Anthocyanins in Ripe Fruit of the Highbush Blueberry, *Vaccinium corymbosum* L.[1]

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Abstract. The 3-monoarabinosides and 3-monogalactosides of delphinidin (Dp), petunidin (Pt), malvidin (Mv), and peonidin (Pn) as well as small amounts of the 3-monoglucosides of Dp, Pt, Mv, Pn, and cyanidin (Cy) were isolated from ripe blueberry fruit, variety 'Croatan'. Small amounts of the 3-monogalactoside of Cy were present also. None of these 14 anthocyanins (Acy) were acylated. The major Acy were (in descending order): Mv-3-galactose, Dp-3-galactose, Dp-3-arabinose, Pt-3-galactose, Pt-3-arabinose and Mv-3-arabinose.

The highbush blueberry industry in the United States has developed rapidly in the past 35 years. To maintain this expansion in times of high labor costs, growers must mechanize production, harvesting, sorting and packaging operations as fast as possible. Recently, a light-transmission difference meter has shown promise as a means of sorting blueberries according to their anthocyanin (Acy) content[2] which increases as the blueberries ripen.[3] A knowledge of the Acy in blueberry fruit might be valuable as a background for future development of light-transmittance instruments which sort according to Acy content. In addition, it might aid plant breeders in determining the inheritance of Acy in blueberry fruit.

Acy in fruit of *V. myrtillus,* L. have been reported but the pigments were not completely identified (9). Fifteen Acy, mainly the 3-monoglucosides and 3-monogalactosides of delphindin, malvidin, petunidin, peonidin and cyanidin, have been detected in lowbush blueberry fruit *(V. angustifolium,* Ait.) (3). Small quantities of the 3-monoarabinosides also were detected. Acy in fruit of *V. corymbosum* have not been identified. The objective of this study, therefore, was to extract, separate, purify, and identify the Acy pigments in fruit of highbush blueberries, *V. corymbosom.*

Methods and Materials

Ripe blueberries of the 'Croatan' variety were harvested in a commercial planting in eastern North Carolina, transported to Raleigh in an ice chest, and frozen until analyzed.

A 50 g sample was blended with cold, acidified methanol (conc HCl, methanol; 1:99 v/v) at high speed. The centrifuged supernatant was decanted and the precipitate extracted and centrifuged twice again. The 3 supernatants were combined and concentrated in vacuo at 40° C. This and all subsequent procedures were carried out under subdued light or in the dark (4).

Paper chromatography. All paper chromatography was of the descending type. Whatman No. 3 paper (18 × 22 inches) was used for isolation and purification. Whatman No. 1 paper of equal size was used to produce R_f data. Solvent systems used (v/v) were:

BAW 1-butanol, glacial acetic acid, water (4:1:5). Upper phase. Aged 24 hr.

Bu HCl 1-butanol, 2*N* hydrochloric acid (1:1). Upper phase. Paper equilibrated for 24 hr in tank containing lower phase.

BBPW 1-butanol, benzene, pyridine, water (5:1:3:3).

Forestal glacial acetic acid, concentrated hydrochloric acid, water (30:3:10).

Formic formic acid, concentrated hydrochloric acid, water (5:2:3).

15% HAc glacial acetic acid, water (15:85).

HAc-HCl glacial acetic, concentrated hydrochloric acid, water (15:3:82).

1% HCl concentrated hydrochloric acid, water (3:97).

Phenol phenol (liq.; Ca 90%), water (4:1).

Pigment separation and purification. The crude extract was streaked on 24 sheets of filter paper and developed with BAW. The resolved bands were eluted with MAW [methanol, acetic acid, water (90:5:5, v/v)], and further purified and separated by redeveloping with BAW followed by 1% HCl and 15% HAc. Twenty-three bands of Acy were isolated (Table 1) and stored under N_2 in the dark for subsequent identification. Band 6 (a very light band) from the first BAW separation contained insufficient pigment for further analysis.

