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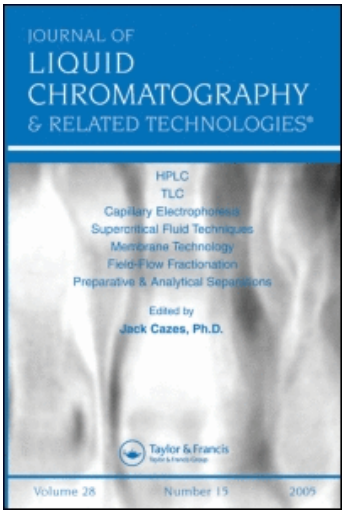

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CHARACTERIZATION OF ACETYLATED ANTHOCYANINS IN LOWBUSH BLUEBERRIES

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ABSTRACT

The anthocyanin pigments of blueberries (*Vaccinium angustifolium* Ait.), previously reported as the 3-glucosides, galactosides and arabinosides of delphinidin, cyanidin, petunidin, peonidin and malvidin have been shown to occur both as non-acylated and acetylated forms by chromatographic, chemical and spectral analyses. In 'Fundy' blueberries, acetylated anthoycanins accounted for over 32% of the anthocyanin content. The two major acetylated anthocyanins were characterized as the 3-acetylglucoside and 3-acetylgalactoside of malvidin. This is the first report of acylated anthoycanins in lowbush blueberries and the first report on the presence of acetylated 3-galactosides of the five commonly occurring anthocyanidins.

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INTRODUCTION

The lowbush blueberry, *Vaccinium angustifolium* Ait., is grown commercially in eastern Canada and northern United States, and used in frozen, canned and fresh markets. The color of the fruit, which is a primary quality attribute, is determined by the composition and concentration of the anthocyanins present (2). There are few reports on the anthocyanin composition of the lowbush blueberry (1,2). Francis *et al.* (1), using paper chromatography for the separation and characterization of the anthocyanins, reported the occurrence of the 3-glucosides and 3-galactosides of delphinidin, malvidin, petunidin, cyanidin, and peonidin, plus a small amount of 3-arabinosides of the same anthocyanidins in an unspecified variety of lowbush blueberry. However, no acylated pigments were identified (1,2).

In this article we report the isolation and characterization of simple and acetylated 3-galactosides, glucosides and arabinosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin in the lowbush blueberries, by reverse phase-high performance liquid chromatography (RP-HPLC) and capillary gas-liquid chromatography (GLC).

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MATERIALS AND METHODS

Materials

The lowbush blueberries (*Vaccinium angustifolium* Ait., cv. 'Fundy'), were harvested in August 1993 from a planting of Agriculture Canada Research Station, Kentville, Nova Scotia. The berries were frozen and kept at -38°C until use.

The 'Cabernet Sauvignon' (*Vitis vinifera*) grape skin was obtained from the Summerland Research Centre, British Columbia, and the other standards were from Sarsynthèse, Merignac, France.

All other chemicals used were of reagent or higher grade.

Extraction

The dark colored blueberry tissue (skin and outer layer pulp) was separated from the less pigmented tissue by squeezing partially thawed berries between the thumb and forefinger. The pigmented tissue (40 g) was blended with 30 mL of MeOH/formic acid/water (70/1/29, MFW) for 10 min in a stainless steel blending bowl equipped with a water jacket for temperature control. The material in the bowl was maintained at 15 ± 1°C by passing refrigerated water through the water jacket throughout the extraction. The resulting slurry was filtered through a 0.45 μM Durapore filter (Millipore), and the filtrate was partially purified by open column chromatography before HPLC analysis.

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Purification and HPLC analysis

The filtrate (20 mL) was applied on to an Amberlite CG-50 (Aldrich, Wilwaukee, WI) column (30 X 500 mm) which had been previously washed with 95% ethanol containing 1% formic acid, and equilibrated with 1% formic acid in water. The loaded column was then washed with 1% formic acid (500 mL) and the anthocyanin pigments were collected by washing the column with 1% formic acid in methanol. The dark colored band was collected (10 mL) and mixed with an equal amount of 1% formic acid in water. The mixture was

filtered, and the filtrate was repeatedly injected in the HPLC column for analysis and preparation of anthocyanin isolates.

