JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Nonanthocyanin Secondary Metabolites of Black Raspberry (*Rubus occidentalis* L.) Fruits: Identification by HPLC-DAD, NMR, HPLC-ESI-MS, and ESI-MS/MS Analyses

Liladhar Paudel,[†,‡] Faith J. Wyzgoski,[*,§] Joseph C. Scheerens,[∥] Ann M. Chanon,[∥] R. Neil Reese,[⊥] Danijela Smiljanic,[‡] Chrys Wesdemiotis,[‡] Joshua J. Blakeslee,[∥] Kenneth M. Riedl,[¶] and Peter L. Rinaldi[‡,*]

[†]School of Chemistry, University of Manchester, Oxford Road, Manchester M13 9PL, United Kingdom

[‡]Department of Chemistry, University of Akron, Akron, Ohio 44325-3601, United States

[§]Department of Chemistry and Biochemistry, The Ohio State University, 1760 University Drive, Mansfield, Ohio 44906, United States

[∥]Department of Horticulture and Crop Science, The Ohio State University, Ohio Agricultural Research and Development Center, 1680 Madison Avenue, Wooster, Ohio 44691, United States

[⊥]Department of Biology and Microbiology, South Dakota State University, Brookings, South Dakota 57007, United States

[¶]Department of Food Science and Technology, The Ohio State University, Columbus, Ohio 43210, United States

Supporting Information

ABSTRACT: Nonanthocyanin secondary metabolites potentially contributing to the antiproliferative bioactivity of black raspberry (*Rubus occidentalis* L.) fruits were extracted in ethyl acetate and isolated by semipreparative and analytical HPLC and analyzed by NMR, HPLC-ESI-MS, and ESI-MS/MS techniques. Here we present complete and partial structures of a variety of the chemical entities such as quercetin 3-glucoside, quercetin 3-rutinoside, myricetin glucoside, dihydrokaempferol glucoside, benzoic acid β-D-glucopyranosyl ester, 3,4-dihydroxybenzoic acid, epicatechin, caffeic acid, *p*-coumaric acid, *p*-coumaryl glucoside, *p*-coumaryl sugar ester, ellagic acid, methyl ellagic acid acetylpentose, methyl ellagic acid valerylpentose, *trans*-piceid, phloretin glucoside (phloridzin), dihydrosinapic acid, salicylic acid β-D-glucopyranosyl ester, a salicylic acid derivative without attached sugar, *p*-alkylphenyl glucoside, and a citric acid derivative. To our knowledge, 15 of these compounds were not previously reported in black raspberry fruits.

KEYWORDS: *nonanthocyanin metabolites, bioactive compounds, NMR, HPLC-DAD, HPLC-ESI-MS, ESI-MS/MS, black raspberry, Rubus occidentalis L.*

INTRODUCTION

Studies using animal and human subjects have indicated that freeze-dried black raspberry (BR) (*Rubus occidentalis* L.) fruits have health benefits, most notably with regard to aero-digestive cancers.[1,2] Anthocyanins such as cyanidin 3-rutinoside (Cy 3-rut), cyanidin 3-xylosylrutinoside (Cy 3-xylrut), cyanidin 3-glucoside (Cy 3-glc), and cyanidin 3-sambubioside (Cy 3-sam) are major phenolic constituents of BR fruits, with 80% or more anthocyanin content being ascribed to Cy 3-rut, and Cy 3-xylrut.[3,4] Many of the chemopreventive properties of BRs have been attributed to these main constituents, including reduced levels of oxidative stress, inflammation, mutagenesis, and cell proliferation and increased activity of inherent enzymatic defense mechanisms including pro-apoptotic activators.[5]

However, anthocyanins alone do not account for all of the carcinostatic properties of BR fruits.[5,6] For example, Wang and coauthors[6] reported that rat diets formulated with lyophilized BR powder, aqueous ethanol extracts of the powder, residue from these extracts and anthocyanin-rich extract fractions affect the development of esophageal cancer in a similar manner. These authors and others[5,7−9] have considered several nonanthocyanin metabolites like ellagic acid, ellagitannins, urolithins, protocatechuic acid, ferulic acid, chlorogenic acid, gallic acid, *p*-coumaric acid, quercetin, flavonols, flavan 3-ols, and their derivatives and β-carotene as being important constituents for controlling the onset and progression of cancer.

Recently, we reported the proliferation of HT-29 colon cancer cells to be differentially inhibited by 75 methanolic extracts of BR fruit obtained from different cultivars, produced on different farms or harvested at different stages of development.[10] Similar to previous reports,[5−9] the dissimilarity in cell assay results among extract treatments in this study could not be attributed solely to variable anthocyanin content of the fruit or the extracts as measured by the total monomeric anthocyanin (TMA) technique.[11] Analysis by nuclear magnetic resonance spectroscopy (^{1}H NMR) indicated the 75 BR methanolic extracts to possess highly variable secondary metabolite profiles. As in our earlier study of similar BR extracts[4] resonances associated with the five predominant BR

Received: May 30, 2013
Revised: November 13, 2013
Accepted: November 14, 2013
Published: November 27, 2013

ACS Publications © 2013 American Chemical Society

anthocyanins were clearly visible in these spectra. Defining ^{1}H resonances of nonanthocyanin BR metabolites in the extracts were also evident, but full structure determination of these constituents was achieved only after additional experimentation described herein.

Here we report the qualitative study of the isolation of BR nonanthocyanin constituents, their fractionation by preparative and analytical HPLC, and their identification by ^{1}H NMR, high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI-MS) and electrospray ionization mass spectrometry-mass spectrometry (ESI-MS/MS) methods. For fractions where sufficient materials could be isolated, we also employed a variety of advanced 1D and 2D NMR techniques. This approach has allowed the identification of several new compounds and new derivatives of known compounds that have the potential to play important roles in control of cancer cell proliferation.

MATERIALS AND METHODS

Reagents, Solvents, and Standards. HPLC-grade methanol was purchased from Fisher Scientific. HPLC-grade water and deionized−distilled (DD) water were purchased from Lonza Walkersville, Inc. (Walkersville, MD). Acetone, concentrated acetic acid, anhydrous sodium acetate, acetonitrile, ethyl acetate, trifluoroacetic acid-*d* (TFA-*d*, 99.5 atom % D), methanol-d_4 (99.8 atom % D), tetramethylsilane (TMS), and standard samples of citric acid, trimethyl citrate, salicylic acid, 3,4-dihydroxybenzoic acid, *p*-coumaric acid, caffeic acid, ellagic acid, quercetin, quercetin 3-glucoside, quercetin 3-rutinoside, kaempferol, resveratrol, *trans*-piceid, myricetin, and epicatechin were purchased from Sigma-Aldrich (St. Louis, MO).

Methanol Extract Preparation. As described previously in detail by Johnson and co-workers,[10] methanol extracts were obtained from the juices of commercially ripe BR fruit samples varying in source, cultivar and maturity by solid phase extraction (SPE). Briefly, juices (25 mL) acidified with 0.1% trifluoroacetic acid were loaded onto prewashed, H_2O-equilibrated Strata C18-E, 5 g/20 mL SPE cartridges (Phenomenex, Torrance, CA) to isolate secondary metabolites of interest from other fruit constituents. The absorbed compounds were washed with acidified H_2O to remove residual sugars, organic acids and other hydrophilic substances present in the juice. Secondary metabolites (predominantly phenolic compounds) were then eluted from the column with acidified methanol, brought to dryness under a nitrogen stream delivered by an N-Evap system (Organomation Associates, Inc., Berlin, MA) equipped with a water bath at 35 °C, lyophilized to remove residual moisture (Model 7960046/7948040, Labconco Inc., Kansas City, MO) and stored at −80 °C until samples were prepared for ^{1}H NMR analysis. Methanol samples are referred to by sample number.

Ethyl Acetate Extract Preparation. The ethyl acetate extraction procedure described by Maatta-Riihinen et al.[12] was adapted for the HPLC collection of nonanthocyanin-rich metabolite fractions. Intact BR fruits resourced similarly to those described above were flash-frozen in liquid N_2, stored at −80 °C, weighed and lyophilized. The freeze-dried BR fruits were manually crushed leaving seeds intact; the crushed drupelet tissue was then sieved to −80 mesh (approximately 0.18 mm particle size) providing a fine powder for extraction. To obtain an array of secondary metabolites, powders were initially extracted with a phenolic extraction solvent comprised of acetone, HPLC-grade water and acetic acid solution (70:29.5:0.5, v/v/v) using a method described by Singleton et al.[13] Initial extracts were concentrated by rotary evaporation (Rotovapor RII; Buchi Corp. USA, New Castle, DE) under partial vacuum at 35 °C until concentrates were essentially free of acetone and acetic acid (approximately 20 mL) as determined by olfactory inspection. This extraction protocol solubilized simple phenolic aglycones and more polar conjugates but did not extract many of the more complex tannins.

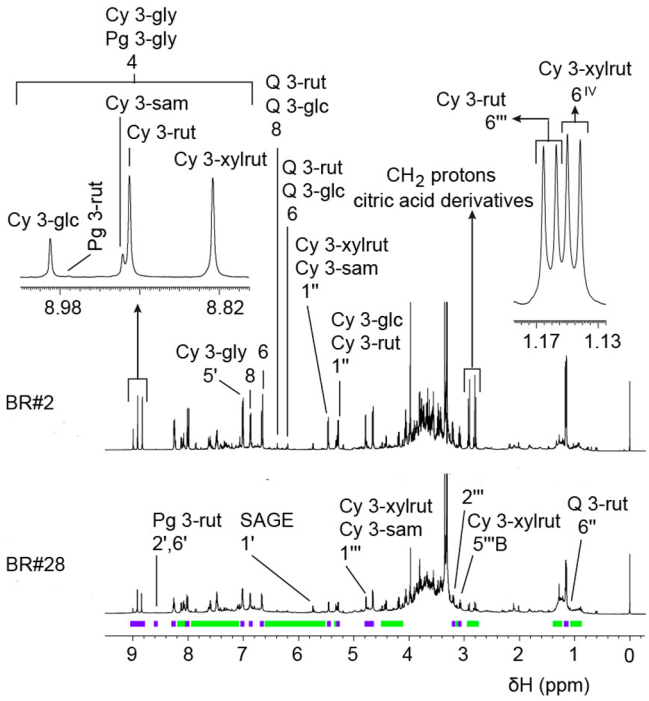

Figure 1. ^{1}H NMR spectra (750 MHz) of two representative BR fruit extracts showing ^{1}H spectral regions (indicated in purple bars above the chemical shift scale) for anthocyanins and resonances (green) from identified nonanthocyanin components of BR fruits.