Acid hydrolysis. Each isolated Acy was heated at 100° C for 30 min with 2*N* HCl. The aglycones were extracted with amyl alcohol and identified by co-chromatography with authentic anthocyanidin.[4] The remaining hydrolysate, containing the sugars, was de-acidified with di-n-octylmethylamine (5), evaporated to dryness under reduced pressure at room temperature and co-chromatographed with authentic sugars with BBPW and Phenol solvents. To locate the sugar spots, the developed papers were air-dried, sprayed with aniline hydrogen phthalate (8) and heated at 100° for 5 min.

Partial hydrolysis. Five mg of purified Acy was dissolved in 2*N* HCl and heated at 100°. Samples were withdrawn after 1, 2, 4, 8, 16, 32, and 60 min (2), spotted directly on papers, and developed with Forestal, Formic, and BAW solvents.

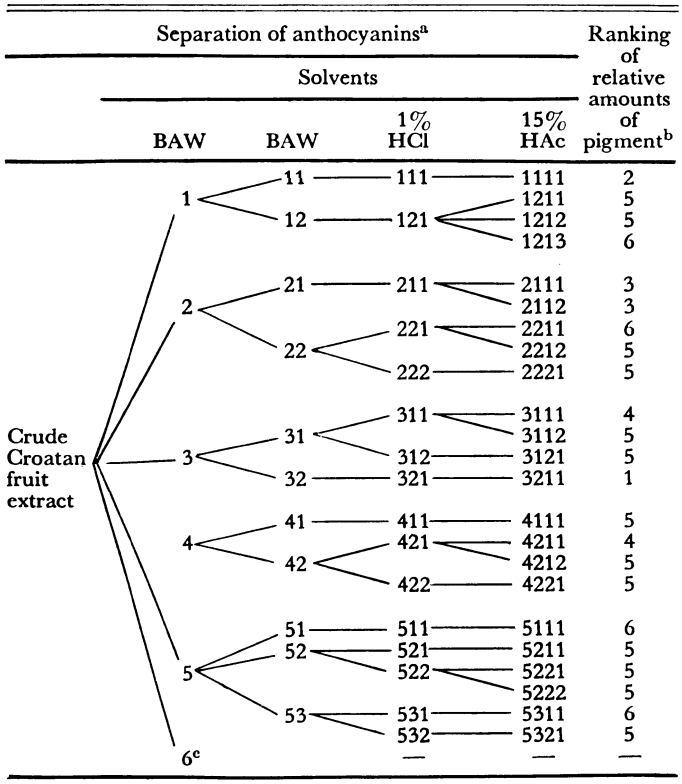
[1]Received for publication September 23, 1969. Contribution from the Horticultural Science Department, North Carolina Agricultural Experiment Station (Paper No. 2974 of the Journal Series), and the ARS, U. S. Department of Agriculture.

[2]Kushman, L. J., J. N. Yeatman, and W. E. Ballinger. 1969. Maturity sorting of blueberries by transmitted light. Manuscript in preparation.

[3]Ballinger, W. E., and L. J. Kushman. 1969. Relationship of stage of ripeness to composition and keeping quality of highbush blueberries. Manuscript in preparation.

[4]Petunidin was isolated from garden huckleberries, *Solanum guineese* (2); the others were obtained commercially.

Table 1. Sequential solvent system for the separation and purification, and ranking of relative amounts of anthocyanins extracted from Croatan blueberries, *Vaccinium corymbosum*.



[a]Band numbers increase with distance from origin.
[b]Relative amounts (visual estimate) of pigments after separation and purification; *1 to 4* indicate major bands with 1 the heaviest; *5* indicates lighter bands; and *6* indicates even smaller amounts present after separation and purification.
[c]Band 6 was present in insufficient quanitity for further analysis.

Hydrogen peroxide oxidation. Sugars attached in the 3-position were identified by the procedure of Chandler and Harper (1). Sugars were removed from the purified Acy by oxidation with hydrogen peroxide (1). The hydrolysate and authentic sugar markers were spotted directly on paper. BBPW and Phenol solvents were used as for sugars above.

The purified pigments also were identified by comparison of their R_f values in 4 solvent systems (BAW, Bu HCl, HAc-HCl, and 1% HCl) with R_f values of Acy reported in the literature (4), and by their ultra-violet absorption spectra (6). Identification indicated that complete separation of glycosides of each anthocyanidin was not always possible with the methods employed. Individual bands were identified as the major Acy found even though a trace of another Acy often was present.

