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Analysis of Anthocyanins in Rat Intestinal Contents—Impact of Anthocyanin Chemical Structure on Fecal Excretion

JIAN HE,[†] BERNADENE A. MAGNUSON,[‡] AND M. MONICA GIUSTI*,[†]
Department of Food Science, The Ohio State University, Columbus, Ohio 43210, and
Department of Nutrition and Food Science, University of Maryland, College Park, Maryland 20742

Absorption of dietary anthocyanins is limited; however, fecal anthocyanin excretion has been rarely studied. We developed a method for extraction and analysis of fecal anthocyanins. Aqueous methanol (60%) maximized extraction efficiency (~88%). Severe anthocyanin degradation (monitored by high-performance liquid chromatography) was observed in feces stored at −18 °C; therefore, storage time should be minimized and lower temperatures used. Fecal and cecal content samples were collected from 32 rats receiving either chokeberry, bilberry, grape-enriched (3.85 g monomeric anthocyanin per kg diet), or control diet for 14 weeks. Fecal anthocyanin concentrations were significantly different among groups (0.7/1.8/2.0 g/kg wet feces, chokeberry/bilberry/grape). Anthocyanin profiles of cecal contents and feces were similar. Losses in the intestinal contents were high for anthocyanin glucosides, moderate for galactosides, and negligible for arabinosides or xylosides. Acylation or diglucosylation enhanced anthocyanin stability. High anthocyanin concentration in the fecal content may favor anthocyanin absorption into the colon epithelial cells, resulting in potential health benefits.

KEYWORDS: Anthocyanins; chemical structure; feces; rat intestinal contents; gut; gastrointestinal tract; microflora; glycosylation; acylation

INTRODUCTION

As the most abundant antioxidants in our diets, polyphenols are receiving increasing interest from consumers and food manufacturers because of their potential health benefits (*1*). Among all of the recognized polyphenols, anthocyanins are especially important because of their high consumption, which has been estimated at 100+ mg per day (*2*, *3*) in the United States. They are very widespread in fruits, vegetables, and processed foods or beverages such as juices and wines (*4*).

To date, a vast number of studies have revealed potential benefits of anthocyanins on human health, but studies also suggest low bioavailability of anthocyanins as indicated by the very low recovery in the plasma and urine after ingestion (*5*−*8*). Health benefits hereby became questionable according to some researchers (*9*). However, we hypothesized that anthocyanins in the intestinal contents may interact with the cells of the gastrointestinal tract (GIT), even without being absorbed given the close contact between them. In vivo and in vitro studies with foods rich in anthocyanins have been associated with prevention of GIT cancers, including oral (*10*), esophageal (*11*), intestinal (*12*), and colorectal (*13*, *14*) cancer, while beyond GIT, anthocyanins showed no inhibition for breast cancer (*15*) or lung cancer (*16*).

Our objective was to evaluate the excretion of anthocyanins through the rat gut. Unlike many other flavonoids, anthocyanins may not be well-degraded by gut microflora, judging by the visual color of feces from animals fed anthocyanin rich diets (*17*). However, to date we found no literature reporting the measurement of the anthocyanin content in feces. This study was conducted as a part of a larger project to evaluate the potential chemopreventive effects of anthocyanin rich extracts on colon cancer prevention using a rat model. Chokeberry, bilberry, and grape anthocyanin-rich extracts (AREs) were chosen as the additives in diets based on our previous finding that they could inhibit colon cancer growth in vitro (*14*, *18*). They were all reported to be good sources of antioxidants (*19*−*21*), yet contained very different and distinctive pigment profiles, including a wide variety of individual anthocyanins. Five aglycones—cyanidin (cy), peonidin (pn), delphinidin (dp), petunidin (pt), and malvidin (mv)—as well as four sugar substitutions—glucoside (glu), galactosides (gal), arabinoside (arab), and xylosides (xyl)—and one cinnamic acid acylation, *p*-coumaroyl (coum), were included in this study. A comparison of the concentration of total and individual anthocyanins in the intestinal contents after feeding with the different extracts may provide valuable information for the impact of structural differences on anthocyanin concentration within GIT, as well as anthocyanin fecal excretion.

MATERIALS AND METHODS

Chemicals and Materials. Commercially available AREs of bilberry (*Vaccinium myrtillus* L.) and chokeberry (*Aronia meloncarpa* E.) extracts were supplied by Artemis International, Inc. (Fort Wayne, IN). Grape extract (*Vitis vinifera*) was supplied by Polyphenolics, Inc.

* To whom correspondence should be addressed. Tel: 614-247-8016. Fax: 614-292-0218. E-mail: giusti.6@osu.edu.
[†] The Ohio State University.
[‡] University of Maryland.

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(Madera, CA). Cy-3-gal standard for high-performance liquid chromatography (HPLC) analysis was purchased from Polyphenols Laboratories (Sandnes, Norway). Acetonitrile, acetic acid, methanol, acetone, and ethyl acetate were HPLC grade reagents from Fisher Scientific (Fair Lawn, NJ). Trifluoroacetic acid (TFA) and azoxymethane (AOM), a potent colon carcinogen, were obtained from Sigma Chemical (St. Louis, MO). AIN-93 powdered diet was from Dyets Inc. (PA). Tissue homogenizer was from Biospec Products, Inc. (Bartlesville, OK). Sep-Pak Vac (6 cm^3, 1 g; 12 cm^3 2 g) C_{18} cartridges for solid phase extraction (SPE) were purchased from Waters (Milford, MA).

