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Cyanidin 3-Rutinoside and Cyanidin 3-Xylosylrutinoside as Primary Phenolic Antioxidants in Black Raspberry

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Anthocyanin constituents in black raspberries (*Rubus occidentalis* L.) were investigated by HPLC-DAD, and their involvement as potent, significant antioxidants in black raspberries was demonstrated by three common antioxidant assays (FRAP, DPPH, ABTS) in this study. Five anthocyanins were present in black raspberries: cyanidin 3-sambubioside, cyanidin 3-glucoside, cyanidin 3-xylosylrutinoside, cyanidin 3-rutinoside, and pelargonidin 3-rutinoside. Their identities and structures, with particular emphasis on cyanidin 3-xylosylrutinoside, were confirmed by NMR spectroscopy. Two of these anthocyanins, cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside, predominated, comprising 24–40 and 49–58%, respectively, of the total anthocyanins in black raspberries. On the basis of both potency and concentration, cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside were found to be the significant contributors to the antioxidant systems of black raspberries. These findings indicate that these two anthocyanin compounds may function as the primary phenolic antioxidants in black raspberries. These two compounds exhibit potential biological activities that may be exploited in conjunction with other naturally occurring bioactive compounds in black raspberry fruit-based products used in clinical trials for the treatment of various types of cancer.

KEYWORDS: Anthocyanins; cyanidin 3-rutinoside; cyanidin 3-xylosylrutinoside; black raspberry; antioxidant capacity; HPLC-DAD; NMR

INTRODUCTION

Anthocyanins, a subgroup of flavonoids, are commonly found in nature. They are widely distributed in fruits and vegetables, such as blueberries, blackberries, raspberries, strawberries, black currants, elderberries, grapes, cranberries, plums, red cabbage, red radish, eggplant, and spinach. Anthocyanin structures (**Figure 1**) are based on the C_{15} skeletons of anthocyanidins (consisting of a chromane ring bearing a second aromatic ring B in position 2) that are glycosylated and/or acylated at specific hydroxylated positions (*1*).

There are over 600 naturally occurring anthocyanins, and most of them are either 3-glycosides or 3,5-diglycosides (*2*). However, only four anthocyanin compounds, cyanidin 3-sambubioside (Cy 3-sam), cyanidin 3-glucoside (Cy 3-glc), cyanidin 3-xylosylrutinoside (Cy 3-xylrut), and cyanidin 3-rutinoside (Cy 3-rut), have been routinely identified and characterized in black raspberries (*3–7*). A fifth pigment has been detected in trace amounts (*3, 6*), and this compound has been identified as pelargonidin 3-rutinoside (Pg 3-rut) (*7*). Recently, a new anthocyanin, tentatively described as cyanidin 3-sambubioside-5-rhamnoside (**Figure 2**), was found in black raspberries by Wu and Prior (*8*) using HPLC-ESI-MS/MS. However, these authors recommended that the structure of this compound be verified by nuclear magnetic resonance (NMR) spectroscopy.

Anthocyanins of fruits and vegetables are important because of their potential health benefits as dietary antioxidants, anti-inflammatory compounds, and/or chemopreventive agents (*9–12*). Black raspberries (*Rubus occidentalis* L.) are of significant interest because they contain high levels of anthocyanins (*13*) but are also a rich natural source of other chemopreventive phytochemicals including ellagic acid, ferulic acid, vitamins C and E, folic acid, calcium, selenium, and β-sitosterol (*14–16*). Black raspberries also demonstrate

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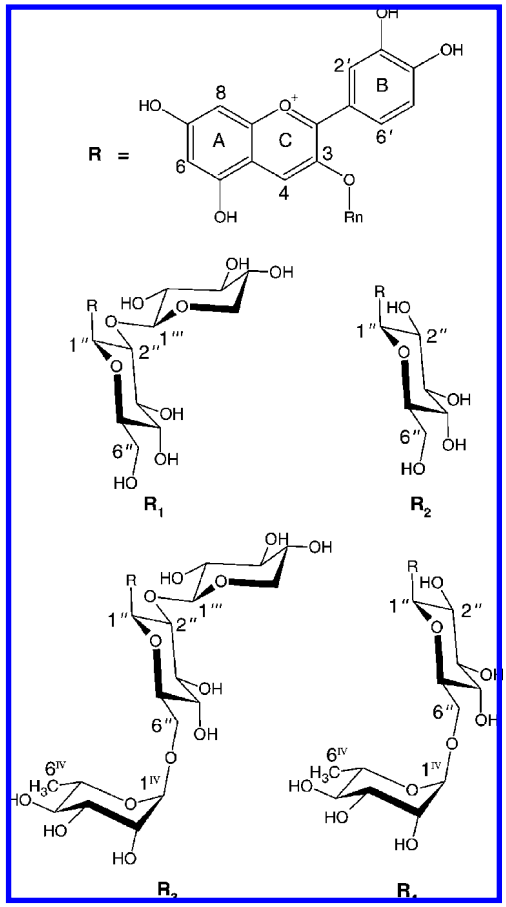

Figure 1. Basic structures of cyanidin and its glycosides in black raspberries: R_1, sambubioside; R_2, glucoside; R_3, rutinoside; R_4, xylosylrutinoside.

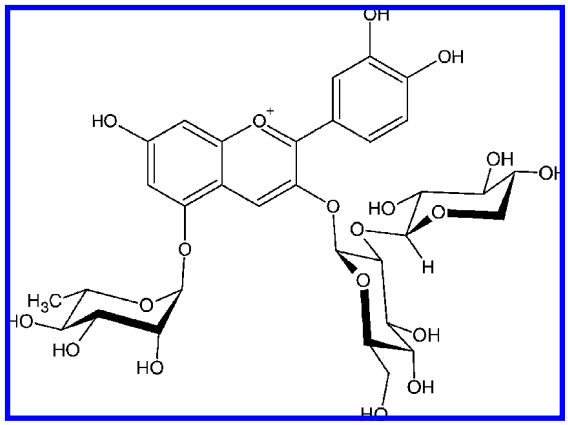

Figure 2. Chemical structure of cyanidin 3-sambubioside-5-rhamnoside tentatively proposed in black raspberries.

high antioxidant capacity (*13, 17, 18*), and this has been attributed to their high concentration of total anthocyanins and total phenolics (*13*).

