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Structural Identification of Two Major Anthocyanin Components of Boysenberry by NMR Spectroscopy

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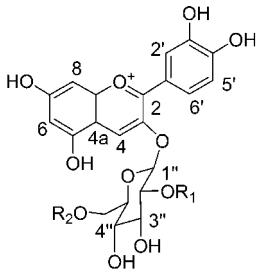
The major anthocyanins of boysenberry fruit, a cross between *Rubus loganbaccus* and *Rubus baileyanus* Britt., were isolated by preparative high-performance liquid chromatography (HPLC). The structures of cyanidin-3-[2-(glucosyl)glucoside] (**1**) and cyanidin-3-[2-(glucosyl)-6-(rhamnosyl)glucoside] (**2**) were determined by NMR in 1% DCOOD/D_2O. An unusually high chemical shift (δ 2.5) is reported for H-5‴ of cyanidin-3-[2-(glucosyl)glucoside].

KEYWORDS: Anthocyanins; boysenberry; NMR spectroscopy; cyanidin-3-[2-(glucosyl)glucoside]; cyanidin-3-[2-(glucosyl)-6-(rhamnosyl)glucoside]; cyanidin-3-*O*-glucoside, cyanidin-3-[6-(rhamnosyl)glucoside]

INTRODUCTION

Anthocyanins are a group of over 500 compounds that are largely responsible for the many blue, purple, red, and orange colors exhibited by plants (*1*). Chemically, they are classified as flavonoids and have a molecular structure based on the 2-phenylbenzopyrylium (or flavylium) cation (**Figure 1**) (*1*). Although anthocyanins have been widely studied because of their central role in determining the color of plant organs, they also have biochemical properties that are believed to be beneficial to human health. Like other flavonoids, anthocyanins are polyphenolic compounds and exhibit substantial in-vitro antioxidant capacity (*2, 3*). Additionally, anthocyanins have been shown to have a range of potential therapeutic properties such as anti-inflammatory, cancer chemoprevention, antiobesity, and vasoprotection (*4*−*7*). Since anthocyanins accumulate to high concentrations in some foods, for example, berry fruit, consumption ranging up to several 100 mg/serving can be achieved. However, despite the identified in-vitro therapeutic properties of anthocyanins and their widespread presence in the diet, studies to date indicate that the apparent bioavailability is very low compared with other polyphenolics and flavonoids (*8, 9*), which suggest that anthocyanins might not exhibit a therapeutic effect when consumed as part of the diet.

As a class of compounds, anthocyanins have a diverse range of molecular structures that are likely to play important roles in determining their biological activity and associated factors such as bioavailability and metabolism. Plant foods contribute a range of anthocyanins and each food type has a particular spectrum of anthocyanins. Recently, Prior and co-workers (*10*, *11*) determined the composition and concentrations of anthocyanins in foods in the United States. One of the foods identified with high anthocyanin concentrations is berry fruit of the genus *Rubus* which includes fruit such as raspberry, blackberry, and black raspberry in addition to hybrid berries like marionberry, boysenberry, and tayberry. In this report, we are particularly interested in the anthocyanin composition of boysenberry, which is considered to be derived from a cross between *Rubus loganbaccus* and *R. baileyanus* Britt. Boysenberry has a particular niche market because of a distinctive aroma and anthocyanin profile (*12*), and although originally was considered to be one genotype, several cultivars are now classified as "boysenberry" types.



	R_1	R_2
1	β-D-glucopyranosyl	H
2	β-D-glucopyranosyl	α-L-rhamnosyl
3	H	H
4	H	α-L-rhamnosyl

Figure 1. Chemical structures of the anthocyanins identified in boysenberry. Cyanidin-3-[2-(glucosyl)glucoside (**1**), cyanidin-3-[2-(glucosyl)-6-(rhamnosyl)glucoside] (**2**), cyanidin-3-glucoside (**3**), and cyanidin-3-[6-(rhamnosyl)glucoside] (**4**).