Animal Care and Feeding Trial. The samples obtained for this study were from rats in a colon cancer chemoprevention study described in detail by Magnuson and co-workers (in preparation). Briefly, weaning male specific pathogen free Fischer 344 rats (Harlan, Indianapolis, IN) were fed either the control AIN-93 powdered diet or a diet added of anthocyanins from chokeberry, bilberry, or grape ARE based on their monomeric anthocyanin content (3.85 g cy-3-gal equivalent/kg diet), at the expense of cornstarch (5, 3.5, and 2.6% of the total diet weight for chokeberry, bilberry and grape treatment respectively). Diets were prepared fresh on a weekly basis and stored at 4 °C until use. After 1 week feeding, all animals received a subcutaneous injection of AOM in saline (20 mg/kg body weight). Rats were fed the powdered diet in standard feeding cups for an additional 13 weeks. Diet and tap water were available ad libitum. Artificial light was supplied from fluorescent tubes, in a 12 h light/12 h dark cycle. The number of air changes was ~10/h. Relative humidity was maintained at 25−60%. Health signs were monitored daily and clinical signs for all the animals were recorded weekly. Body weight was recorded twice/week and 3 day food intake was measured twice during the 14 week study. The University of Maryland Institutional Animal Care and Use Committee approved all animal protocols.

Sample Collection. Fecal samples were collected from eight animals per diet group 1 week before the final sacrifice. Each animal was placed individually in a Nalgene metabolic cage (Mini Mitter Inc., Bend, OR) over a period of 12 h of dark cycle. The powdered diet was not provided during this time to prevent any contamination of samples. Feces were collected twice at 6 h and 12 h, sealed in polypropylene bags, and immediately stored at −80 °C until analyzed. At the end of the study, all cecum were collected, wrapped in foil, rapidly frozen in liquid nitrogen, and stored at −80 °C.

Method Development. Four solvents, methanol, acetone, methanol water mixture (60:40; v/v), and double distilled (DD) water, all acidified with 1% TFA (*22*), were studied for the extraction efficiency. The experiment was done in duplicate. For each replication, one frozen fecal sample from each ARE diet group was randomly selected, homogenized and divided to four equal weighted aliquots of 0.100 g and then placed in four 40 mL centrifuge tube. After breaking the sample into smaller pieces with a spatula, 20 mL of extracting solvent was added. A tissue homogenizer was used to facilitate the feces extraction. Then the suspension was sonicated for 3 min and centrifuged for 10 min at 4000 rpm, 4 °C to precipitate the protein and other insoluble materials. The supernatant was decanted and the pellet was re-extracted twice with 10 mL of extracting solvent following the same procedure described above. The combination of supernatants was evaporated in a Büchii rotovapor at 40 °C. After evaporation to almost dryness, the solution was diluted with small amount of acidified water, and then applied to a Sep-Pak Vac C_{18} (12 cm^3, 2 g) cartridge preconditioned with 1 volume of methanol followed by 1 volume of water, both acidified by 1% TFA. After washing with 1 volume of acidified water, anthocyanins were eluted with one volume of acidified methanol. The eluate was carefully evaporated in a Büchii rotovapor at 40 °C, and the sample was taken to 5 mL with water containing 1% TFA.

Recovery and Degradation Test. On day one, four fecal samples from the group receiving non anthocyanin diet were randomly selected. A portion of each sample was taken out by spatula and pooled. After mixing the pooled feces with a spatula, 18 equal weighted aliquots (0.1 g) were put into 18 caped tubes and randomly assigned to three treatments. Within each group, known amounts (similar level as detected in the fecal samples fed ARE diets) of chokeberry, bilberry, and grape AREs were spiked into duplicate samples. Samples in treatment 3 were pasteurized in 95 °C water bath for 2 min before spiking (*23*). After brief blending, all the tubes were stored at −18 °C to allow anthocyanins to bind to fecal matrix. Samples in treatments 1 and 3 were frozen for 24 h and samples in treatment 2 were frozen for 72 h. Sample were then extracted with 60% methanol and semipurified following the procedures described above, and immediately analyzed by HPLC.

Comparison of Anthocyanins in the Feces and Cecal Contents of Rats Fed Different Anthocyanin Diets. Fecal samples from eight rats per diet group were defrosted in polypropylene bags at room temperature. An aliquot of 0.1 g was extracted with aqueous methanol and then semipurified with the procedures described above. Cecal contents were obtained from the defrosted cecum and then extracted and semi-purified in exactly the same way as feces.

HPLC Analysis. All the samples were filtered through 0.45 μm Whatman polypropylene filter before HPLC injection. Analyses were conducted on a Waters HPLC system (Waters Delta 600) equipped with a photodiode array detector (Waters 996), Millennium32 software (Waters Corp., Milford, MA), and autosampler (Waters 717 plus). Separation of anthocyanins was accomplished on a Symmetry C_{18} column (5 μm; 4.6 mm × 150 mm). Mobile phases and gradients were modified from Rodríguez-Saona and co-workers (*24*). Mobile phases were A, 10% acetic acid and 1% phosphoric acid in deionized water and B, acetonitrile. The gradient condition was 0−5 min, 0% B isocratic and 5−40 min linear increase to 35% B. Spectral data (260−650 nm) was collected during the whole run. Elution of compounds of interest was monitored at wavelength 520 nm for anthocyanins, 280 nm for phenolics, and 320 nm for hydroxyl-cinnamic acids. Other chromatographic conditions were as follows: flow rate, 1 mL/min; injection volume, 300 μL. Anthocyanin peak identification was based on comparison of relative retention times, percentage peak area, and spectral data with data provided by the suppliers, known anthocyanin cocktails, literatures (*19*−*21*, *25*) and unpublished data from our lab (Tian and co-workers).