Lyophilized black raspberries have been suggested as a food-based approach to chemoprevention for a number of aerodigestive tract cancers. Recent animal studies have shown the chemopreventive properties of black raspberries in esophageal (*11*), colon (*14*), and oral cancers (*12*) including embryo fibroblasts (*15*). Black raspberry extracts also inhibited premalignant and malignant growths in human oral cell lines (*16*) and suppressed tumorigenic phenotypes associated with human oral squamous cell carcinoma (*19*) and may play a key role in the regulation of cell proliferation in other cancer cell lines.

Although whole black raspberries and raspberry extracts have putative antioxidative and chemopreventive effects, the contribution of individual anthocyanins to black raspberry antioxidant capacity has yet to be examined. Hence, this study was undertaken to characterize the anthocyanin constituents from black raspberries using high-performance liquid chromatography coupled with photodiode array detector (HPLC-DAD) and to demonstrate the contribution of these common anthocyanins to black raspberry antioxidant capacity. The structures and identities of these anthocyanins, with particular emphasis on the Cy 3-xylrut compound in question, were investigated and subjected to further verification using NMR analysis. The relative antioxidant capacity of the individual black raspberry anthocyanins was determined from commercial standards or purified compounds. Lastly, sample extract fractions sequentially collected from HPLC column eluates were analyzed for their antioxidant capacity to confirm the putative role of anthocyanins as a primary source of antioxidants in black raspberries.

MATERIALS AND METHODS

Chemicals and Standards. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from EMD Biosciences, Inc. (San Diego, CA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), and trifluoroacetic acid (TFA) were supplied by Sigma Chemical Co. (St. Louis, MO). Methanol-d_4 (99.8 atom % D), trifluoroacetic acid-*d* (99.5 atom % D), and tetramethylsilane were purchased from Sigma-Aldrich. HPLC grade water, acetonitrile, methanol, acetone, glacial acetic acid, and formic acid were obtained from Fisher Scientific Co. (Hanover Park, IL). Ethyl acetate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Anthocyanin standards, Cy 3-sam (FW = 617), Cy 3-glc (FW = 485), and Cy 3-rut (FW = 631) were purchased as chloride salts from Polyphenols Laboratories AS (Sandnes, Norway).

Plant Materials and Sample Preparation. Black raspberry (*R. occidentalis* L. cv. Jewel) samples were obtained from four commercial berry growers in Ohio. Three field replicates of each sample were harvested at full maturity stage, transported under chilled conditions to Ohio Agricultural Research and Development Center, and stored at –20 °C. For each triplicate, 25 g of frozen berries was homogenized in 12.5 mL of distilled water using a Polytron (Brinkmann Instruments, Inc. Westbury, NY) for 3 min. The homogenates were squeezed through three-layered cheesecloth and then centrifuged at 10000*g* for 15 min at 4 °C. The supernatant was collected and stored at −20 °C.

Preparation of Sample Extracts. For subsequent analytical HPLC, preparative HPLC, and antioxidant assay procedures, the concentration of acidic methanol-soluble compounds from crude extracts was carried out using solid-phase extraction (SPE) cartridge. Approximately 3 mL of the crude extract was passed through a 1000 mg, 8 mL, C_{18} Extract Clean cartridge (Alltech, Deerfield, IL) previously activated with methanol and then water, both acidified with 0.1% TFA. The cartridge was eluted with acidified water (0.1% TFA) to remove sugars, acids, and other polar compounds that may interfere with HPLC and/or NMR analyses. Compounds of interest were then eluted with acidified methanol (0.1% TFA). The methanolic eluate was concentrated using a rotary evaporator under vacuum (40 °C) and then transferred to a 1.5 mL amber-colored vial. The remaining solvent was removed using a nitrogen flush, and all vials were capped. The samples were then placed in a desiccator until analyzed by analytical and semipreparative HPLC or subjected to antioxidant assays. The concentrated extract was resuspended in 1 mL of acidified methanol prior to analyses.

Analytical HPLC-DAD Analysis. HPLC characterization of anthocyanins was performed using a reversed-phase HPLC System Gold (Beckman Coulter, Inc., Fullerton, CA) 406A liquid chromatograph equipped with diode array detector (model 168) and an autosampler

(model 508) interfaced to an IBM computer with 32 Karat V.8.0 software (Beckman Coulter, Inc.). Separation was carried out on a Prodigy ODS-3 100 Å column, 250 × 4.60 mm i.d., 5 μm (Phenomenex, Torrance, CA) fitted with a SecurityGuard analytical cartridge (4 × 3.0 mm i.d., 5 μm; Phenomenex) following the procedure of Wu et al. with modifications (*20*). Elution was performed using mobile phase A (5% formic acid aqueous solution) and mobile phase B (100% methanol). The gradient system was 0–2 min, 5% B; 2–10 min, 5–24% B; 10–15 min, 24% B; 15–30 min, 24–35% B; 30–32 min, 35–45% B; 32–35, 45% B; 35–38 min, 45–5% B; 38–45 min, 5% B. The flow rate was 1 mL/min, and the injection volume was 10 μL. The detection wavelength was 520 nm. Solvents were filtered through a 0.45 μm nylon Zapcap-CR disposable bottle-top filter (Schleicher & Schuell, Keene, NH). Anthocyanin peaks were identified and quantified using standards and purified samples compared with published data (*3–7*). Results were expressed as micrograms of specific anthocyanin per milliliter of black raspberry extracts.