The anthocyanin composition of boysenberry was previously found by Torre and Barritt (*13*, *14*) to contain four major anthocyanins identified as cyanidin-3-[2-(glucosyl)glucoside], cyanidin-3-glucoside, cyandin-3-[2-(glucosyl)-6-(rhamnosyl)-glucoside], and cyanidin-3-[6-(rhamnosyl)glucoside] (**Figure 1**). These identifications were based on classical methods of analysis

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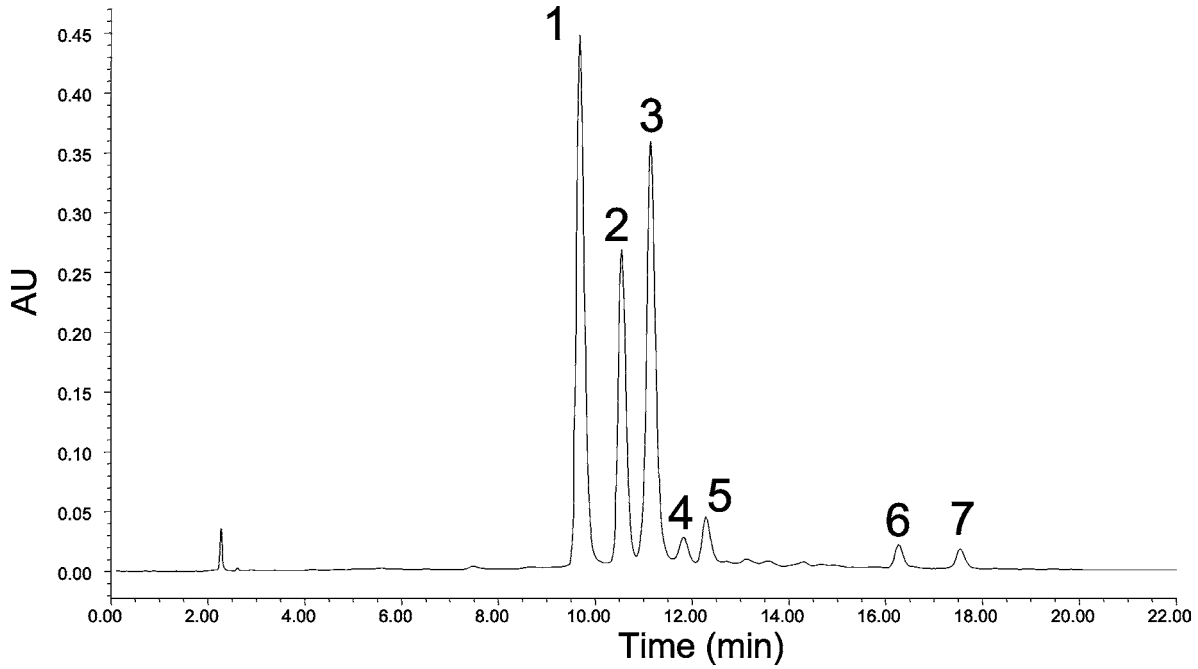

Figure 2. Chromatogram trace of the crude polyphenolic extract of boysenberry, showing the anthocyanins detected. Detection is at 530 nm. See **Figure 1** for peak identities.

