PAPER III

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Anthocyanins in Caprifoliaceae

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Abstract

The qualitative and relative quantitative anthocyanin content of 19 species belonging to the genera *Sambucus*, *Lonicera* and *Viburnum* in the family Caprifoliaceae has been determined. Altogether 12 anthocyanins were identified; the 3-*O*-glucoside (**2**), 3-*O*-galactoside (**5**), 3-*O*-(6″-*O*-arabinosylglucoside) (**7**), 3-*O*-(6″-*O*-rhamnosylglucoside) (**9**), 3-*O*-(2″-*O*-xylosyl-6″-*O*-rhamnosylglucoside) (**10**), 3-*O*-(2″-*O*-xylosylgalactoside) (**11**), 3-*O*-(2″-*O*-xylosylglucoside) (**12**), 3-*O*-(2″-*O*-xylosylglucoside)-5-*O*-glucoside (**14**), 3-*O*-(2″-*O*-xylosyl-6″-*O*-*Z*-*p*-coumaroylglucoside)-5-*O*-glucoside (**15**) and 3-*O*-(2″-*O*-xylosyl-6″-*O*-*E*-*p*-coumaroylglucoside)-5-*O*-glucoside (**16**) of cyanidin, in addition to the 3-*O*-glucosides of pelargonidin and delphinidin (**1** and **3**). Pigment **7** is the first complete identification of the disaccharide vicianose, 6″-*O*-α-arabinopyranosyl-β-glucopyranose, linked to an anthocyanidin.

Despite differences in colour from orange to black, the berries in the genus *Sambucus* are characterized by pigments **14**–**16**, or by other cyanidin derivatives containing xylose. Simple anthocyanin 3-monoglucosides (mainly **2**) dominate in berries of *Lonicera* species, while the examined species of *Viburnum* contained one or more cyanidin 3-glycoside, however, with differences in their anthocyanin pattern.

Keywords: Caprifoliaceae; *Sambucus*; *Lonicera*; *Viburnum*; Anthocyanins; Vicianose; Cyanidin 3-*O*-(6″-*O*-α-arabinopyranosyl-β-glucopyranoside); Chemotaxonomy

1. Introduction

Plants in the family Caprifoliaceae are perennial and mostly woody plants that include vines, shrubs, and small trees with berries taking colours from orange to black. Fruit characters are found to be particularly important in the classification of individual genera (Manchester and Donoghue, 1995). Previously recorded information about the anthocyanin content of berries of species in the genera *Sambucus*, *Lonicera* and *Viburnum* are collected in Table 1. Caused by potential health benefits, the anthocyanin content in berries of some Caprifoliaceae species has received attention, in particular juices and extracts from elderberry, *Sambucus nigra*, which have been used in clinical studies (Abuja et al., 1998; Netzel et al., 2002; Wu et al., 2002; Bitsch et al., 2004).

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Table 1
Previously reported anthocyanins from species in the family Caprifoliaceae

Species	Anthocyanin[a]											References
	1	**2**	**4**	**6**	**8**	**9**	**12**	**13**	**14**	**15**	**16**	
Sambuceae												
S. canadensis		X					X		X	X	X	Johansen et al. (1991), Nakatani et al. (1995), Inami et al. (1996)
S. nigra		X					X	X	X			Shin and Ahn (1980), Inami et al. (1996)
S. racemosa									X		X[b]	Lamaison et al. (1979)
Lonicereae												
L. caerulea	X	X	X	X	X	X		X	X			Terahara et al. (1993), Chavoanalikit et al. (2004)
Viburneae												
V. dilatatum		X							X			Kim et al. (2003)
V. lantana		X										Guichard et al. (1976)
V. sargentii									X			Kaminskaya et al. (1994)
V. tinus		X							X			Godeau et al. (1979)