Purification of Cy 3-Xylrut and Other Anthocyanin Fractions by Semipreparative HPLC-DAD. Purification of anthocyanins was performed using a Beckman Coulter HPLC System Gold (Beckman Coulter, Inc.) semipreparative liquid chromatograph. The System Gold 126P solvent module was equipped with System Gold 168 photodiode array detector (DAD), a System Gold 508 autosampler, and a manual injector interfaced to an IBM computer with 32 Karat V.8.0 software. Separation was carried out on a Prodigy ODS-3 100 Å semipreparative column, 250 × 10.00 mm i.d., 5 μm (Phenomenex) fitted with a semipreparative SecurityGuard cartridge C18 (10 × 10 mm i.d., 5 μm Phenomenex) following the procedure of Wu et al. with modification (*20*). Elution was performed using mobile phase A (5% formic acid aqueous solution) and mobile phase B (100% methanol). The gradient system was 0–2 min, 5% B; 2–10 min, 5–24% B; 10–15 min, 24% B: 15–30 min, 24–35% B; 30–32 min, 35–45% B; 32–35 min, 45% B; 35–38 min, 45–5% B; 38–55 min, 5% B. The flow rate was 4 mL/min, and the injection volume was 100 μL. The detection wavelength was 520 nm.

Approximately 100 μL of anthocyanin extracts were injected in the semipreparative HPLC. Individual fractions of the four characterized anthocyanin compounds in black raspberries were collected at an interval of 15 s using ProteomeLab SC-100 fraction collector module (Alltech, Deerfield, IL). Repeated injections were made, and isolated fractions were combined until a mass of 6–10 mg per anthocyanin was obtained. The purified fractions were concentrated on a rotary evaporator and flushed with N_2 prior to NMR analysis. A representative sample was reinjected to confirm the purity of each compound collected; a single peak was obtained and then compared with the retention time and spectral characteristics of the standard anthocyanin. The antioxidant capacity of each purified pigments was also analyzed prior to NMR analysis.

Collection of Extract Fractions by HPLC-DAD. Extract fractions were collected sequentially over the entire range of the chromatographic program (approximately 40 min). Methanol solution (100%) was injected to generate a blank sample with sequential fractions representing the change in solvent strength throughout the chromatographic process. Approximately 10 μL of extract from a representative black raspberry sample was injected in the HPLC-DAD, and sequential fractions were collected at intervals of 30 s using ProteomeLab SC-100 fraction collector module (Alltech). The antioxidant capacity of each extract fraction was analyzed using the FRAP assay method. This procedure was repeated three times.

NMR Analysis. After repeated collection and concentration of the HPLC column eluate under N_2, the identity and purity of the peaks were confirmed by high-field NMR spectroscopy using a Varian INOVA 750 MHz NMR spectrometer (Varian Inc., Palo Alto, CA) with a 5 mm Varian triple resonance $^1H\{^{13}C/^{15}N\}$ pulsed field gradient probe. Solid material (0.5–8 mg) derived from the column eluate was redissolved in 250 μL of a solution containing methanol-d_4/trifluoroacetic acid-d (95:5 v/v) and tetramethylsilane as an internal reference standard. These samples were stable for at least 2 weeks if stored at 4 °C, and under the NMR experimental conditions the samples were stable for at least several days (no change was observed in the NMR spectra). Sample solutions were transferred to NMR tubes with a 3 mm diameter, and the ^1H NMR spectra were collected at 25 °C over a spectral width of 9.5K and a 10.7 μs (90°) pulse. The acquisition time was 2.5 s, and 128 transients were accumulated with presaturation of the HDO resonance at ca. 4.9 ppm using 0.5 W of continuous wave decoupling during the 3 s relaxation delay. Data were processed with 0.5 Hz exponential line broadening and zero filled to 128K points before Fourier transformation. The anthocyanin content of the chromatographic material was confirmed by comparison with the ^1H NMR spectra of standard materials and from known literature values (*7, 21, 22*).

Two-dimensional NMR experiments, gradient assisted heteronuclear single quantum correlation (gHSQC) (*23*), and heteronuclear multiple bond correlation gHMBC (*24*) were conducted using an 8 mg sample. The spectra were obtained at 25 °C with 90° pulse widths for ^1H and ^{13}C of 10.7 and 16.0 μs, respectively. For the phase-sensitive gHSQC spectrum, the following conditions applied: the acquisition time was 0.105 s [with ^{13}C GARP (*25*) decoupling]; a delay of 1.8 s, $\Delta = 2 \times 1.8$ ms (based on $^1J_{CH} = 140$ Hz); and coherence selection gradients of 0.20 and 0.10 T/m with durations of 2.0 and 1.0 ms, respectively. During t_1 128 transients were averaged for each of 2 × 128 increments. The experiment time was ca. 18 h. Data were zero filled to a 2048 × 4096 matrix and weighted with a sinebell function before Fourier transformation.

The gHMBC spectrum was obtained in a similar manner, except that the acquisition time was 0.217 s; 80 transients were averaged for each of 512 increments, coherence selection gradients of 0.20 and 0.15 T/m with a duration of 2.0 ms each were applied, and a fixed delay of 0.080 s was used to allow the long-range heteronuclear antiphase magnetization to evolve for multiple-bond correlations. The data were zero filled to a 1024 × 4096 matrix and weighted with a sinebell function before Fourier transformation. The experiment time was ca. 18 h.

Determination of Molar Absorptivity of Cy 3-Xylrut. The molar absorptivity (ϵ) of Cy 3-xylrut was determined by dissolving a known quantity of purified Cy 3-xylrut in 5% formic acid. Five solutions were prepared by diluting different volumes of the Cy 3-xylrut solution with 5% formic acid in methanol (15:85). The absorbance of the Cy 3-xylrut in this dilution series was measured in a DU-600 spectrophotometer (Beckman Coulter, Inc.) using 1 cm path length quartz cells at λ_{max}. The molar absorptivity (ϵ) of Cy 3-xylrut (FW = 763) was calculated to be 25354 L/(mol·cm) from the slope of the plot between λ_{max} and molar concentration.