by thin-layer chromatography and hydrolysis followed by chemical analysis to identify the aglycone and attached sugars. More recently, liquid chromatography−mass spectrometry (LC-MS) has been used to investigate the identity of boysenberry anthocyanins. Wada and Ou (*15*) detected four anthocyanin peaks and confirmed the identity of three of the peaks as cyanidin-3-[2-(glucosyl)glucoside], cyanidin-3-glucoside, and cyanidin-3-[6-(rhamnosyl)glucoside], consistent with the previous identifications. However, the second peak that eluted between cyanidin-3-[2-(glucosyl)glucoside] and cyanidin-3-*O*-glucoside using a reversed-phase high-performance liquid chromatography (HPLC) system was found to have a mass of *m*/*z* 757, and the structure was assigned as cyanidin-3-[6′-(*p*-coumaryl)glucoside]-5-glucoside. Further analysis of boysenberry anthocyanins by Cooney et al. (*16*) using LC-MS also reported this second eluting peak to have a mass of *m*/*z* 757, but this peak was considered to be cyanidin-3-[2-(glucosyl)-6-(rhamnosyl)glucoside], consistent with the previous identifications of Barritt and Torre. The molecular weight of *m*/*z* 757 is consistent with both the cyanidin-3-[6′-(*p*-coumaryl)glucoside]-5-glucoside and cyandin-3-[2-(glucosyl)-6-(rhamnosyl)glucoside] structures. More recently, Wu and Prior (*11*) used LC-MS to investigate the anthocyanin composition of red raspberry, which has a similar anthocyanin composition to boysenberry. They found that the second eluting peak had an *m*/*z* of 757 (*11*), but they tentatively identified this peak as cyanidin-3-[2-(glucosyl)glucoside]-5-rhamnoside because of the presence of *m*/*z* 433 in the MS/MS spectrum of *m*/*z* 757.

Thus, the molecular structure of the anthocyanin that elutes between cyanidin-3-[2-(glucosyl)glucoside] and cyanidin-3-*O*-glucoside using a reversed-phase HPLC system in both boysenberry and red raspberry is uncertain. In this study, we have isolated the four major anthocyanins present in boysenberry by preparative HPLC and have determined the structures of the first two eluting peaks by nuclear magnetic resonance (NMR) spectroscopy.

MATERIALS AND METHODS

Anthocyanin Extraction and Purification. Frozen boysenberry fruit (*Rubus loganbaccus* and *R. baileyanus* Britt. cultivar Riwaka Choice) was kindly provided by Berryfruit Export NZ Ltd (Richmond, New Zealand). A crude extract was prepared by macerating 800 g of fruit with 2400 mL of acetone in a Waring blender and by recovering the extract by filtration through filter paper (GF/A). The solid residue was re-extracted with 2000 mL 70% acetone and was filtered. The combined extracts were evaporated on a rotary evaporator (45 °C) to about 500 mL and then were partitioned with 500 mL heptane in a glass separating funnel to remove lipids. The aqueous phase was concentrated further to about 100 mL, and 400 mL of 1% formic acid was added.

XAD-7 resin (30 g) was soaked overnight in methanol and then was washed with methanol and 1% formic acid. The XAD-7 was then added to the boysenberry extract, and the phenolic compounds were allowed to adsorb for 1 h. The XAD-7 was recovered by filtration, was washed with 1% formic acid, and the phenolics were removed by washing with methanol. The methanol extract was concentrated and dried to yield a dark red powder (1.4 g).

Reversed-Phase HPLC Analysis. To isolate the individual anthocyanins, the extract prepared above was dissolved in methanol/water (50:50), was filtered through a 0.22-μm filter, and was injected into a preparative Shimadzu HPLC system comprised of two LC-8A pumps, SIL-10AP autosampler, CTO-20A column oven, SPD-20A detector, FRC-10A fraction collector, and a CBM-20A controller. The separation column was a 250 × 15 mm i.d., 4 μm, Synergi Hydro-RP (Phenomenex, Torrance, CA) with solvents (A) 5% formic acid in water and (B) 100% methanol run isocratically at 78% A and 22% B with the column maintained at 40 °C. The injection volume was 50−200 μL, and anthocyanins were detected at 530 nm. Fractions were collected separately from multiple chromatographic runs and like samples were combined, evaporated by rotary evaporator (45 °C), and dried under vacuum.