Acylation. Two mg of isolated pigment was dissolved in 1 ml of 2*N* NaOH under N_2 and stored in the dark under N_2 for 2 hr at room temperature (2). The solution was neutralized with 2*N* HCl. Aliquots, taken before and after alkaline hydrolysis, were co-chromatographed on paper using BAW solvent; R_f changes were noted.

Spectral analysis. Spectral curves (6) of the isolated Acy were determined with a Beckman DB-G spectrophotometer using 0.01 per cent HCl in methanol as solvent. $AlCl_3$ shifts were determined by adding 1 drop of 5 per cent $AlCl_3$ in ethanol (w/v) to the 1 ml of Acy solution in the microcuvette. Presence or absence of peaks in the 300-330 nm region of the curve was recorded.

Other varieties. To confirm the presence of arabinosides in fruit of other varieties of *V. corymbosum*, the crude extracts from 'Angola', 'Berkeley', and 'Bluecrop' fruit were streaked on 8 sheets each of Whatman No. 3 filter paper and developed sequentially with BAW, 1% HCl and 15% HAc. The resolved bands from each variety were combined, dried and hydrolyzed with 2*N* HCl at 90° C for 90 min. Anthocyanindin hydrolysates were extracted with amyl alcohol and identified using 2-way TLC (7). The remaining hydrolysate containing the sugars was neutralized with di-n-octylmethylamine and the sugars identified using co-chromatography with standard markers. The varieties 'Angola' and 'Croatan' are sibs while the 'Berkeley' and 'Bluecrop' varieties originated from different parentage.

Results

The major Acy detected in the ripe fruit of highbush blueberries, variety 'Croatan', were arabinosides and galactosides of delphinidin, petunidin, and malvidin (Table 1, 2). Lighter bands contained glucose derivatives of delphinidin, petunidin, malvidin, and peonidin; and peonidin arabinoside. Glucose and galactose derivatives (monoglycosides) of cyanidin were found together in 2 bands; most sub-bands from the original BAW band 5 contained

Table 2. Products of acid and peroxide hydrolyses, and $AlCl_3$ spectral shifts of anthocyanins isolated from ripe Croatan blueberries.

Pigment band	Products of hydrolysis[a]			$AlCl_3$ shift
	Acid		Peroxide	
	Aglycone[b]	Sugar[c]	Sugar[c]	
1111[d]	Dp	Gal	Gal	yes
1211	Dp	Glc	—[e]	yes
1212	Pt	Glc	—	yes
1213	—[e]	—[e]	—	—[e]
2111[d]	Dp	Arab	Arab	yes
2112[d]	Pt	Gal	Gal	yes
2211	—	—	—	—
2212	Mv	Glc	—	no
2221	Mv	Gal	Gal	no
3111[d]	Pt	Arab	Arab	yes
3112	Mv &/or Pn	Gal	Gal	no
3121	Mv	Gal	Gal	no
3211[d]	Mv	Gal	Gal	no
4111	Mv &/or Pn	Gal	Gal	no
4211[d]	Mv	Arab	Arab	no
4212	Mv &/or Pn	Gal	Gal	no
4221	Pn	Gal	Gal	no
5111	—	—	—	—
5211	Cy[f]	Glc & Gal	—	yes
5221	Cy[f]	Glc & Gal	—	yes
5222	Mv &/or Pn	Glc	—	no
5311	—	—	—	—
5321	Pn	Arab	—	no
—	—	—	—	—

[a]Delphinidin (Dp), Petunidin (Pt), Malvidin (Mv), Cyanidin (Cy), Peonidin (Pn), Galactose (Gal), Glucose (Glc), Arabinose (Arab).
[b]Aglycones were identified by co-chromatography with authentic markers on (a) Whatman 1 filter paper using Forestal, Formic acid BAW solvents and (b) TLC plates coated with cellulose using an E:F:2*N* HCl solvent.
[c]Sugars were identified by co-chromatography on Whatman 1 filter paper using BBPW and Phenol solvents for the acid hydrolysates and BAW and BBPW solvents for the peroxide hydrolysates.
[d]Major bands.
[e]Insufficient pigment for test.
[f]Trace of Pn also present.

so little Acy that identification of sugars from all subbands except band 5311 was not positive.