A subsequent extraction of the concentrate into ethyl acetate was employed to isolate simple phenolics, flavonols and other compounds of intermediate polarity from anthocyanins. For this procedure, each concentrate, was treated with an equal volume of 0.2 M aqueous sodium acetate and then this aqueous solution was partitioned against three to four sequential aliquots of ethyl acetate (i.e., until the ethyl acetate layer was colorless). The ethyl acetate aliquots were combined and then the solvent was evaporated under N_2 to obtain a dried, yellow-red colored, phenolic-rich solid. Solids were stored at −20 °C for chromatographic fractionation.

Chromatography. Individual phenolic components or groups of constituents contained in the phenolic-rich solids were separated using semipreparative and/or analytical high performance liquid chromatography (HPLC). Prior to injection, solids were redissolved in 1.0 mL of 30% acidic acetonitrile (30.0 acetonitrile: 69.8 HPLC-grade water: 0.2 acetic acid, v/v/v), then filtered through a 0.45 μm nylon filter (Fisher Scientific, Pittsburgh, PA).

The nonanthocyanin rich fractions were initially separated using semipreparative HPLC (Beckman Coulter Inc., Fullerton, CA) equipped with a Phenomenex (Torrance, CA) Gemini C6-phenyl column (250 × 10 mm). The column was held at 30 °C (Alltech Assoc., Deerfield, IL; column heater Model 631). Compounds were eluted using a programmed gradient of solvent consisting of Solvent A = acidified water (0.2% acetic acid in HPLC-grade water); Solvent B = 100% CH_3CN. The solvent composition ramp followed the gradient program: hold at 9% B from 0 to 10 min; transition to 22% B from 10 to 20 min; transition to 30% B from 20 to 35 min; transition to 60% B from 35 to 40 min; hold at 60% B from 40 to 45 min; transition 9% B from 45 to 50 min; hold at 9% B from 50 to 55 min. The solvent flow (2.0 mL/min) was maintained at a constant rate; the injection volume for each collection run was 50 μL. Column eluate fractions (105) were collected from 19 to 54 min at 20 s intervals using a ProteomeLab FC fraction collector (Beckman Coulter). Several series of collections were made. To obtain enough material for subsequent compound identification, injections were repeated as many as 49 times during a series and corresponding eluate fractions from all collection runs

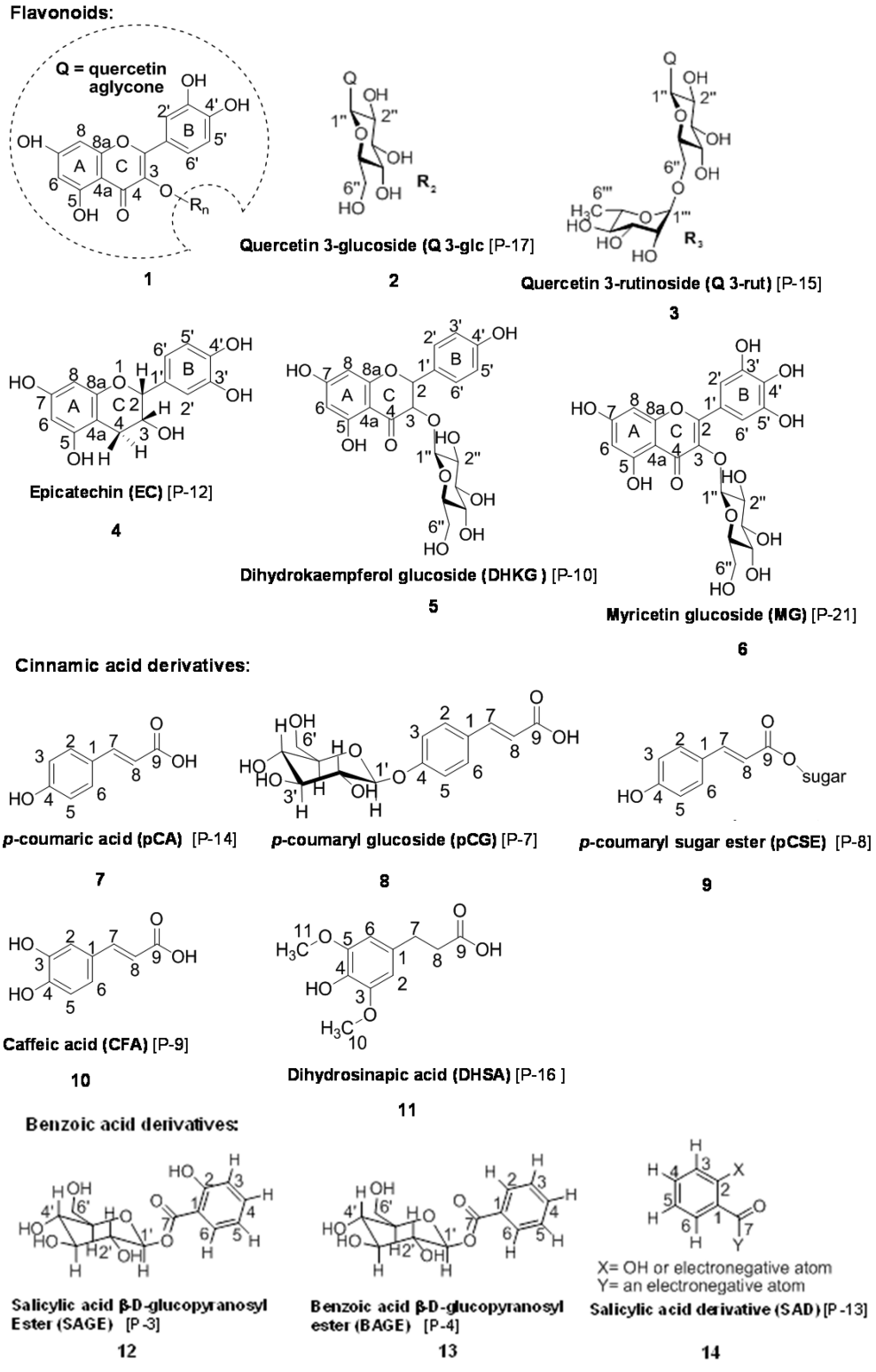

Figure 2. continued

dx.doi.org/10.1021/jf4039953 | J. Agric. Food Chem. 2013, 61, 12032−12043

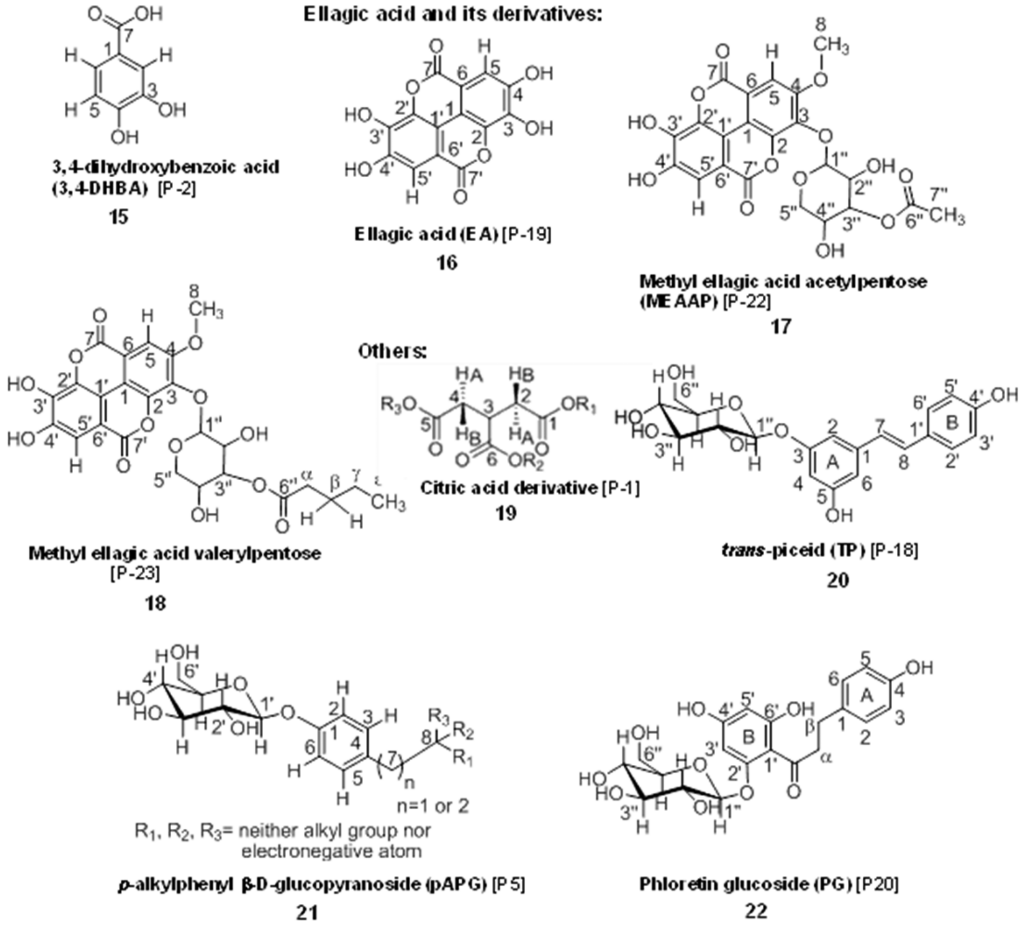

Figure 2. Chemical structures of nonanthocyanin compounds in BR fruits.

within the series were combined. Acetonitrile levels in fractions were reduced under an N_2 stream, and the remaining aqueous fractions were frozen at −20 °C, lyophilized, and stored at −20 °C until analyzed by ^{1}H NMR, HPLC-ESI-MS, and/or ESI-MS/MS.

