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Isolation and Structural Identification of the Anthocyanin Components of Red Kiwifruit

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The anthocyanins responsible for the red color of red kiwifruit were extracted in acidified ethanol and isolated by solid phase extraction (SPE) followed by preparative HPLC. Five anthocyanins were obtained and subsequently identified as delphinidin 3-[2-(xylosyl)galactoside], delphinidin 3-galactoside, cyanidin 3-[2-(xylosyl)galactoside], cyanidin 3-galactoside, and cyanidin 3-glucoside by a combination of LC-MS/MS, GC-MS, and 2D NMR. Delphinidin 3-[2-(xylosyl)galactoside] and delphinidin 3-galactoside have not previously been reported in the genus *Actinidia*.

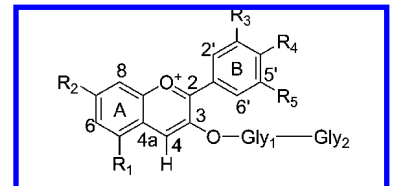
KEYWORDS: Anthocyanins; red kiwifruit; *Actinidia*; delphinidin 3-[2-(xylosyl)galactoside]; delphinidin 3-galactoside; cyanidin 3-[2-(xylosyl)galactoside]; cyanidin 3-galactoside; cyanidin 3-glucoside

INTRODUCTION

The compounds that give fruits, vegetables, and flowers their many colors continue to be of interest because of their attractive pigmentation, potential as natural food colorants (*1*), and benefits to human health (*2*−*4*). Anthocyanins are a large subgroup of flavonoids, responsible for many of the red, and blue colors present in nature. Anthocyanins consist of an aglycone (anthocyanidin), which is responsible for the observed color, with one or more *O*-glycosides attached (*5, 6*). At low pH (<3), the anthocyanidin is present as the red 2-phenylbenzopyrylium or flavylium cation (**Figure 1**), and at higher pH (>6), it exists as the less stable blue quinonoidal base form. Varying hydroxylation and methylation of the A and B rings gives rise to several naturally occurring anthocyanidins with slightly differing spectroscopic properties. Glycoside or acylglycoside moieties most commonly occur at the C3 position; however, C5, C7, C3′, C5′, and multiple substitutions are also possible. Varying anthocyanidins and patterns of sugar substitution lead to a large number of possible anthocyanin structures, with over 500 natural anthocyanins identified in plants (*5*).

Anthocyanins are believed to play an important role in plant protection as well as animal attraction (seed dispersal and pollination) (*6, 7*). Commercially, anthocyanin extracts of various fruits and vegetables are used as the food colorant/additive "Grape skin extract" (E 163, red to mauve-blue), and anthocyanins from black carrots are "Vegetable juice" (red) (*1*). Additionally, the role anthocyanins play in human nutrition is continually being investigated. Individual anthocyanin compounds and fruit with high anthocyanin content, e.g. berryfruit, exhibit a substantial *in vitro* antioxidant capacity (*8, 9*), and studies have shown anthocyanins to exhibit a wide range of potential therapeutic effects (*4, 10*), including anti-inflammatory, cancer chemoprevention, and antiobesity.

The ripe fruits of various kiwifruit species can be green, red, purple, yellow, or orange; however, until recently only the green kiwifruit *Actinidia deliciosa* were cultivated commercially throughout most of the world. In recent decades, fruit flesh color



	R_{1-4}	R_5	Gly_1	Gly_2
1	OH	OH	β-galactosyl	β-xylosyl
2	OH	OH	β-galactosyl	-
3	OH	H	β-galactosyl	β-xylosyl
4	OH	H	β-galactosyl	-
5	OH	H	β-glucosyl	-

Figure 1. Structures of the anthocyanins identified in red kiwifruit. Delphinidin 3-*O*-[2-*O*-(β-xylosyl)-β-galactoside] (**1**), delphinidin 3-*O*-β-galactoside (**2**), cyanidin 3-*O*-[2-*O*-(β-xylosyl)-β-galactoside (**3**), cyanidin 3-*O*-β-galactoside (**4**), and cyanidin 3-*O*-β-glucoside (**5**).

