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NMR-Based Metabolomic Investigation of Bioactivity of Chemical Constituents in Black Raspberry (*Rubus occidentalis* L.) Fruit Extracts

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Supporting Information

ABSTRACT: Black raspberry (*Rubus occidentalis* L.) (BR) fruit extracts with differing compound profiles have shown variable antiproliferative activities against HT-29 colon cancer cell lines. This study used partial least-squares (PLS) regression analysis to develop a high-resolution ^{1}H NMR-based multivariate statistical model for discerning the biological activity of BR constituents. This model identified specific bioactive compounds and ascertained their relative contribution against cancer cell proliferation. Cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside were the predominant contributors to the extract bioactivity, but salicylic acid derivatives (e.g., salicylic acid glucosyl ester), quercetin 3-glucoside, quercetin 3-rutinoside, *p*-coumaric acid, epicatechin, methyl ellagic acid derivatives (e.g., methyl ellagic acetyl pentose), and citric acid derivatives also contributed significantly to the antiproliferative activity of the berry extracts. This approach enabled the identification of new bioactive components in BR fruits and demonstrates the utility of the method for assessing chemopreventive compounds in foods and food products.

KEYWORDS: *NMR, black raspberries, Rubus occidentalis L., cancer, anthocyanins, nonanthocyanins, citric acid, salicylic acid, phenolics, partial least-squares regression, metabolomics, chemoprevention*

INTRODUCTION

Secondary compounds (phytonutrients) in plant-based foods include well-known antioxidants[1] that function on several metabolic levels to reduce oxidative stress and to regulate signal transduction pathways, transcription factor function, gene expression, enzyme activity, and other cellular and subcellular processes that moderate inflammatory responses associated with the onset and development of chronic diseases.[2−8] To accomplish their roles, these bioactive compounds likely act in concert rather than independently.[4−6] Moreover, even simple fruit extracts may contain hundreds of secondary plant compounds from several compound classes, of which a select few may interact to induce a therapeutic response.[9] Therefore, using metabolomics or metabolomics-based approaches[10,11] involving partial least-squares (PLS) regression analysis and/or other statistical procedures[12] to identify specific bioactive phytonutrients and to define their role in moderating disease may have significant advantages over more traditional investigative techniques by which compound bioactivities are investigated sequentially.

Clinical research with animal/human models and subjects provides compelling evidence that black raspberries (BR, *Rubus occidentalis* L.) contain bioactive compounds that have complex chemopreventive properties with regard to aerodigestive (oral, esophageal, and colorectal) cancers[2,5−8] and other cancers.[2,13,14] Although these classical studies have identified several important chemopreventive constituents of BR derivatives including anthocyanins[2,7,8] and nonanthocyanin phenols such as ellagic acid, ferulic acid, gallic acid, *p*-coumaric acid, flavonols, and their derivatives,[5] the total impact of the treatments could not be accounted for by any single compound or single group of constituents.[2,7,15] Therefore, the body of evidence confirming the potential efficacy of BR-based clinical treatments and the current level of knowledge about their apparent bioactive constituents offered a unique opportunity to

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develop analytical model systems that delineate plant constituent−therapeutic response relationships.

To meet this challenge, we developed a model system that compares the ^{1}H NMR spectra of relatively crude BR extracts with the cellular proliferation of HT-29 human colon cancer cells.

Our approach to modeling linked variable bioassay results to variation in plant sample extract secondary metabolite complements and employed multivariate statistical analyses to identify specific regions of the extract NMR spectra that were associated with the array of biological responses. Once spectral regions responsible for these relationships were identified by the model, compound structures were verified using 2D NMR methods, such as heteronuclear single-quantum coherence (HSQC)[16] and heteronuclear multiple-bond correlation (HMBC)[17] experiments.

To validate this approach, we first ensured that BR extract profile variability could be achieved by capitalizing upon the genetic and environmental influences on fruit composition.[18,19] In addition, we verified that specific signals within complex NMR profiles responsible for known chemical relationships could be identified through multivariate statistical analyses. Specifically, we regressed BR extract NMR profiles against standard biochemical analyses [total monomeric anthocyanin content (TMA) and antioxidant capacity assays, ferric-reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazl (DPPH)].[20] This verification model successfully recognized the specific protons within the NMR spectra associated with anthocyanins that played significant roles in the activity of extracts in these assays. Furthermore, the model found structure−activity relationships to be statistically identifiable.

Here we have applied our system to model the biological relationship between bioactive compound variability among 73 BR extracts and their ability to inhibit HT-29 colon cancer cell proliferation. We previously reported the effects of these extracts on cancer cell cultures in this study.[21] The variable BR juice extracts inhibited HT-29 colon cancer cell growth broadly from 33 to 118% when included in the culture media at low and high doses of 0.6 or 1.2 mg extract/mL, respectively (Table S1 in the Supporting Information). Cell responses were dose-dependent, and the response arrays between doses were highly correlated. Although anthocyanins are known to contribute substantially to BR's chemoprotective properties,[2,5−8] extract anthocyanin concentrations as measured by TMA were not correlated to levels of growth inhibition, suggesting that nonanthocyanin components also contribute to BR extract bioactivity in the HT-29 colon cancer cell assay. Recently, we identified a cadre of nonanthocyanins present in our extracts, some of which had yet to be reported as BR constituents.[22]

In this paper, we present the description of anthocyanin as well as nonanthocyanin compounds identified by our modeling system that are responsible for the antiproliferative properties of BR extracts within the HT-29 colon cancer cell bioassay. Our model also indicates that compounds of various classes present in extracts over a broad concentration range may act together to affect cancer cell growth.