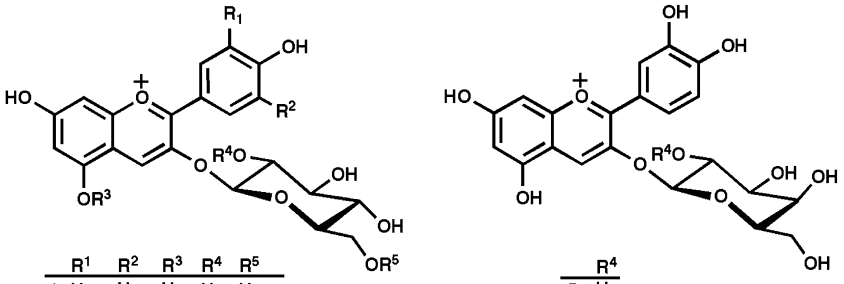
[a] See Fig. 1 for structures.
[b] *E*/*Z*-configuration of the *p*-coumaric acid is not determined.

The aim of this paper is to report the complete identification of a new type of disaccharide linked to an anthocyanidin, to present the qualitative and relative quantitative anthocyanin content of 19 species belonging to the genera *Sambucus*, *Lonicera* and *Viburnum*, and to reveal the chemotaxonomic potential of the anthocyanins in these species.

2. Materials and methods

2.1. Plant material

Berries (or autumn leaves in the case of *Viburnum furcatum*) from the examined species are collected in the period 1998–2005 at The Norwegian Arboretum at Milde (Bergen, Norway). Accession numbers are as follows: *Sambucus*



	R1	R2	R3	R4	R5
1:	H	H	H	H	H
2:	OH	H	H	H	H
3:	OH	OH	H	H	H
7:	OH	H	H	H	ara
9:	OH	H	H	H	rha
10:	OH	H	H	xyl	rha
12:	OH	H	H	xyl	H
14:	OH	H	glc	xyl	H
15:	OH	H	glc	xyl	*Z*-co
16:	OH	H	glc	xyl	*E*-co

	R4
5:	H
11:	xyl

Fig. 1. Structures of the anthocyanins found in the genera *Sambucus*, *Lonicera* and *Viburnum* in the family Caprifoliaceae. **1** = pelargonidin 3-glucoside, **2** = cyanidin 3-glucoside, **3** = delphinidin 3-glucoside, **5** = cyanidin 3-galactoside, **7** = cyanidin -(6″-arabinosylglucoside), **9** = cyanidin 3-(6″-rhamnosylglucoside), **10** = cyanidin 3-(2″-xylosyl-6″-rhamnosylglucoside), **11** = cyanidin 3-(2″-xylosylgalactoside), **12** = cyanidin 3-(2″-xylosylglucoside), **14** = cyanidin 3-(2″-xylosylglucoside)-5-glucoside, **15** = cyanidin 3-(2″-xylosyl-6″-*Z*-*p*-coumaroylglucoside)-5-glucoside, **16** = cyanidin 3-(2″-xylosyl-6″-*E*-*p*-coumaroylglucoside)-5-glucoside. Pigments **4** (peonidin 3-glucoside), **6** (peonidin 3-(6″-rhamnosylglucoside)), **8** (cyanidin 3-(6″-glucosylglucoside)) and **13** (cyanidin 3,5-diglucoside) have previously been reported to occur in species of the Caprifoliaceae (Table 1), however, not found in the present study.

sachalinensis Pojark (1990.1206), *Sambucus sibirica* Nakai (1991.1358), *Sambucus racemosa* L. ssp. *pubens* (1990.0399), *S. racemosa* L. (1983.0023), *S. racemosa* ssp. *sieboldii* (1990.1207), *S. racemosa* L. ssp. *callicarpa* (1990.0376), *Sambucus canadensis* L. (1992.0167), *S. nigra* L. (1991.0123), *Sambucus caerulea* Raf. (1991.1272), *Lonicera maximowiczii* Maxim var. *sachalinensis* Schmidt (1994.0799), *Lonicera maakii* Maxim (1997.0812), *Lonicera alpigena* L. (1991.0119), *Lonicera henryi* Hemsley (1996.0999), *Lonicera involucrata* Richards (1972.1034), *Lonicera caucasica* Pallas (1987.0262), *Lonicera caerulea* L. 'Kirke' (1991.1881), *Viburnum trilobum* Marshall (1992.0400), *Viburnum lantana* L. (1983.0268), *Viburnum opulus* L. (1996.0752) and *Viburnum furcatum* Blume ex Maxim (1977.1609).