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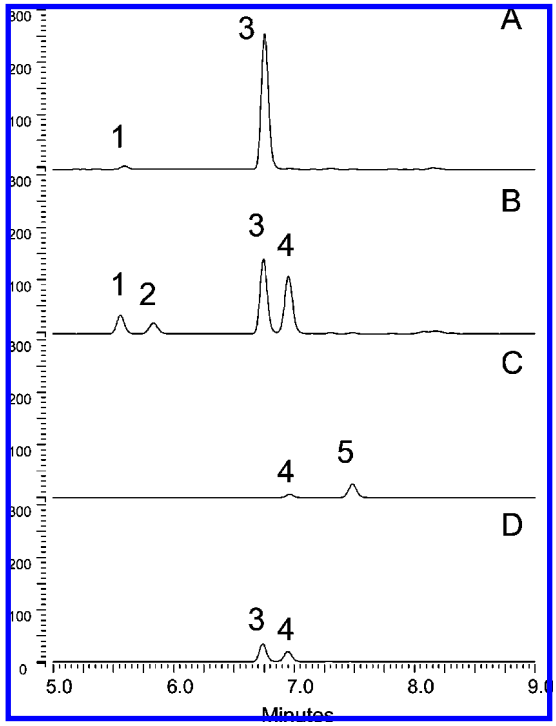

Figure 2. HPLC chromatograms (530 nm) from four *Actinidia* species: (**A**) *A. purpurea* (PU 33), (**B**) *A. melanandra* (ME 12), (**C**) *A. deliciosa* (DE 02), (**D**) *A. chinensis* (CH 77). For peak assignment, see **Table 1**.

has become an important breeding target, which has led to the introduction of yellow-fleshed *A. chinensis* "Hort16A" (marketed as ZESPRI GOLD Kiwifruit) in the late 1990s. More recently, *A. chinensis* "Hongyang" ("Red Sun") became the first red-fleshed kiwifruit cultivar to be grown on a commercial scale (*11*), and efforts to develop further red-fleshed varieties continue (*12*). Despite the interest in red-fleshed kiwifruit, little has being reported on the compounds responsible for their pigmentation; Montefiori et al. (*13*) examined the green, yellow, and red pigments present in a selection of red-fleshed *A. deliciosa* and *A. chinensis* genotypes, including the cultivar "Hongyang", and they tentatively identified three major and two minor anthocyanins present in the flesh. In our current study, anthocyanins present in several *Actinidia* species were isolated by preparative high-performance liquid chromatography (HPLC) and identified by a combination of liquid chromatography−tandem mass spectrometry (LC-MS/MS), gas chromatography−mass spectrometry (GCMS), and nuclear magnetic resonance (NMR).

MATERIALS AND METHODS

Chemicals and Reagents. Amberlite XAD7HP (XAD-7) and methoxyamine were purchased from Sigma (Sydney, Australia). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Aldrich (Sydney, Australia). Dowex 50W-X8 20−50 U.S. mesh (H) ion-exchange resin and the sugar standards (glucose, galactose, and xylose) were purchased from BDH Chemicals Ltd. (Poole, England). Cyanidin 3-galactoside and cyanidin 3-glucoside standards were purchased from Extrasynthese (Genay, France). Deuterated solvents—formic acid (d_2-formic acid, 98% D) and water (D_2O, 99.9% D)—were purchased from Aldrich. Solvents used for extraction and chromatography were AR grade.