Relative Antioxidant Capacity of Black Raspberry Anthocyanins. The antioxidant capacity of black raspberry standard compounds and the isolated fraction of purified Cy 3-xylrut were determined using the three antioxidant assays: ferric reducing antioxidant power (FRAP), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH). In brief, the FRAP assay was performed as previously described by Benzie and Strain (*26*). The analysis was conducted at 25–30 °C under pH 3.6 condition with a blank sample in parallel at 593 nm. The ABTS assay was performed according to the modified method of Ozgen et al. (*27*). Levels of reduced ABTS reactants were measured at 734 nm. The DPPH assay was performed according to the method of Brand-Williams et al. (*28*), and the absorbance was determined at 517 nm. All assay reactions were kept in the dark for 60 min prior to measurement of the absorbance using a Beckman Coulter DU-600 spectrophotometer (*27*). The results were expressed as micromoles of Trolox equivalents (TE) per micromole of anthocyanin.

Antioxidant Capacity of Black Raspberry Extracts and Extract Fractions. The antioxidant capacities of sample extracts were determined by the FRAP assay as described above. Subsequently, the antioxidant capacities of samples were compared with the concentration of individual anthocyanins in their anthocyanin profiles.

To quantify the relative antioxidant capacity of acidic methanol-soluble black raspberry constituents, sequential HPLC eluate fractions from a representative black raspberry sample were subjected to the FRAP assay procedure. Antioxidant values of sequential fractions were compared to corresponding regions of the analytical HPLC chromatographic data collected at 254 and 520 nm to ascertain the contribution of anthocyanins and other acidic methanol-soluble (potentially phenolic) compounds to the overall antioxidant capacity of sample extracts. The

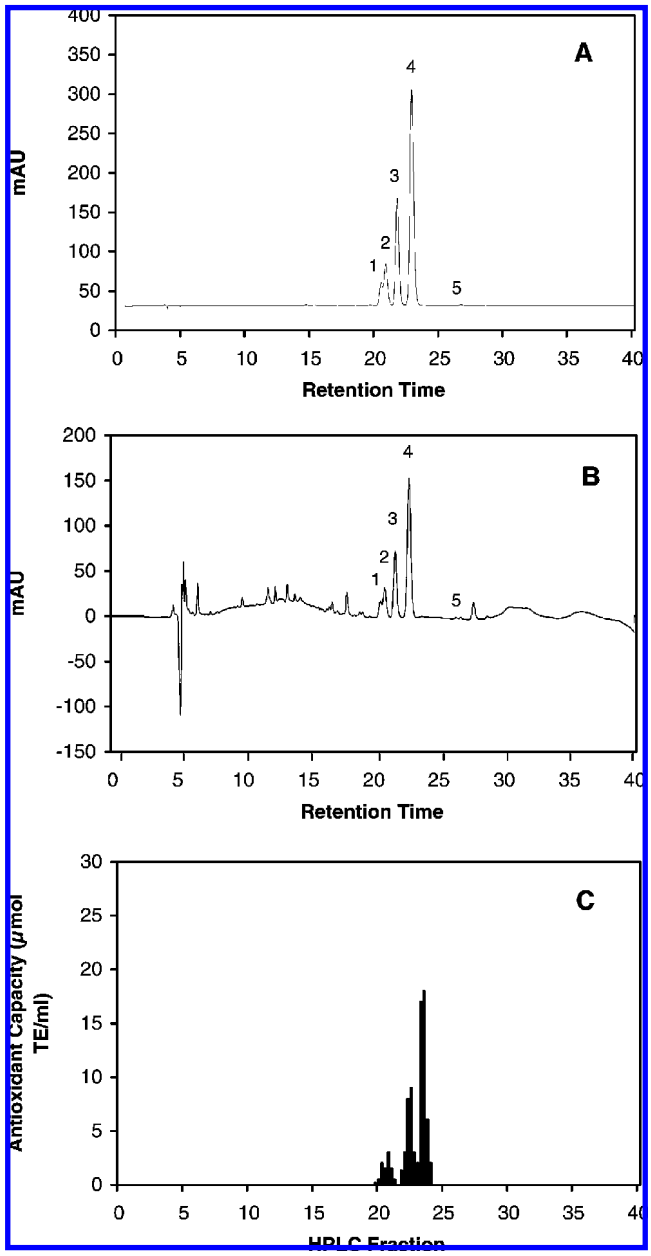

Figure 3. HPLC chromatograms of anthocyanins recorded at 520 nm (**A**) and 254 nm (**B**) and antioxidant capacity of extract sequential fractions (**C**) in black raspberry sample. TE, Trolox equivalents. Peaks: 1, cyanidin 3-sambubioside; 2, cyanidin 3-glucoside; 3, cyanidin 3-xylosylrutinoside; 4, cyanidin 3-rutinoside; 5, pelargonidin 3-rutinoside.

fractions collected from the chromatographic blank described above did not exhibit antioxidant activity, indicating that gradient differences in solvent composition had a negligible effect on the assay of extract fractions.

Statistical Analyses. Means, standard errors, regression equations, and Pearson's correlation coefficients were calculated using Microsoft Excel (Microsoft International, Redmond, WA) spreadsheet functions.

RESULTS AND DISCUSSION

Separation and Characterization of Anthocyanins. Using reversed-phase HPLC-DAD, one pelargonidin and four cyanidin pigments were separated and characterized from the Jewel cultivar of black raspberries grown in Ohio (**Figure 3A**). These anthocyanins were identified as peak 1, Cy 3-sam; peak 2, Cy 3-glc; peak 3, Cy 3-xylrut; peak 4, Cy 3-rut; and peak 5, Pg 3-rut (**Table 1**). Classification of these anthocyanins was based

Table 1. Chromatographic and Spectroscopic Characteristics of Anthocyanins from Black Raspberry Extracts

peak	t_R (min)	UV (nm)	vis (nm)	identity
1	20.1	278	517	cyanidin 3-sambubioside (Cy 3-sam)
2	20.5	279	516	cyanidin 3-glucoside (Cy 3-glc)
3	21.4	280	531	cyanidin 3-xylosylrutinoside (Cy 3-xylrut)
4	22.5	281	519	cyanidin 3-rutinoside (Cy 3-rut)
5	26.4	272	519	pelargonidin 3-rutinoside (Pg 3-rut)

on their retention times and spectral characteristics compared to those of known standards and published data (*3–7*). Identities of these anthocyanins were verified further by collecting peaks as corresponding fractions using semipreparative HPLC.