2.2. Isolation of pigments

Fresh fruits of *V. opulus*, *V. lantana*, *L. caucasica*, *S. canadensis* and *S. racemosa* ssp. *pubens* were extracted with MeOH containing 0.1% HCl, while the rest of the samples were extracted with MeOH containing 0.5% TFA.

The anthocyanins in acidified, methanolic extracts of *V. opulus*, *V. lantana* and *V. trilobum* were after concentration under reduced pressure purified by partition against ethyl acetate before application on an Amberlite XAD-7 column (Andersen, 1988). Further purification and pigment separation were performed on XK 50-columns (Amersham Biosciences) of different sizes packed with Toyopearl HW-40F (TOSOH) material, using H_2O–MeOH–TFA (80:19.6:0.4, v/v) as eluent. The structures of the anthocyanins within these samples were elucidated by means of various spectroscopic techniques (Section 2.3).

The anthocyanins in the rest of the samples were determined by on-line UV–vis spectra and co-chromatography (HPLC and TLC) with authentic anthocyanins. Authentic anthocyanins were from the following sources: *Fragaria ananassa*: **1** (Nerdal et al., 1992); *Ribes nigrum*: **2**, **3** and **9** (Frøytlog et al., 1998); *Vaccinium vitis-idaea*: **5** (Andersen, 1985); *Sambucus nigrum*: **12** (Andersen et al., 1991); *S. canadensis*: **14**, **15** and **16** (Johansen et al., 1991; Nakatani et al., 1995).

Analytical HPLC was performed with a HP-1050 module system (Hewlett–Packard) using an ODS Hypersil column (25 × 0.4 cm, 5 μm). Two solvents; A (H_2O:HCOOH; 9:0.5, v/v) and B (H_2O:MeOH:HCOOH; 4:5:0.5, v/v) were used for elution. The elution profile consisted of initial conditions with 90% A and 10% B followed by the subsequent linear gradient conditions; 3 min (30% B), 22 min (60% B), 24 min (100% B), isocratic elution 24–33 min, and final linear gradient elution 33–34 min (10% B). The flow-rate was 0.75 ml min^{-1}, and aliquots of 15 μl were injected. TLC was carried out on 0.1 mm cellulose F (Merck) with the solvent FHW (HCO_2H–conc. HCl–H_2O; 25:24:51, v/v).

2.3. Spectroscopy

UV–visible 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. Relative amounts of each anthocyanin are reported as percentage of the total peak area in HPLC chromatograms. The HPLC peak areas are recorded as the average value of

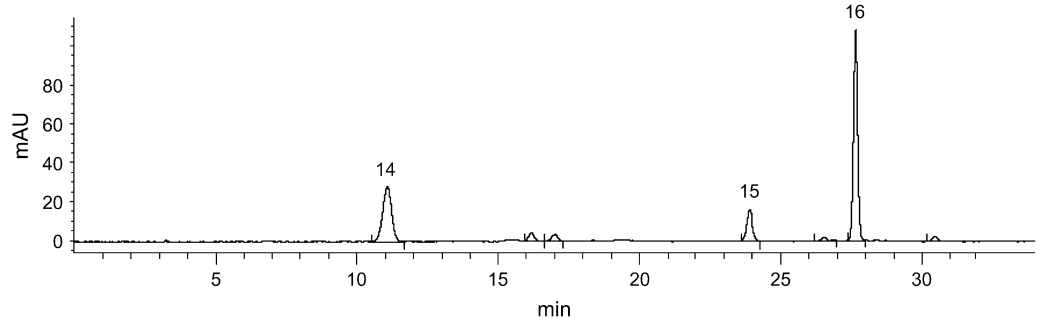


Fig. 2. The HPLC-profile of the anthocyanins in fruits of *Sambucus sachalinensis*. The labelled peaks refer to the 3-*O*-(2″-*O*-xylosylglucoside)-5-*O*-glucoside (**14**), 3-*O*-(2″-*O*-xylosyl-6″-*O*-*Z*-*p*-coumaroylglucoside)-5-*O*-glucoside (**15**) and 3-*O*-(2″-*O*-xylosyl-6″-*O*-*E*-*p*-coumaroylglucoside)-5-*O*-glucoside (**16**) of cyanidin.