NMR Analysis. Samples (5 mg) were dissolved in 0.65 mL 1% DCOOD/D_2O (Aldrich), and nuclear magnetic resonance (NMR) spectra were recorded using a Bruker Avance 500 MHz spectrometer equipped with a probe optimized for inverse detection. TOCSY and selective TOCSY experiments (mixing time 100 ms) were used to identify individual spin systems of glycoside units. Directly and indirectly coupled ^{13}C resonances were inferred from phase-sensitive heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments, and the presence of these signals was confirmed by direct measurement of the ^{13}C spectrum. Double quantum filtered correlation spectroscopy (DQF COSY) and nuclear Overhauser and exchange spectroscopy (NOESY) experiments (mixing time 1 s) were then used to order coupling networks within glycoside residues

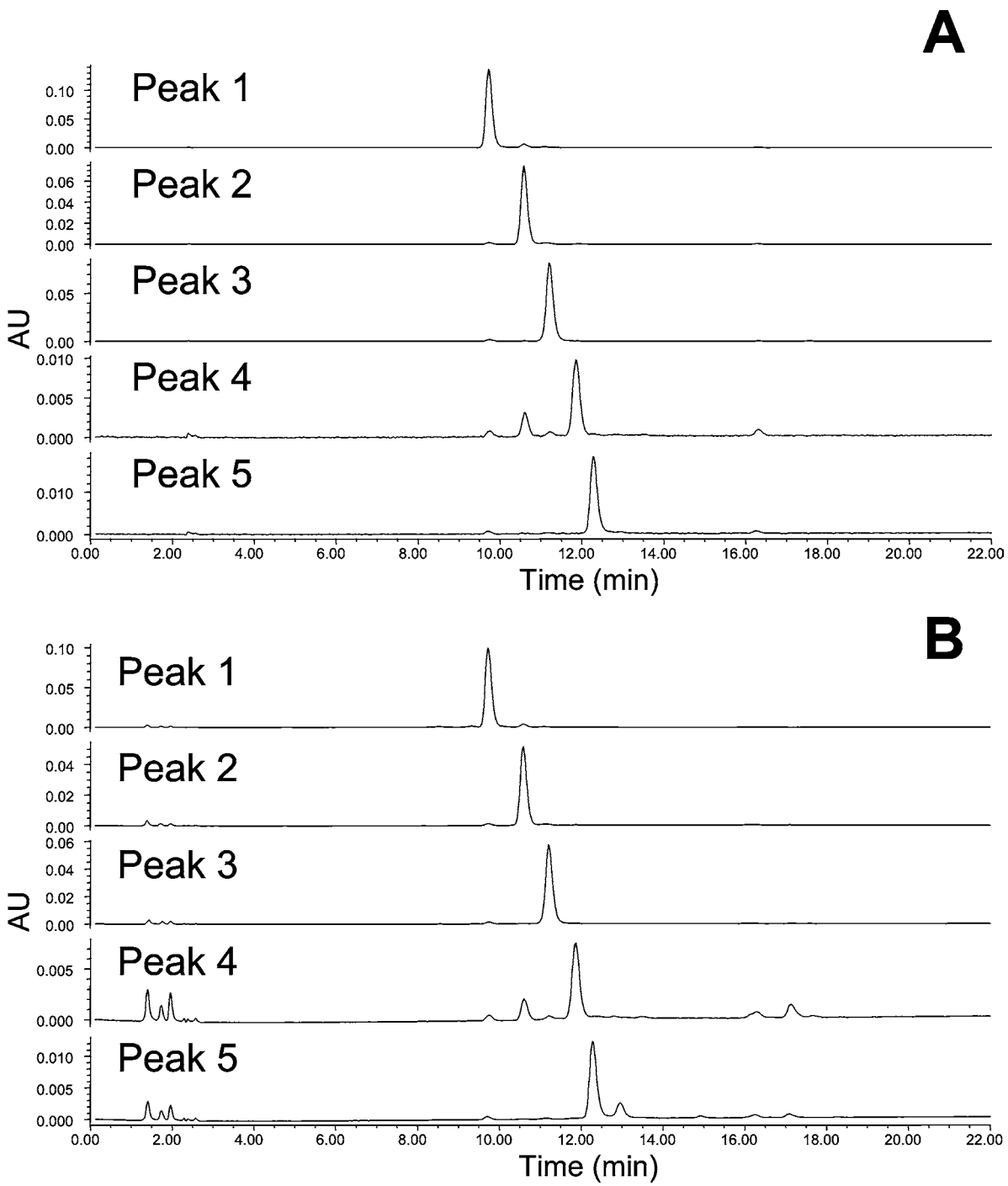

Figure 3. Chromatogram traces of each purified fraction recorded at (A) 530 nm and (B) 280 nm.