Fruit Extraction. Fruits from four species displaying red color (in the flesh or skin). *Actinidia arguta* var. *purpurea* (Rehd.) C. F. Liang, *A. melanandra* Franch., *A. deliciosa* (A. Chev.) C. F. Liang et A. R. Ferguson, and *A. chinensis* Planch were collected from the HortResearch *Actinidia* germplasm collection held at Te Puke (Bay of Plenty, New Zealand) and Riwaka (Nelson, New Zealand). The following extraction procedure was typical. Whole fruit of *A. arguta* var. *purpurea* and *A. melanandra* or the flesh of larger fruit, *A. deliciosa* and *A. chinensis* (500 g), was homogenized using a Waring blender and Ultra Turrax in acidified aqueous EtOH (1 L of EtOH, 500 mL of H_2O, 40 mL of HCOOH). After 24 h at 1 °C, the extract was filtered and the solid residue re-extracted (1 L of EtOH, 40 mL of HCOOH) for a further 24 h at 1 °C. Combined extracts were concentrated with a rotary evaporator (50 °C) to 100 mL before partitioning with hexane (3 × 300 mL) to remove lipids and brief *in vacuo* evaporation to remove residual hexanes. 400 g of XAD-7, conditioned with 5% HCOOH/MeOH followed by H_2O, was then added and the polyphenols were extracted for 24 h at 4 °C. The XAD-7 was washed extensively with H_2O (at least 5 × 3 L) to remove sugars, followed by MeOH (2 × 1.5 L) to elute the polyphenolics. MeOH was removed with a rotary evaporator (50 °C), and the extract was dried overnight in a desiccator under vacuum, yielding 0.3−1.8 g of a deep red powder.

Ion Exchange Removal of Nonanthocyanin Polyphenolics. 60 g of Dowex ion-exchange resin was conditioned with 10% HCl/MeOH followed by water. 400 mg of powdered extract was dissolved in 10 mL of 1% HCOOH/MeOH and loaded onto a column of the resin. Nonanthocyanin polyphenolics were eluted first with water (500 mL) and then with 1% HCOOH/MeOH (500 mL). The anthocyanins were eluted with 10% HCl/MeOH (1500 mL); solvent was removed with a rotary evaporator (50 °C).

Analytical HPLC. Component analysis of extracts and the monitoring of preparative HPLC fraction purity was performed on a Waters Alliance 2690 HPLC system equipped with a 996 photodiode array detector. The separation column was a 150 mm × 4.6 mm i.d., 1.8 μ, Zorbax SB-C18 HHRT (Agilent Technologies, Santa Clara, CA) protected with a guard column containing C18 packing. The solvents—(A) 5% formic acid in water and (B) 100% acetonitrile—were applied as follows: flow 0.80 mL/min; 0 min, B 5%; 10 min, B 20%; 13 min, B 80%; 17 min, B 80%; 20 min, B 5%. The sample injection volume was 5 μL, and the column was maintained at 40 °C. Anthocyanins were monitored at 530 nm.

Preparative HPLC. Extracts were dissolved in EtOH/H_2O/HCOOH (80/20/1) and injected onto a preparative Shimadzu HPLC system comprised of two LC-8A pumps, an SIL-10AP autosampler, a CTO-20A column oven, an SPD-20A detector, an FRC-10A fraction collector, and a CBM-20A controller. The separation column was a 250 mm × 15 mm i.d., 4 μm, Synergi Hydro-RP (Phenomenex, Torrance, CA) with solvents (A) 5% FA in water and (B) 100% acetonitrile applied as follows: 0 min, B 5%; 5 min, B 5%; 15 min, B 28%; 18 min, B 80%; 20 min, B 80%. And the column was maintained at 30 °C. The injection volume was 150−250 μL, and anthocyanins were monitored at 530 nm. Fractions were collected separately from multiple chromatographic runs, and like samples were combined, evaporated with a rotary evaporator (45 °C), and dried under vacuum.

Methoxyamine and MSTFA Derivatization of Sugars. The prep-HPLC fractions were taken up in 500 μL of 1% HCOOH/MeOH, 50 μL of which was hydrolyzed in 1 mL of 1 M HCl (80 °C, 30 min). After hydrolysis, the solvent was removed under a stream of N_2 at 50 °C and the residue derivatized with 50 μL of methoxyamine solution (20 mg/mL in pyridine, 80 °C, 30 min), followed by 50 μL of MSTFA (80 °C, 30 min). Ten microliters of the sugar standard solutions (~5 mg/mL in water) were evaporated to dryness under a stream of N_2 at 50 °C, and the residue was derivatized as above.