Standards and Calibration Curves. Commercially available cy-3-gal standard was dissolved in double distilled water containing 1% TFA to 0.100 g/L, and thereafter diluted (0.78−50 μg/L) to make a standard curve ($R^2 \geq 0.99$). All anthocyanins analyzed fell within the standard curve range and were expressed as cy-3-gal equivalent in weight. Total anthocyanins recovered from fecal samples were calculated by adding the area under curve of individual anthocyanin peaks together and using a calibration curve.

Statistical Analysis. One-way analysis of variance (ANOVA) was conducted using SPSS (version 10, 1999, SPSS Inc., Chicago, IL) to compare fecal anthocyanin concentrations in different diet groups, and values were given as means ± SEM. When appropriate, significance of differences between values was determined by LSD. Differences of $P < 0.05$ were considered significant. The precision of duplicated data in the fecal anthocyanin stability study was evaluated by calculating the relative percent difference (RPD) of each pair of measurements for each individual peak.

RESULTS AND DISCUSSION

Anthocyanin Extraction Method Development. We report, for the first time, the development of a method for rapid analysis of various anthocyanins in feces. Felgines and co-workers (*26*) reported anthocyanin concentration in the cecal contents of rats fed blackberry, using HCl acidified water/acetone (1/1) as extraction solvent. However to date little information was available on methodology for analysis of fecal anthocyanins. We developed such a method based on reported procedures for cecal content anthocyanin extraction (*26*) as well as the fecal quercetin extraction (*27*) in order to systematically study anthocyanins in the gut contents. Our anthocyanin extraction method for fecal samples incorporates three steps designed to prevent enzymatic degradation of anthocyanin. First, centrifugation (4000 rpm, 10 min) facilitated by organic solvent favors enzyme (proteins) precipitation, as evidenced by the increased stability of centrifuged crude extracts (data not shown). Second, controlled low temperature (4 °C) during extraction is aimed at

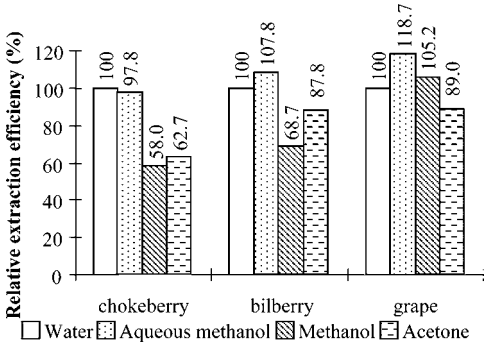

Figure 1. Relative efficiency of four solvents on fecal anthocyanin extraction as compared to acidified water efficiency (arbitrarily assigned 100%). Values are the means of two replications.

reducing enzyme activity. Third, the short processing time (1 h) compared to traditional long (6 h) extractions (*28*) also is a safeguard against degradation.

Solvents Comparison. Methanol (*22*, *28*), acetone (*29*, *30*), and water have been widely used for anthocyanin extraction. Aqueous methanol (*31*, *32*) has also been recommended. We compared the extraction efficiency of these four solvent systems, all acidified with 1% of TFA. Anthocyanin recovery by water extraction was arbitrarily assigned 100%, and relative recoveries by all the other solvents were calculated based on the relative efficiency to water extraction. Results are summarized in **Figure 1**.

The extraction efficiency with organic solvents (methanol and acetone) or organic−inorganic solvent mixture (aqueous methanol) was affected by the hydrophilicity of the anthocyanins present in the extract, increasing in the order of chokeberry > bilberry > grape. This was reasonable since the distribution of more or less hydrophilic anthocyanins in those three extracts was different. In the chokeberry ARE only hydrophilic cy monoglycosides were present, yet in bilberry ARE, there were large amount of less hydrophilic anthocyanins such as pn-3-glycosides and mv-3-glycosides. In grape ARE, there was a significant amount of acylated anthocyanins, which were even less hydrophilic, resulting in a higher affinity for organic solvents. Overall, aqueous methanol (60% methanol) gave the best extraction efficiency. This mixture of inorganic and organic solvents seemed to have good efficiency for a wide range of individual anthocyanins. In addition, methanol would likely help to precipitate enzymes (proteins) during centrifugation. Traditionally, organic solvents were favored for anthocyanin extraction from fruits and vegetables (*30*, *33*, *34*), partly because organic solvents have the advantage of easily penetrating the cell membranes and consequently extracting the pigments from vacuolar compartments. However, anthocyanins present in feces could have been released from the food matrix due to digestion, and therefore the advantage of organic solvents was not apparent.

Water is a good alternative for aqueous methanol, not only because it is an environmental friendly solvent, but also because water extraction can be directly applied to the C_{18} cartridge, and therefore eliminates the need for evaporation prior to C_{18} SPE purification. Typically the vacuum evaporation is conducted at 35−40 °C for anthocyanin solutions, and may accelerate potential anthocyanin degradation in the presence of fecal microflora. For our study, we chose to use acidified methanol water mixture (60:40, v/v) for anthocyanin extraction from gut contents because of the higher extraction efficiency obtained.