The HPLC system (Waters Chromatography, Milford, MA) used consisted of a Waters 990 photodiode array detector and a SuperPac Pep-S column (5 μM, 4 x 250 mm; guard column, 4 x 10 mm; Pharmacia) which was placed in thermostated control system (Waters) and operated at 26.0 ± 0.1 °C throughout the analysis. The following solvents and elution profiles were used: solvent A, 5% formic acid in water; solvent B, methanol; elution profile: 0-4 min, 10-12% B (linear gradient); 4-10 min, 12-15% B; 10-20 min, 15-20% B; 20-23 min, 20% B; 23-32 min, 20-30% B; 32-40 min, 30-35% B; 40-48 min, 35-37% B; 48-50 min, 37-70% B; 50-53 min, 70% B; 53-55 min, 70-10% B. The flow rate was 1.2 mL/min. The same equipment and elution profile was used for the preparation of anthocyanin fractions, and the characterization of the anthocyanins.

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Compositional analysis of anthocyanins

The chemical composition of anthocyanin fractions accumulated from the HPLC system was determined by HPLC and GC as described by Gao and Mazza (1994). A micro amount (0.1-0.2 mg) of anthocyanin accumulated from the analytical HPLC was hydrolyzed in MeOH-2N HCl at 100°C for 2 h. An aliquot (10 μL) of the hydrolyzate was injected on the HPLC for detecting aglycones and possible acylating phenolic acids, simultaneously monitored at 280 and 525 nm. An aliquot (0.5 μL) of a chloroform extract of the remaining hydrolyzate was analyzed by GC for aliphatic acylation in the anthocyanin.

The hydrolyzate was then used for the analysis of the sugar moieties in the anthocyanin molecule, as described previously (3).

Spectral analysis

Spectral data for purified individual anthocyanins and for pigments subjected to alkaline hydrolysis were recorded by the on-line photodiode array detector, and were compared with those of the anthocyanin standards. The anthocyanin standards were from a *Vitis vinifera*, cv. 'Cabernet Sauvignon', which is known to contain simple and acetylated 3-glucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin (4). Alkaline hydrolysis was carried out according to Markham (5).

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RESULTS AND DISCUSSION

Analytical RP-HPLC of the methanolic extracts from lowbush blueberries revealed the presence of 25 anthocyanin peaks (Fig. 1). Each peak was collected, concentrated under reduced pressure, subjected to acid hydrolysis in HCl-MeOH and analyzed by HPLC for anthocyanidins and acylating phenolic acids. The results of these analyses are presented in Table 1. Acylating phenolic acids were detected in none of the hydrolyzate from the fractions containing the 25 peaks, which is consistent with the spectral characteristics of the peaks shown in Table 2. The presence of cinnamic acid acylation in anthocyanin molecules would produce a pronounced shoulder at 300-320 nm beside the anthocyanin absorption peak at 280 nm. The fraction

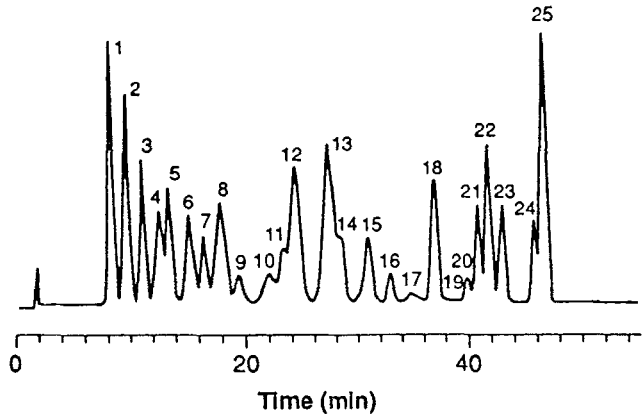

Fig. 1. RP-HPLC of anthocyanins in the methanolic extract of lowbush blueberries (*Vaccinium angustifolium* Ait. cv. 'Fundy'). Elution monitored at 525 nm. Pk numbers refer to anthocyanin fractions in Tables 1 through 4. Conditions for HPLC analysis are given in Materials and Methods.

