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Stability of Black Raspberry Anthocyanins in the Digestive Tract Lumen and Transport Efficiency into Gastric and Small Intestinal Tissues in the Rat

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The fate of black raspberry anthocyanins in the gastrointestinal tract (GI) was evaluated. Fasted male rats ($n = 30$) were administered either 27 ± 6.7 nmol of cyanidin 3-glucoside equivalent of anthocyanins and euthanized at 30, 60, 120, and 180 min or vehicle only and killed at 30 min (control) to collect bladder urine, GI contents, stomach, and small intestine. HPLC-MS analysis showed that anthocyanins in the gastric lumen decreased linearly over time ($t_{1/2} = 120$ min). Anthocyanins in small intestinal tissue and lumen peaked at 120 min. Uptake by small intestinal tissue reached 7.5% of the administered dose, much higher than the reported bioavailability of these pigments. Ingested anthocyanin glycosides remain relatively stable in the GI contents (75−79% of administered dose). Selective decrease of cyanidin 3-glucoside in the small intestinal content likely resulted from β-glucosidase activity. Urinary anthocyanin profiles reflected profiles present in the GI at the time of absorption.

KEYWORDS: Anthocyanin; black raspberry; digestion; gastrointestinal tract; tissue uptake; absorption; β-glucosidase; stability; metabolism

INTRODUCTION

Anthocyanins are a group of natural pigments that belong to the large family of flavonoids and are responsible for the red, purple, and blue colors of many plant materials. Consumption of anthocyanins is high compared to that of other flavonoids, with the daily intake estimated to be 12.5 mg/day/person in the United States (*1*). Berries are generally high in anthocyanin content and contribute a major portion of anthocyanins consumed.

Increasing evidence shows that anthocyanins are potent antioxidants and are associated with protective effects against many chronic diseases such as cancer, cardiovascular diseases, and even obesity (*2*). However, such postulated health-promoting effects are largely based on in vitro evidence, whereas data related to the in vivo absorption and metabolism of anthocyanins remain limited (*3*). Recent animal and clinical studies have focused on the bioavailability of anthocyanins by monitoring the concentration in plasma and urine, as well as distribution in organs (*4*−*6*). In contrast, information about the fate of anthocyanins in the gastrointestinal tract (GI) is scarce. The lack of such information impairs our understanding of their dietary value. First, because the stomach and small intestine have been considered as potential absorption sites of anthocyanins (*7*), the availability of anthocyanins in these segments would likely be a major factor influencing absorption. Existing data suggest rapid absorption and elimination of a small percentage of ingested anthocyanins (*6*). Therefore, the emptying time in the stomach and transit rate in the small intestine are expected to determine the duration of mucosal cell uptake and circulation in the blood. Second, intact anthocyanins in the GI lumen may directly interact with GI tissues, where these antioxidants may protect the GI against acute distress and chronic diseases associated with oxidative stress (*8*−*10*). Third, metabolism of anthocyanin glycosides has been proposed to be possible before absorption (*11*). It was suggested that less hydrophilic aglycones could be produced in the small intestine by the action of β-glucosidase, with the resulting aglycone being transported across the mucosal epithelium via passive diffusion in a manner analogous to that of some other flavonoids (*3*). Further derivatives from the ring fission of aglycones may also be absorbed and contribute to the health-promoting effects (*11, 12*). Direct evidence of anthocyanin deglycosylation in the GI is required to support such speculations.

Black raspberry (*Rubus occidentalis*) can provide 845 mg of anthocyanins in a single serving and was selected as a representative anthocyanin-rich source for the present study (*1*). Its phenolic content, primarily anthocyanins, has been shown to effectively inhibit proliferation of human oral, breast, prostate,

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and colon cancer cells in vitro (*13*). Here we investigated the kinetics of black raspberry anthocyanin flux in the stomach and intestine of fasted rats, focusing on the stability, rate of emptying, uptake into GI tissues, and selective deglycosylation by intestinal β-glucosidase activity.