Anthocyanin Recovery from Feces. To study the anthocyanin recovery from feces, AREs were spiked into fecal samples from rats receiving no anthocyanin diet, briefly blended, and left at −18 °C overnight to allow binding. Anthocyanin profiles of the spiked AREs are shown in **Figures 2A−4A**. Anthocyanin degradation during storage was evidenced by a rapid disappearance of red color. We suspected that the fecal microflora played an important role in the color degradation. To test this hypothesis, pasteurization of feces was performed to kill microflora and inactivate enzymes, which may be involved in anthocyanin degradation. Our hypothesis was supported by the observation that samples spiked with ARE had identical shapes of anthocyanin profiles as the dietary AREs used for spiking only when the feces were pasteurized (data not shown). Thus the pasteurized samples were assumed to have no enzymatic activity on anthocyanins and differences between the spiked AREs and those recovered from pasteurized samples were attributed to losses during extraction and semi-purification. The recovery rate of fecal anthocyanins was calculated based on the amount recovered from pasteurized fecal samples and the amounts being spiked (**Tables 1−3**). The average recovery rate (aqueous methanol as extraction solvent) for chokeberry ARE, bilberry ARE, and grape ARE was 88.1 ± 2.3% ($n = 6$). Certainly recovery of spiked samples may not represent the extraction from matrix, but when extracting the fecal samples

Table 1. Individual Anthocyanins Recovered from Feces Spiked with Chokeberry ARE[a]

		recovered		
			unpasteurized feces	
anthocyanins	amount spiked[b]	pasteurized feces	day 1	day 3
cy-3-gal	105 (1.98)[c]	90.1 (1.57)	61.1 (3.62)	20.8 (10.07)
cy-3-glu	6.2 (1.38)	5.3 (0.39)	5.6 (6.82)	3.6 (9.35)
cy-3-arab	49.2 (2.03)	45.5 (0.28)	45.6 (8.86)	36.8 (11.72)
cy-3-xyl	7.0 (2.30)	6.2 (0.48)	6.7 (13.19)	6.2 (15.54)
total	170.5	147.7	120.2	69.5

[a] Fecal anthocyanins (μg cy-3-gal equivalent) recovered from 0.1 g of feces, expressed as means of two replications. [b] Amounts were chosen based on preliminary results of anthocyanin concentration in fecal samples. [c] In parentheses is the RPD for each pair of individual replications.

Table 2. Individual Anthocyanins Recovered from Feces Spiked with Bilberry ARE[a]

		recovered		
			unpasteurized feces	
anthocyanins	amount spiked[b]	pasteurized feces	day 1	day 3
dp-3-gal	17.5 (4.92)[c]	15.6 (4.92)	13.9 (4.54)	7.7 (19.35)
dp-3-glu	26.9 (4.53)	25.2 (4.46)	25.8 (0.82)	21.0 (1.61)
cy-3-gal and dp-3-arab[d]	26.6 (5.84)	23.2 (6.66)	21.0 (0.75)	15.8 (5.24)
cy-3-glu	20.8 (4.80)	18.7 (4.94)	18.2 (7.27)	11.9 (3.18)
pt-3-gal and cy-3-arab[d]	17.7 (4.12)	16.2 (4.78)	15.5 (0.79)	11.3 (4.10)
pt-3-glu	19.6 (3.58)	18.6 (4.42)	18.8 (2.52)	14.2 (2.68)
pn-3-gal and pt-3-arab[d]	6.8 (5.25)	05.9 (3.05)	5.8 (0.92)	5.3 (4.17)
pn-3-glu and mv-3-gal[d]	15.4 (4.43)	13.4 (5.47)	13.3 (6.54)	8.5 (2.12)
pn-3-arab	1.4 (1.50)	1.3 (3.54)	1.4 (1.13)	1.8 (2.37)
mv-3-glu	20.9 (4.59)	18.3 (5.57)	18.3 (9.67)	10.9 (1.17)
mv-3-arab	4.4 (3.67)	3.9 (0.01)	4.3 (3.83)	4.1 (7.04)
total	178.0	160.3	156.3	112.5

[a] Fecal anthocyanins (μg cy-3-gal equivalent) recovered from 0.1 g of feces, expressed as means of two replications. [b] Amounts were chosen based on preliminary results of anthocyanin concentration in fecal samples. [c] In parentheses is the RPD for each pair of individual replications. [d] Coeluting peaks.

Table 3. Individual Anthocyanins Recovered from Feces Spiked with Grape ARE[a]

		recovered		
			unpasteurized	
anthocyanins	spiked[b]	pasteurized	day 1	day 3
dp-3,5-glu	3.9 (0.07)[c]	2.6 (4.42)	2.9 (7.64)	3.3 (0.92)
cy-3,5-glu	2.1 (2.70)	1.8 (1.29)	1.8 (0.31)	2.3 (0.05)
dp-3-glu	5.4 (1.74)	4.7 (11.40)	4.8 (8.44)	5.2 (14.94)
pt-3,5-glu	7.3 (1.65)	5.7 (7.60)	6.1 (4.41)	6.3 (3.63)
cy-3-glu	3.1 (1.93)	2.6 (7.50)	2.9 (4.14)	2.8 (1.32)
pn-3,5-glu	32.2 (0.21)	26.2 (5.04)	26.9 (2.71)	22.3 (0.46)
mv-3,5-glu and pt-3-glu[d]	52.4 (0.49)	44.9 (3.77)	45.1 (6.49)	39.7 (2.11)
pn-3-glu	4.1 (0.93)	3.3 (3.94)	4.1 (17.04)	4.6 (1.31)
mv-3-glu	7.8 (1.00)	6.5 (7.76)	8.1 (15.59)	10.7 (14.61)
unknown	3.3 (0.31)	3.2 (4.91)	3.1 (22.08)	3.9 (5. 09)
dp-3-glu-*p*-coum[e]	4.1 (0.21)	4.1 (5.83)	4.0 (21.02)	4.8 (1.71)
pt-3-glu-*p*-coum[e]	18.4 (2.39)	19.5 (5.52)	18.6 (23.20)	17.7 (1.55)
mv-3-glu-*p*-coum[e]	2.3 (11.26)	0.030 (2.66)	0.023 (77.47)	0.040 (4.23)
total	146.4	125.1	128.4	123.6

[a] Fecal anthocyanins (μg cy-3-gal equivalent) recovered from 0.1 g of feces, expressed as means of two replications. [b] Amounts were chosen based on preliminary results of anthocyanin concentration in fecal samples. [c] In parentheses is the RPD for each pair of individual replications. [d] Pt-3-glu is a coeluting minor peak. [e] Tentative peak identity.

from anthocyanin fed rats, not much red color could be seen in the precipitate by naked eyes after the extraction. This also is an indication of the good extraction efficiency of our method.