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which contained peaks 13 and 14 had two aglycones, which indicates that there is more than one anthocyanin present in the fraction although they eluted as almost a single peak on RP-HPLC (Fig. 1). In subsequent analyses for sugars and aliphatic acylating acids by capillary GC (3), acetic acid, in the form of methyl acetate, was detected in the fraction containing peaks 13 and 14, and each fraction containing peaks 16 through 25 (Table 3), indicating extensive acetylation of anthocyanins in blueberry anthocyanins. The HPLC fraction which was found to contain more than one aglycone also contained two types of sugars. There were a total of three types of sugars detected in all fractions, and these were identified as galactose, glucose, and arabinose (Table 3).

Table 1. Retention times on RP-HPLC of the aglycones from anthocyanins in lowbush blueberries cv. 'Fundy'

Anthocyanin peaks or standards		t_R on HPLC (min)	Anthocyanidin t_R(min)	Phenolic acid
Pk	1*	8.5	22.8	--‡
	2	10.0	22.8	--
	3	11.4	30.9	--
	4	13.0	22.8	--
	5	13.8	30.8	--
	6	15.4	35.5	--
	7	16.8	30.8	--
	8	18.2	35.5	--
	9	19.9	38.7	--
	10	22.4	35.5	--
	11	23.7	38.8	--
	12	24.6	41.8	--
	13/14	27.7/28.7†	41.7/22.7†	--
	15	31.2	41.7	--
	16	33.2	30.8	--
	17	35.2	30.8	--
	18	37.1	22.7	--
	19	38.8	35.4	--
	20	40.9	38.7	--
	21	41.0	30.8	--
	22	42.0	41.8	--
	23	43.1	35.5	--
	24	45.9	38.6	--
	25	46.7	41.7	--
Delphinidin			22.7	
Cyanidin			30.8	
Petunidin			35.5	
Peonidin			38.7	
Malvidin			41.8	

* The fraction number refers to the peaks in the HPLC chromatogram shown in Fig. 1.

† In the order of decreasing peak area on HPLC if more than one peak found in the acid hydrolyzate of the collected peak.

‡ -- denotes "not detected".

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Table 2. Anthocyanins in lowbush blueberry and their spectral characteristics

Anthocyanin fraction	Peak area (%)	HPLC t_R (min)	Alkali Hydrolysis product†	λ_{max} (nm)	$E_{440}/E_{vis\ max}$ (%)	$E_{UVmax}/E_{vis\ max}$ (%)	$AlCl_3$
1*	7.12	8.53	--	278, 528	29.0	63.3	+
2	7.69	10.0	--	279, 528	26.4	58.1	+
3	4.47	11.4	--	280, 518	30.7	62.3	+
4	3.42	13.0	--	278, 526	26.6	56.0	+
5	4.42	13.8	--	280, 518	31.2	69.7	+
6	3.88	15.5	--	279, 528	26.6	55.1	+
7	2.69	16.8	--	280, 518	31.6	64.0	+
8	5.52	18.2	--	280, 528	25.2	57.1	+
9	1.19	19.9	--	280, 519	30.8	61.5	-
10	1.50	22.4	--	278, 540	15.8		‡
11	2.41	23.7	--	280, 520	30.5	78.5	-
12	8.19	24.6	--	279, 530	24.7	56.6	-
13	9.26	27.7	--	278, 529	25.0	56.2	-
14	2.84	28.7	1	280, 529	28.1	62.9	‡
15	3.24	31.2	--	278, 530	25.4	57.1	-
16	0.88	33.2	3	280, 520	28.2	47.6	+
17	0.45	35.2	7	280, 520	32.4	66.0	+

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18	4.83	37.2	2	279, 530	25.2	59.7	+
19	0.08	38.8	6	278, 525	26.6	62.2	‡
20	0.77	40.9	9	280, 522	29.0	69.0	-
21	3.06	41.1	5	281, 508	30.6	64.5	+
22	5.57	42.0	12	280, 532	25.5	63.7	-
23	3.36	43.1	8	280, 530	24.4	58.1	+
24	2.17	45.9	11	280, 520	31.0	73.3	-
25	10.6	46.7	13	280, 530	25.4	61.8	-
Dp 3-Gl-Ac		37.3		280, 530	25.8	59.0	
Dp 3-Gl		10.0		279, 528	27.5	57.7	
Cn 3-Gl-Ac		41.1		280, 520	30.0	66.0	
Cn 3-Gl		13.9		281, 520	30.6	65.5	
Pt 3-Gl-Ac		43.2		280, 528	27.0	55.8	
Pt 3-Gl		18.1		279, 528	27.5	60.0	
Pn 3-Gl-Ac		46.1		281, 518	31.4	66.6	
Pn 3-Gl		23.6		280, 519	31.8	67.3	
Mv 3-Gl-Ac		46.7		280, 530	27.3	60.3	
Mv 3-Gl		27.5		280, 528	27.8	60.6	