and to confirm some long-range HMBC connectivities. Standard instrument parameters were used throughout. Two-dimensional (2D) experiments were performed using the echo−antiecho method. Each spectrum contained 256 rows of 1024 points. Linear prediction followed by zero filling was applied to the indirect dimension giving a final size of 1024 × 1024 points. The data were subjected to shifted sinebell apodization prior to Fourier transformation. A sample of cyanidin-3-[6-(rhamnosyl)glucoside] (10 mg), isolated from black currant, was similarly measured and used to aid signal assignment. ^{1}H NMR spectra were referenced to residual HOD at δ 4.70 ppm.

RESULTS AND DISCUSSION

Boysenberry has been reported to contain four major anthocyanins (*14*, *16*). Analytical HPLC analysis of the crude extract prepared for this study revealed seven anthocyanins in total, four major and three minor components (**Figure 2**). Five of these components (peaks 1−5) were isolated using preparative HPLC. Isolated fractions were examined by analytical HPLC, which demonstrated that fractions 1−3 were relatively pure with respect to both anthocyanins (530 nm) and other phenolics (280 nm) (**Figure 3**). Peak 4 (a minor peak) was not pure and was not further analyzed. NMR analysis of peak 5 (0.8 mg) confirmed the presence of additional phenolic glycosides. Selective TOCSY and HSQC experiments indicated the presence of a major rutinosyl and a sophorosyl glycoside, but the full structure of the major anthocyanin in this sample could not be determined.

Complete ^{1}H and ^{13}C NMR assignments for peaks 1 (**1**) and 2 (**2**) are given in **Tables 1** and **2**. The proton and carbon signals

Table 1. 500 MHz ^{1}H NMR Assignments for Cyanidin-3-[2-(glucosyl)glucoside (**1**), Cyanidin-3-[2-(glucosyl)-6-(rhamnosyl)glucoside] (**2**), and Cyanidin-3-[6-(rhamnosyl)glucoside] (**4**) Measured in 1% DCOOD/D_2O[a]

	δ_H (mult., J_{Hz})		
position	cyanidin-3-[2-(glucosyl)-glucoside] (**1**)	cyanidin-3-[2-(glucosyl)-6-(rhamnosyl)glucoside] (**2**)	cyanidin-3-[6-(rhamnosyl)-glucoside] (**4**)
4	8.63 (s)	8.60 (s)	8.21
6	6.52 (d, 1.9)	6.52 (d, 1.8)	6.37
8	6.68 (d, 1.3)	6.66 (bs)	6.38
2′	7.54 (d, 2.2)	7.62 (d, 2.1)	7.25
5′	6.86 (d, 8.7)	6.89 (d, 8.7)	6.54
6′	7.79 (dd, 8.7, 2.2)	7.80 (dd, 8.7, 2.0)	7.49
1″	5.33 (d, 7.5)	5.31 (d, 7.5)	5.01 (s)
2″	4.02 (dd, 9.0, 7.5)	3.99 (dd, 9.0, 7.5)	3.52 (m)
3″	3.76 (t, 9.1)	3.73 (t, 9.3)	3.57 (m)
4″	3.60 (t, 9.5)	3.55 (t, 9.6)	3.45 (bt, 8.4)
5″	3.69 (m)	3.78 (m)	3.66 (bt, 7.9)
6a″	3.99 (dd, 12.5, 1.9)	4.02 (d, 12)	3.99 (d, 10.8)
6b″	3.84 (dd, 12.5, 5.5)	3.645 (bd, 12)	3.58 (m)
1‴	4.61 (d, 7.8)	4.63 (d, 7.8)[b]	
2‴	3.08 (dd, 9.3, 7.9)	3.09 (bt, 9.6)	
Glc2-3	3.16 (t, 9.2)	3.17 (t, 9.2)	
Glc2-4	3.10 (t, 9.4)	3.13 (t, 9.4)	
Glc2-5	2.49 (ddd, 9.4, 5.7, 2.2)	2.56 (ddd, 9.1, 5.5, 2.3)	
Glc2-6a	3.23 (dd, 12.4, 5.6)	3.27 (dd, 12.5, 5.4)	
Glc2-6b	3.19 (dd, 12.3, 2.0)	3.24 (dd, 12.4, 2.1)	
Rha-1		4.74[b]	4.71[b]
Rha-2		3.88 (dd, 3.3, 1.5)	3.85 (dd, 3.5, 1.5)
Rha-3		3.68 (dd, 9.7, 3.5)	3.60 (dd, 9.6, 3.5)
Rha-4		3.32 (t, 9.6)	3.30 (t, 9.5)
Rha-5		3.58 (m, 9.5, 6.3)	3.51 (dq, 9.6, 6.6)
Rha-6		1.09 (d, 6.2)	1.06 (d, 6.6)