MATERIALS AND METHODS

Sample Acquisition and Extract Preparation. Marketable BR fruit samples of three cultivars ('Bristol', 'Jewel', or 'MacBlack') varying in ripeness (slightly under-ripe, ripe, or slightly over-ripe)[21] from seven Ohio commercial farms were obtained for this study. Fruit were hand-harvested in fiber-board containers and transported on ice to the Ohio Agricultural Research and Development Center. Sample extracts from 75 samples for both NMR and cell assay procedures were prepared as described by Johnson and coauthors.[21] This process included the following steps: sample handling, over-ripening fruit, sample storage, preparation of BR juices, isolation of secondary product constituent-rich methanolic extracts via solid phase extraction (SPE), evaporation of solvents under an N_2 stream, and freeze-drying of solvent-free SPE column eluates.

NMR Data Collection. All NMR studies of BR extract samples were performed with a Varian INOVA 750 MHz NMR spectrometer (Varian Inc., Palo Alto, CA, USA) using a Varian triple-resonance ^{1}H{^{13}C/^{15}N} pulsed field gradient cryoprobe. Solid materials derived from the column eluate were diluted with appropriate amounts of methanol-d_4/trifluoroacetic acid-d (95:5 v/v) and 5 mM 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) 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 and placed in 5 mm NMR tubes. All samples were stored at 4 °C prior to analysis. Two of the 75 samples were prepared incorrectly for NMR analysis and were excluded from further examination.

The spectra were collected at 25 °C over a spectral width of 9023 Hz using a 6.2 μs (90°) pulse. The acquisition time was 2.7 s, and 128 transients were accumulated with presaturation of the HDO resonance in the 4.91−5.07 ppm region. Data were processed with 0.5 Hz exponential line broadening and zero filled to 131,072 points before Fourier transformation.

NMR Data Binning. ^{1}H NMR free induction decays (FIDs) were processed as a batch, using KnowItAll Metabolomics software 8.1 (Bio-Rad Laboratories, Informatics Division, Philadelphia, PA, USA) with a Lorentzian apodization of 0.3 Hz, baseline correction with automatic base point detection, and spline fitting. The DSS reference peak was manually set to 0 ppm for each spectrum. After the region containing solvent (CD_3OD) resonances (3.257 −3.373 ppm) had been flat-lined, each spectrum was normalized against the DSS peak. Alignment macros were applied as a batch process to numerous specific locations along the spectrum to compensate for peak shifting due to variation in pH and in the constituent concentrations among samples. Prior to statistical analysis, binning/bucketing was conducted from 0.550 to 9.200 ppm using the Bio-Rad IntelliBucket method, where the bin width was automatically adjusted on the basis of the overlap density heatmap (ODH) consensus spectrum and a bin width setting of 0.004 ± 0.002 ppm. Regions that were excluded from binning were as follows: (1) 4.910−5.070 ppm (presaturation region for HDO); (2) 3.257−3.373 ppm (flat-lined for solvent); (3) highly overlapping peaks (3.220−4.000 ppm) of the saccharide region; and (4) regions throughout the spectrum of low signal/noise. However, all regions containing resonances for anomeric protons were included in the binning process. This resulted in a data set of 1003 bins from each of 73 BR extract samples that was further reduced to 464 bins by selecting bins with intensities above the analytically justified limit of quantitation[23] (LOQ, i.e., ≥10 times the standard deviation of intensity of bins from data representative of spectral noise).

PLS Modeling. The PLS regression-based predictive model building using Pirouette 4.0 (Infometrix, Bothell, WA, USA) started with the 464 bin data sets. Peak intensities of bins for each of the 73 BR samples were regressed against the bioassay responses[21] as shown in Table S1 in the Supporting Information. A maximum number of 15 factors and a probability threshold of 0.95 were set. Two-component orthogonal signal correction[24] (OSC) was used to remove components in our large X block that were orthogonal to the Y block, thus segregating the portion of the X block that was correlated. To aid the interpretation of chemical information from the regression models, one subset of relevant variables (NMR bins) was selected from the entire block of X-variables. The criteria for variable selection were software-dependent measures of fit [prediction residual error sum of squares (PRESS) and r]. The regression vector values from this subset of bins were then ranked from highest to lowest for the low- and high-dose bioassays, respectively.

RESULTS AND DISCUSSION

Classical biochemical studies of chemoprevention and cancer cell antiproliferation, in response to berry fruits and BR in particular, are challenging because several berry fractions and many specific secondary products have been shown to have some effect on the cancer cell performance, but no single fraction or compound can account for the efficacy of whole fruit or crude fruit extracts.[2,7] Our approach uses "omics" and bioinformatic procedures to identify active components by NMR spectroscopy and to link them to their impact on cell proliferation in HT-29 colon cancer cells in a manner similar to that described by Leiss et al.[25] and Gavaghan et al.[26] to determine important effectors of host-plant resistance and maize salt stress, respectively. Because many BR secondary compounds have been shown to have roles in BR regulation of cancer cell development, there exists a large body of data for the potential verification of the compounds identified by the model.

Modeling of secondary product chemistry, in conjunction with medical efficacy, provides an opportunity to examine complex mixtures of secondary products that are present in plant materials and determine how they affect cell health, chemoprevention, and/or cancer cell proliferation. NMR is a powerful method used to elucidate chemical structures of molecules; here we have used it to identify components of complex mixtures of small molecule metabolites simultaneously, at a high resolution. Moreover, samples were readily prepared in a reproducible manner without the need for additional separation techniques. Processing the data with KnowItAll statistical software permitted the BR components that play significant roles in the control of HT-29 cell proliferation to be identified.