MATERIALS AND METHODS

Chemicals and Materials. Reagent grade trifluoroacetic acid (TFA), high-performance liquid chromatography (HPLC) grade acetone and methanol for extraction, and HPLC−mass spectrometer (MS) grade water and acetonitrile for chromatography were purchased from Fisher Scientific (Fair Lawn, NJ). Formic acid (99%) was from Acros Organics (Morris Plains, NJ). Cyanidin aglycone was prepared from cyanidin 3-glucoside standard (ChromaDex Inc., Irvine, CA) by acid hydrolysis (*14*). Protocatechuic acid standard was from MP Biomedicals (Solon, OH).

Black Raspberry Extract. Lyophilized black raspberry powder was a generous gift from Dr. Laura Kresty, Ohio State University Comprehensive Cancer Center. The powder was extracted following a modified procedure of Jing et al. (*15*). One part of the dry powder was mixed with three parts of acetone/water (70:30, v/v) containing 0.1% formic acid, briefly homogenized by a hand-held tissuemiser (Fisher Scientific), and sonicated in a Fisher FS30 ultrasonic bath for 15 min. The slurry was then centrifuged at 1800*g* for 10 min, and the supernatant was collected. The pellet was re-extracted twice for further removal of pigmentation from the matrix. The pooled supernatant was mixed (1:1, v/v) with chloroform and centrifuged at 1800*g* for 15 min. The red aqueous fraction was transferred to a round-bottom flask and evaporated with a rotary evaporator at 35 °C to remove organic solvents. The extract was lyophilized to powder form and stored at −80 °C under N_2.

Animals and Experimental Design. Male Fischer 344 rats (8 weeks of age; ∼200 g) were purchased (Taconic Farms, Germantown, NY) and maintained in the University Laboratory Animal Resources facility on The Ohio State University campus in accordance with NIH Laboratory Animal Use Guidelines. The Institutional Animal Care and Use Committee at The Ohio State University approved the experimental protocol (OSU Protocol 2004A0139). Animals were group housed with two animals per cage and maintained under standard conditions (22 ± 2 °C, 50 ± 10% relative humidity, 12 h light/dark cycle). Commercial diet and water were available ad libitum prior to the experiment.

Rats ($n = 30$) at 11 weeks of age were randomly assigned to one of five groups. After being fasted overnight, animals were lightly anesthetized for the delivery of 1.2 ± 0.3 mL of water either containing 0.1% citric acid alone (control) or including black raspberry extract (treatment) by stomach tube. The precise volume delivered was determined by weighing the syringe and intubation tube before and after gavage. The administered extract contained 22.3 nmol (10 mg) of cyanidin 3-glucoside equivalent/mL as measured by pH differential method (*16*). Treatment groups ($n = 6$) were asphyxiated with CO_2 after 30, 60, 120, and 180 min. The control group ($n = 6$) was asphyxiated at 30 min after intubation. Contents from stomach and small intestine were separately collected by perfusing the lumen with 25 mL of ice-cold 20% formic acid in phosphate-buffered saline (PBS). Urine samples (when present) were collected from the bladder and acidified immediately with 20% (v/v) formic acid. The stomach and small intestine were then removed and immediately frozen by liquid nitrogen. All samples were stored at −80 °C until analysis.

Sample Preparation. Urine samples were thawed and weighed with the container. The weight of the container was later recorded and subtracted from the total weight. Urine volume was calculated on the basis of weight assuming a density of 1.0 g/mL. A 50 μL aliquot was added to 550 μL of 0.1% TFA and centrifuged at 16000*g* for 15 min prior to HPLC analysis of the supernatant (*17*). The remaining samples were pooled and semipurified by a 1 cm^3 Sep-Pak C_{18} cartridge (Waters, Milford, MA) for HPLC-MS analysis to confirm peak identities.