Subsequent series of fractionations were conducted for individual fractions if samples with higher purity were desired. These fractionations were conducted using an analytical HPLC system (Beckman Coulter Inc., Fullerton, CA) equipped with a Phenomenex (Torrance, CA) Gemini C6-phenyl column (250 × 4.6 mm). All other chromatographic parameters were identical to those described above except that the flow rate was reduced to 0.7 mL/min and eluate fractions were collected at 15 s intervals. Prior to NMR and LC-MS and/or MS/MS analyses, analytical chromatogram, and UV−vis absorption profiles of each of the individual fractions were recorded in the similar manner. Compounds isolated in chromatographic fractions are referred to by peak (P) number.

NMR Analyses. All NMR investigations of methanolic BR extractions and of ethyl acetate extracted (predominately anthocyanin-free) fractions of BR were conducted at 25 °C on a Varian INOVA 750 MHz NMR spectrometer (Varian, Inc., Palo Alto, CA) using a Varian triple-resonance ^{1}H{^{13}C/^{15}N} pulsed field gradient cryoprobe and operating with VNMRJ 3.2a software unless otherwise specified. Solid materials from the column eluate of methanolic BR extractions were diluted with appropriate amounts of methanol-d_4/trifluoroacetic acid-d (95:5 v/v) and 5 mM DSS (2,2-dimethyl-2-silapentane-5-sulfonate sodium salt) in methanol-d_4 to give 800 μL aliquots that contained 20 mg of dried berry extract and 0.5 mM DSS. The samples were filtered through glass wool then placed in 5 mm NMR tubes. Samples for NMR analyses of each nonanthocyanin fraction were prepared as follows: solid material (2.75−4.60 mg) was solubilized in an appropriate volume of a mixture of methanol-d_4 (99.8 atom % D)/trifluoroacetic acid-d (99.5 atom % D) (95:5, v/v), and tetramethylsilane (TMS) was added as an internal chemical-shift reference standard. The solution was transferred to a 5 mm micro NMR tube tapered to a 2.5 mm sample holding stem. Prior to running NMR experiments, all samples were stored at 4 °C.

^{1}H 1D NMR spectra of all samples were collected with a 90° pulse width of ca. 8 μs, a spectral width of 8609.6 Hz and a relaxation delay of 3.0 s. The data were acquired for an acquisition time of 2.7 s and 128 transients were averaged with presaturation of the HDO resonance at ca. 5.0 ppm. Data were processed with 0.5 Hz exponential line broadening, zero filled to 128k points and Fourier transformed. The spectra of the methanolic BR extracts were referenced to DSS, and the spectra of the nonanthocyanin extracts were referenced relative to the CH_3 resonance of TMS set to −0.016 ppm [the value relative to CH_3 resonance of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) set to 0]. For the nonanthocyanin samples, 1D selective excitation total correlation spectroscopy (selective TOCSY)[14,15] spectra were collected in the same manner using clean-MLEV17[14] spin locking pulse cycle with varying mixing time in the range 20−200 ms and spin locking field of ca. 8 kHz. For the gradient assisted double quantum filtered correlation spectroscopy (DQCOSY) homonuclear 2D experimental data collection, the acquisition time of 0.238 s and a ^{1}H window of 8609.6 Hz, each for f_2 and f_1 dimension was used. The coherence selection gradients of −0.09 and 0.18 T/m, each for duration of 1 ms, were used; 8 transients were averaged for each of 2 × 256 free induction decays in which t_1 was incremented to provide the spectral window of 8609.6 in the f_1 dimension using the States[16] method of phase sensitive detection. Linear prediction was used in the f_1 dimension to forward extend the data three times its original length; the data were zero filled to a 4096 × 4096 matrix, and weighted with a sinebell function before Fourier transformation. For some of the fractions, 2D rotating frame nuclear Overhauser effect spectroscopy (ROESY), nuclear Overhauser

effect spectroscopy (NOESY), and 1D NOE difference experiments were also collected using mixing times in the range 100−500 ms. In addition, 2D heteronuclear NMR experiments were conducted which used Varian's multiplicity-edited broad band selective adiabatic gHSQC (gHSQCAD) sequence and Varian's absolute value mode gradient assisted HMBC (gHMBC) sequence. Additional information on the methodology for these 2D experiments can be found in Part 1 of the Supporting Information.

LC-MS and MS/MS Analyses. Samples for LC-MS or MS/MS analyses were prepared by dissolving dry solid material (ca. 0.8 mg) from each of the BR fractions in an appropriate volume of 30% acidic acetonitrile (30.0 acetonitrile:69.8 HPLC-grade water:0.2 acetic acid, v/v/v) so as to obtain solutions in the range 0.5−1.0 mg/mL. The sample solutions were vortexed for a few minutes and left at room temperature for 30−60 min. Prior to analyses, the insoluble materials in the samples were removed by filtering through a 0.2 μm Whatman Syringe filter (Fisher Scientific, Pittsburgh, PA).

Most of the LC-MS and MS/MS experiments were conducted using an Esquire-LC (Bruker Daltonics, Billerica, MA) quadrupole ion trap (QIT) equipped with electrospray ionization (ESI) source unless otherwise mentioned. For direct injection MS and MS/MS measurements, the sample solution was delivered using a syringe pump at a rate of 250 μL/min. The ESI source was operated at atmospheric pressure and was equipped with a grounded spray needle. The capillary voltage was set to ca. 4 kV, the offset potential between the plate electrode and capillary entrance was 0.5 kV, and the nebulizing nitrogen gas pressure was 10 psi. The drying gas used was also nitrogen, which was delivered at 8 L/min and the temperature of the drying gas was maintained to 250 °C. For LC-MS, the ESI source of the QIT instrument was interfaced with an Agilent HP 1100 HPLC system. The HPLC separation was conducted in the same manner as described above, except that the injection volume was 20 μL and the solvent flow (0.7 mL/min) was maintained at constant rate. To maintain the flow rate compatible with mass spectrometry, the column eluates were passed to the ESI source of the mass spectrometry system through a split flow controller. All LC-MS data collection was conducted in negative ion mode, unless otherwise mentioned.

RESULTS AND DISCUSSION

In our previous research,[10] we demonstrated that 75 BR methanol extracts supplied in culture media to HT-29 colon cancer cells at high (1.2 mg/mL) or low (0.6 mg/mL) doses resulted in antiproliferative activity ranging from 33 to 118%. Concomitantly, the ^{1}H NMR spectra of these extracts, some of which are illustrated herein (Figure 1), suggested potential bioactive compound concentrations to vary substantially among cell bioassay treatments. Resonances associated with cyanidin glycosides are prominent in these spectra (as indicated by purple bars above the scale) because these compounds are present in fruits at levels that are two to 3 orders of magnitude greater than other BR secondary metabolites.[2] Among other chemoprotective functions, anthocyanins are well-known drivers of antiproliferation via their inhibition of cell-cycle mediators and promotion of caspase-induced apoptosis.[17] Specifically, BR anthocyanins have been reported to play a significant chemopreventive role in reducing tumor growth as well as in a number of other metabolic processes associated with cancer development.[1,2,5,6] However, the lack of a significant correlation between extract anthocyanin concentrations and their performance in our HT-29 colon cancer cell bioassay[10] and the reports of others[5−9] suggested the potential of additional extract components, the nonanthocyanin metabolites, as important cell cycle inhibitors or apoptosis promoters. Herein we have identified a cadre of these nonanthocyanin metabolites also evident in the ^{1}H NMR spectra of the 75 BR extracts (Figure 1, indicated by green bars above the scale).

Overlapping signals in the NMR spectra can be a hurdle in the identification of individual metabolites. An even more challenging task is to identify the nonanthocyanin components in our extracts in which free phenolics contribute only a fraction of the total content of the fruits. J-correlated ^{1}H 1D (e.g., TOCSY) and ^{1}H−^{1}H homonuclear 2D NMR such as gDQCOSY can be used to ascertain some of the structural information from these complex signals. More difficult structural assignments can be assessed when additional information from ^{1}H−^{13}C heteronuclear 2D NMR such as HSQC and HMBC are combined. However, these studies using whole fruit extracts cannot provide sufficient information about the structures of minor components. To resolve these problems, we collected ethyl acetate-soluble BR fractions rich in one or more of the nonanthocyanin phenolic components (but relatively free of anthocyanins) by semipreparative and analytical HPLC (multiple injections). Collection of these individual fractions enormously reduced spectral overlap problems and allowed the identification of a number of nonanthocyanin metabolites in the BR extracts using ^{1}H and 2D (^{1}H−^{1}H and/or ^{1}H−^{13}C) NMR, together with HPLC-ESI-MS, and MS/MS analyses. These metabolites included flavonoids, benzoic acid derivatives, cinnamic acid derivatives, ellagic acid and its derivatives, and other classes of compounds such as citric acid, phloretin, and resveratrol derivatives (Figure 2).

Figure 3 shows HPLC-DAD typical chromatographic profiles of ethyl acetate soluble fractions of nonanthocyanin secondary metabolites of BR fruits, where identifiable chromatographic signals associated with peaks P-1 to P-23 subjected to identification are labeled. The traces at 256 nm emphasize the detection of flavonol glycosides (e.g., P-15 and P-17) and

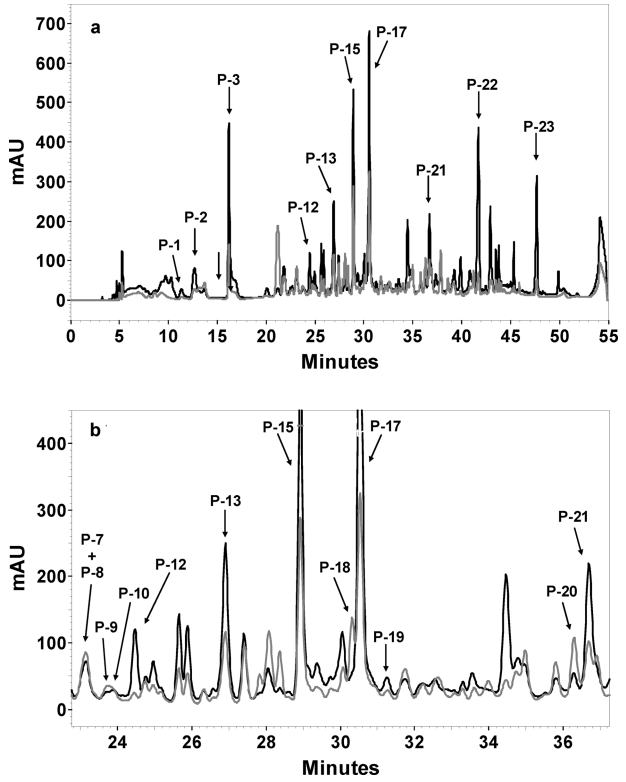

Figure 3. C6-phenyl HPLC-DAD (a) complete and (b) detail chromatographic profiles of ethyl acetate soluble compounds from black raspberry fruits at two wavelengths 256 nm (black traces) and 306 nm (gray traces).

