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Characterization of a new anthocyanin in black raspberries (*Rubus occidentalis*) by liquid chromatography electrospray ionization tandem mass spectrometry

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Abstract

Anthocyanin composition of black raspberry (*Rubus occidentalis*) was studied using high-performance liquid chromatography coupled to photodiode array (PDA) detection and electrospray ionization mass spectrometry (LC-ESI/MS) and tandem mass spectrometry (MS/MS). Pelargonidin 3-rutinoside was isolated and identified in black raspberries using HPLC, UV–Vis spectroscopy, MS, and NMR spectroscopy. No pelargonidin derivative had been previously found in *Rubus occidentalis*. In addition, the presence and identities of four previously reported anthocyanins (cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside) were confirmed by HPLC/MS and MS/MS analyses.

Keywords: Black raspberries (*Rubus occidentalis*); Anthocyanins; Pelargonidin 3-rutinoside

1. Introduction

Anthocyanins are a group of flavonoids responsible for the red, violet, and blue colors of most of berries and fruits. Berries are one of the richest dietary sources of anthocyanins for humans because of the substantial quantities of anthocyanins they contain. Black raspberries (*Rubus occidentalis*) have the highest anthocyanin content among berries (Torre & Barritt, 1977; Wada & Ou, 2002), and total anthocyanin content has been reported as high as 1770 mg/100 g of freeze-dried black raspberries (Harris et al., 2001). The high antioxidative capacity of raspberries has been attributed to the high concentration of anthocyanins in these fruits (Kahkonen, Hopia, & Heinonen, 2001; Zheng & Wang, 2003). The anthocyanin composition of black raspberries has been extensively studied, and cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-rutinoside, and cyanidin 3-xylosylrutinoside have been previously identified and quantified (Harborne & Hall, 1964; Torre & Barritt, 1977). Hong and Wrolstad (1990) confirmed the identities of four anthocyanins in black raspberries using HPLC coupled photodiode array (PDA) detection and reported the presence of an unidentified anthocyanin (Mazza & Miniati, 1993).

Electrospray ionization mass spectroscopy (ESI) and tandem mass spectrometry (MS–MS) are powerful techniques for identification and characterization of anthocyanins (Giusti, Rodriguez-Saona, Griffin, & Wrolstad,

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1999; Glaessgen, Seitz, & Metzger, 1992). The aim of this paper is to present the identification of an unknown anthocyanin in black raspberries by LC-PDA-ESI/MS and MS/MS. In addition, the identities of the four previous reported anthocyanins in black raspberries were confirmed by LC/MS and MS/MS analyses.

2. Materials and methods

2.1. Anthocyanin extraction

Ripe black raspberries (*Rubus occidentalis*) used for a clinical study were purchased from Dale Stokes Berry Farm (Wilmington, OH). The berries were picked, washed immediately on-site, and stored frozen at −20 °C. The frozen berries were lyophilized using a Virtis freeze-drying unit (VirTis Company, Gardiner, NY). After they were dried, the berries were ground into powder using a Brown pulper-finisher. An aliquot of berry powder (10 g) was extracted using three volumes of methanol containing 0.1% formic acid and sonicated 10 min for three times. The combined extracts were filtered and dried at 40 °C using a rotary evaporator. An aliquot of the extract was dissolved in deionized water (containing 0.1% formic acid) and purified using a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA). After washing with deionized water (acidified with 0.1% formic acid), anthocyanins were eluted with methanol containing 0.1% formic acid.

2.2. Chemicals and standards

HPLC grade acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, NJ). ACS grade formic acid was also acquired from Fisher Scientific. Cyanidin 3-glucoside, cyanidin 3-sambubioside, and cyanidin 3-rutinoside were purchased from Polyphenols Laboratories (Hanaveien, Norway). Pelargonidin 3-rutinoside, identified previously in red raspberries (Mullen, Lean, & Crozier, 2002), was used as a reference since no commercial pure standard of this compound was available.

2.3. HPLC

High-performance liquid chromatography was performed on a Waters C_{18} symmetry column (4.6 × 75 mm, 3.5 μm, Waters Assoc., Milford, MA) using a Waters 2696 separation module equipped with a 996 photodiode array detector. A gradient mobile phase was used for elution: A, water containing 10% formic acid; B, acetonitrile. The elution profile was 0–15 min, 5–15% B in A (linear gradient), 15–20 min, 15% B in A (isocratic), 20–25 min, 15–5% B in A (linear gradient), the flow rate was 1 ml min^{-1}.