Gastric and small intestinal contents were thawed, sonicated for 15 min in an ice−water bath, and centrifuged at 1800*g* for 10 min. An aliquot of the supernatant was filtered (0.45 μm Whatman polypropylene filter) for HPLC analysis. A second aliquot of the remaining sample was pooled by group and semipurified by a 6 cm^3 Sep-Pak C_{18} cartridge for HPLC-MS analysis to confirm peak identities. The C_{18} semipurification method was modified from our previous studies (*8, 18*). Briefly, the sample was loaded onto a preconditioned C_{18} cartridge and washed with 2 volumes of 0.1% TFA acidified water before anthocyanins were eluted with 1 volume of 0.1% TFA acidified methanol. The methanolic eluate of urine was dried under N_2 and redissolved in an aliquot of acidified water for HPLC analyses. To preserve the labile anthocyanidin aglycone in gastrointestinal samples, the methanolic eluate was not evaporated but instead diluted in acidified water (1:9, v/v) to maintain retention of anthocyanins on the reverse-phase HPLC column.

Extractions of anthocyanins from the stomach (1.43 ± 0.032 g, $n = 30$) and small intestinal tissues (7.1 ± 0.12 g, $n = 30$) were carried out separately. The samples and solutions were kept on ice during the entire process to prevent enzymatic and/or heat degradation. Mucus and epithelial layer were removed from the small intestine because dark food particles adhered to the surface. The remaining muscular tissue was homogenized at high speed for approximately 3 min in 10 mL (stomach) or 15 mL (intestine) of methanol (with 0.1% formic acid) using a hand-held tissuemizer followed by sonication in an ice−water bath for 20 s. The methanolic extract was then centrifuged at 1800*g* for 10 min. Supernatants were collected, pellets were re-extracted and centrifuged at 1800*g* for 10 min, and the pooled supernatants were diluted 6-fold using water containing 0.1% formic acid. Diluted supernatants were then passed through a Sep-Pak C_{18} (5 g, 20 cm^3) cartridge and recovered with 10 mL of 0.1% formic acid in methanol, which was evaporated using a rotary evaporator. The remaining anthocyanins were redissolved and made up to 5 mL using 10% formic acid prior to centrifugation at 16000*g* for 10 min, filtration with 0.45 μL polypropylene syringe filters, and HPLC-MS analysis.

Recovery. Black raspberry extract (11 nmol of cyanidin 3-glucoside equivalent) was spiked into effluents of gastric and small intestinal contents from control animals ($n = 4$) to simulate the concentration found in the 30 min treatment groups. The slurry was sonicated in an ice−water bath for 15 min to facilitate anthocyanin penetration into particulate materials. Spiked samples were frozen and analyzed on the following day according to the procedures described above. Recovery was calculated as (total amount recovered/total amount added) × 100%. Profiles of recovered anthocyanins from the spiked samples were compared to that from the gastrointestinal contents of treatment groups to identify specific changes not attributable to nonspecific binding.

HPLC-MS Analysis of Anthocyanins and Anthocyanin Metabolites. Samples were analyzed using a Shimadzu LCMS-2010 EV HPLC-MS (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with an SPD-M20A photodiode array (PDA) detector and a single-quadrupole electron spray ionization (ESI) MS detector. Separation was accomplished on a 150 mm × 4.6 mm i.d., 3.5 μm, Symmetry C_{18} column (Waters Corp., Milford, MA) with a flow rate of 0.8 mL/min. Conditions for urine samples were as follows: injection volume, 200 μL; mobile phases, A, 4.5% formic acid in HPLC-MS grade water, and B, HPLC-MS grade acetonitrile; gradient, 0−5 min, 2−7% B; 5−20 min, 7−12% B; 20−25 min, 12−17% B; and 25−40 min, 17−25% B. Conditions for gastric and small intestinal content/tissue samples were the following: injection volume, 20 μL; mobile phases, A, 10% formic acid in HPLC-MS grade water, and B, HPLC-MS grade acetonitrile; gradient, 0−6 min, 5−8% B; 6−15 min, 8−10% B; 15−20 min, 10−25% B; and 20−25 min, 25% B. After each run, the column was equilibrated for 5 min under the initial condition. Due to the fact that anthocyanins undergo reversible structure interconversion at pH >2, it is critical to keep the mobile phase pH <2 to achieve sharp peaks, even though the Symmetry C_{18} column does not really tolerate pH conditions <2. Spectroscopic data (250−700 nm) were collected during the entire separation procedure. When the MS was coupled to the HPLC, spectra were obtained under positive ion condition using SCAN (from *m*/*z* 200−1200) and selective ion monitoring (SIM) modes. Six channels including *m*/*z* 287 for cyanidin and *m*/*z* ratios for other common anthocyanin aglycones were monitored in the SIM mode. Anthocyanin concentrations in the urine, perfusate of gastrointestinal contents, and tissues were calculated using peak areas of HPLC chromatograms at 520 nm and a standard calibration curve. Anthocyanin amount was calculated by multiplying the concentration in the aliquot with the