Table 1. ^{1}H (750 MHz) and ^{13}C (188 MHz) Chemical Shifts of Resolved Resonances from Nonanthocyanin Constituents of Black Raspberry Fruits from ^{1}H 1D and $^{1}H-^{13}C$ Heteronuclear 2D NMR

compound	$\delta^{1}H$ (ppm)	$\delta^{13}C$ (ppm)
citric acid derivative (**19**)	2.92 (m, 2H, H-2A, 4A); 2.78 (m, 2H, H-2B, 4B)	177.0 (C-6); 173.5 (C-1, 5); 74.0 (C-3); 44.0 (C-2, 4)
3.4-diydroxybenzoic acid (**15**)	7.439 (d, 1H, J = 2 Hz, H-2); 7.422 (dd, 1H, J = 8.2, 2.0 Hz, H-6); 6.798 (d, 1H, J = 8.2 Hz, H-5)	151.4 (C-4); 145.9 (C-3); 123.8 (C-6); 123.2 (C-1); 117.6 (C-2); 115.7 (C-5)
salicylic acid β-D-glucopyranosyl ester (**12**)	8.066 (dd, 1H, J = 7.5, 1.5 Hz, H-6); 7.484 (ddd, 1H, J_{avg} = 7.5, 1.5 Hz, H-4); 7.091 (dd, 1H, J_{avg} = 8.24 Hz, H-3); 7.021 (ddd, 1H, J_{avg} = 7.5 Hz, H-5); 5.724 (d, 1H, J = 8.24 Hz, H-1′); 3.862 (dd,1H, J = 12.0, 2.3 Hz, H-6′A); 3.699 (dd, 1H, J = 12.0, 5.2 Hz, H-6′B); 3.53−3.39 (m, 4H, H-2′ to 5′)	167.8 (C-7); 150.6 (C-2); 135.8 (C-4); 132.5 (C-6); 118.8 (C-3); 118.4 (C-5); 112.1 (C-1); 95.5 (C-1′); 78.8 (C-5′); 78.0 (C-3′); 73.9 (C-2′); 70.1 (C-4′); 62.2 (C-6′)
benzoic acid β-D- glucopyranosyl ester (**13**)	8.087 (d, 2H, J = 7.5 Hz, H-2, 6); 7.619 (t, 1H, J = 6.7 Hz, H-4); 7.482 (t, 2H, J = 6.7 Hz, H-3, 5); 5.725 (d, 1H, J = 7.5 Hz, H-1′); 3.873 (m, overlap, H-6′A); 3.723−3.689 (m, overlap, H-6′B); 3.539−3.380 (m, overlap, H-2′ to H-5′)	166.9 (C-7); 134.7 (C-4); 130.9 (C-2, 6); 129.6 (C-3, 5); 96.2 (C-1′)
p-alkylphenyl β-D- glucopyranoside (**21**)	7.103 (d, 2H, J = 6.7 Hz, H-3, 5); 6.996 (d, 2H, J = 6.7 Hz, H-2, 6); 4.857 (d, 1H, J = 6.0 Hz, H-1′); 3.873 (m, overlap, H-6′A); 3.723−3.689 (m, overlap, H-6′B); 3.539−3.380 (m, overlap, H-2′ to H-5′); 2.82−2.74 (m, side-chain methylenes)	157.5 (C-1); 136.4 (C-4); 130.2 (C-3, 5); 117.8 (C-2, 6); 102.4 (C-1′); 29.8 (C-7, side-chain methylenes); 45.1 (C-8)
epicatechin (**4**)	6.923 (d, 1H, J = 1.65 Hz, H-2′); 6.786 (dd, 1H, J = 8.10, 1.65 Hz, H-6′); 6.751 (d, 1H, J = 8.10 Hz, H-5′); 5.934 (H-6); 5.907 (H-8); 4.805 (m, 1H, H-2ax); 4.168 (m, 1H, H-3eq); 2.844 (dd, 1H, J = 16.5, 4.5 Hz, H-4ax); 2.722 (dd, 1H, J = 16.5, 2.7 Hz, H-4eq)	157.3 (C-8a, 5); 146.2 (C-3′); 145.9 (C-4′); 132.4 (C-1′); 119.4 (C-6′); 115.9 (C-5′); 115.4 (C-2′); 100.3 (C-4a); 79.9 (C-2); 67.4 (C-3); 29.2 (C-4)
salicylic acid derivative (**14**)	8.099 (dd, 1H, J = 7.5, 1.5 Hz, H-6); 7.600 (ddd, 1H, J_{avg} = 9.0, 1.5 Hz, H-4); 7.294 (dd, 1H, J_{avg} = 7.5 Hz, H-3); 7.282 (ddd, 1H, J_{avg} = 8.2 Hz, H-5)	169.7 (C-7); 139.7 (C-2); 135.3 (C-4); 133.2 (C-6); 126.3 (C-3); 123.3 (C-5); 120.63 (C-1)
quercetin 3-rutinoside (**3**)	7.667 (d, 1H, J = 2.2 Hz, H-2″); 7.651 (dd, 1H, J = 8.4, 2.2 Hz, H-6′); 6.873 (d, 1H, J = 8.4 Hz, H-5′); 6.407 (d, 1H, 1.5 Hz, H-8); 6.214 (d, 1H, J = 1.5 Hz, H-6); 5.087 (d,1H, J = 7.8 Hz, H-1″); 4.511 (d, 1H, J = 1.5 Hz, H-1‴); 3.81−3.14 (m, nonanomeric saccharide); 1.111 (d, 3H, J = 6.2 Hz, rhamnose CH_3)	147.8 (H-3); 123.5 (C-6′); 117.6 (C-2′); 116.0 (C-5′); 100.5 (H-6); 95.2 (H-8); 104.6 (C-1″); 102.4 (C-1‴); 68.5 (C-6″); 17.0 (rhamnose CH_3)
quercetin 3-glucoside (**2**)	7.716 (d, 1H, J = 2.2 Hz, H-2′); 7.583 (dd, 1H, J = 8.2, 2.2 Hz, H-6′); 6.874 (d, 1H, J = 8.2 Hz, H-5′); 6.406 (d, 1H, J = 2.0 Hz, H-8); 6.214 (d, 1H, J = 2.0, H-6); 5.227 (d, 1H, J = 8.2 Hz, H-1′); 3.72−3.19 (m, glucose nonanomeric)	
ellagic acid (**16**)	7.555 (s, H-5,5′)	161.9 (C═O, C-7, 7′); 141.2 (C-3, 3′); 114.6 (C-1, 1′); 111.88 (C-5, 5′); 110.1 (C-6, 6′)
trans-piceid (**20**)	7.011 (d, J = 16.5 Hz, H-7); 6.611 (d, J = 2.0 Hz, H-6); 6.445(d, J = 2.0, H-4)	
methyl ellagic acid acetylpentose (**17**)	7.883 (s, 1H, H-5); 7.696 (s, 1H, H-5′); 4.050 (s, 3H, H-8:OCH3 bonded to ellagic acid ring); 2.159 (s, 3H, H-7″: $OCOCH_3$ bonded to pentose)	172.8 (C-6″); 161.5 (C-7); 161.1 (C-7′); 152.1 (C-4); 148.4 (C-3); 113.9 (C-5); 108.1 (H-5′); 110.3 (C-1′); 57.3 (C-8); 21.2 (C-7″)
p-coumaric acid (**7**)	7.599 (d, 1H, J = 15.8 Hz, H-8); 7.440 (d, 2H, J = 9.0 Hz, H-2,6); 6.806 (dd, 2H, J = 9.0 Hz, H-3,5); 6.272 (d, 1H, J = 15.8 Hz, H-7)	117.1 (C-2,6); 131.3 (C-3,5); 129.1 (C-1); 160.9 (C-4)

ellagic acid derivatives (e.g., P-22, P-23) whereas those at 306 nm show *trans*-piceid (P-18), phloretin glucoside (P-20), and hydroxycinnamic acid derivatives (e.g., P-7 and P-8) with greater sensitivity.

NMR and MS Approaches for Compound Identification. ^{1}H NMR spectra of all fractions were obtained and the chemical shifts of the resonances and their spectral patterns are reported in Table 1. In addition, ^{1}H 1D NMR (e.g., selective TOCSY and NOE difference) spectra, and J-correlated (gDQCOSY) and NOE/ROE based (NOESY/ROESY) $^{1}H-^{1}H$ homonuclear, 2D NMR experiments were used as well as heteronuclear 2D NMR techniques to determine connectivities and compound structures present in the fractions.

In MS analyses, the molecular ions were determined by full scan HPLC-ESI-MS or direct injection ESI-MS. ESI of nonanthocyanin-rich fractions often produced pseudo molecular ions of the form $(M-H)^-$ (negative ion mode) or $(M+H)^+/(M+Na)^+$ (positive ion mode). The negative ion mode MS was found to be more sensitive, and therefore the method of choice. Further structure verification was accomplished by tandem mass spectrometry (MS/MS), where fragment ions and loss of neutral species were important sources of structural information. For example, the neutral loss of hexose (e.g., glucose or galactose), deoxyhexose (e.g., rhamnose), pentose (e.g., xylose) and acetylpentose were indicated by the loss of 162, 146, 132, and 174 Da respectively.

Whenever possible, the HPLC-DAD, NMR, and MS data were also compared with literature values[18−31] and those of authentic standards. Examples of the application of these various methods to characterize specific compounds are described below.