2.4. Spectroscopy

UV–Vis absorption spectra of anthocyanins were recorded on-line from 200–600 nm using a photodiode array detector (Waters Assoc., Milford, MA). Mass spectra were obtained using a Micromass triple quadrupole ion-tunnel mass spectrometer equipped with a Z-spray ESI source (Micromass UK Ltd., Manchester, UK). Approximately 100 μl of the eluate from HPLC was delivered to the ESI source by a micro splitter valve for ESI/MS and MS/MS analyses. Standard cyanidin 3-rutinoside was used to tune the instrument. For precursor ion scan, the instrument was tuned to maximum abundance of the daughter ion (*m*/*z* 287) and molecular cation (*m*/*z* 595) signals (approximately in equal abundance). For product ion scan, the precursor ion (*m*/*z* 595) was attenuated to ~50%. The quadrupole instrument was operated at the following settings: capillary voltage, 3.0 kV; cone voltage, 35 V; RF lense 1, 50 V; desolvation gas temperature, 500 °C at a flow of 17 L min^{-1}; source temperature, 105 °C; collision gas (argon) pressure, 7 psi; collision energy was set at 18 eV. The relative amount of each anthocyanin was reported as the mean of three replicates. The detection limits ($S/N > 3$) approximately 1 femtomol were obtained during LC/MS/MS analysis of cyanidin 3-glucoside, cyanidin 3-sambubioside, and cyanidin 3-rutinoside. ^{1}H NMR spectra were acquired on a Bruker DMX 600 MHz spectrometer, solvent was CD_3OD–CF_3OOD (9:1).

3. Results and discussion

Freeze-dried black raspberries have been previously evaluated for inhibition of azoxymethane-induced colon cancer in rat (Harris et al., 2001) and benzo(α)pyrene induced cell transformation in vitro (Huang et al., 2002). It is important to identify potential bioactive compounds within the freeze-dried black raspberries, such as anthocyanins. HPLC analysis using ultraviolet visible detection (520 nm) showed four major anthocyanins (**1**–**4**) and one minor anthocyanin **5** (Fig. 1) in the acidic methanol extract of black raspberries.

The four major anthocyanins were further identified by HPLC-ESI/MS and MS/MS using full scan, precursor ion scan, and product ion scan experiments. Full scan data showed that the four major anthocyanins (**1**–**4**) exhibited molecular cations of *m*/*z* 449, 581, 727, and 595, respectively. Precursor ion scan demonstrated that the four molecular cations were the precursors of cyanidin (*m*/*z* 287), indicative of cyanidin anthocyanins. MS/MS of compound **1** using product ion scan produced a fragment ion of cyanidin (*m*/*z* 287, $[M\text{-hexose}]^+$) from the molecular cation at *m*/*z* 449 (Table 1). Identical retention time, UV–Vis spectra (Table 2), and mass

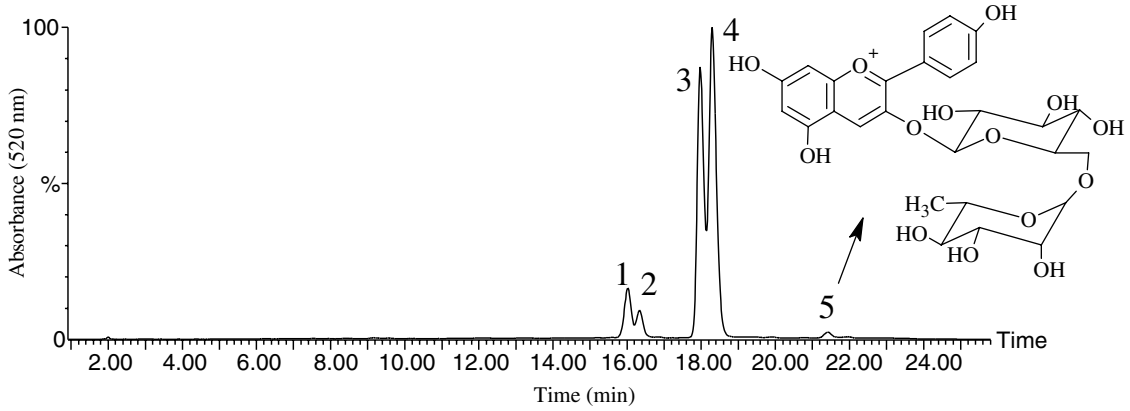

Fig. 1. HPLC profile of anthocyanin extract of black raspberries of *Rubus occidentalis* detected at 520 nm.