corresponding volume. On the basis of the imperial rule that the signal-to-noise ratio needs to be greater than 10:1 for accurate quantitation, the detection limit for our HPLC instrument is 0.1 nmol/mL using a 200 μL injection volume.

Calibration Curve. Commercially available cyanidin 3-glucoside standard (22.3 nmol) was dissolved in 10 mL of double-distilled water containing 0.1% TFA, and a series of dilutions (0.1−35 nmol/mL) were prepared to generate a standard curve ($R^2 > 0.99$). All anthocyanins analyzed fell within the range of the standard curve and were expressed as nmol cyanidin 3-glucoside equivalents. The total amount of recovered anthocyanins was calculated by summing the peak areas of individual anthocyanin peaks at 520 nm and using this calibration curve.

Statistical Analysis. One-way ANOVA or its nonparametric analogue Kruskal−Wallis test (equal variance not assumed) was conducted using SPSS (version 13, 2004, SPSS Inc., Chicago, IL) to compare amounts of total and individual anthocyanins in different treatment groups, and data are given as means ± SE. When appropriate, significance of differences between means was determined by Tukey's HSD or Dunnett's T3 (equal variance not assumed). Differences of $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Flux of Anthocyanins in the GI Lumen. Information regarding the kinetic flux of anthocyanins in the GI is critical for understanding anthocyanin absorption. Recent studies have suggested that the stomach is a potential site for the absorption of anthocyanins (*7*, *19*), in addition to the small intestine, which is a known common absorption site for other structurally related flavonoids (*17*). Therefore, absorption may begin immediately after ingested anthocyanins reach the stomach. The rate of gastric emptying and transit through the small intestine, as well as anthocyanin stability, will determine the duration and extent of anthocyanin accessibility to the mucosal epithelia for uptake and possible transfer to circulation. Combining kinetic flux through the GI with pharmacokinetic data is necessary to determine the primary site(s) of anthocyanin absorption.

A preliminary examination using spiked perfusate samples from fasted rats demonstrated recovery rates of 93 ± 2% (n = 4) and 84 ± 10% (n = 4) for black raspberry anthocyanins added to gastric and small intestinal contents, respectively. We observed that contents from the small intestine had a gel-like property causing difficulty with passage through a 0.45 μm polypropylene filter. The extent of pigmentation on the filters was greater for samples with more viscous or gel-like consistency. The viscosity variability of luminal contents from the small intestine samples contributed to the relatively large variation in recovery of anthocyanins from the small intestinal contents.

Anthocyanins in the black raspberry extract and collected biological materials were identified by comparing retention times and UV−vis spectra to known standards and by comparing m/z ratios of molecular ions and fragments to established values in black raspberry (*20*). The four major anthocyanins identified in the crude extract of black raspberry, as well as gastric and intestinal contents (**Figure 1**), included cyanidin 3-sambubioside, cyanidin 3-glucoside, cyanidin 3-xylosylrutinoside, and cyanidin 3-rutinoside. Anthocyanin total concentrations in the gastric and small intestinal contents were adjusted for the efficiency of extraction and are presented in **Figure 2**. Approximately 52% of the total amount of black raspberry anthocyanins delivered to the stomach was present in gastric luminal contents 30 min after administration of the bolus. Anthocyanin content in the gastric lumen decreased linearly (R^2 = 0.67) during the 180 min study. The estimated time to deplete half of the anthocyanin content in the gastric lumen ($t_{1/2}$) of the fasted rat was approximately 120 min, suggesting that minimal amounts of