* The fraction number refer to the peaks in the HPLC chromatogram shown in Fig. 1.
† Numbers refer to the corresponding retention time of the anthocyanin peak on HPLC (shown in Fig. 1).
‡ Data not available or no clear-cut response.

Table 3. Retention time on capillary gas chromatography of sugars and aliphatic acylating acids from anthocyanin peaks in the lowbush blueberry cv. 'Fundy'

Anthocyanin peaks or standards		Sugar(s) t_R (min)	Acylating acid t_R (min)
Pk	1*	10.42/11.65	--‡
	2	11.15/12.90	--
	3	10.43/11.66	--
	4	6.78/7.03/7.53/7.78	--
	5	11.15/12.89	--
	6	10.45/11.68	--
	7	6.78/7.03/7.54/7.77	--
	8	11.16/12.89	--
	9	10.43/11.66	--
	10	6.78/7.03/7.54/7.77	--
	11	11.15/12.89	--
	12	10.40/11.63	--
	13/14	11.12/12.85; 10.40/11.63†	3.50(small)
	15	6.77/7.02/7.53/7.76	--
	16	10.40/11.62	3.51
	17	6.76/7.01/7.51/7.75	3.51
	18	11.12/12.85	3.51
	19	10.40/11.62	3.51
	20	10.45/11.69	3.51
	21	11.15/12.89	3.51
	22	10.43/11.67	3.51
	23	11.14/12.87	3.51
	24	11.14/12.87	3.51
	25	11.14/12.88	3.51
Arabinose		6.78/7.04/7.55/7.78	
Rhamnose		6.94/8.12	
Xylose		7.14/8.37/9.04	
Galactose		10.42/11.66	
Glucose		11.14/12.88	
Acetic acid			3.51
Oxalic acid			8.26
Malonic acid			9.20
Succinic acid			10.28

* Fraction number refers to the peaks in HPLC chromatogram shown in Fig. 1.

† For fraction(s) that contained more than one sugar, the sugar displaying the larger total peak area is listed first.

‡ "--" denotes "not detected".

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To further characterize the anthocyanin pigments corresponding to each of the 25 HPLC peaks, small amounts (0.05 mg) of each of the fractions collected from the HPLC column outlet were treated with NaOH (2N) according to Markham (5). The hydrolyzate from the alkaline treatment was re-injected onto the HPLC; the retention times and the spectral characteristics were recorded for the hydrolysis product(s), and compared to the anthocyanin standards from an extract of 'Cabernet Sauvignon' grape skins (Table 2).

Fraction 13, containing peaks 13 and 14, produced, upon alkali hydrolysis, a small de-acylated fraction, which was eluted earlier than the original peak while a major peak having the original retention time reoccurred in the chromatogram. The results indicate that the fraction is a mixture of acylated and simple anthocyanins, which is consistent with the relatively small acetic acid peak in the GC analyses for these fractions.

Although the spectral characteristics were not readily determined for the acetylated anthocyanins in the fraction which contained peaks 13 and 14, the spectral data for the corresponding de-acylated anthocyanin were easily obtained from the HPLC peak(s) of the alkaline hydrolyzate, and the spectral data could easily be matched to that of the acetylated pigment in the sample and in the standard pigment (Table 2).

All the spectra for the individual anthocyanins showed a shoulder at around 440 nm of the visible maxima. This is characteristic of 3-glycosylation (6), and indicates that the anthocyanins of lowbush blueberries are 3-glycosylated.