GC-MS. Gas chromatography−mass spectrometry was performed on a Shimadzu QP5050A, the separation column was a 30 m × 0.25 mm i.d. × 0.25 μm film thickness Phenomenex ZB-5 ms (Phenomenex, Torrance, CA). The temperature was programmed from 40 °C (held for 1 min) to 300 at 7 °C/min (held for 4 min). The derivatized sugar residues were identified by comparison of retention times and fragmentation patterns with authentic standards. Hexoses (glucose or galactose) *m/z* (rel. intensity) 319(36), 205(26), 147(30), 73(100); pentose (xylose) *m/z* (rel. intensity) 307(19), 217(39), 147(20), 103(53), 73(100).

LC-MS/MS. The instrument used was an LCQ Deca ion trap mass spectrometer (ThermoQuest, Finnigan, San Jose, CA) coupled to a Surveyor HPLC. The analytical column used was a 250 mm × 2 mm i.d. LiChroCart Superpher 100 RP-18 end-capped column (Merck,

Table 1. HPLC-DAD, LC-MS/MS, and GC-MS Data for Kiwifruit Anthocyanins

	HPLC-DAD		LC-MS/MS				GC-MS	
no.	rt (min)	λ_{max} (nm)	M^+	MS^2	aglycone	glycoside	glycoside	anthocyanin
1	5.6	527	597	303	delphinidin	hexose pentose	galactose xylose	delphinidin 3-*O*-[2-*O*-(β-xylosyl)-β-galactoside]
2	5.8	526	465	303	delphinidin	hexose	galactose	delphinidin 3-*O*-β-galactoside
3	6.8	518	581	287	cyanidin	hexose pentose	galactose xylose	cyanidin 3-*O*-[2-*O*-(β-xylosyl)-β-galactoside]
4	7.0	517	449	287	cyanidin	hexose	galactose	cyanidin 3-*O*-β-galactoside
5	7.5	517	449	287	cyanidin	hexose	glucose	cyanidin 3-*O*-β-glucoside

Darmstadt, Germany), maintained at 35 °C. The injection volume was 10 μL, and elution solvents—(A) 15:3.75:81.25 MeOH/HCOOH/H_2O and (B) 100% MeOH—were applied as follows: flow rate 250 μL/min; 0 min, B 25%; 40 min, B 60%; 45 min, B 100%; 50 min, B 100%. ESI-MS data were acquired in the positive mode using a data-dependent LC-MS*n* method. The ESI voltage, capillary temperature, sheath gas pressure, and auxiliary gas pressure were 39 V, 300 °C, 448 kPa, and 138 kPa, respectively.