Anthocyanin Stability in Fecal Samples. Storage of fecal samples at −18 °C did not completely inhibit anthocyanin degradation, most likely due to the enzymatic activity by microflora. **Tables 1−3** show that anthocyanins underwent significant degradation in unpasteurized fecal samples when stored at −18 °C. As compared to the pasteurized samples, the total chokeberry anthocyanins recovered from unpasteurized samples decreased by ∼16% (86.4−70.3%) after 1 day, and by ∼46% (86.4−40.7%) after 3 days.

A comparison of individual anthocyanins detected in pasteurized feces and unpasteurized feces stored for up to 3 days at −18 °C (**Tables 1−3**), revealed severe degradation of anthocyanin gals, followed by anthocyanin glus, while little degradation was observed for anthocyanin arabs or xyls. Among the four cy-glycosides present in chokeberry, the greatest reduction occurred in cy-3-gal from 90.1 μg in the pasteurized feces (assumed no fecal enzymatic activity) to 61.1 μg on day 1, and then to 21.8 μg on day 3 in the feces without pasteurization (**Table 1**). After 3 days of storage, only cy-3-xyl and cy-3-arab showed minor degradation as compared to the others. Bilberry anthocyanin profile (**Table 2**) was much more complex than that of chokeberry, but the data demonstrate a similar degradation trend. After 3 days, the level of dp-3-gal was reduced the greatest, by more than 55%. Dp-3-glu, cy-3-glu, pn-3-glu, and mv-3-glu levels were decreased by ∼ 22 to ∼48%, while levels of anthocyanin arabs (pn-3-arab and mv-3-arab) showed no significant change. In contrast, grape anthocyanins, glycosylated only with glucose (*20*, *21*), showed little degradation following addition to fecal matter, suggesting these anthocyanins are highly resistant to the fecal microflora (**Table 3**). However, two major compounds, pn-3,5-diglu and mv-3,5-diglu still degraded significantly after 3 days of storage. Their corresponding 3 mono glus, pn-3-glu and mv-3-glu, both increased to levels even higher than the spiked amount and the decreases of 3,5-diglucosides closely correlated to the increases of 3 monoglucosides, suggesting the partial hydrolysis of 3,5-diglucosides to 3-glucosides. Some minor peaks did not follow the trend described above, probably because of large baseline variation (**Figure 4B**). We propose that the selective degradation is attributed largely to microbial enzyme mediated degradation.

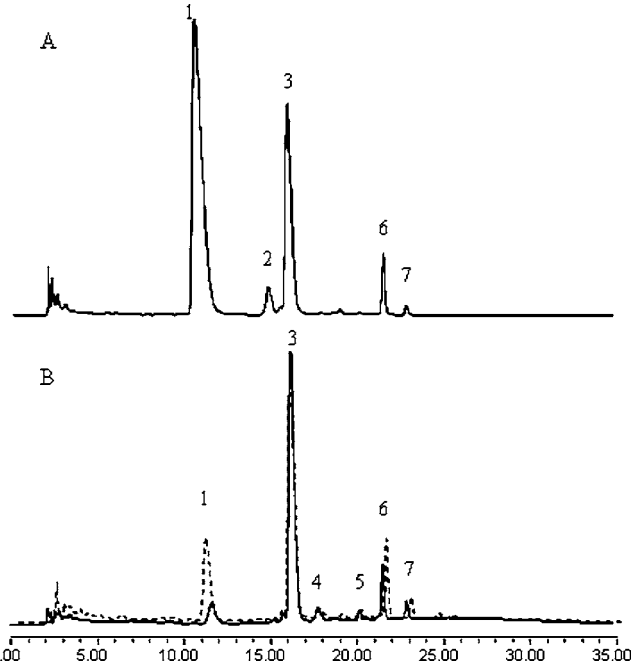

Figure 2. Comparison of anthocyanins in chokeberry ARE (**A**) to the anthocyanins in cecal (**B**, dotted line) and fecal contents (**B**, solid line) of chokeberry diet fed rat. Detection wavelength: 520 nm. Peak identities: 1, cy-3-gal; 2, cy-3-glu; 3, cy-3-arab; 4, 5, unknown; 6, cy-3-xyl; and 7, unknown (cy glycoside acylated with an aliphatic acid).