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Table 4. Identification of anthocyanins in lowbush blueberry cv. 'Fundy'

Anthocyanin peak		Alkali hydrolysis product†‡	Sugar	Aglycone	Anthocyanin standards position§	Anthocyanins identified[J]
Pk	1*	--	Ga	Dp	--	Dp 3-Ga
	2	--	Gl	Dp	Dp 3-Gl	Dp 3-Gl
	3	--	Ga	Cn	--	Cn 3-Ga
	4	--	Ar	Dp	--	Dp 3-Ar
	5	--	Gl	Cn	Cn 3-Gl	Cn 3-Gl
	6	--	Ga	Pt	--	Pt 3-Ga
	7	--	Ar	Cn	--	Cn 3-Ar
	8	--	Gl	Pt	Pt 3-Gl	Pt 3-Gl
	9	--	Ga	Pn	--	Pn 3-Ga
	10	--	Ar	Pt		Pt 3-Ar
	11	--	Gl	Pn	Pn 3-Gl	Pn 3-Gl
	12	--	Ga	Mv	--	Mv 3-Ga
	13/14	1	Gl;Ga‡	Mv; Dp‡	Mv 3-Gl	Mv 3-Gl; Dp 3-Ga-Ac
	15	--	Ar	Mv	--	Mv 3-Ar
	16	3	Ga	Cn	--	Cn 3-Ga-Ac

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17	7	Ar	Cn	--	Cn 3-Ar-Ac
18	2	Gl	Dp	Dp 3-Gl-Ac	Dp 3-Gl-Ac
19	6	Ga	Pt	--	Pt 3-Ga-Ac
20	9	Ga	Pn	--	Pn 3-Ga-Ac
21	5	Gl	Cn	Cn 3-Gl-Ac	Cn 3-Gl-Ac
22	12	Ga	Mv	--	Mv 3-Ga-Ac
23	8	Gl	Pt	Pt 3-Gl-Ac	Pt 3-Gl-Ac
24	11	Gl	Pn	Pn 3-Gl-Ac	Pn 3-Gl-Ac
25	13	Gl	Mv	Mv 3-Gl-Ac	Mv 3-Gl-Ac

* The peak number refer to the peaks in the HPLC chromatogram shown in Fig. 1.
† Number refers to the retention time of the corresponding peak on HPLC chromatogram (Fig. 1).
‡ In the order of decreasing peak(s) areas for sugars and aglycones in fraction(s) which contained more than one anthocyanin.
§ The anthocyanin standards are positioned by their retention time which corresponds to that of the respective anthocyanin peaks.
[J] Abbreviation: Ga, Galactose; Gl, Glucose; Ar, Arabinose; Dp, Delphinidin; Cn, Cyanidin; Pt, Petunidin; Pn, Peonidin; Mv, Malvidin; Ac, Acetylated.

Based on the results of this study, lowbush blueberries contain at least 25 anthocyanins, including the acetylated glucosides and galactosides of delphinidin, cyanidin, petunidin, peonidin and malvidin, as well as the arabinosides of delphinidin, cyanidin, petunidin and malvidin (Table 4). The identity of the majority of the anthocyanins listed in Table 4 was further confirmed by co-chromatography of authentic pigments obtained from commercial sources (Sarsynthèse, Merignac, France) and/or from 'Cabernet Sauvignon ' grape skins which are known to contain simple and acylated forms of delphinidin, cyanidin, petunidin, peonidin and malvidin (2,4). The results reported here are consistent with the finding of Francis *et al.* (1) that the lowbush blueberries contain a number of 3-glucosides and galactosides of the five commonly occurring anthocyanidins, plus a small amount of arabinosides. However, extensive acetylation of the anthocyanins was found in the present study. The discrepancy is probably due to the use of different blueberry varieties in the two studies, similar to the compositional differences which are common in grapes (e.g. 'Cabernet Sauvignon ' vs. 'Pinot Noir ', with the later variety containing no acylated pigments) (2). A follow-up study from this laboratory (7) has indicated that the anthocyanin composition of the lowbush blueberries are variety-dependent. It is also possible that the acetylated pigments were not detected by paper chromatography which was used in the study by Francis *et al.* (1).

This is also the first report documenting the presence of acetylated galactosides of delphinidin, cyanidin, petunidin, peonidin and malvidin. A study on the compositional differences among various cultivars and hybrid varieties of lowbush blueberries is reported elsewhere (7).

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