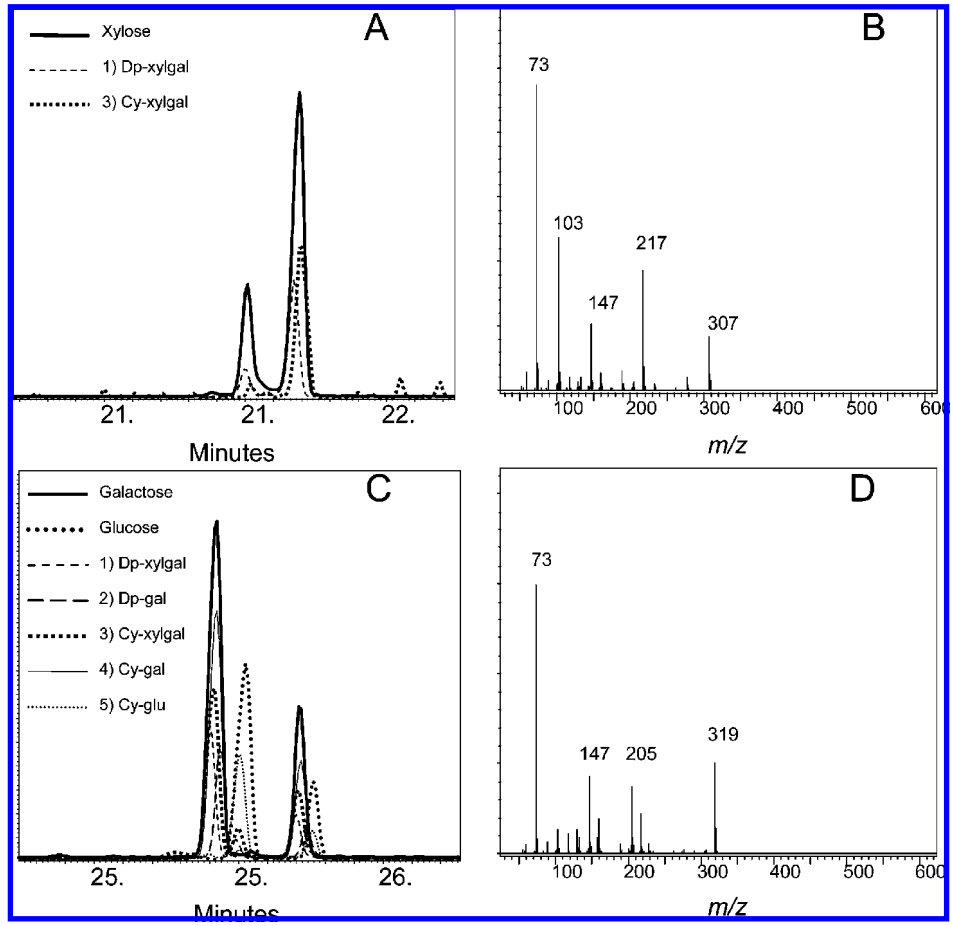

Figure 3. Gas chromatograms (**A**) and mass spectrum (**B**) of derivatized pentose residues and standards. Gas chromatograms (**C**) and mass spectrum (**D**) of derivatized hexose residues and standards.

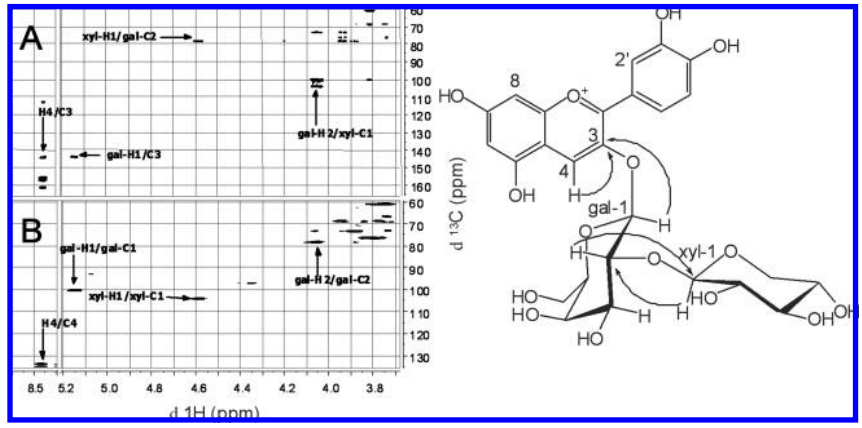

Figure 4. 2D NMR (**A**) HMBC and (**B**) HSQC correlations important for structural determination of anthocyanin **3**.

NMR. Samples were dissolved in 5% d_2-HCOOH/D_2O and NMR spectra recorded using a Bruker Avance 500 MHz spectrometer equipped with a probe optimized for inverse detection. For two samples (delphinidin 3-[2-(xylosyl)galactoside] and delphinidin 3-galactoside), spectra were recorded using a Bruker Avance 700 MHz spectrometer equipped with a cryoprobe. HSQC-TOCSY, TOCSY, and selective TOCSY experiments (with mixing times between 20 and 200 ms) were used to identify individual spin systems (glycoside units). ^{13}C chemical shifts were obtained from phase-sensitive HSQC and HMBC experiments. Signals were completed with the aid of DQF COSY and H2BC experiments (*14*). Standard instrument parameters were used throughout.