Identification of Methyl Ellagic Acid Derivatives. The ^{1}H NMR spectrum (Figure 4) of a fraction containing P-22 shows resonances at 7.883 (s, H-5) and 7.696 (s, H-5′) ppm, each integrating to one proton, were consistent with the asymmetrically substituted ellagic acid (EA) moiety of methyl ellagic acid acetylpentose [(**17**), MEAAP]. Based on ^{1}H NMR resonances, their integrated areas, 2D NMR (Figure 5), and mass spectrometry data (Figures 6 and 7), the ^{1}H resonances at 4.050 and 2.159 were attributed to methoxy (H-8) and acetyl (H-7″) groups. The resonance at 3.784 ppm (s) from a methoxy group, and two aromatic resonances at 7.878 and 7.858 ppm could be from an additional methyl ellagic acid derivative. HMBC 2D NMR spectra (Figure 5) of P-22 showed multiple bond correlations from H-5 ($\delta^{13}C$ = 113.9 ppm) to

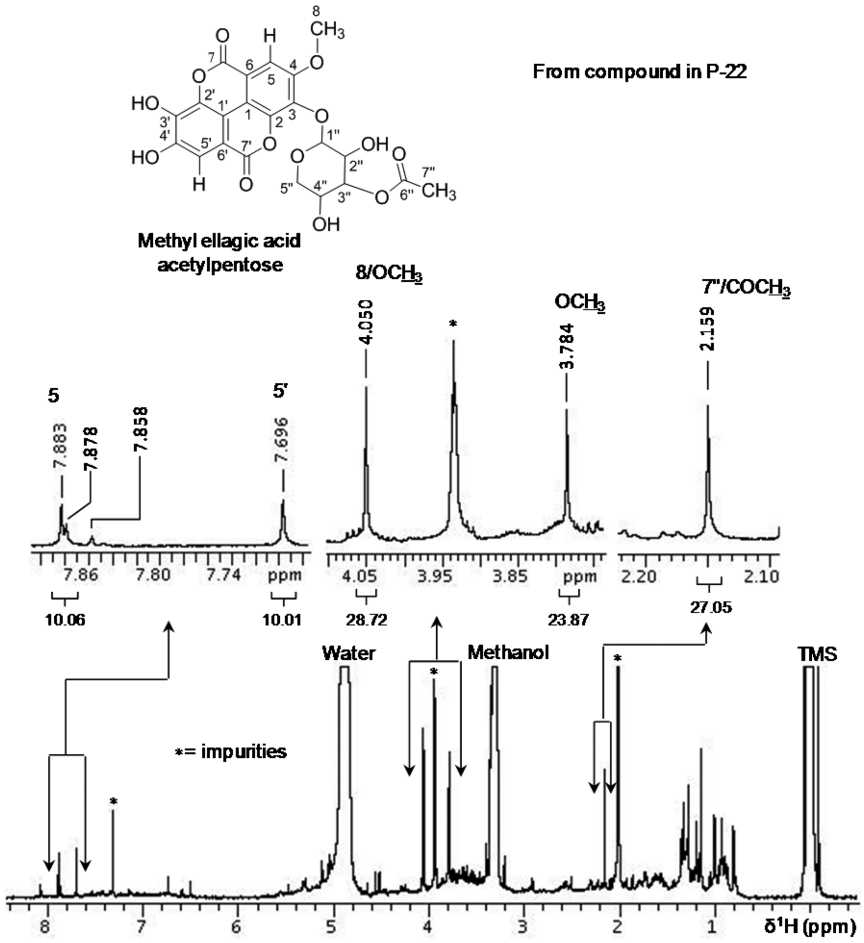

Figure 4. ^{1}H NMR spectrum and its expansions from a fraction containing P-22 showing the assignments of resonances from methyl ellagic acid acetylpentose (MEAAP, **17**).

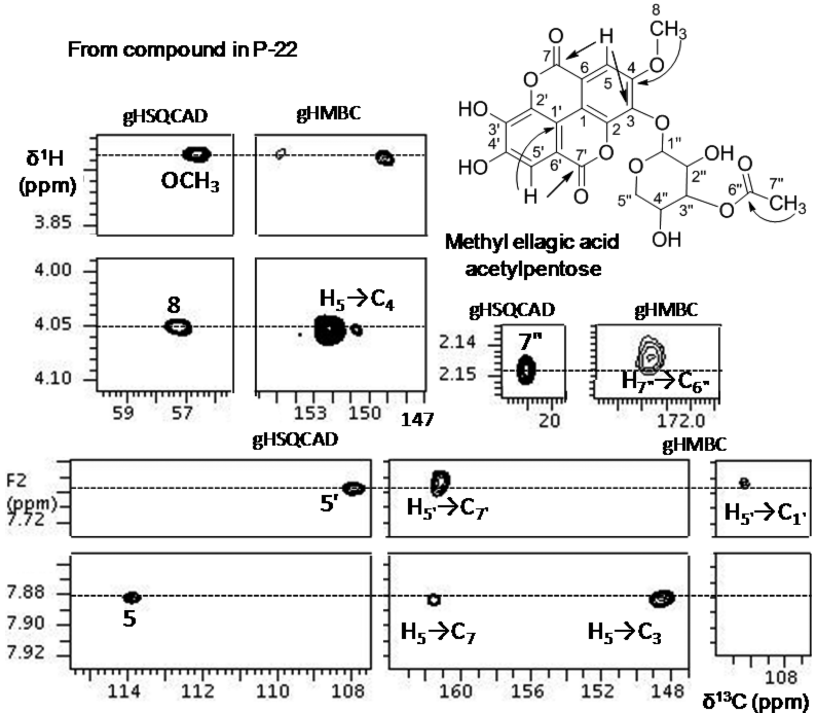

Figure 5. Selective regions from gHSQCAD and gHMBC spectra from the fraction containing P-22 showing single bond (HSQC) and multiple bond (HMBC) correlations from methyl ellagic acid acetylpentose (MEAAP, **17**).

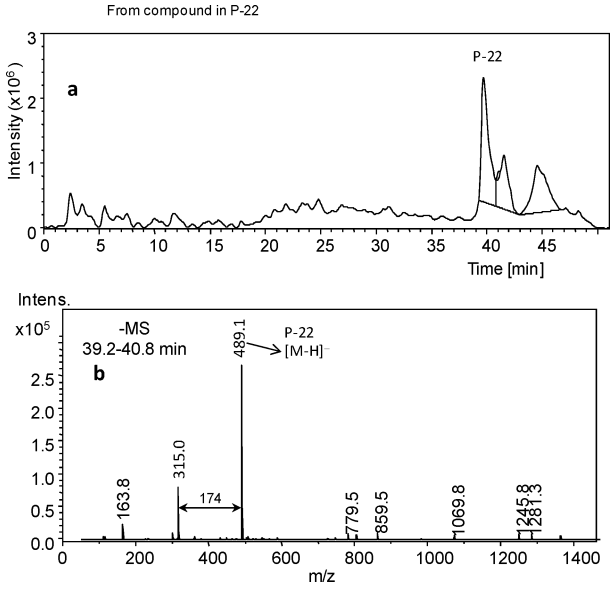

Figure 6. Negative ion mode (a) HPLC-ESI-MS total ion chromatogram (TIC) from the fraction containing P-22 and (b) full scan MS of P-22 (eluting between 39.2 and 40.8 min).

C-7 (δ^{13}C = 161.5 ppm) and C-3 (δ^{13}C = 148.4 ppm) and H-5′ (δ^{13}C = 108.1 ppm) to C-7′ (δ^{13}C = 161.1 ppm), and C-1′ (δ^{13}C = 110.3 ppm), consistent with the ellagic acid moiety. Protons from OCH_3 with a chemical shift of 4.050 ppm (with HSQC correlation to δ^{13}C = 57.3 ppm) are also correlated to another carbon in the HMBC spectrum at δ^{13}C = 152.1 ppm indicating a methoxy group attached to an aromatic carbon. Likewise, the multiple bond correlation from CH_3 (δ^{13}C = 21.2 ppm) protons at 2.159 ppm to a carbon at 172.8 ppm (consistent with a carbonyl carbon) indicated its attachment to the carbonyl carbon. The attachment of the acetyl (CH_3CO-) group to the sugar was confirmed on the basis of mass spectrometry.

Negative ion mode HPLC-ESI-MS total ion mode chromatogram (TIC) (Figure 6a) shows a peak (P-22) that elutes between 39.0 and 40.8 min. The full scan MS (Figure 6b) of P-22 shows two significant anions at m/z 489 [corresponding to MEAAP (**17**)], and m/z 315 (methyl ellagic acid, MEA). The MS^2 spectrum (Figure 7a) of the anion at m/z 489 shows a daughter anion at m/z 315 produced by the loss of an acetylpentose group (174 Da). The additional daughter anion at m/z 300, corresponding to the loss of CH_3 group from methyl ellagic acid, is confirmed from its own MS^2 spectrum (Figure 7b). These data are consistent with MEAAP in which an OCH_3 group is attached to ellagic acid (most probably on C-4, but could also be 3′ or 4′) and an acetyl group is attached to a pentose sugar (most probably C-3″, but could also be C-2″ or C-4″).

The presence of methyl ellagic acid valerylpentose (**18**, P-23) was confirmed by mass spectrometry. Negative ion mode HPLC-ESI-MS TIC (Figure 8a) shows a main peak (P-23) between 45.2 and 47.1 min. The full scan MS spectrum of P-23 shows three significant anions at m/z 531, 315, and 300 (Figure 8b). The MS^2 spectrum of the anion at 531 (Figure 8c) is very similar to the full scan MS of P-23; close inspection also shows a daughter anion at m/z 489 corresponding to methyl ellagic acid acetyl pentose, which is formed by the loss of 42 Da. Therefore, it is hypothesized that the anion at 531 m/z is methyl ellagic acid valerylpentose with a chain of three additional carbon atoms.