Our model generated large blocks of data concerning the chemical nature of the extracts, which were evaluated against a complex series of chemical reactions that we monitored as cell proliferation. The impact of these secondary compounds on cell proliferation represents the sum of all of the reactions controlling cell growth, metabolism, and apoptosis that have been demonstrated by a host of research.[2,5,7,8,15]

^{1}H 1D-NMR Analyses. Model development was initiated by obtaining the ^{1}H NMR spectra of the 73 BR extracts. The spectra of three representative BR extract samples are shown in Figure 1 [a, slightly under-ripe sample (BR-2); b, ripe sample (BR-47); and c, slightly over-ripe sample (BR-28)]. In all ^{1}H spectra of BR extract samples, the HDO resonance from water that had been observed in the 4.91−5.07 ppm region was suppressed using a presaturation pulse sequence. The singlet peak at 0.0 ppm is from DSS, which was added as an internal standard for chemical shift measurement as well as a calibration standard for quantitation. The strong peaks centered at 3.31 ppm (quintet), 3.35 ppm (singlet), and 3.97 ppm (singlet) are attributed to the resonances of residual $C\underline{H}D_2OD$ from methanol-d_4, a solvent-like impurity, and an artifact compound, respectively. The resonances in the complex region, 4.00−3.22 ppm, are attributed to nonanomeric resonances from the saccharides that are attached to the phenolic structures. The suppression of these signals and the regions of low signal-to-noise ratios (see Materials and Methods) in the initial data sets were made to ensure a robust analysis.

The spectra of BR extract samples are dominated by the resonances from cyanidin 3-glycosides (**1**, Figure 2) as well as defining peaks for cyanidin 3-rutinoside (Cy 3-rut, **2**), cyanidin 3-xylosylrutinoside (Cy 3-xylrut, **3**), cyanidin 3-glucoside (Cy 3-glc, **4**), and cyanidin 3-sambubioside (Cy 3-sam, **5**), the major phenolic antioxidants of BR fruits.[19] The unique resonances from the minor anthocyanin, pelargonidin 3-rutinoside, are also detected. In Figure 1, the resonances from these anthocyanin phenolics are indicated in purple bars above the chemical shift scale.

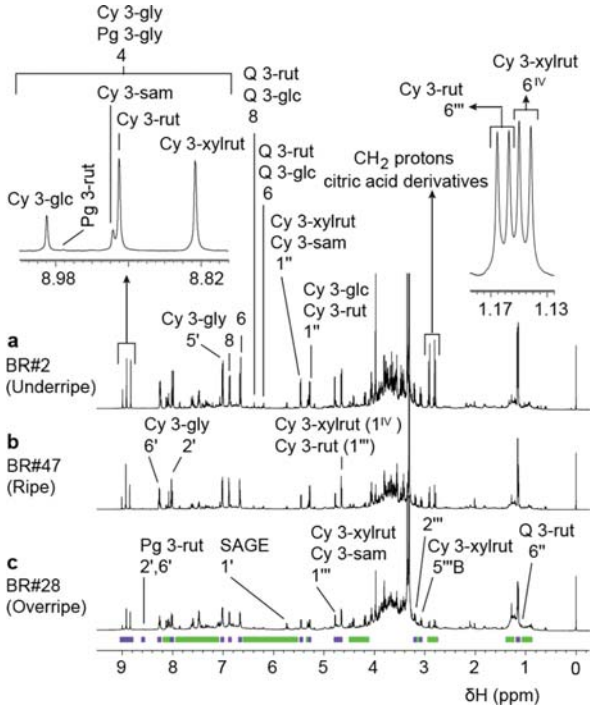

Figure 1. 750 MHz ^{1}H NMR spectra of three representative methanolic BR juice extract samples: (a) sample BR-2 from slightly under-ripe berries; (b) sample BR-47 from ripe berries; (c) sample BR-28 from slightly over-ripe berries. Resonances from anthocyanins are indicated in purple and those from nonanthocyanins are indicated in green bars above the chemical shift scale.

Several resonances from nonanthocyanin metabolites and the aliphatic moieties associated with citric acid derivatives (**6**) were also observed (Figure 1, indicated in green bars above the chemical shift scale). The structures of nonanthocyanin phenolics, which were readily detected in the ^{1}H NMR spectra of BR extracts, are shown in Figure 3. Compounds based on the quercetin moiety (**7**, Q, where R_n = H) included quercetin 3-glucoside (Q 3-glc, **8**) and quercetin 3-rutinoside (Q 3-rut, **9**). Compound **11**, salicylic acid glucosyl ester (SAGE), where the glucose moiety is attached to a salicylic acid (SA, **10**), was also evident. Additional nonanthocyanin compounds that have been detected in the ^{1}H NMR spectra of BR extract samples are epicatechin (**12**), benzoic acid β-D-glucopyranosyl ester (BAGE, **13**), a salicylic acid derivative (SAD, **14**), *p*-coumaric acid (**15**), and methyl ellagic acid acetylpentose (MEAAP, **16**).

Although these minor metabolites are present at concentrations approximately 10^{-2}−10^{-3} times the concentrations of the cyanidin 3-glycosides,[5] some of their proton resonances are clearly visible in the ^{1}H NMR spectra of the BR extracts (Figure 1). For example, the H-8 and H-6 resonances from the quercetin glycosides, namely, Q 3-rut and Q 3-glc, are seen at 6.39 and 6.20 ppm, respectively. Likewise, for SAGE, one of the readily detected resonances is H-1′ (5.73 ppm), the anomeric proton resonance from the glucose attached to the phenolic structure via an ester linkage.