[a] Cyanidin-3-[6-(rhamnosyl)glucoside] (**4**) was obtained from blackcurrant berries and was measured at 15 mg/mL. [b] Peak obscured or partially obscured by HDO.

Table 2. 126 MHz ^{13}C NMR Assignments for Cyanidin-3-[2-(glucosyl)glucoside (**1**), Cyanidin-3-[2-(glucosyl)-6-(rhamnosyl)glucoside] (**2**), and Cyanidin-3-[6-(rhamnosyl)glucoside] (**4**) Obtained in 1% CDOOD/D_2O[a]

	δ_C		
position	cyanidin-3-[2-(glucosly)-glucoside] (**1**)	cyanidin-3-[2-(glucosyl)-6-(rhamnosyl)glucoside] (**2**)	cyanidin-3-[6-(rhamnosyl)-glucoside] (**4**)
2	161.1	161.6	159.4
3	143.3	143.2	143.1
4	133.7	133.7	133.4
4a	112.0	111.9	111.3
5	156.6	156.6	156.5
6	102.5	102.4	102.3
7	167.9	168.1	168.2
8	94.0	94.5	94.5
8a	155.3	155.4	154.7
1′	119.5	119.7	118.7
2′	117.1	117.3	116.5
3′	144.9	144.9	144.6
4′	152.9	152.9	153.3
5′	116.6	116.5	116.1
6′	126.9	126.8	126.5
1″	100.0	100.0	101.2
2″	80.0	80.0	72.6
3″	75.5	75.4	75.8
4″	68.8	68.8	69.2
5″	76.2	75.1	75.5
6″	60.4	66.0	66.3
Glc2-1	102.2	102.3	
Glc2-2	73.7	73.6	
Glc2-3	75.0	75.3	
Glc2-4	69.0	69.0	
Glc2-5	75.8	75.8	
Glc2-6	60.3	60.2	
Rha-1		100.2	100.2
Rha-2		69.8	69.8
Rha-3		70.1	70.3
Rha-4		71.8	71.8
Rha-5		68.5	68.5
Rha-6		16.4	16.4

[a] Cyanidin-3-[6-(rhamnosyl)glucoside] (**4**) was obtained from blackcurrant berries.

of the aglycones were assigned on the based of coupling constants, HSQC, and HMBC experiments and are in general agreement with those of Fossen and Andersen (*17*) and Mikanagi et al. (*18*), allowing for the different solvents used by these authors.