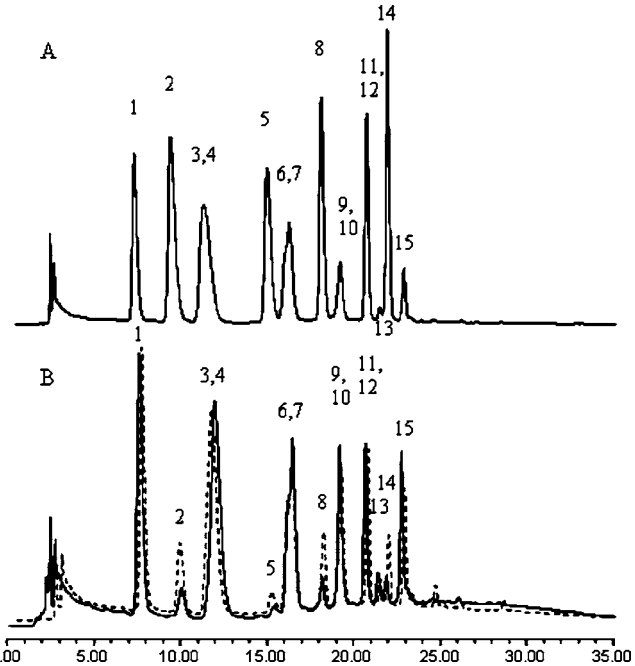

Figure 3. Comparison of anthocyanins in bilberry ARE (**A**) to the anthocyanin in cecal (**B**, dotted line) and fecal contents (**B**, solid line) from bilberry diet fed rat. Detection wavelength: 520 nm. Peak identities: 1, dp-3-gal; 2, dp-3-glu; 3, cy-3-gal; 4, dp-3-arab; 5, cy-3-glu; 6, pt-3-gal; 7, cy-3-arab; 8, pn-3-glu; 9, pn-3-glu; 10, pt-3-arab; 11, pn-3-glu; 12, mv-3-gal; 13, pn-3-arab; 14, mv-3-glu; and 15, mv-3-arab.

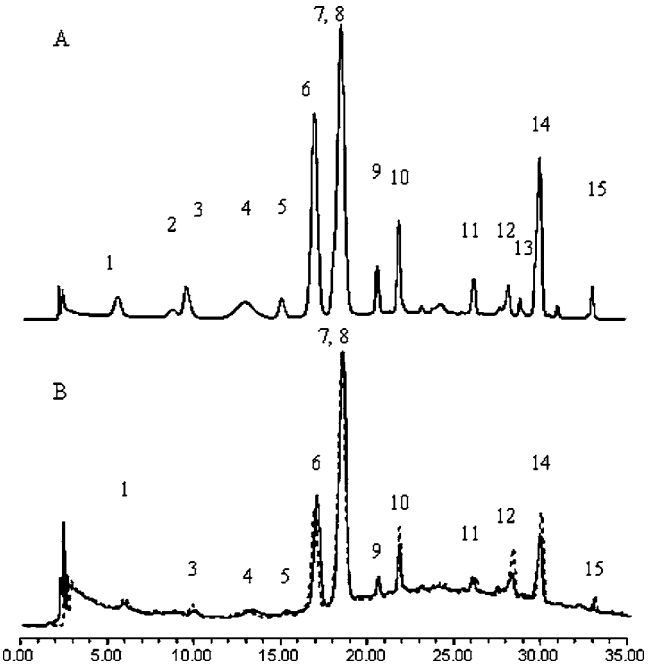

Figure 4. Comparison of anthocyanins in grape ARE (**A**) to the cecal (**B**, dotted line) and fecal contents (**B**, solid line) from a rat fed grape diet. Detection wavelength: 520 nm. Peak identities: 1, dp-3,5-diglu; 2, cy-3,5-diglu; 3, dp-3-glu; 4, pt-3,5-diglu; 5, cy-3-glu; 6, pn-3,5-diglu; 7, pt-3-glu; 8, mv-3,5-diglu; 9, pn-3-glu; 10, mv-3-glu; 11, unknown; 12, dp-3-glu-*p*-coum (tentative); 13, unknown; 14, pt-3-glu-*p*-coum (tentative); and 15, mv-3-glu-*p*-coum (tentative).

Comparing the anthocyanin glus present on unpasteurized feces spiked with grape ARE to those spiked with bilberry and chokeberry AREs, we found that even the same cy-3-glu displayed much lower resistance to degradation in the chokeberry and bilberry groups. Considering the large baseline variation in the grape ARE spiked feces (**Figure 4B**), the relatively small amount of cy-3-glu in grape anthocyanins may have been biased in the feces. But caution must be given that the degradation products of other anthocyanins (anthocyanin 3-gal in particular) in bilberry and chokeberry might accelerate the degradation of anthocyanin 3-glu in those samples. A similar mechanism had been suggested when polyphenol oxidase (PPO) was involved in anthocyanin degradation (*35*), where anthocyanins themselves were not substrates for PPO, yet the secondary quinones from other degraded flavonoids induced pigment degradation.

These data suggested that collection of fecal samples must be done as quickly as possible and the storage of fecal anthocyanins must be under a much lower temperature than −18 °C. Although centrifugation may reduce enzyme activity by precipitating enzymes, the extraction and semi-purification procedure to evaluate anthocyanin from fecal samples should be done quickly under controlled low temperatures. Therefore, it is reasonable to infer that our results (discussed below) may underestimate fecal anthocyanin levels (chokeberry treatment in particular because of the high gals percentage) since there was a gap (up to several hours) between rat excretion and sample collection at room temperature that may have resulted in anthocyanin degradation before samples were frozen.

Gut Content Anthocyanin. The intense coloration of the fecal and cecal content samples from anthocyanin fed rats suggested that anthocyanins existed in high concentration in the gut. At the time of collection, feces from anthocyanin treated rats appeared dark purple to black, and red color was observed as soon as acidified solvent was used for extraction. This color change was attributed to the equilibrium of anthocyanin structure conversion. Under neutral or slightly acidic conditions anthocyanins may exist in colorless pseudobase form or purple to violet colored quinonoidal base form. Feces might not have sufficient water to hydrate flavylium anthocyanins to form pseudobases, resulting in predominance of the quinonoidal bases, and intense dark color. Cecal content also seemed very dark.