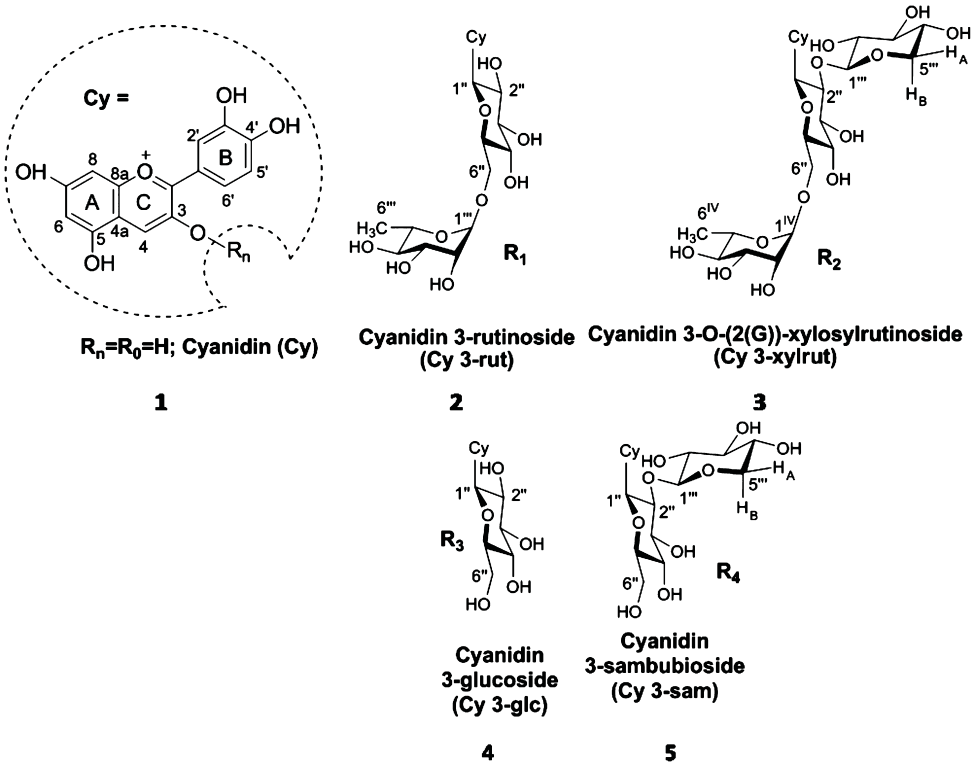

Figure 2. Structures of the cyanidin aglycone and four major anthocyanin phenolic compounds of black raspberry. **1**, cyanidin aglycone (Cy); **2**, cyanidin 3-rutinoside (Cy 3-rut); **3**, cyanidin 3-xylosylrutinoside (Cy 3-xylrut); **4**, cyanidin 3-glucoside (Cy 3-glc); and **5**, cyanidin 3-sambubioside (Cy 3-sam).

Our model simultaneously analyzed a number of regions of the 1D ^{1}H NMR spectra to ensure that participating components of the BR could be identified. This approach was demanding, because several proton resonances can be associated with one compound and intensities may differ depending on the number of protons in the compound structural unit. In addition, similarities in structures (e.g., phenolics) can result in overlapping peaks yielding complex spectral patterns. To verify structures, especially for compounds present at low concentrations, additional NMR experiments such as 1D selective total correlation spectroscopy, TOCSY, and 2D-NMR (gDQCOSY, gHSQCAD, and gHMBC) were conducted (Figures S1−S11 in the Supporting Information). Furthermore, during the statistical analysis, the NMR data were subjected to Pareto scaling,[27] where the square root of the standard deviation was used as a scaling factor. This minimized the effect of larger intensities in the presence of much smaller intensities.

To make regression modeling possible, we needed large amounts of variation in the extracts with a corresponding amount of variability in the cancer cell responses. This methodology differs from traditional medicinal chemistry approaches in which consistency in treatments is essential to understanding experimental outcomes. To verify the variability among our samples, the concentrations (μM) of cyanidin structures were obtained for all 73 spectra on the basis of the manual integration of the H-4 resonances using Bio-Rad's ProcessIt NMR application. Normalized peak areas were obtained relative to the integration of the CH_3 peak of DSS calibration standard at 0.0 ppm set to 100. These data revealed that the concentrations of the anthocyanin components in the 73 BR extract samples exhibited the desired high level of variability needed for our modeling efforts (Table S2 in the Supporting Information). In this study, Cy 3-xylrut was the most abundant phenolic component of the berry extracts (mean = 58 μM; CV = 34%), and the second most abundant component was Cy 3-rut (mean = 51 μM; CV = 31%). In general, the amounts of Cy 3-xylrut and Cy 3-rut were higher in ripe samples than in the under-ripe samples, whereas the amounts of Cy 3-glc and Cy 3-sam (mean = 24 μM, CV = 52%; and mean = 13 μM, CV = 40%, respectively) both decrease with increasing ripeness.

Bioactivity Model Development. Initially, a model of the low-dose assay was generated with a data set of 464 bins determined using Pareto scaling and leave-one-out cross-validation. NMR bins were ranked according to their regression vector values. Subsets of the highest ranked bins were then created and modeled against the low-dose bioassay to determine the best fit of the NMR spectra to the extract impact on cell proliferation. Figure 4a shows a plot of PRESS as well as r values against model sets with various numbers of X-variables. Figure 4b shows the corresponding line plot between model factors and number of bins. The arrows A_4 and A_5 represent the regions of minimum PRESS and maximum r, respectively. Because the maximum number of bins does not exceed 2 times the number of samples, the minimum value of PRESS, maximum value of r, and minimum number of model factors are the quality indices of a good model. A compromise among these quality indices indicated that the optimum number of bins for the low-dose bioassay was 125 (Figure 4, indicated by arrow A_5). The indicators of the fit for this five-factor model are shown in Table 1. This model has r and slope values approaching 1 and values of the standard error of cross-validation (SECV) and PRESS that approached their minima.