the absorptions obtained for every second nanometer between 500 and 540 nm. Fig. 2 shows the HPLC profile of the anthocyanins in fruits of *S. sachalinensis*. The labelled peaks constitute together 90% of the total anthocyanin content. Minor amounts of unidentified anthocyanins constitute the rest of the anthocyanin content. The other examined plant samples contain similar or lesser amounts of unidentified anthocyanins. For the novel pigment, **7**, UV–vis absorption spectrum was also recorded between 240 and 700 nm on a Varian Cary3 UV–vis Spectrophotometer using MeOH containing 0.1% conc. HCl (v/v) as solvent.

The 1-D ^{1}H and 2-D HSQC experiments of **12**, and ^{13}C SEFT experiment of **7**, were obtained at 400.13 and 100.61 MHz for ^{1}H and ^{13}C, respectively, on a Bruker DMX 400 MHz instrument at 25 °C. The 1-D ^{1}H and 2-D HSQC experiments of **12** were performed on a 5 mm inverse-probe. The 1-D SEFT experiments of **7** were performed on a 5 mm ^{1}H-^{13}C dual-probe. The other experiments were obtained at 600.13 MHz and 150.92 MHz for ^{1}H and ^{13}C, respectively, on a Bruker DRX 600 MHz instrument at 25 °C. The 1-D ^{1}H experiment, the 2-D homonuclear correlation experiment (DQF-COSY) and the 2D heteronuclear experiments (HMBC and HSQC) were performed using a 5 mm TBI-probe.

The electrospray mass spectrometry analysis was performed on a Quattro II MS/MS (Micromass, UK) with API source and flow injection. The instrument was operated in the positive ion mode with cone voltage 35 V and capillary voltage 3 kV. The mobile phase carrier was a methanol–water (1:1) mixture. The carrier was pumped into the source at a flow-rate of 100 μl min^{-1}. The samples were dissolved in the methanol containing 3% formic acid (v/v) prior to analysis.

3. Results and discussion

3.1. Structure elucidation

The HPLC profile of the acidified methanolic extract of *V. opulus* fruits detected at 520 ± 20 nm revealed three anthocyanins (Table 3). After purification of the concentrated extract by partition against ethyl acetate and Amberlite XAD-7 chromatography followed by isolation of individual anthocyanins by Sephadex LH-20 chromatography, the UV–vis spectrum of **7** showed visible maximum at 528 nm with A_{440}/A_{528} of 27% in agreement with a cyanidin or peonidin 3-glycoside. A molecular ion at *m/z* 581 in the ESI-MS spectrum of **7** was in accordance with cyanidin connected to one hexose and one pentose unit. On the basis of the signals in the 1D ^{1}H NMR, ^{1}H–^{1}H COSY, ^{1}H–^{1}H TOCSY, ^{13}C SEFT, ^{1}H–^{13}C HSQC and ^{1}H–^{13}C HMBC spectra, the chemical shifts (^{1}H and ^{13}C) of **7** (Table 2) were in agreement with cyanidin linked to one β-glucopyranose and one α-arabinopyranose unit. A possible way to determine the ring size of monosaccharides was to use the carbon shift values (Bock and Thøgersen, 1982). In arabinosides the ^{13}C shifts of C-2 and C-4 in arabinofuranoside were found to be 10–15 ppm downfield for C-2 (72.9 ppm) and C-4 (69.6 ppm) in arabinopyranoside. The carbon shifts observed for the arabinoside sugar unit in **7** (Table 2) were thus in agreement with the arabinopyranoside form. The cross-peak at δ 5.33/145.59 (H-1″/C-3) in the HMBC spectrum revealed that the glucose unit was attached to the aglycone 3-hydroxyl position. The high-field position of the anomeric proton of the arabinose unit (δ 4.26) indicated a sugar–sugar linkage. The pronounced downfield shift of C-6″ (δ 69.51), and the cross-peak at δ 4.26/69.51 in the HMBC spectrum between H-1″ and C-6″ confirmed that the arabinose residue was connected to C-6″of the glucose ring, in accordance with cyanidin 3-*O*-(6″-*O*-α-arabinopyranosyl-β-glucopyranoside).