When the purified anthocyanin fractions from semipreparative HPLC were subjected to ^{1}H NMR analysis, the following anthocyanins were identified: Cy 3-sam (fraction 1), Cy 3-glc (fraction 2), Cy 3-xylrut (fraction 3), and Cy 3-rut (fraction 4). **Figure 4** shows some relevant regions from the ^{1}H NMR spectra of these fractions. Distinctive chemical shifts for the H4 proton of the aglycone were observed as singlets for Cy 3-sam (8.969 ppm), Cy 3-glc (9.038 ppm), Cy 3-xylrut (8.857 ppm), and Cy 3-rut (8.959 ppm). The chemical shifts for the glucosyl proton (H1″) for fractions 1–4 were observed as doublets centered at 5.453, 5.300, 5.470, and 5.287 ppm, respectively. In fractions 1 and 3, the chemical shifts of H1‴ of the xylosyl proton attached to the 2″ position of the glucosyl moiety were observed as doublets centered at 4.749 and 4.776 ppm. Distinguishing resonances for the rhamnosyl residues include $H1^{IV}$ of fraction 3 (δ 4.647) and H1‴ of fraction 4 (δ 4.656), as well as those for the CH_3 protons of fractions 3 and 4 that were observed as doublets centered at 1.152 and 1.164 ppm, respectively.

According to a recent HPLC-DAD-ESI-MS/MS study by Wu and Prior (*8*), an HPLC fraction (peak 3) of black raspberry, previously identified as Cy 3-xylrut (**Figure 1**) by other researchers (*3–7*), was tentatively reassigned as cyanidin 3-sambubioside-5-rhamnoside with their proposed structure shown in **Figure 2**. However, the authors stipulated that their structure assignment needed to be verified by NMR spectroscopy. Therefore, a more concentrated NMR sample (3.2% w/v) of the corresponding fraction (peak 3) in our HPLC study was further subjected to NMR analysis to confirm compound structure. The ^{1}H NMR assignments (**Table 2**) for this sample were similar to those for Cy 3-xylrut cited in previous work (*22*), and the Cy 3-xylrut structure was further confirmed by

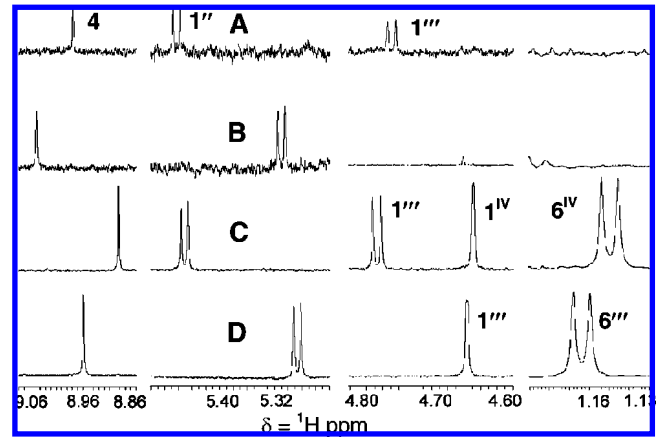

Figure 4. Selected regions from ^{1}H NMR spectra of HPLC fractions of black raspberry extracts: fraction 1, cyanidin 3-sambubioside (**A**); fraction 2, cyanidin 3-glucoside (**B**); fraction 3, cyanidin 3-xylosylrutinoside (**C**); fraction 4, cyanidin 3-rutinoside (**D**).

Table 2. ^{1}H NMR Chemical Shifts and ^{1}H−^{1}H Coupling Constants for HPLC Fraction 3 Containing Cyanidin 3- Xylosylrutinoside Obtained from Black Raspberry Extracts

H no.	δ (*J*, Hz)
aglycone	
4	8.857 s
6	6.675 d (1.8)
8	6.894 d (1.8)
2′	8.019 d (2.3)
5′	7.029 d (8.7)
6′	8.269 dd (8.7, 2.3)
3-*O*-glucoside	
1″	5.470 d (7.6)
2″	3.982 dd (7.6, 8.9)
3″	3.801 t (8.9)
4″	3.477 t (8.9)
5″	3.758 m
6″A	4.054 dd (1.7, 11.3)
6″B	3.602 dd (11.3, 4.5)
2″-*O*-xylosyl	
1‴	4.776 d (7.7)
2‴	3.200 dd (7.7, 9.0)
3‴	3.326[a]
4‴	3.433 m
5‴A	3.728 dd (11.5, 5.4)
5‴B	3.078 dd (11.5, 10.6)
6″-*O*-rhamnosyl	
1^{IV}	4.647 d (1.3)
2^{IV}	3.775 dd (1.3, 3.3)
3^{IV}	3.637 m
4^{IV}	3.325[a]
5^{IV}	3.566 m
6^{IV}	1.152 d (6.2)

[a] Overlaps with methanol signal.

heteronuclear correlation experiments: gHSQC and gHMBC. Some of the relevant HC-correlations are shown in **Figures 5A**,**B**. The cross signal H1″/C-3 (5.47/144.2 ppm) clearly shows attachment of the glucosyl moiety to the aglycone at C-3, whereas the correlations H1‴/C2″ (4.78/82.4 ppm) and H1‴/C2‴ (4.78/80.7 ppm) confirm attachment of the xylosyl residue at the 2″ position of the glucosyl moiety. In addition, the correlations H1^{IV}/C6″ (4.65/68.7 ppm) and H1^{IV}/C2^{IV} (4.65/71.6 ppm) establish that the rhamnosyl residue is attached at the 6″ carbon of the glucosyl moiety. Therefore, cyanidin 3-sambubioside-5-rhamnoside is not evident in Jewel black raspberry samples.