The ^{1}H NMR of peak 1 (**1**) showed two anomeric signals at δ 5.33 (d, $J = 7.5$ Hz) and 4.61 (d, $J = 7.8$ Hz) with coupling constants indicative of β-glycosides (*17*). TOCSY and selective TOCSY experiments showed that the remaining proton signals for each sugar residue were fully separated from each other. Initial analysis focused on those glycoside signals at lower field (H-2″ to H-6″) and associated with H-1″ at δ 5.33. A DQF COSY experiment and analysis of J couplings identified two overlapping signals at $\sim\delta$ 4.00. The first of these H-6a″ (δ 3.99) was coupled to a methylene carbon (δ 60.4), to a second proton at δ 3.84 (H-6b″, $J = 12.5$ Hz), and hence to a proton at δ 3.69 (H-5″). The low-field second proton (δ 4.02, H-2″) was coupled to H-1″ (δ 5.33) and to H-3″ (δ 3.76) and sequentially to H-4″ (δ 3.60) and H-5″ (δ 3.69). These assignments were confirmed by HSQC and by HMBC which identified an additional coupling between H-1″ and a signal at δ 143.3, assigned as C-3 of the aglycone (**Figure 4**). Glycosylation at C-3 was confirmed by a NOESY experiment which connected H-4 of the aglycone with the three axial protons (H-1″ (δ 5.33), H-3″, and H-5″) of the first glucose residue.

That C-2″ was the site of attachment of the second sugar residue was demonstrated by an HMBC correlation between H-2″ (δ 4.02) and the carbon at δ 102.2 (C-1‴) and from the observation of n.O.e's between H-2″ (δ 4.02) and H-1‴ (δ 4.61)

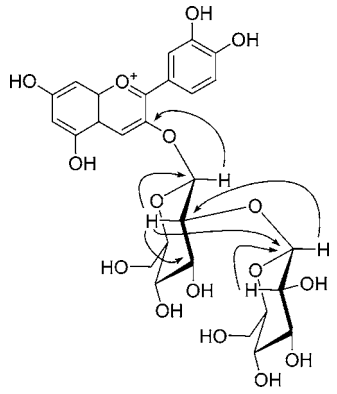

Figure 4. ^{1}H NMR HMBC connectivities important for structural determination observed for peak 1 (**1**).

and signals at δ 3.16 and 2.48, independently identified as the H-3‴ and H-5‴, respectively, the axial protons of the second glucose residue. A second HMBC correlation between the proton at δ 4.61 (H-1‴), directly bonded to the carbon (δ 102.2) and C-2″ (δ 80.0) (**Figure 4**), confirmed these results. A complete assignment of the second glucose residue was achieved by careful consideration of coupling constants and of the two-dimensional data. The ^{13}C NMR assignments were consistent with the near symmetry of the two glucose residues.

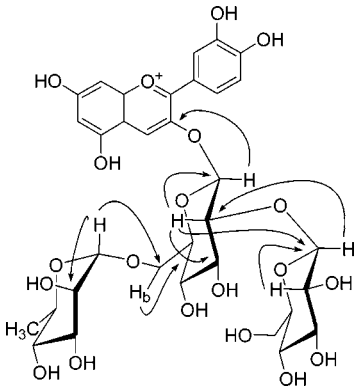

Figure 5. ^{1}H NMR HMBC connectivities important for structural determination observed for peak 2 (**2**).

To aid the NMR assignment of **2**, the NMR spectrum of cyanidin-3-[6-(rhamnosyl)glucoside] (**4**) (obtained from blackcurrant) was used as a model. The ^{1}H NMR of the aglycone resonances of **4** (**Table 1**) gave poor agreement with previously obtained values for **1** and **2**, and the glucosidic protons were also somewhat broadened, attributable to this being a more concentrated sample. The H-1‴ signal for rhamnose was obscured by the water signal. The NMR spectrum was fully assigned as described previously, using 2D and selective TOCSY experiments to assign the glycosidic protons to particular sugar residues and by using COSY experiments to order proton signals within each glycosidic residue. An HMBC experiment established the connectivity of glucose H6a″ (δ 3.99, J 10.8 Hz) to C-1‴ of rhamnose (δ 100.2). A NOESY experiment confirmed these assignments linking the rhamnose signals assigned to H-2‴ and H-1‴ to signals assigned to glucose H6a″ and H6b'' (and to H-1″, H-3″, and H-5″). The complete ^{13}C NMR assignments for **4** are given in **Table 2** and show good agreement with those measured in 5% CF_3COOD/d4-methanol (*17*).