BR Secondary Metabolite Array. The rest of the non-anthocyanin constituents were identified in a similar manner (see Supporting Information Parts 1 and 2 and Figures S1–S39). The structures identified are shown in Figure 2, and the results of the HPLC-ESI-MS, ESI-MS/MS and NMR analyses of all compounds in this research are summarized in Tables 1 and 2. Within this set, quercetin 3-rutinoside [(**3**), Q 3-rut),[32] caffeic acid (**10**),[9] 3,4-dihydroxybenzoic (protocatechuic) acid (**15**),[7,9] epicatechin (**4**),[8] ellagic acid (**16**),[2,7,9,32] and *p*-coumaric acid (**7**)[32] have been previously reported in BR fruit. Casto and colleagues,[7] Khanal and colleagues,[8] Wu and

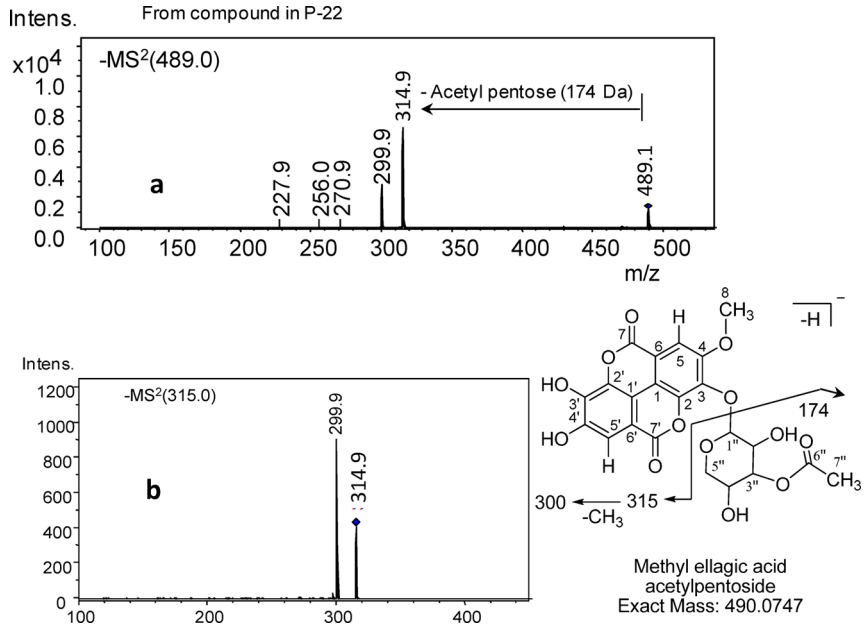

Figure 7. Negative ion mode MS^2 spectra of anions obtained from P-22 at (a) m/z 489 and (b) m/z 315.

dx.doi.org/10.1021/jf4039953 | J. Agric. Food Chem. 2013, 61, 12032–12043

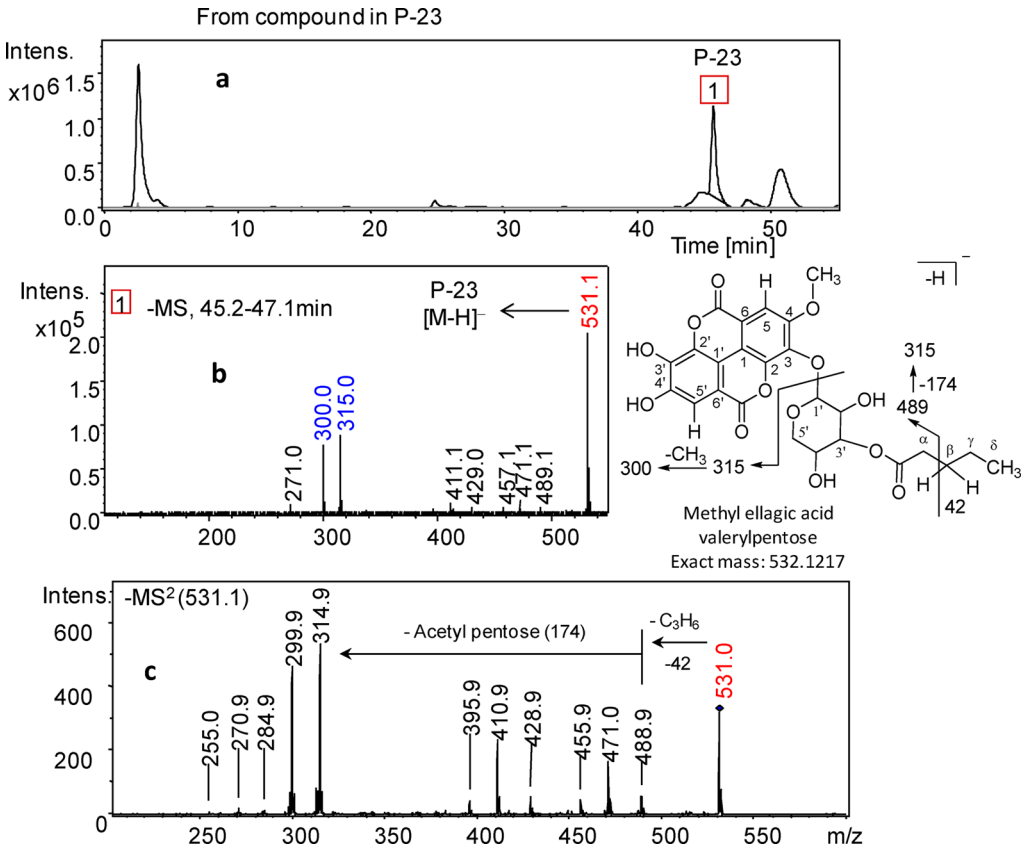

Figure 8. Negative ion mode MS: (a) HPLC-ESI-MS total ion chromatogram (TIC) of a BR fraction containing P-23, (b) full scan MS of P-23 (elution time = 45.2−47.1 min), and (c) MS/MS spectrum of the P-23 anion at 531 *m/z* (corresponding to methyl ellagic acid valerylpentose).

colleagues,[9] and Stoner and colleagues[2] also reported the phenolic metabolites, catechin, ferulic acid, chlorogenic acid, and quercetin aglycone (**1**) to be present in freeze-dried whole fruit powders.

Identified compounds not previously reported as non-anthocyanin metabolites of BR fruit include: quercetin 3-glucoside[(**2**) Q 3-glc], myricetin 3-glucoside (**6**), dihydrokaempferol glucoside (**5**), benzoic acid glucosyl ester [(**13**) BAGE], *p*-coumaryl glucoside (**8**), *p*-coumaryl sugar ester (**9**), MEAAP and methyl ellagic acid valerylpentose (**18**), phloretin glucoside (**22**), dihydrosinapic acid (**11**), salicylic acid glucosyl ester [(**12**), SAGE], and salicylic acid derivative [(**14**), SAD]. However, in their review, Lee and colleagues[33] cited studies reporting the presence of Q 3-glc, *p*-coumaryl sugar esters, kaempferol glycosides and other derivatives, sinapic acid, salicylic acid, methyl ellagic acid pentoses in other *Rubus* species. Also, Milivojevic and colleagues[34] reported myricetin to be present in a wild strain and two cultivars of red raspberry (*Rubus ideaus*) and Humpf and Schreier[35] identified free benzoic acid and benzyl alcohol in evergreen blackberry (*Rubus laciniata*). Cho and colleagues[36] found phloretin glucoside (phloridzin) in fruits of Korean black raspberry (*Rubus coreanus*) and Wu and colleagues[9] detected phloretic acid in the gastrointestinal tract of pigs fed freeze-dried BR fruit powder, but not in the powder itself.

To our knowledge, *p*-alkylphenyl *β*-D-glucopyranoside, *trans*-piceid and citric acid derivative have yet to be reported as constituents of *Rubus* fruits. The identities of the R-groups associated with the citric acid derivative (Figures S1, S4, S7, and S8 in Supporting Information) are currently under investigation. However, to verify that this compound was not an artifact of extraction, additional LC-MS/MS analyses ascertained that neither free citric acid nor methyl citrate were present in SPE-isolated, methanolic extracts of BR juice [Supplemental text (Part 3) in Supporting Information].

Although anthocyanins are major berry constituents that effectively impede multiple cancer developmental processes (e.g., chronic inflammation, cell proliferation, invasiveness) and promote apoptosis,[5] they likely act with other secondary metabolites to orchestrate these effects.[37] Nonanthocyanin secondary compounds such as flavonols[38] (e.g., Q 3-glc), flavan-3-ols[39] (e.g., epicatechin), flavanonols[40] (e.g., dihydrokaempferol), hydroxybenzoic acids[41,42] (e.g., protocatechuic acid, salicylic acid), hydroxycinnamic acids[43] (e.g, *p*-coumaric acid), ellagic acid and its derivatives and metabolites,[44] stilbenes[45] (e.g., piceid), dihydrochalcones[46] (e.g., phloretin) and citrate[47] have demonstrated definitive chemopreventive activity against various forms of cancer. In this study we found a wide variety of nonanthocyanin secondary metabolites with potential bioactivity present in BR fruits; fifteen of these compounds have not been reported previously to be BR fruit constituents. Since these metabolites are present at concentrations approximately 10^{-2} to 10^{-3} less than those of the major anthocyanins,[2] their structural characterization required the separation of components into individual or groups of compounds via HPLC followed by ^{1}H NMR, additional 1D and 2D NMR, HPLC-ESI-MS, and MS/MS analyses of compounds within fractions. Structures were resolved by combining complementary information from these analyses. Investigations similar to our BR study that identify the minor components in complex bioactive mixtures derived from fruits and vegetables are fundamental to metabolomic approaches exploring the potential synergistic