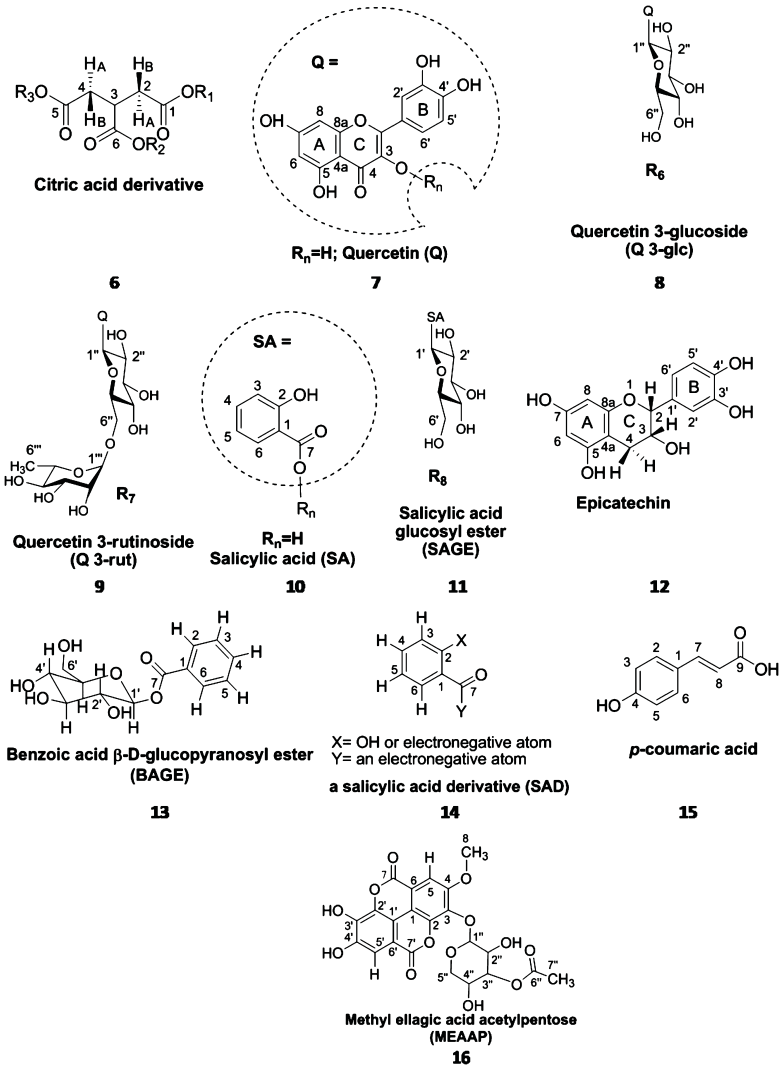

Figure 3. Structures of a citric acid derivative and nonanthocyanin phenolics detected in BR juice extract samples and their aglycones. **6**, citric acid derivatives; **7**, quercetin aglycone (Q); **8**, quercetin 3-glucoside (Q 3-glc); **9**, quercetin 3-rutinoside (Q 3-rut); **10**, salicylic acid aglycone; **11**, glucose ester of salicylic acid (SAGE); **12**, epicatechin; **13**, glucose ester of benzoic acid (BAGE); **14**, salicylic acid derivative (SAD); **15**, *p*-coumaric acid; and **16**, methyl ellagic acid acetylpentose (MEAAP).

In addition, Figure 5a shows the goodness of the *Y*-fit between predicted and measured values. The random distribution of the residuals (Figure 5b) also indicates that the assumption of linearity between the *X*- and *Y*-variables is warranted.

When the top 125 bins of this model were ranked according to their regression vector values, the contribution of specific compounds became evident. Because regression vectors are the weighted sum of loadings included in the model, they reveal the importance of each independent variable. These predictor variables are the basis for interpreting the effect of NMR bins (our independent variables), which carry the information about the chemical components, on the dependent variable, percent inhibition of cell proliferation. For the low-dose bioassay, Table 2 shows some of these compounds by their defining atom assignments and their rank/bin number, with corresponding atomic assignments being illustrated in Figures 2 and 3. The complete table of all 125 bin assignments can be found in Table S3 in the Supporting Information. In Table 2, specific bins corresponding to each of the four major anthocyanins in BR are shown to be important with Cy 3-xylrut and Cy 3-rut as the top-ranked. Cy 3-sam and Cy 3-glc also have an impact on bioassay results. A large number of bins were attributed to resonances of these cyanidin glycosides.

Bins corresponding to nonanthocyanin compounds were also shown to augment inhibition of cell proliferation in the HT29 low-dose bioassay (Table 2). BR anthocyanins and nonanthocyanin phenolic compounds share some similarities in structure (Figures 2 and 3); therefore, their ^{1}H NMR spectra (Figure 1) would be expected to contain some overlapping resonances. Although this overlap confounded compound identification, defining bins were found to be important for MEAAP, epicatechin, Q 3-rut, Q 3-glc, *p*-coumaric acid, SAGE, a salicylic acid derivative (SAD), BAGE, and citric acid derivatives.

When a model was calculated for the high-dose bioassay, similar but not identical results were obtained. This model was optimized at 100 bins (Figure S12 in the Supporting Information), and indicators of fit are shown in Table 1 and Figure 5c,d. When the 100 bins were ranked according to their regression vector values, a trend similar to the low-dose results was evident (Table 3 and Table S4 in the Supporting Information). The highest ranked

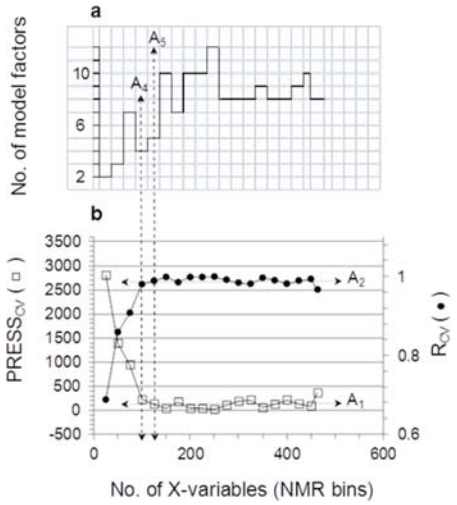

Figure 4. Partial least-squares (PLS) plots for the selection of *X*-variables (NMR bins): (a) number of factors; (b) indicators of fit. Arrow A_1 represents the regions of minimum prediction residual error sum of squares (PRESS) values, and arrow A_2 represents the regions of maximum *r* values. The vertical arrow indicated by A_5 represents the subset of optimum of *X*-variables and its PLS-derived low-dose model factors.