The disaccharide, arabinosylglucoside, has previously been reported to occur in the 3-arabinosylglucoside and 3-arabinosylsambubioside of cyanidin isolated from *V. trilobum* (Wang and Francis, 1972; Du et al., 1974). In our examination of the individual anthocyanins in acidified methanolic extracts of fruits of *V. trilobum*, we did not find any trace of anthocyanins containing arabinose. However, the major anthocyanin was determined to be cyanidin 3-*O*-(2″-*O*-α-xylopyranosyl-β-glucopyranoside), cyanidin-3-sambubioside (**12**). The arabinosylglucoside moiety has also been reported to occur in cyanidin 3-arabinosylglucoside from petals of *Adonis aestivali* (Hammouri et al., 2001) and *Polygonum* species (Yoshitama et al., 1984), and in the 3-arabinosylglucoside-5-glucosides of cyanidin and malvidin from some species belonging to Gesneriaceae (Lowry, 1972). In all these pigments the linkage position and/or the ring size of the arabinosyl moiety has not been determined. In two rather complex anthocyanins from *Zebrina pendula* (Idaka et al., 1987) and *Tradescantia pallida* (Baublis and Berber-Jimenez, 1995) (both Commelinaceae), the linkage position of the arabinose unit was established to be in the glucose 6-position. However, in these cases the arabinose was found to occur as arabinofuranosyls, and not as arabinopyranosyl as in **7**.

Table 2
^{1}H and ^{13}C spectral data for cyanidin 3-*O*-vicianoside, **7**, dissolved in CD_3OD:CF_3COOD (19:1) at 25 °C

	^{1}H δ (ppm)[a]	^{13}C δ (ppm)
Cyanidin		
2		164.47
3		145.59
4	9.06	137.65
5		159.24
6	6.74	103.52
7		170.71
8	6.97	95.35
9		157.86
10		113.46
1′		121.24
2′	8.11	118.51
3′		147.41
4′		155.79
5′	7.09	117.46
6′	8.33	128.35
3-*O*-β-glucopyranoside		
1″	5.33	104.28
2″	3.77[b]	74.81
3″	3.63[b]	77.93
4″	3.56[b]	71.13
5″	3.80[b]	77.73
6A″	4.23[b]	69.51
6B″	3.86[c]	
6″-*O*-β-arabinopyranosyl		
1‴	4.26[b]	105.28
2‴	3.63[b]	72.38
3‴	3.50[b]	74.14
4‴	3.85[b]	69.58
5A‴	3.90[b]	66.90
5B‴	3.50[b]	

d, doublet.
[a] Coupling constants (Hz): H-6 (d, 1.8); H-8 (d, 1.4); H-2′ (d, 2.3); H-5′ (d, 8.7); H-6′ (dd, 2.3, 8.9); H-1″ (d, 7.7); H-1‴ (d, 7.1).
[b] Chemical shifts achieved from COSY spectra.
[c] Chemical shifts achieved from HSQC spectra.