Relative Distribution of Anthocyanins. Sample anthocyanin profiles are depicted in **Figure 6**. Cy 3-rut and Cy 3-xylrut were the major anthocyanins, representing 46 and 39% on average, respectively, of the total anthocyanins in the Jewel cultivar. These results are consistent with previous findings that Cy 3-rut and Cy 3-xylrut were the major anthocyanin constituents in black raspberry (*5, 6*). Cy 3-sam and Cy 3-glc were found in relatively smaller and almost identical amounts. These two minor anthocyanins comprised 5–6% of the total anthocyanins. Pg 3-rut was detected in negligible amounts.

The overall level of anthocyanins and the relative level of individual anthocyanins in concentrated black raspberry extracts, especially those of Cy 3-rut and Cy 3-xylrut, varied with production sites, corroborating our earlier studies (*29*). In the present study, the average ratio of Cy 3-rut to Cy 3-xylrut in black raspberry samples was 1.6:1, but this ratio varied significantly from 1.2:1 to 2.4:1. Whether or not production site variability has dietary or clinical significance has yet to be determined. In vitro and in vivo bioactivities of black raspberry extracts (*12, 14, 15, 19, 30*) and other fruits (*30, 31*) have been shown to be concentration-dependent. Although black raspberry HPLC subfractions containing Cy 3-glc, Cy 3-xylrut, or Cy 3-rut all demonstrated substantial chemopreventive activity in mouse epidermal cell bioassays (*32*), the relative inhibitory effects of individual anthocyanins on various carcinogenic processes are known to be dependent on their chemical structures (*10, 31, 33*). Important structural features included the aglycone moiety itself (i.e., the degree of hydroxylation and methoxylation of the B ring), and the nature or extent of glycosylation or acylation present. The bioactivities of Cy 3-rut and Cy 3-xylrut have yet to be directly compared, but Seeram et al. (*10*) found the inhibition of COX (cyclooxygenase) to decrease with increasing complexity of glycosylation in cyanidin-based anthocyanins (i.e., cyanidin > cyanidin 3-rutinoside > cyanidin 3-glucosylrutinoside).

Antioxidant Capacity of Standards. The antioxidant capacities of the anthocyanin standards obtained from commercial sources and purified Cy 3-xylrut were analyzed using three antioxidant assays: FRAP, DPPH, and ABTS (**Figure 7**). The antioxidant capacity of Cy 3-rut based on these antioxidant assays was considerably higher than that of the other two commercial standards, Cy 3-sam and Cy 3-glc. In addition, the antioxidant capacity of the purified Cy 3-xylrut was less powerful than that of Cy 3-rut, but slightly more potent than that of either Cy 3-sam or Cy 3-glc. Cy 3-glc was the least effective antioxidant, particularly with the FRAP assay method. The hierarchy of antioxidant capacity among the anthocyanin standards tested was independent of the assay method. The results obtained in this study were comparable to those of our previous paper (*29*). In addition to their structural effects on bioactivity, Seeram et al. (*10*) reported that the antioxidant activity of Cy 3-rut was higher than those of the other pure cyanidin glycosides.

Antioxidant Capacity of Samples. The antioxidant capacity of the black raspberry extracts varied nearly 2-fold, with samples A, B, C, and D averaging 41, 23, 34, and 35 μmol of TE/mL of extracts, respectively. The antioxidant capacity of black raspberries in this study was lower than those reported by Moyer et al. (*17*) but similar to our previous work on black raspberry (*29, 34*). The total anthocyanin contents of fruit samples of *Rubus* species are often highly correlated with their antioxidant capacities (*13, 17, 35*), with r values ranging from 0.74 to 0.95. However, because the antioxidant capacities of individual anthocyanins differ, the relative distribution of moieties within a sample's anthocyanin profile is also likely to influence the antioxidant capacity of the sample. The relationship between total antioxidant capacity of black raspberry extracts and individual anthocyanin levels within their profile is shown in **Figure 8**. The antioxidant capacity of black raspberries was highly correlated to Cy 3-rut ($r = 0.63$) and Cy 3-xylrut ($r = 0.76$). In contrast, Cy 3-sam ($r = 0.22$) and Cy 3-glc ($r = 0.47$) exhibited weak correlations with the antioxidant capacity of black raspberries. The relative strength of these relationships in black raspberry extracts corroborate our assertions concerning antioxidant capacity differences among black raspberry anthocyanin standards and the relative distribution of individual anthocyanins within sample profiles.

Antioxidant Capacity of Extract Fractions. To ascertain the contribution of anthocyanins and other acidic (potentially phenolic) compounds to the overall antioxidant capacity of sample extracts, regions of the analytical HPLC chromatographic data collected at 520 and 254 nm (**Figures 3A**,**B**, respectively) were compared with antioxidant values of

