acknowledgements

We thank Joyce Edwards, Erin Deaton, and Jack Lotito for technical assistance.

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