3.2. Chemotaxonomy

Even though the berries of species belonging to *Sambucus* vary from orange through red to black, most of them are characterized by the same anthocyanins; cyanidin 3-sambubioside-5-glucoside (**14**) and its (*E*)- and (*Z*)-*p*-coumaroyl derivatives (**15** and **16**) (Table 3). In some papers the (*Z*)-*p*-coumaroyl moiety of anthocyanins has been considered as an artefact created during exposure to UV-light in acidic methanolic solutions (Yoshida et al., 2003), however, the data presented in Table 3 are based on HPLC profiles recorded on fresh extracts. Black berries of *S. nigra* contained, however, only minor amounts of **14** (9%), and had cyanidin 3-sambubioside (**12**) as the major pigment. *S. nigra* is the only examined species in *Sambucus* containing cyanidin 3-glucoside (**2**). The blue berries of *S. caerulea* included cyanidin 3-lathyroside, **11**, in addition to **12**. Pigment **11** differs from **12** by having the xylosyl moiety in the 2-position on a galactose instead of a glucose moiety.

When it comes to species in the tribes *Lonicera* and *Viburnum*, the chemotaxonomic importance of their anthocyanin content is more limited (Table 3). The anthocyanin, cyanidin 3-glucoside, **2**, dominates in berries of most *Lonicera* species, however, this is the most common anthocyanin distributed in nature (Andersen and Jordheim, 2006). *L. maximowiczii* var. *sachalinensis* contained 86% of pelargonidin 3-glucoside (**1**) in addition to cyanidin 3-rutinoside, **9**, (11%), and *L. maakii*, contained delphinidin 3-glucoside, **3**, (25%) and cyanidin 3-sambubioside-5-glucoside, **14**, (75%). Three of the species (*L. maakii*, *L. henryi* and *L. caucasica*) contained **14**, containing the

Table 3
Qualitative and relative quantitative anthocyanin content of berries in the genera *Sambucus*, *Viburnum* and *Lonicera*

Species	Berry colour	Proportions of anthocyanins (%)[a,b]											
		1	**2**	**3**	**5**	**7**	**9**	**10**	**11**	**12**	**14**	**15**	**16**
Sambuceae													
S. sachalinensis	Orange										23	12	55
S. sibirica	Orange										18	11	68
S. racemosa	Red										46	6	47
S. racemosa ssp. *pubens*	Red										26	11	57
S. racemosa ssp. *sieboldii*	Red										21	13	66
S. racemosa ssp. *callicarpa*	Red-orange										18	14	64
S. canadensis	Black										66	10	12
S. nigra	Black		32							59	9		
S. caerulea	Blue								63	28			
Lonicereae													
L. maximowiczii var. *sachalinensis*	Red	86					11						
L. maakii	Red			25							75		
L. alpigena	Red		100										
L. henryi	Blue		51	7							38		
L. involucrata	Blue-black		83				8						
L. caucasica	Black		73				11				8		
L. caerulea 'kirke'	Blue		89										
Viburneae													
V. trilobum	Red									98			
V. lantana	Red/black				7				93				
V. opulus	Red		42			37		21					
V. furcatum	Red[c]		99										

[a] See Fig. 1 for structures.
[b] In some species the proportions are not summarized to 100% due to small amounts of unidentified pigments.
[c] Autumn leaves.

disaccharide sambubiose, which has been found in all examined *Sambucus* species. *L. maximowiczii* var. *sachalinensis*, *L. involucrata* and *L. caucasica* were the only species in Caprifoliaceae, which contained cyanidin 3-rutinoside, **9**. All the examined species of *Viburnum* contained one or more cyanidin 3-glycosides, however, the qualitative anthocyanin varied between the species (Table 3). Berries of *V. opulus* were rather outstanding containing the novel pigment cyanidin 3-vicianoside (**7**) and cyanidin 3-(2″-xylosyl-6″-rhamnosylglucoside), **10**, while berries of *V. lantana* had cyanidin 3-lathyroside (**11**) as the major pigment together with small amounts of cyanidin 3-galactoside (**5**).

In a detailed study of floral anatomy and morphology it is suggested that the tribe *Lonicera* has a different origin than *Sambucus* and *Viburnum* (Wilkinson, 1949). These studies indicated that *Sambucus* and *Viburnum* shared several characteristics not found in the rest of the family, and a segregation of these genera into two or more families has been discussed. However, this type of segregation is not supported by the anthocyanin content revealed in the examined species of this study (Table 3).

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