Relative anthocyanin concentrations found in fecal samples from different diet groups are summarized in **Tables 4**−**6**. Also, three cecal samples collected from each diet group were analyzed to compare the anthocyanin in cecal content to that in the colon. As cecum is the starting point of large intestine, and feces are collected at the end of large intestine, the comparison of cecal anthocyanin to fecal anthocyanin was conducted to better understand if any changes had occurred in anthocyanins in the large intestine, where most of the microflora in the entire body resides. Anthocyanin profiles in the cecal contents were very similar to those in the fecal samples (**Figures 2B**−**4B**). Our results demonstrate that feces are a major excretion route of ingested anthocyanins. The anthocyanin concentrations in feces from rats fed chokeberry and bilberry AREs were higher than those from the grape ARE diet group, with no significant difference ($P < 0.05$) between chokeberry and bilberry groups (**Figure 5**). Since food and water consumption were not significantly different among the dietary groups (data not shown), we can speculate that grape anthocyanins either experienced more degradation in the gut or were absorbed to a greater extent.

Table 4. Distribution of Individual Anthocyanins in Chokeberry ARE and Fecal Samples from Rats Fed Chokeberry ARE

anthocyanin components	abundance (% peak area)		proportion to ARE
	ARE[a]	feces[b] ($n = 7$)	
cy-3-gal	61.0	23.2 ± 3.8	0.38
cy-3-glu	3.40	0.84 ± 0.33	0.25
cy-3-arab	28.0	63.7 ± 4.5	2.27
cy-3-xyl	3.91	5.93 ± 0.94	1.52
unknown	3.61	1.57 ± 0.30	0.44

[a] Percentage areas are expressed as means of two replications. [b] Percentage areas are expressed as means ± SEM.

Table 5. Distribution of Individual Anthocyanins in Bilberry ARE and Fecal Samples from Rats Fed Bilberry ARE

anthocyanin components	abundance (% peak area)		proportion to ARE
	ARE[a]	feces[b] ($n = 5$)	
dp-3-gal	10.13	19.35 ± 1.25	1.91
dp-3-glu	15.75	2.98 ± 0.54	0.19
cy-3-gal and dp-3-arab[c]	15.48	31.61 ± 1.23	2.04
cy-3-glu	11.18	0.71 ± 0.14	0.06
pt-3-gal and cy-3-arab[c]	8.90	17.81 ± 0.80	2.00
pt-3-glu	11.35	1.99 ± 0.21	0.18
pn-3-gal and pt-3-arab[c]	4.06	9.53 ± 0.59	2.35
pn-3-glu and mv-3-gal[c]	8.96	6.87 ± 0.84	0.77
pn-3-arab	0.23	1.02 ± 0.20	4.52
mv-3-glu	11.61	1.09 ± 0.15	0.09
mv-3-arab	1.44	7.03 ± 0.83	4.88

[a] Percentage areas are expressed as means of two replications. [b] Percentage areas are expressed as means ± SEM. [c] Coeluting peaks.

Table 6. Distribution of Individual Anthocyanins in Grape ARE and Fecal Samples from Rats Fed Grape ARE

anthocyanin components	abundance (% peak area)		proportion to ARE
	ARE[a]	feces[b] ($n = 7$)	
dp-3,5-diglu	2.91	ND[c]	
cy-3,5-diglu	0.85	ND	
dp-3-glu	3.03	ND	
pt-3,5-diglu	4.83	ND	
cy-3-glu	1.71	ND	
pn-3,5-diglu	21.2	22.1 ± 0.51	1.04
mv-3,5-diglu + pt-3-glu[d]	35.5	47.8 ± 1.52	1.35
pn-3-glu	2.38	1.80 ± 0.07	0.76[e]
mv-3-glu	4.79	4.31 ± 0.18	0.90[e]
dp-3-glu-*p*-coum	2.61	1.63 ± 0.43	0.62
pt-3-glu-*p*-coum	14.1	19.5 ± 1.59	1.38
mv-3-glu-*p*-coum	1.50	0.63 ± 0.08	0.42

[a] Percentage areas are expressed as means of two replications. [b] Percentage areas are expressed as means ± SEM. [c] ND means not detected or below quantitation limit. [d] Pt-3-glu is a coeluting minor peak. [e] Pn-3-glu and mv-3-glu in feces were boosted by the partial hydrolysis of pn-3,5-diglu and mv-3,5-diglu.

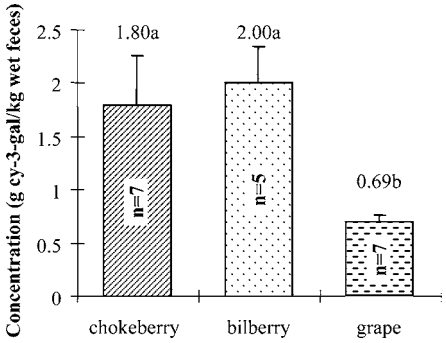

Figure 5. Anthocyanin concentration in fecal samples from rats fed AREs. Values are the mean ± SEM of seven replications for chokeberry and grape treatment, five replications for bilberry treatment (one-way ANOVA followed by the LSD test). Means with different letters are significantly different at $P < 0.05$.

High levels of anthocyanins in the GIT may have health benefits. In a parallel study conducted with the same rats used in this study, Magnuson and co-workers (in preparation) found that rats exposed to diets enriched with AREs developed less and smaller aberrant crypt foci (ACF), the early stage biomarker of colon cancer, than rats on a control diet. They also found more inhibition of ACF in the rats fed chokeberry and bilberry diets than in the rats fed grape diet. The inhibition positively correlated to the anthocyanin concentration in feces across diet, but not correlated to the anthocyanin concentration in the urine, suggesting that the colon cancer preventive effect was related to the concentration of anthocyanins in the GIT, rather than the amount in the blood, probably due to direct absorption into the colon epithelial cells. The same AREs that were used in this study inhibited the growth of colon cancer but not nontransformed colonic cells in vitro (*18*).