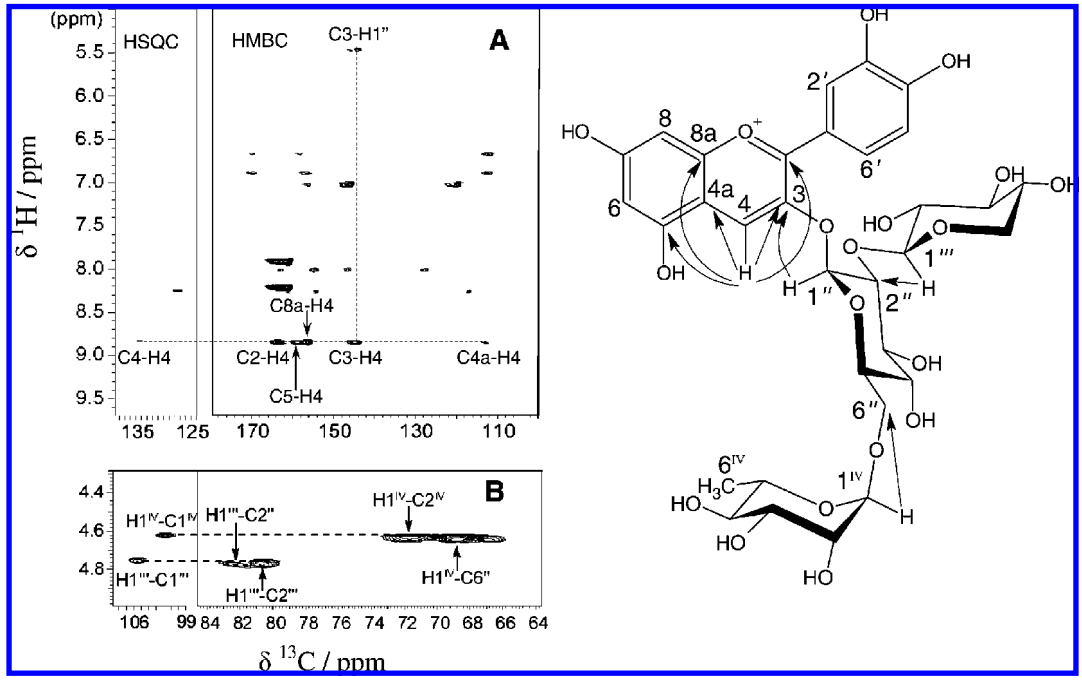

Figure 5. HSQC and HMBC correlations for downfield (**A**) and upfield (**B**) regions from the 2D-NMR spectra of HPLC fraction 3 containing cyanidin 3-xylosylrutinoside in black raspberry extracts.

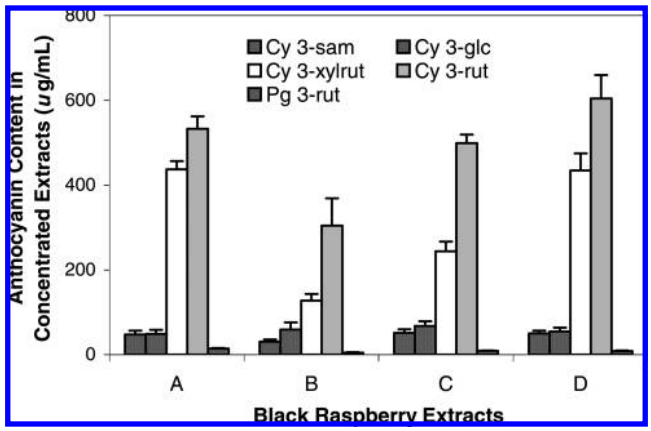

Figure 6. Relative anthocyanin content in concentrated extracts of black raspberries. Cy 3-sam, cyanidin 3-sambubioside; Cy 3-glc, cyanidin 3-glucoside; Cy 3-xylrut, cyanidin 3-xylrutinoside; Cy 3-rut, cyanidin 3-rutinoside; Pg 3-rut, pelargonidin 3-rutinoside. A−D refer to the four berry farm growers in Ohio. $n = 3$.

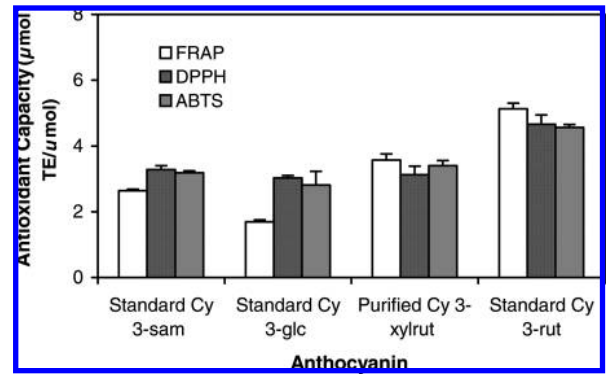

Figure 7. Antioxidant capacity of commercial anthocyanin standards and purified cyanidin 3-xylosylrutinoside measured by FRAP, DPPH, and ABTS. Cy 3-sam, cyanidin 3-sambubioside; Cy 3-glc, cyanidin 3-glucoside; Cy 3-xylrut, cyanidin 3-xylrutinoside; Cy 3-rut, cyanidin 3-rutinoside. TE, Trolox equivalents. $n = 3$.

sequential fractions as depicted in **Figure 3C**. Very few of the fractions exhibited antioxidant activity. Fractions that did exhibit substantial antioxidant capacity corresponded directly with the retention times for Cy 3-sam, Cy 3-glc, Cy 3-xylrut, Cy 3-rut, and Pg 3-rut (**Figures 3A**,**C**). Correspondingly, the fractions with the highest percentage anthocyanin content (**Figure 6**) and the highest activity in pure form (**Figure 7**) were found to contain the highest antioxidant capacity. Therefore, on the basis of both potency and concentration, the proportional contribution of the individual anthocyanins to the antioxidant capacity in black raspberry was Cy 3-rut > Cy 3-xylrut > Cy 3-sam > Cy 3-glc. Levels of Pg 3-rut in black raspberry extracts were insignificant; hence, antioxidant capacity could not be detected.

The lack of antioxidant activity in any other fraction suggested other acidic methanol-soluble compounds present under the conditions of this study individually contributed little to the antioxidant potential of black raspberry. In a study by Seeram et al. (*30*), acidic methanol extracts of black raspberry examined by HPLC-UV and LC-ESI-MS proportionally displayed high detector response levels for anthocyanin peaks, modest levels of response for a quercetin derivative, and several ellagic acid derivatives at very low response levels. Pure quercetin exhibited substantial antioxidant activity in our previous studies (*27*), but its putatively low concentration likely precludes it from being a major antioxidant in black raspberries. Admittedly, our extracts may not have contained proanthocyanidins and other phenolic compounds, which are poorly soluble or insoluble in acidic methanol (*30*), but may add in aggregate to the total antioxidant capacity of this fruit. In addition, fresh black raspberries have been reported to contain 26.2 mg/100 g of vitamin C (*36*), a concentration similar to those of grapefruits and tangerines. Although its antioxidant potential was found to be one-third that of quercetin and similar to that of Trolox (*27*), the relatively high levels of vitamin C reported suggest that it, also, is potentially a major antioxidant of black raspberry. Black raspberries were not acknowledged as good