None of the Acy subjected to partial-hydrolysis yielded intermediate products, a characteristic of 3-monoglycosides. Peroxide oxidation (Table 2) and comparison of R_f data from the literature (4) with those of the 6 major Acy (Table 3) also indicated that the sugars were attached in the 3-position.

In summary, all possible combinations of galactose, arabinose, and glucose with delphinidin, petunidin, malvidin, cyanidin, and peonidin except the arabinoside of cyanidin were isolated from the 'Croatan' blueberry fruit. The relative amounts of the 6 major Acy present in the separated bands, according to visual estimates, were: malvidin galactoside, delphinidin galactoside, delphinidin arabinoside, petunidin galactoside, petunidin arabinoside and malvidin arabinoside, in descending order (Table 1, 2). Small amounts of the glycosides of cyanidin and peonidin were identified.

None of the pigments were acylated, as evidenced by the absence of peaks in the 300–330 nm region of the spectral curves. This was confirmed by alkaline hydrolyses (data not presented). No diglycosides were detected.

The additional tests, made with fruit of other varieties of highbush blueberries, 'Bluecrop', 'Berkeley', and 'Angola', confirmed the presence of fairly large amounts of Acy arabinosides in highbush fruit. Arabinose was present in as large or larger quantities than galactose or glucose in the hydrolysates of the Acy of the fruit of all 3 varieties (Table 4). The ratio of quantities of delphinidin, malvidin, petunidin, cyanidin, and peonidin in these hydrolysates was approximately the same as that found for the 'Croatan' fruit (data not shown).

Table 3. R_f values for 4 solvent systems for the 6 major anthocyanins of ripe Croatan blueberries, *Vaccinium corymbosom*.[a]

Pigment band	Anthocyanins			
	R_f in solvent:			
	BAW	Bu HCl	1% HCl	HAc-HCl
1111	25	10	3.7	18
2111	31	17	3.3	17
2112	30	13	4.0	22
3111	40	19	2.9	19
3211	40	15	4.0	26
4211	45	22	2.2	20

[a]Insufficient pigment for testing of other bands given in Tables 1 and 2.

Table 4. Hydrolysates of gross anthocyanin extracts from Angola, Berkeley, and Bluecrop blueberry fruit.[a]

Hydrolysates	Variety		
	Angola	Berkeley	Bluecrop
Aglycones			
Delphinidin	+ + + + +[b]	+ + + +	+ + +
Malvidin	+ + + +	+ + + +	+ + + +
Petunidin	+ + + +	+ + +	+ + +
Cyanidin	+ + +	+ +	+ +
Peonidin	+ +	+	+
Sugars			
Arabinose	+ + +	+ + +	+ + +
Galactose	+ +	+ + +	+ +
Glucose	+ + +	+	+

[a]See text for methods.
[b]Visual estimates of relative quantities of hydrolysates.

Discussion

Acy content of ripe fruit of highbush blueberries appears to be quite similar to that of lowbush blueberries (3). Fruit of both species contain 3-monogalactosides, 3-monoglucosides and 3-monoarabinosides of delphinidin, malvidin, petunidin, cyanidin, and peonidin. Both fruits have little or no diglycosides. The main difference appears to be in the proportions of sugars, one to another, on the aglycones. Acy arabinosides were among the major Acy identified in highbush fruit. Only small amounts were found in lowbush fruit (3).

These studies further confirm the theory of Harborne (4) that the arabinosides of these 5 anthocyanidins probably occurs in fruit of *Vaccinium* species.

The rarely found complexity of Acy in *Vaccinium corymbosum* fruit, paralleling that in *V. angustifolium* (3), and the difference in ratios of sugars among the hydrolysates of the Acy of the fruit of these 2 species, indicates a great potential for variation of Acy contents of blueberries. Detailed identification of the Acy of fruits of other blueberry species as well as clones within species perhaps would be extremely valuable to plant breeders as a background for development of blueberry varieties with optimum coloration. Additional studies would be desirable to determine the ontogeny of these Acy as blueberry fruits develop and ripen.

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