RESULTS AND DISCUSSION

The four extracts from red kiwifruit species were analyzed by HPLC for anthocyanin content. Five major anthocyanins were observed in total; however, no single kiwifruit species contained all five compounds as major components. **Figure 2** shows the variation in anthocyanin profiles observed. Anthocyanin fractions were obtained by solid phase extraction (SPE) on XAD-7 followed by preparative HPLC. The five anthocyanin fractions were relatively pure with respect to other anthocyanin components; however, anthocyanin **2**, observed exclusively in *A. melanandra* (ME 12), coeluted with another nonanthocyanin polyphenolic compound under both the analytical and preparative HPLC conditions. To obtain anthocyanin **2** at higher purity, a second SPE step on Dowex ion-exchange resin was employed to separate the positively charged anthocyanin from the neutral polyphenolic compounds in the XAD-7 treated *A. melanandra* extract. HPLC analysis of the extract after ion exchange chromatography showed that the ratio of anthocyanins **1** and **2** and of anthocyanins **3** and **4** had increased from 1:0.6 to 1:1.8 and 1:0.9 to 1:1.9, respectively, indicating a significant loss of **1** and **3** under the more acidic conditions required to elute anthocyanins from the Dowex resin. Furthermore, two late-eluting peaks with anthocyanin like UV/vis spectra, presumably the delphinidin and cyanidin aglycones, were also observed. The relatively short retention times of the five anthocyanin components on C18 reverse phase silica and an absence of peaks in the 300−350 nm region indicated a lack of acylation. All five fractions had in-line UV/vis λ_{max} values around 520 nm, but the presence of two groups of fractions, with λ_{max} at ∼526 nm and ∼518 nm, respectively, indicated two aglycones with varying degrees of hydroxylation/methoxylation (**Table 1**).

LC-MS/MS results (**Table 1**) confirm the presence of two aglycones, delphinidin (MS^2 m/z = 303, **1** and **2**) and cyanidin (MS^2 m/z = 287, **3**−**5**), glycosylated with either a hexose−pentose moiety ($M^+ - MS^2$ m/z = 294, **1** and **3**) or a single hexose moiety ($M^+ - MS^2$ m/z = 162, **2**, **4**, and **5**) (*15*). The retention times (HPLC) and in-line UV/vis spectra of anthocyanins **4** and **5** were compared against available cyanidin hexose standards to give tentative identifications of cyanidin 3-galactoside and cyanidin 3-glucoside, respectively. Similarly, anthocyanin **2** was tentatively identified as delphinidin 3-galactoside by comparison with a blueberry extract known to contain delphinidin 3-galactoside, delphinidin 3-glucoside, and several other anthocyanins (*16*).

Further evidence to complete the structures was obtained by GC-MS (**Figure 3**), NMR, and 2D NMR techniques (**Figure 4**). Acid hydrolysis with 1 M HCl gave free hexose and pentose sugars that were derivatized with methoxyamine and MSTFA and identified by GC-MS. The hexose of anthocyanins **1**−**4** was confirmed as galactose and the hexose of anthocyanin **5** as glucose. The pentose of anthocyanins **1** and **3** was confirmed as xylose. The ^1H NMR of anthocyanin **1** was consistent with delphinidin xylosylgalactoside; HMBC correlations between gal-H1 and C3 and between xyl-H1 and gal-C2 confirmed a 1→3 galactose to delphinidin linkage and a 1→2 xylose to galactose linkage. From these results, **1** was identified as delphinidin 3-*O*-[2-*O*-(*β*-xylosyl)-*β*-galactoside], also known as delphinidin 3-*O*-lathyroside. The ^1H NMR data of anthocyanins **2** (*17*), **3** (*18*), and **4** (*17*) were consistent with previously reported NMR data, taking into account the different solvents used, and HMBC correlations confirmed their structures as (**2**) delphinidin 3-*O*-*β*-galactoside, (**3**) cyanidin 3-*O*-[2-*O*-(*β*-xylosyl)-*β*-galactoside] (cyanidin 3-*O*-lathyroside), and (**4**) cyanidin 3-*O*-*β*-galactoside. Insufficient quantities of pure anthocyanin **5** were obtained for NMR analysis; however, **5** was identified as cyanidin 3-*O*-*β*-glucoside from the above mass spectrometric data and HPLC coelution with an authentic standard.

To the authors' knowledge, these two delphinidins identified here have not previously been reported in the genus *Actinidia*, and the cyanidin structures confirm the tentative identifications of the three major anthocyanins previously reported by Montefiori et al. (*13*). Red kiwifruit are novel fruits that have substantial consumer appeal, and consumption will probably increase similar to that experienced after the introduction of the yellow-fleshed *A. chinensis* "Hort16A". The results presented here establish the identity of the anthocyanins that provide the red color and add to the potential health benefits of red kiwifruit.

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