Table 1. Pirouette PLS-Derived Summary of Fit Measurements for the Low- and High-Dose Bioassay Activity of BRs

statistical parameter	low-dose bioassay[a]	high-dose bioassay[b]
no. of BR samples	73	73
no. of independent variables (NMR bins)	125	100
SECV[c]	1.3353	0.9338
PRESS[d]	130.1561	63.6579
r[e]	0.9878	0.9953
factors	5	5
slope	0.9897	0.9949
intercept	0.6496	0.4723

[a]Data are taken from the best PLS model with 125 highest ranked *X*-variables (NMR bins) from the first test model. [b]Data are taken from the best PLS model with 100 highest ranked *X*-variables (NMR bins) from the first test model. [c]SECV, standard error of cross-validation. [d]PRESS, prediction residual error sums of squares. [e]*r*, correlation coefficient.

bin corresponded to Cy 3-xylrut and a defining bin for Cy 3-rut was ranked 11th. Bins consistent with Cy 3-sam and Cy 3-glc were also evident as well as a large number of bins that corresponded to resonances of cyanidin glycosides. The same nonanthocyanin compounds that affected the low-dose model were also evident. However, the rankings of these compounds, especially a citric acid derivative, differed from those in the low-dose model (Table 3). In general, many of the nonanthocyanin constituents appeared to gain in predictive value in the high-dose model.

Overall, the importance of the nonanthocyanins was evident. As shown in Tables 2 and 3, of our original 1003 bins for each of 73 samples, the rankings of the NMR bins based on their regression vector values ranged from 30 to 121 for the low-dose bioassay and from 6 to 81 for the high-dose bioassay. Furthermore, error analyses of PLS models that were calculated using only anthocyanin-related bins proved to be less than optimal when compared to our PLS models containing both anthocyanin- and nonanthocyanin-related bins.

Model-Identified Cell Antiproliferative Compounds. As with other "omics" approaches, the cell assay (our *Y*-variable), provided a simple phenotypic-like response to the treatments, here, control of the HT-29 colon cancer cell proliferation. However, just as a simple phenotypic response is controlled by changes in many overlapping and/or independent biochemical pathways, cell proliferation reflects the combined impacts of the extract components on the stimulation of apoptosis, regulation of cell cycle gene expression, and the impact of BR extract components on overlapping and independent biochemical pathways. Therefore, the model must integrate all of these interactions to determine the impact of the extracts on cancer cell growth. This integration then provides data to identify compounds that affect any of a myriad of chemical reactions.

Cy 3-xylrut and Cy 3-rut were identified by the model as the most important contributors to the inhibition of proliferation of HT-29 colon cancer cells. Anthocyanins constitute a major group of phenolic antioxidants produced in most small fruits.[28] According to Stoner,[5] anthocyanins are present in BR at concentrations that exceed those of ellagic acid by approximately 15-fold and of all other phenolic materials by factors of 10^2 or 10^3. BR anthocyanins are known to effectively intercede against various forms of aerodigestive cancers[2,5−8] at several stages by initiating and maintaining the enzymatic defenses against oxidative stress, reducing inflammation, and inhibiting mutagenesis, as well as decreasing cell proliferation and stimulating apoptosis.[8] BR anthocyanins or anthocyanin-rich fractions affect these chemopreventive processes at cellular and subcellular levels by direct interaction or interaction with controlling elements of kinase-based signaling cascades that influence gene expression, mRNA translation, or enzymatic activity of key cancer-related factors.[2,4−8]

However, the model data also support our previous conclusion that the antiproliferative effectiveness of these BR extracts is not directly correlated with their TMA contents.[21] When we compared the NMR-determined concentrations of individual anthocyanins herein with the low- and high-dose bioassay data using a simple Pearson correlation analysis, only the Cy 3-rut correlation values ($r = -0.32$ and $r = -0.31$, respectively) were significant ($p < 0.01$). However, this relationship was weak, suggesting that the effects of BR extracts on HT-29 colon cancer cell proliferation arise from the activity of multiple compounds in concert. Moreover, bins associated with protons of nonanthocyanin constituents appeared to gain in importance in the anthocyanin-rich extract/high-dose assay PLS model over their position in the model constructed with low-dose assay data developed with extracts containing lower levels of anthocyanins. Together, these findings suggest that anthocyanins may reach a maximum effect on cells and that further increases in their total content or alterations in anthocyanin profiles may not increase the activity of the specific extract.

Supporting this contention, Seguin and co-workers[29] recently reported four temperate berries (BR, blueberry, red raspberry, strawberry) and a cyanidin-containing tropical fruit acai (*Euterpe oleraceae*), presumably with differing anthocyanin profiles, to be similar in their antiproliferative activities. In a study by Wang and co-workers,[7] dietary treatments with freeze-dried BR powder, an aqueous ethanol extract, and an anthocyanin-enriched subfraction of the extract were found to affect rat esophageal papilloma formation and mass, proteins associated with proliferation, and markers of apoptosis similarly.