Table 1
Mass spectral data of anthocyanins in black raspberries (*Rubus occidentalis*)

Peak label[a]	Anthocyanin[b]	MS/MS	
		Molecular ion	Fragment ions[b]
1	cy 3-glucoside	449	287 (cy)
2	cy 3-sambubioside	581	287 (cy)
3	cy 3-xylosylrutinoside	727	581 (M^+-rham), 287 (cy)
4	cy 3-rutinoside	595	449 (M^+-rham), 287 (cy)
5	pg 3-rutinoside	579	579 (M^+-rham), 271 (pg)

[a] See Fig. 1.
[b] cy, cyanidin; pg, pelargonidin; rham, rahamnose.

Table 2
Chromatographic, spectral data, and relative quantity of anthocyanins (**1**–**5**) in black raspberries

Compound[a]	UV–Vis spectroscopy			HPLC (t_R) (min)	Relative quantity (%)
	Vis-Max (nm)	$A_{440}/A_{\text{Vis-max}}$ (×100)	$A_{280}/A_{\text{Vis-max}}$ (×100)		
1	516	32	32	16.03	7.1
2	515	31	31	16.35	3.5
3	519	31	31	17.99	36.8
4	515	32	32	18.30	51.8
5	500	44	44	21.40	0.8

[a] See Fig. 1.

spectrometric fragmentation patterns were observed and compared to the authentic standard cyanidin 3-glucoside confirming the identity of compound **1**. Compounds **2**–**4** were characterized as cyanidin 3-sambubioside, cyanidin 3-xylosylrutinoside and cyanidin 3-rutinoside, respectively, based on their observed molecular cations, major fragment ions (Table 1), and characteristic UV–Vis spectral data (Table 2). In addition, the identities of cyanidin 3-rutinoside and cyanidin 3-sambubioside were further confirmed by comparison with authentic anthocyanin standards. These anthocyanins are consistent with the previously reported composition of anthocyanins in black raspberries (Harborne & Hall, 1964; Hong & Wrolstad, 1990; Torre & Barritt, 1977).

ESI/MS of the unknown anthocyanin, peak **5**, showed a molecular cation of *m*/*z* 579. Precursor ion scan demonstrated that *m*/*z* 579 was the precursor of *m*/*z* 271, showing that this anthocyanin contained pelargonidin within its structure. MS/MS product ion scan produced a fragment ion of *m*/*z* 433 that corresponded to loss of rhamnose ([M-rhamnose]$^+$) and the aglycone at *m*/*z* 271 (pelargonidin). The UV–Vis spectra of **5** showed a maximum absorption around 500 nm (in 10% formic acid–MeOH) with $E_{440}/E_{\max}$ ratio of 44% (Table 2), indicative of glycosylation of pelargonidin at the C-3 position only (Francis, 1982; Harborne, 1958). The compound was further purified and characterized by NMR spectroscopy. ^{1}H NMR spectrum of compound **5** is shown in Table 3. The chemical shifts corresponded to previous reports of pelargonidin (Pedersen, Andersen, Aksnes, & Nerdal, 1993) and rutinose (Norbaek & Kondo, 1999; Tatsuzawa et al., 2003). Additionally, identical mass spectrometric fragmentation pattern (Table 1), HPLC retention time, and UV–Vis spectra (Table 2), in comparison to a reference standard of pelargonidin 3-rutinoside from red raspberries (Mullen

Table 3
^{1}H NMR spectral data of anthocyanin (**5**) from black raspberry (600 MHz, in CD_3OD-TFA-d (9:1), TMS as internal standard, *J* Hz in parentheses)

H No.	Pelargonidin (ppm)	3-Glucoside (ppm)	Rhamnoside (ppm)
4	9.01 s		
6	6.72 s		
8	6.93 br s		
2′ and 6′	8.61 d (9.0)		
3′ and 5′	7.08 d (9.0)		
1		5.27 d (8.0)	4.68 br s
2		3.68 m	3.85 br s
3		3.45	3.65 m
4		3.45 t (9.0)	3.33[a]
5		3.68 m	3.45 m
6		3.50 dd, 4.08 m	1.18 d (6.0)

[a] Overlapped with methanol signal.

et al., 2002), further confirmed the identity of this compound.

4. Conclusion

Pelargonidin 3-rutinoside was isolated and identified for the first time in black raspberries (*Rubus occidentalis*) by ESI/MS/MS, UV–Vis, and NMR spectroscopy. In addition, the previously reported four cyanidin anthocyanins were also confirmed by ES/MS and MS/MS analyses.

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