Two tiny anthocyanin peaks were detected in the fecal content and feces from chokeberry diet group (**Figure 2**). However, neither of them was detected in the feces spiked with chokeberry anthocyanins in the fecal anthocyanin stability study. Therefore it seems that these two compounds were not generated by colonic microflora. Instead, they might be the anthocyanin metabolites excreted into the GIT by enterohepatic circulation via the bile duct, as recently proposed by Ichiyanagi et al. (*36*).

Effects of Sugar Moieties and Acylation on Gut Content Anthocyanins. The type of sugar substitution on anthocyanins affected the relative amounts of anthocyanins that were excreted in the rat gut. Very low amounts of anthocyanin monoglucosides were detected in cecal contents and feces (**Figures 2B−4B**), suggesting their degradation or absorption before entering the large intestine. As shown in **Tables 4** and **5**, every anthocyanin glu peak except pn-3-glu decreased to 6−25% of its original proportion in ARE. Indeed, the coelution of pn-3-glu with another anthocyanin prevented accurate quantitation of that particular peak. The predominance of anthocyanin glus in grape ARE may explain the low concentration of total grape anthocyanins found in gut content. Information on the deglycosylation of anthocyanins is scarce (*37*−*39*). However, the broad-specificity liver β-glucosidase as well as substrate specific β-glucosidase such as lactase phlorizin hydrolase (LPH), which is present on the luminal side of the brush border in the small intestine, are potential factors responsible for the flavonoids deglucosylation (*40*−*43*). It's reasonable to speculate that anthocyanins likewise may undergo deglycosylation procedures as the similarly structured flavonoids such as quercetin glus (*44*). Further studies on the anthocyanin absorption and degradation in the small intestine content would be needed to confirm this hypothesis.

Considerable reduction in the levels of gals was also observed. As shown in **Table 4**, fecal cy-3-gal decreased to 38% of its percentage in chokeberry ARE. There was large variability in the cy-3-gal level and hence total anthocyanin level in feces from rats fed chokeberry ARE, because in chokeberry ARE 70% anthocyanins was cy-3-gal. The large variation raises concern about uncontrolled fecal excretion time, because the proposed enzymatic degradation might proceed further, when remained in the intestine for longer time. An alternative explanation is that rats may have varied ability to break down or absorb gals.

In bilberry-fed rats, the proportion of 2 of the well-separated 3-arab anthocyanins (pn-3-arab and mv-3-arab) increased to greater than 4.5 times in feces as compared to their original proportion in ARE. This again supports the hypothesis that anthocyanin arabs was relatively stable under physiological conditions. If we consider the proportion of individual anthocyanins in feces versus in ARE, we can see that the proportion of arabs to gals increased by more than 2-fold. Even if we assume no degradation of anthocyanin 3-arabs, the anthocyanin gals must have degraded or been absorbed at least by half through the GIT.

Acylation with *p*-coumaric acid also increased the anthocyanin resistance to absorption or degradation in the gut. In the grape diet group, most minor anthocyanin peaks disappeared in the fecal sample extracts, however the minor acylated peaks remained (**Table 6**). Pt-3-(coum-glu), a major peak, increased the most among all peaks in terms of percent area. The protecting effect was probably attributed to the acylated group, which reduced the contact between anthocyanin aglycone and water, and consequently the contact between anthocyanin aglycone and hydrophilic enzymes. Acylated flavonoids were conjectured susceptible to hydrolysis in stomach under low pH. However, acylated anthocyanins in cecal content maintained their percentage in ARE (**Figure 4**), indicating that acylated anthocyanins could survive the strong acidic condition.

Anthocyanin 3,5-diglu extracted from rat feces from the grape diet group (**Table 6**) demonstrated less loss than the corresponding monoglucosides. Both pn-3,5-diglu and mv-3,5-diglu exhibited more than 36% increase in terms of their proportion to pn-3-glu and mv-3-glu respectively, even though diglucosides may undergo partial hydrolysis to produce corresponding glus, as mentioned earlier.

Our experiment was part of a project designed to study the chemopreventive properties of anthocyanins, hence rats were treated with colon carcinogen. However the results of this experiment are likely to be representative of samples from untreated rats as samples were collected much earlier than the stage of colon tumor development.

In conclusion, to date this is the first systematic study of anthocyanins in feces. The method we developed for fecal anthocyanin analysis allowed us to accurately measure anthocyanins in cecal contents and feces, and therefore made it possible to depict the pattern of anthocyanin degradation in the large intestine. Overall in the gut anthocyanin glus were the least stable, probably due to the β-glucosidase present in the small intestine. However in feces anthocyanin gals were also very unstable, probably influenced by large intestine microflora. Anthocyanin arabs and xyls seemed to be quite stable in the gut. Acylation with *p*-coumaric acid or a second sugar moiety protected anthocyanins in the gut. These findings provide information for the screening of more stable anthocyanins in vivo. Given the correlation of anthocyanin concentration on the feces with lower incidence of ACF, it is possible to speculate that anthocyanin pentosides, which are more resistant to degradation in the gut, may play an important role on the health benefits of anthocyanin in the colon.

ABBREVIATIONS USED

ACF, aberrant crypt foci; AOM, azoxymethane; arab, arabinoside; coum, *p*-coumaroyl; cy, cyanidin; dp, delphinidin; gal, galactosides; GIT, gastrointestinal tract; glu, glucoside; LPH, lactase phlorizin hydrolase; mv, malvidin; pn, peonidin; pt, petunidin; PPO, polyphenol oxidase; RPD, relative percent difference; SPE, solid phase extraction; xly, xylosides.

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