Table 2. Characterization of Nonanthocyanin Constituents of Black Raspberry Fruits using HPLC-ESI-MS and ESI-MS/MS

peak	compound	retention time (min)[a,b]	MS (m/z)[c]	MS/MS (m/z)[d]
P-1	citric acid derivative (**19**)[e]	n.d.	n.d.	n.d.
P-2	3.4-diydroxybenzoic acid (**15**)[f]	13.9	153/$[M-H]^-$ d.f.i. (−): 150/136/92	n.d.
P-3	salicylic acid β-D-glucopyranosyl ester (**12**)[f]	18.9	d.f.i. (+): 138/120	n.d.
P-4	benzoic acid β-D- glucopyranosyl ester (**13**)[f]	n.k.	n.d.	n.a.
P-5	*p*-alkylphenyl β-D- glucopyranoside (**21**)[f]	n.k.	n.d.	n.a.
P-7	*p*-coumaryl glucoside (**8**)[g]	22.7	325/$[M-H]^-$ d.f.i. (−): 163	n.a.
P-8	*p*-coumaryl sugar ester (**9**)[g]	22.7	355/$[M-H]^-$ d.f.i. (−): 175	n.a.
P-9	caffeic acid (**10**)[g]	23.1	179/$[M-H]^-$	n.a.
P-10	dihydrokaempferol glucoside (**5**)[g]	23.7	449/$[M-H]^-$ d.f.i. (−): 287/259/125	n.a.
P-12	epicatechin (**4**)[h]	24.1	289/$[M-H]^-$	271/245/227/205/179/151/125
P-13	salicylic acid derivative (**14**)[f]	n.d.	n.d.	n.a.
P-14	*p*-coumaric acid (**7**)[h]	14.0[i]	d.f.i. (+): 147	n.a.
P-15	quercetin 3-rutinoside (**3**)[h]	n.a.	609/$[M-H]^-$ 633/$[M+Na]^+$	343/301/271/255/179 487/331/325/243/185
P-16	dihydrosinapic acid (**11**)[j]	n.a.	475/$[2M+Na]^+$ 451/$[2M-H]^-$	457/431/365 433/333/225
P-17	quercetin 3-glucoside (**2**)[h]	n.a.	463/$[M-H]^-$	301/283/271/255/179/151
P-18	*trans*-piceid (**20**)[h]	29.8	389/$[M-H]^-$	227/185/183/159/157/143
P-19	ellagic acid (**16**)[e]	29.3	301/$[M-H]^-$	257/229
P-20	phloretin glucoside (**22**)[j]	35.4	435/$[M-H]^-$	273/217/167
P-21	myricetin glucoside (**6**)[j]	35.9	479/$[M-H]^-$	317/163
P-22	methyl ellagic acid acetylpentose (**17**)[h]	40.0	489/$[M-H]^-$	315/300/271/256/228
P-23	methyl ellagic acid valerylpentose (**18**)[h]	46.1	531/$[M-H]^-$	489/315/300/271 285/271

[a]Retention times using Bruker HPLC/MS detection; P-2, P-3 and P-6 to P-12 were determined using negative ion mode MS; all other values were acquired from positive MS scans. [b]MS detection (total ion mode); n.d. = not detected; n.a. = not analyzed; n.k. = not known. [c]Precursor ion/ion mode; d.f.i. = diagnostic fragment ion(s). [d]Product ions/MS. [e]Tentative identification based on NMR. [f]Identification based on NMR, pseudomolecular ion $[M-H]^-$ and/or in-source fragmentation. [g]Tentative identification based on pseudomolecular ion $[M-H]^-$ and/or ^{1}H NMR. [h]Identification based on NMR, pseudomolecular ions $[M-H]^-$/ $[M+H]^+$/ $[M+Na]^+$, and fragmentation pattern. [i]Measurement conditions differed from those described in Materials and Methods: mobile phases − (A) 0.1−1.0% formic acid in H_2O, (B) 0.1−1.0% formic acid in ACN; 1.5 mL/min; 35 °C; gradient −0−30% B/0−17 min, 30−95%B/17−22 min, 95−0%B 22−26 min; and QTof Premier HPLC. [j]Identification based on pseudomolecular ions $[M-H]^-$/ $[2M-H]^-$/[$M+H]^+$/ $[M+Na]^+$, and fragmentation pattern.

mechanisms that underlie the health-beneficial dietary effects of fresh and processed produce.

■ ASSOCIATED CONTENT

Supporting Information

Part 1 (methanolic extracts): ^{1}H selective TOCSY spectra of BR methanolic extracts and corresponding ^{1}H NMR spectral regions delineating structures or partial structures for compounds in P-1, P-3, P-4, P-5, P-13, P-15, and P-17 (Figures S1−S3). Parameters for 2D heteronuclear experiments and HSQC and HMBC spectra of BR methanolic extracts showing ^{1}H−^{13}C single bond and multiple bond correlations of compounds in P-1, P-3, P-4, P-15 and P-17 (Figures S4−S6); Part 2 (ethyl acetate extracts): the ^{1}H NMR spectra and the ^{1}H−^{13}C heteronuclear correlated 2D NMR spectra of a fraction containing P-1 and P-2 (Figures S7−S8); ^{1}H NMR spectrum, the gDQCOSY spectrum showing ^{1}H−^{1}H correlations (Figure S9) and HSQC and HMBC spectra (Figure S10) of the BR extract showing correlations of compounds in P-3; ^{1}H NMR spectrum, the gDQCOSY spectrum showing ^{1}H−^{1}H correlations (Figures S11−S12), and HSQC and HMBC spectra of BR extracts showing correlations of compounds in P-5 (Figure S13); LC-MS total ion chromatograph (TIC) in negative ion mode for P-6, P-7, P-8, P-9, P-10, and P-11 (Figure S14); the ^{1}H NMR spectrum, sections from gHSQCAD and gHMBC spectra, the HPLC-ESI-MS TIC in negative ion mode, and the MS/MS spectrum for P-12 (Figures S15−S18); the ^{1}H presaturation 1D NMR spectra, ^{1}H selective TOCSY 1D NMR spectra, and the gDQCOSY 2D NMR spectrum showing ^{1}H−^{1}H correlations for P-13 (Figure S19); the ^{1}H NMR spectrum and the 2D heteronuclear NMR gHSQCAD and gHMBC data for P-13 (Figure S20); the ^{1}H NMR spectrum, the selective 1D TOCSY spectra and cycled NOE spectrum, gHSQCAD and gHMBC spectra for P-14 (Figures S21−S24); the ^{1}H NMR spectrum, gHSQCAD and gHMBC spectra, the HPLC-ESI-MS TIC in negative and positive ion modes, and the positive ion MS/MS spectrum for P-15 (Figures S25−S28); HPLC-ESI-MS TIC in negative and positive ion modes for P-16 (Figure S27) and MS/MS spectra for P-16 (Figure S29); the ^{1}H NMR spectrum, the HPLC-ESI-MS TIC in negative ion mode and the positive ion MS/MS spectrum for P-17 (Figures S30−S31); the ^{1}H NMR spectrum, gHSQCAD and gHMBC spectra, the HPLC-ESI-MS TIC in negative ion mode, and the negative ion MS/MS spectrum for P-17, P-18 and P-19 (Figures S32−S36); the HPLC-ESI-MS TIC in negative ion mode and the negative ion MS/MS spectrum for P-20 and P-21 (Figures S37−S39). Part 3 (additional methanolic extracts): supplemental text verifying the absence of citric acid or methyl citrate. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +01(330)-972-5990. Fax: +01(330)-972-5256. E-mail: rinaldi@uakron.edu (P.L.R.). Phone: +01(419)-755-4342. Fax: +01(419)-755-4241. E-mail: wyzgoski.1@osu.edu (F.J.W.).

Funding

We thank the Kresge Foundation and the donors to the Kresge Challenge Program at the University of Akron for funds used to purchase the 750 MHz NMR instrument used in this work. Salaries and research support were provided in part by state and federal funds appropriated by the Ohio State University and the Ohio Agricultural Research and Development Center, and by U.S. Department of Agriculture Special Research Grants for Dietary Intervention 2005−38903−02313, 2006−38903−03560, 2007−38903−03560, 2008−38903−19245, 2009−38903−19901, and 2010−38903−20977. Salaries and supports were also provided in part by the South Dakota Agricultural experiment station.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge Deborah A. Miller for preparing the black raspberry methanolic extracts and Jinshan Lin for technical LC-MS/MS assistance.

■ REFERENCES

(1) Stoner, G. D.; Seeram, N. P. *Berries and cancer prevention*; Springer: New York, 2011.

(2) Stoner, G. D. Foodstuffs for preventing cancer: the preclinical and clinical development of berries. *Cancer Prev. Res.* **2009**, *2*, 187−194.

(3) Tulio, A. Z., Jr.; Reese, R. N.; Wyzgoski, F. J.; Rinaldi, P. L.; Fu, R.; Scheerens, J. C.; Miller, A. R. Cyanidin 3-rutinoside and cyanidin 3-xylosylrutionside as primary phenolic antioxidants in black raspberry. *J. Agric. Food Chem.* **2008**, *56*, 1880−1888.

(4) Wyzgoski, F. J.; Paudel, L.; Rinaldi, P. L.; Reese, R. N.; Ozgen, M.; Tulio, A. Z., Jr.; Miller, R.; Scheerens, J. C.; Hardy, J. K. Modeling relationships among active components in black raspberry(*Rubus occidentalis* L.) fruit extracts using high-resolution ^{1}H nuclear magnetic resonance(NMR) spectroscopy and multivariate statistical analysis. *J. Agric. Food Chem.* **2010**, *58*, 3407−3414.

(5) Wang, L.-S.; Stoner, G. D. Anthocyanins and their role in cancer prevention. *Cancer Lett.* **2008**, *269*, 281−290.

(6) Wang, L-S; Hecht, S. S.; Carmella, S. G.; Yu, N.; Larue, B.; Henry, C.; McIntyre, C.; Rocha, C.; Lechner, J. F.; Stoner, G. D. Anthocyanins in black raspberries prevent esophageal tumors in rats. *Cancer Prevention Res.* **2009**, *2*, 84−93.

(7) Casto, B. C.; Knoblock, T. J.; Weghorst, C. M. Inhibition of oral cancer in animal models by black raspberries and berry components. In *Berries and cancer prevention*; Stoner, G. D., Seeram, N. P., Eds.; Springer: New York, 2011; pp 189−207.

(8) Khanal, R. C.; Howard, L. R.; Wilkes, S. E.; Rogers, T. J.; Prior, R. L. Urinary excretion of (epi) catechins in rats fed different berries or berry products. *J. Agric. Food Chem.* **2010**, *58*, 11257−11264.

(9) Wu, X.; Pittman, H. E., III; Hager, T.; Hager, A.; Howard, L.; Prior, R. L. Phenolic acids in black raspberry and in the gastrointestinal tract of pigs following ingestion of black raspberry. *Mol. Nutr. Food Res.* **2009**, *53*, S76−S84.