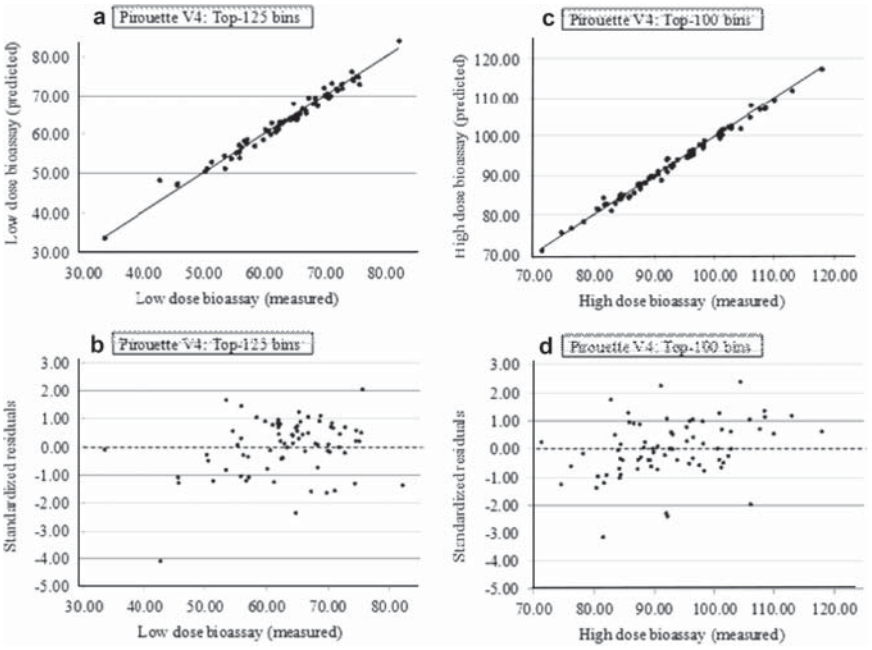

Figure 5. *Y*-fits and residual plots for the models produced with low-dose (a, b) and high-dose (c, d) bioassays.

Table 2. Pirouette PLS Model Developed with the Top 125 Bins Showing Bin Assignments, Chemical Shifts, and Rankings Based on Regression Vector Values for the Assessment of Low-Dose Bioassay Activity of Black Raspberry Extracts

species	group (atom no.)	δ^1H range (ppm)	rank/bin no.
Cy 3-xylrut (cyanidin 3-xylosylrutinoside)	CH_3 (6^{IV})	(1.147−1.142) (1.139−1.134)	1/425, 2/427
		(1.134−1.128) (1.142−1.139)	18/428, 36/426
	CH (4)	(8.848−8.842)	10/9
Cy 3-rut (cyanidin 3-rutinoside)	CH_3 (6‴)	(1.155−1.149) (1.163−1.158)	4/423, 9/421
		(1.158−1.155)	41/422
	CH (4)	(8.923−8.918)	19/5
Cy 3-sam (cyanidin 3-sambubioside)	CH (2‴)	(3.154−3.148)	99/313
Cy 3-glc (cyanidin 3-glucoside)	CH(1″)	(5.302−5.296)	103/196
SAGE (glucose ester of salicylic acid)	CH (3, 5)	(7.070−7.064) (7.099−7.097)	30/132, 79/130
		(7.061−7.059)	112/134
	CH (1′)	(5.739−5.733)	71/174
	CH (6)	(8.065−8.059) (8.069−8.065)	106/40, 119/39
		(8.055−8.049)	120/43
BAGE (glucose ester of benzoic acid)	CH (2,6)	(8.083−8.078) (8.089−8.083)	33/36, 59/35
	CH (4)	(7.626−7.622)(7.622−7.616)	53/70, 73/71
citric acid derivatives	CH_2 (2B,4B)	(2.806−2.800) (2.785−2.779)	42/350, 57/356
	CH_2 (2A, 4A)	(2.899−2.893) (2.920−2.915)	43/341, 62/335
methyl ellagic acid derivative	OCH_3	(4.055−4.050)	48/292
SAD (salicylic acid derivative)	CH (6)	(8.099−8.093) (8.128−8.125)	67/32, 109/25
epicatechin	CH (3 equiv)	(4.172−4.166) (4.189−4.183)	66/271, 85/265
p-coumaric acid	CH (8, olefinic)	(7.608−7.602)	94/75
Q 3-rut (quercetin 3-rutinoside)	CH (1‴)	(4.526−4.521)	50/238
	CH_3 (6‴)	(1.110−1.104) (1.104−1.098)	64/432, 80/433
		(1.118−1.112) (1.122−1.118)	91/430,121/429
	CH (6′)	(7.630−7.626)	63/69
Q-3-glc (quercetin 3-glucoside)	CH (6′)	(7.598−7.592)	100/78

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Table 3. Pirouette PLS Model Developed with Top 100 Bins Showing Bin Assignments, Chemical Shifts, and Rankings Based on Regression Vector Values for the Assessment of High-Dose Bioassay Activity of Black Raspberry Extracts

species	group (atom no.)	δ^1H range (ppm)	rank/bin no.
Cy 3-xylrut (cyanidin 3-xylosylrutinoside)	CH_3 (6^{IV})	(1.147−1.142) (1.139−1.134)	1/425, 3/427
		(1.134−1.128) (1.142−1.139)	30/428, 41/426
	CH (4)	(8.848−8.842)	10/9
Cy 3-rut (cyanidin 3-rutinoside)	CH_3 (6‴)	(1.155−1.149) (1.163−1.158)	11/423, 19/421
		(1.158−1.155)	90/422
	CH (4)	(8.923−8.918)	22/5
Cy 3-sam (cyanidin 3-sambubioside)	CH (2‴)	(3.154−3.148)	88/313
Cy 3-glc (cyanidin 3-glucoside)	CH (1″)	(5.302−5.296)	98/196
SAGE (glucose ester of salicylic acid)	CH (3, 5)	(7.070−7.064)	33/132
	CH (6)	(8.065−8.059)	77/40
BAGE (glucose ester of benzoic acid)	CH (2, 6)	(8.083−8.078) (8.089−8.083)	81/36, 57/35
citric acid derivatives	CH_2 (2B, 4B)	(2.806−2.800) (2.785−2.779)	6/350 12/356
	CH_2 (2A, 4A)	(2.899−2.893)	9/341
methyl ellagic acid derivative	OCH_3	(4.055−4.050)	60/292
SAD (salicylic acid derivative)	CH (6)	(8.099−8.093) (8.128−8.125)	51/32, 72/25
epicatechin	CH (3 equiv)	(4.172−4.166) (4.189−4.183)	56/271, 52/265
p-coumaric acid	CH (8, olefinic)	(7.608−7.602)	50/75
Q 3-rut (quercetin 3-rutinoside)	CH (6′)	(7.630−7.626)	68/69
Q-3-glc (quercetin 3-glucoside)	CH (6′)	(7.598−7.592)	45/78