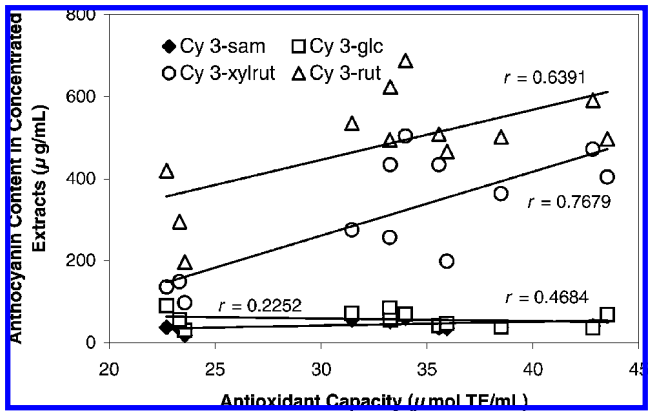

Figure 8. Relationship between the anthocyanin content in concentrated extracts and antioxidant capacity of the four samples of black raspberries in triplicates. Cy 3-sam, cyanidin 3-sambubioside; Cy 3-glc, cyanidin 3-glucoside; Cy 3-xylrut, cyanidin 3-xylrutinoside; Cy 3-rut, cyanidin 3-rutinoside; Pg 3-rut, pelargonidin 3-rutinoside. TE, Trolox equivalents. $n = 3$.

dietary sources of common lipophilic antioxidants such as vitamins A and E (*36*).

On the basis of potency and concentration, the data collectively imply that Cy 3-rut is the major methanol-soluble phenolic antioxidant compound in the black raspberry samples characterized in this study. However, Cy 3-xylrut, another major component of black raspberries, may also be considered as a highly important source of antioxidant in black raspberries. Kuhnau (*37*) ascribed the antioxidant effect of anthocyanins, in part, to their metal chelation properties, whereas Chimi et al. (*38*) attributed this to their ability to scavenge peroxyl and alkoxyl radicals. The most compelling mechanism of antioxidant action of anthocyanins is attributed to their chemical structures, which are highly reactive due to electron deficiency of the C ring (**Figure 1**) (*39*). Specifically, the antioxidant capacity exhibited by anthocyanins, including Cy 3-rut and Cy 3-xylrut, is modulated by the pattern of hydroxyl (OH) substitutions and the degree of methylations on the aromatic B ring (*18, 40, 41*) and the number of sugars attached to the molecule (*10*).

Furthermore, Fukumoto and Mazza (*42*) and Seeram et al. (*10*) reported that adding a sugar moiety diminishes the antioxidant capacity of the aglycone and that the second addition of sugar moiety further diminishes the capacity, which they tentatively attributed to steric impediment of sugar moieties. For example, when the antioxidant capacity of cyanidin 3,5-diglucoside was compared with those of the standards commonly found in black raspberries (*29*), it was very low. In fact, the antioxidant capacity of the cyanidin 3,5-diglucoside was 10 times lower than that of Cy 3-rut, which might be due to the sugar attachments on 3 and 5 configurations of the chromane ring (**Figure 1**). The tentative structure (**Figure 2**) proposed by Wu and Prior (*8*) is similar to cyanidin 3,5-diglucoside; hence, it is highly unlikely that this compound would have the demonstrated antioxidant capacity of the peak 3 compound. Indeed, NMR studies that were conducted herein (**Figures 4** and **5**; **Table 2**; Supplemental **Table 3**) yielded convincing evidence that the structure of this compound is unambiguously identified as Cy 3-xylrut (**Figure 1**), which is consistent with previous work.

Black Raspberries as a Source of Dietary Antioxidants. In general, phenolic compounds are responsible for a substantial portion of antioxidant capacity in many fruits and vegetables (*43*). However, because black raspberries contain high levels of anthocyanins, they may yield potential health benefits in addition to their strong antioxidant effects, such as the regulation of oncogene expression and enzymes controlling cell cycling and proliferation, the promotion of cancer cell apoptosis, and the inhibition of tumor cell invasiveness (*33, 44*). The identity and mode of action of the specific compounds mediating the chemopreventive effects reported in previous studies (*11, 12, 14–16, 19*) are still not yet fully understood. However, anthocyanins, and specifically Cy 3-rut, have been shown herein and elsewhere (*10, 43*) to exhibit potent antioxidant capacity, which is a typical characteristic of a food-based chemopreventive agent (*11, 14, 33*). In addition, a very recent study using Cy 3-rut isolated from black raspberries showed its putative antioxidative potential by selectively inhibiting leukemic cells by reactive oxygen species activation (*44*).

In conclusion, we observed that Cy 3-rut and Cy 3-xylrut were the predominant anthocyanins and highly potent antioxidants in black raspberries, suggesting that these anthocyanins may function as the primary phenolic antioxidants in this fruit. Cy 3-xylrut, the structure of which was verified by NMR, may also exhibit potential bioavailability and bioactivity similar to those of Cy 3-rut in conjunction with other naturally occurring bioactive compounds in black raspberry fruit-based products that may be utilized in clinical trials for cancer chemoprevention studies. Because genetic and environmental factors affect the antioxidant profiles of fruits and vegetables, it is very important that screening of the antioxidant profiles and anthocyanin levels is carried out prior to measuring biological and medicinal activities of these foods, their extracts, or the clinical products derived from them. For black raspberries specifically, because the antioxidant capacity of individual anthocyanins varies considerably and their concentrations are subject to genetic and environmental influences, it is imperative that anthocyanin profiles of these fruits are examined before their biological and medicinal activities are evaluated.

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Supporting Information Available: Tables containing additional NMR data are provided. 750 MHz ^{1}H NMR chemical shifts and ^{1}H−^{1}H coupling constants for HPLC fraction 1 containing Cy 3-sam from black raspberry extracts are given in Supplemental **Table 1**, whereas those for HPLC fractions 2 and 4 containing Cy 3-glc and Cy 3-rut, respectively, are provided in Supplemental **Table 2**. Supplemental Table 3 details ^{13}C chemical shifts obtained by the HMBC NMR experiment for HPLC fraction 3 containing Cy 3-xylrut from black raspberry. This material is available free of charge via the Internet at http://pubs.acs.org.

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