(10) Johnson, J. L.; Bomser, J. A.; Scheerens, J. C.; Giusti, M. M. Effect of black raspberry (*Rubus occidentalis* L.) extract variation conditioned by cultivar, production site, and fruit maturity stage on colon cancer cell proliferation. *J. Agric. Food Chem.* **2011**, *59*, 1638−1645.

(11) Wrolstad, R.; Acree, T.; Decker, E.; Penner, M.; Reid, D.; Schwartz, S.; Shoemaker, C.; Smith, D.; Sporns, P. Characterization and measurement of anthocyanins by UV-visible spectroscopy. In *Handbook of Food Analytical Chemistry*; Wrolstad, R., Schwartz, S., Eds.; John Wiley & Sons, Inc.: Hoboken, NJ, 2005; pp 19−31.

(12) Maatta-Riihinen, K. R.; Kamal-Eldin, A.; Torronen, A. R. Identification and quantification of phenolic compounds in berries of *Fragaria* and *Rubus* species (family Rosaceae). *J. Agric. Food Chem.* **2004**, *52*, 6178−6187.

(13) Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymol.* **1999**, *299*, 152−178.

(14) Griesinger, C.; Otting, G.; Wuthrich, K.; Ernst, R. R. Clean TOCSY for ^{1}H spin system identification in macromolecules. *J. Am. Chem. Soc.* **1988**, *110*, 7870−7872.

(15) Kupce, E.; Freeman, R. Multiple Hartmann-Hahn coherence transfer in nuclear magnetic resonance spectroscopy. *J. Am. Chem. Soc.* **1992**, *114*, 10671−10672.

(16) States, D. J.; Haberkorn, R. A.; Ruben, D. J. A two-dimensional nuclear Overhauser experiment with pure absorption phase in 4 quadrants. *J. Magn. Reson.* **1982**, *48*, 286−292.

(17) Domitrovic, R. The molecular basis for the pharmacological activity of anthocyans. *Curr. Med. Chem.* **2011**, *18*, 4454−4469.

(18) Han, Y. N.; Choo, Y.; Lee, Y.-C.; Moon, Y.-I.; Kim, S.-D.; Chop, J.-W. Monoamine oxidase B inhibitors from the fruits of *Opuntia ficus-indica* var *saboten*. *Arch. Pharm. Res.* **2001**, *24*, 51−54.

(19) Anet, F. A. L.; Park, J. Proton chemical shift assignments in citrate and trimethyl citrate in chiral media. *J. Am. Chem. Soc.* **1992**, *114*, 411−416.

(20) Lee, I.-C.; Bae, J.-S.; Kim, T.; Kwon, O. J.; Kim, T. H. Polyphenolic constituents from the aerial parts of *Thymus quinquecostatus* var. *japonica* collected on Ulleung Island. *J. Korean Soc. Appl. Biol. Chem.* **2011**, *54*, 811−816.

(21) Khallouki, F.; Haubner, R.; Hull, W. E.; Erben, G.; Spiegelhalder, B.; Bartsch, H.; Owen, R. W. Isolation, purification and identification of ellagic acid derivatives, catechins, and procyanidins from the root bark of *Anisophyllea dichostyla* R. Br. *Food Chem. Toxicol.* **2007**, *45*, 472−485.

(22) Duennas, M.; Gonzalez-Manzano, S.; Surco-Laos, F.; lez-Paramas, A. G.; Santos-Buelga, C. Characterization of sulfated quercetin and epicatechin metabolites. *J. Agric. Food Chem.* **2012**, *60*, 3592−3598.

(23) Stoggl, W. M.; Huck, C. W.; Bonn, G. K. Structural elucidation of catechin and epicatechin in sorrel leaf extracts using liquid-chromatography coupled to diode array-, fluorescence-, and mass spectrometric detection. *J. Sep. Sci.* **2004**, *27*, 524−528.

(24) Keki, S.; Deak, G.; Zsuga, M. Fragmentation study of rutin, a naturally occurring flavone glycoside cationized with different alkali metal ions, using post-source decay matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* **2001**, *36*, 1312−1316.

(25) Hvattum, E. Determination of phenolic compounds in rose hip (*Rosa canina*) using liquid chromatography coupled with electrospray ionization tandem mass spectrometry and diode-array detection. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 655−622.

(26) Lim, S.-Y.; Hwang, J.-Y.; Yoon, H.-R.; Paik, Y.-S. Antioxidative phenolics from petals of *Carthamus tinctorius*. *J. Appl. Biol. Chem.* **2007**, *50*, 304−307.

(27) Moreno-labanda, J. F.; Mallavia, R.; Rez-fons, L. P.; Lizama, V.; Saura, D.; Micol, V. Determination of piceid and resveratrol in Spanish wines deriving from Monastrell (*Vitis vinifera L.*) grape variety. *J. Agric. Food Chem.* **2004**, *52*, 5396−5403.

(28) Justesen, U.; Knuthsen, P.; Leth, T. Quantitative analysis of flavonols, flavones, and flavanones in fruits, vegetables and beverages by high-performance liquid chromatography with photo-diode array and mass spectrometric detection. *J. Chromatogr., A* **1998**, *799*, 101−110.

(29) Aaby, K.; Ekeberg, D.; Skrede, G. Characterization of phenolic compounds in strawberry (*Fragaria* X *ananassa*) fruits by different HPLC detectors and contribution of individual compounds to total antioxidant capacity. *J. Agric. Food Chem.* **2007**, *55*, 4395−4406.

(30) Hilt, P.; Schieber, A.; Yildirim, C.; Arnold, G.; Klaiber, I.; Conrad, J.; Beifuss, U.; Carle, R. Detection of phloridzin in strawberries (*Fragaria* X *ananassa* Duch.) by HPLC-PDA-MS/MS and NMR spectroscopy. *J. Agric. Food Chem.* **2003**, *51*, 2896−2899.

(31) Jokapic, J.; Petkovsek, M. M.; Likojar, A.; Solar, A.; Stampar, F.; Veberic, R. HPLC−MS identification of phenols in hazelnut (*Corylus avellana* L.) kernels. *Food Chem.* **2011**, *124*, 1100−1106.
(32) Wada, L.; Ou, B. Antioxidant activity of phenolic content of Oregon caneberries. *J. Agric. Food Chem.* **2002**, *50*, 3494−3500.
(33) Lee, J.; Dossett, M.; Finn, C. E. Rubus fruit phenolic research: the good, the bad, and the confusing. *Food Chem.* **2012**, *130*, 785−796.
(34) Milivojevic, J.; Maksimovic, V.; Nikolic, M.; Bogdanovic, J.; Maletic, R.; Milatovic, D. Chemical and antioxidant properties of cultivated and wild *Fragaria* and *Rubus* berries. *J. Food Qual.* **2011**, *34*, 1−9.
(35) Humpf, H.-U.; Schreier, P. Bound aroma compounds from the fruit and the leaves of blackberry (*Rubus laciniata*). *J. Agric. Food Chem.* **1991**, *39*, 1830−1832.
(36) Cho, J.-Y.; Yoon, I.; Jung, D.-H.; Sook, H.-H.; Lee, K.-H.; Moon, H.-H.; Park, K.-H. Jaboticabin and flavonoids from the ripened fruit of black raspberry (*Rubus coreanus*). *Food Sci. Biotechnol.* **2012**, *21*, 1081−1086.
(37) Lila, M. A. From beans to berries and beyond − teamwork between plant chemicals for protection of optimal human health. In *Healthy aging and longevity*, Weller, N. J., Rattan, S. I. S., Eds.; Ann. NY Acad. Sci., Vol. *1114*; Wiley: Hoboken, NJ, 2007; pp 372−380.
(38) Warren, C. A.; Paulhill, K. J.; Davidson, L. A.; Lupton, J. R.; Taddeo, S. S.; Hong, M. Y.; Carroll, R. J.; Chapkin, R. S.; Turner, N. D. Quercetin may suppress rat aberrant crypt foci formation by suppressing inflammatory mediators that influence proliferation and apoptosis. *J. Nutr.* **2008**, *139*, 101−105.
(39) Butt, M. S.; Sultan, M. T. Green tea: nature's defense against malignancies. *Crit. Rev. Food Sci. Nutr.* **2009**, *49*, 463−473.
(40) Weidmann, A. E. Dihydroquercetin: more than just an impurity. *Eur. J. Pharmacol.* **2012**, *684*, 19−26.
(41) Masella, R.; Santangelo, C.; D'Archivio, M.; Li Volti, G.; Giovannini, C.; Galvano, F. Protocatechuic acid and human disease prevention: biological activities and molecular mechanisms. *Curr. Med. Chem.* **2012**, *19*, 2901−2917.
(42) Elwood, P. C.; Gallagher, A. M.; Duthie, G. G.; Mur, L. A. J.; Morgan, G. Aspirin, salicylates, and cancer. *Lancet* **2009**, *373*, 1301−1309.
(43) Zhang, Y.; Seeram, N. P.; Lee, R.; Feng, L.; Heber, D. Isolation and identification of strawberry phenolics with antioxidant and human cancer cell antiproliferative properties. *J. Agric. Food Chem.* **2008**, *56*, 670−675.
(44) Landete, J. M. Ellagitannins, ellagic acid and their derived metabolites: a review about source, metabolism, functions and health. *Food Res. Intl.* **2011**, *44*, 1150−1160.
(45) Juan, M. E.; Alfaras, I.; Planas, J. M. Colorectal cancer chemoprevention by trans-resveratrol. *Pharmacol. Res.* **2012**, *65*, 584−591.
(46) Wang, J.; Chung, M.-H.; Xue, B.; Ma, H.; Ma, C.; Hattori, M. Estrogenic and antiestrogenic activities of phloridzin. *Biol. Pharmacol. Bull.* **2010**, *33*, 592−597.
(47) Kruspig, B.; Nilchian, A.; Orrenius, S.; Zhivotovsky, B.; Gogvadze, V. Citrate kills cells through activation of apical caspases. *Cell. Mol. Life Sci.* **2012**, *69*, 4229−4237.