Wang and co-workers[7] also noted similar antiproliferative properties associated with a diet formulated with the insoluble residue of the extraction with anthocyanin concentrations reduced by approximately 200-fold. These authors noted that among the potential nonanthocyanin constituents of the extract insoluble residue, ellagitannins (polymers of ellagic acid) were likely responsible for the chemoprotective effects of the residue diet. Due to their low solubility in methanol, ellagitannins were likely to be present at minimal concentrations in our extracts. However, our model clearly identified MEAAP as one of the important antiproliferative components of BR extracts. The metabolism and chemoprotective properties of ellagic acid and its polymers, derivatives, and colonic microflora-generated metabolites (urolithins, a series of bioactive dibenzopyran-6-one biomarkers of ellagic acid consumption) were recently reviewed by Landete.[30] Similar to other polyphenols, these compounds act chemoprotectively by reducing the activity of signaling cascades, limiting inflammatory responses, controlling cell cycling and proliferation, reducing angiogenesis, and stimulating apoptosis.[8,30]

Our model also identified epicatechin, Q 3-glc, Q 3-rut, SAGE, SAD, BAGE, and *p*-coumaric acids as important BR extract components in the control of HT-29 cell proliferation. Flavan-3-ols (e.g., epicatechin), flavonol glycosides (e.g., Q 3-glc, Q 3-rut), and salicylates (e.g., SAGE, SAD) are powerful phenolic antioxidants that have been shown to have chemopreventive properties against colon cancer[31−34] and other types of cancers.[3,6,31,32,34,35] In vitro cell assays and animal studies have shown these compounds inhibit proliferation or tumor growth by controlling cell cycle genes and enzymes and by promoting genes and enzymes associated with apoptosis. In a study of bioactive constituents isolated from freeze-dried strawberry, Zhang and coworkers[34] demonstrated that a *p*-coumaric acid glucose ester strongly inhibited proliferation in two colon cancer cell line assays. There is also evidence that hydroxycinnamic acids may play an active role in cancer cell regulation when acylated to anthocyanins.[36] The chemopreventive activity of hydroxybenzoic acids and their derivatives (e.g., protocatechuic acid) has also been extensively studied.[37] However, to our knowledge, this is the first report of a benzoic acid glucose ester (BAGE) showing antiproliferative potential against cancer cells.

Altered metabolism favoring cytoplasm-centered "aerobic glycolysis" is an "emerging hallmark" of cancer,[38] leading to the production of metabolites necessary for continued proliferation and sufficient energy for tumor growth.[39,40] However, aerobic glycolysis and its effects can be curtailed by high levels of cytosolic citrate.[39−41] Elevated citrate levels in cancer cells may lead to cell cycle arrest, metabolic changes favoring pro-apoptotic over anti-apoptotic proteins, and increased cytosolic- or mitochondrial-induced caspase activity leading to cell death[39−42] in a threshold-dependent manner. The increased importance of citric acid derivatives in the high-dose model (Table 3 and Table S4 in the Supporting Information) perhaps reflects this concentration threshold dependency.

The ^{1}H NMR-based model that was developed here has made a significant contribution to our understanding of the impact of the crude BR extracts in controlling HT-29 cell proliferation and provides insights as to the chemopreventive nature of BR fruits. The nonanthocyanin components of the fruit that affect cell proliferation represent a very small percentage of the total phenolic and organic acid derivatives found in the BR extracts, but both the low- and high-dose models indicated their importance in curtailing cancer cell growth. Among the model-identified nonanthocyanin components, Q 3-glc, Q 3-rut, *p*-coumaric acid, and various ellagic acid derivatives have been previously considered as bioactive

constituents of BR.[4−8,30,43,44] However, the bioactivity of MEAAP specifically, BR-derived salicylates, flavan-3-ols (e.g., epicatechin), citric acid derivatives, and/or esters of benzoic acid in controlling colon cancer cell proliferation have not been cited to our knowledge in previous work. Recognition, by the model, of the contributions of these low-concentration constituents on the impact of the extracts on cell growth clearly provides paths for new lines of research into the mechanisms of BR constituents. The data will lead to studies on the interactive effects of the extracts on the chemoprotective nature of berry fruits in general and have demonstrated the utility of using this type of approach to the study of the bioactivity of natural products.

Further advanced NMR analysis with unassigned bins (Tables S3 and S4 in the Supporting Information) may lead to the identification of additional important compounds. As this study was focused on model development and viewed as a proof of concept, recognition by the model of all of the important BR bioactive compounds was limited by the use of acidic methanol extracts and the in vitro HT-29 cell proliferation assay. However, our overall approach is readily adaptable to modeling the effects of a series of extracts including those containing compounds that differ substantially in solubility from that of phenols (e.g., carotenoids, steroids)[5] and can be adapted to accommodate other continuous *Y*-variables such as control of tumor development, enzymatic activity, or biomarker levels, which would lead to a richer understanding of the complex mechanisms by which BR compounds may affect carcinogenesis. Likewise, our approach can also be applied to assess the activity of other natural products with complex NMR spectra, not only against cancer but also for any complex interaction that can be evaluated using a biological assay.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table S1, variability in HT-29 colon cancer cell % inhibition following exposure to 73 BR extracts at two concentrations; Table S2, ^{1}H NMR-determined concentrations of cyanidin glycosides in BR extracts; Tables S3 and S4, regression vector values, bin rankings, compounds, associated protons (number and type), δ^1H range (ppm), and bin numbers for low- and high-dose PLS models; Figures S1−S11, structure data resulting from compound analysis using additional 1D selective TOCSY and 2D NMR techniques; Figure S12, selection criteria for the 100 bin model of the high-dose bioassay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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