The ^{1}H NMR of **2** showed only one anomeric signal (H-1″, δ 5.31 d, J 7.5 Hz), the other two being obscured by the residual water signal. TOCSY experiments were used to assign overlapping glycosidic signals to their particular sugar residues. A phase-sensitive HSQC experiment found the two obscured anomeric signals and identified the two glycosidic methylene residues at δ 66.0 and 60.2 and the C-6‴ methyl of a rhamnose sugar at δ 16.4. A combination of DQF COSY, HSQC, and HMBC experiments allowed a full assignment of the proton and carbon spectra (**Tables 1** and **2**). In particular, the HMBC experiment established connectivities between H-1″ of the first glucose and the cyanidin C-3, between H-2″ of the first glucose and C-1‴ of the second glucose, and between H-1‴ of the rhamnose sugar and C-6‴ of the first glucose residue (**Figure 5**). A NOESY experiment confirmed the proximity of H-4 and H-1″. The ^{13}C NMR of **2** showed excellent agreement with those of anthocyanins **1** and **4**.

The ^{1}H NMR of both **1** and **2**, but not **4**, contained a resonance at an unusually high chemical shift (δ 2.49 and 2.56, respectively) which was assigned to H-5‴, the second glucose residue of a sophoroside. In both **1** and **2**, this high-field resonance shows an HSQC correlation to a methine carbon at δ 75.8. While the signal at δ 2.5 gave no HMBC correlations in either **1** or **2**, careful analysis of peak intensities and coupling constants, and TOCSY and NOESY experiments, confirmed this assignment. It is possible that these protons are located near the shielding zone of the aromatic system of the aglycone and are subjected to diamagnetic current effects. A similar chemical shift (δ 2.78) has been reported in d_6-DMSO for H-5′ of a substituted sophoroside anthocyanin from *Pharbitis nil* (*19*).

The data presented here show that the two major anthocyanins of boysenberry, which elute first and second by reversed-phase HPLC, are cyanidin-3-[2-(glucosyl)glucoside] (**1**) and cyanidin-3-[2-(glucosyl)-6-(rhamnosyl)glucoside] (**2**), respectively. Although previous studies have concluded that peak two (**2**) was cyanidin-3-[6-(coumaryl)glucoside]-5-glucoside (*15*), and tentatively as cyanidin-3-[2-(glucosyl)glucoside]-5-rhamnoside (*11*), we found no indication of the presence of coumaryl acylation and the NMR data clearly showed the presence of only one aglycone 3-glycosyl linkage and showed that all the sugar units are linked to each other. However, Wu and Prior (*11*) found that for the corresponding compound from red raspberry (peak 2), the MS/MS fragmentation of the molecular ion (*m*/*z* 757) produces an ion of *m*/*z* 433 that corresponds to a cyanidin 5-rhamnosyl structure. In compound **2** isolated here from boysenberry, there is no evidence of substitution at the 5 position as all three compounds examined have identical ^{13}C NMR shifts (**Table 2**). Substitution of the 5 H with a sugar group would produce an upfield shift at the β position carbon. Although the anthocyanin composition of boysenberry and red raspberry has been considered to be similar (*14*), the differences in the data from this study and the study of Wu and Prior suggest that the compounds represented as peak 2 in boysenberry and raspberry may be structurally distinct anthocyanins.

The therapeutic value of berry fruit is due to the molecular structures of the phytochemical components, and accurate determination of the molecular structures of the major anthocyanin components of boysenberry will result in better assessment of the health value of boysenberry.

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