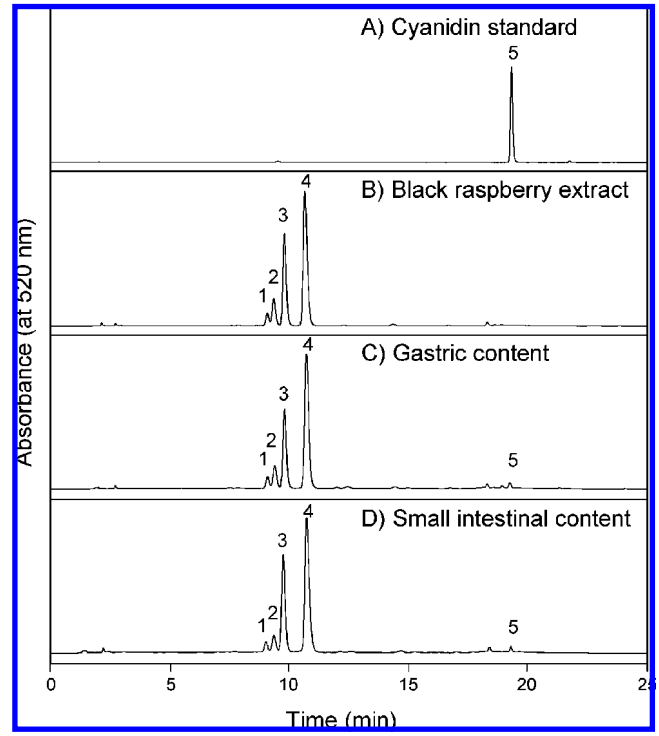

Figure 1. Chromatographic profiles of (**A**) cyanidin standard and (**B**) black raspberry anthocyanins in the administered crude extract and of representative samples of luminal contents from (**C**) the stomach and (**D**) the small intestine of a rat killed 120 min after gavage. Chromatograms are monitored at 520 nm for detection of anthocyanins. Peak identities are as follows: cyanidin 3-sambuoside (1); cyanidin 3-glucoside (2); cyanidin 3-xylosylrutinoside (3); cyanidin 3-rutinoside (4); cyanidin (5).

anthocyanins would be present in the stomach after 4 h. Our result agrees with a previous study by Borges et al. (*21*), who reported almost no raspberry anthocyanins remained in the gastric lumen of fasted rats 4 h after gavaging raspberry juice. However, these investigators observed a nonlinear decrease of the anthocyanin content in the stomach, with a more rapid decrease during the first hour. This rapid decrease in their study may be a result of the high anthocyanin dose administered (920 vs 27 nmol of cyanidin 3-glucoside equivalent in our experiment).

The amount of anthocyanins in the small intestinal lumen increased between 30 and 120 min after administration of the black raspberry extract and decreased by 180 min (**Figure 2**). This suggests that the amount entering the small intestine prior to 120 min exceeded the total amount of small intestinal mucosa uptake, exiting to the lower intestine, and perhaps degradation. Combining such information with the fact that plasma anthocyanin concentration usually is maximal within 15−30 min in rats after gastric intubation of anthocyanin extract (*6*), it is reasonable to speculate that the stomach has an important role in anthocyanin absorption. Otherwise, the plasma concentration of anthocyanins would be maintained at the same or a higher concentration after 30 min as anthocyanin content in the small intestine continues to increase from 30 to 120 min.

The total amount of administered anthocyanins recovered from gastric and small intestinal contents was 75−79% (~20 nmol of cyanidin 3-glucoside equivalent) from 30 to 120 min after delivery (**Figure 2**), demonstrating that the anthocyanins delivered in the absence of a food/meal are stable within the lumen of the upper GI of fasted rats. In contrast, losses of >50% of anthocyanins have been reported during in vitro digestion (*22*, *23*). Anthocyanins are not stable at neutral pH, forming

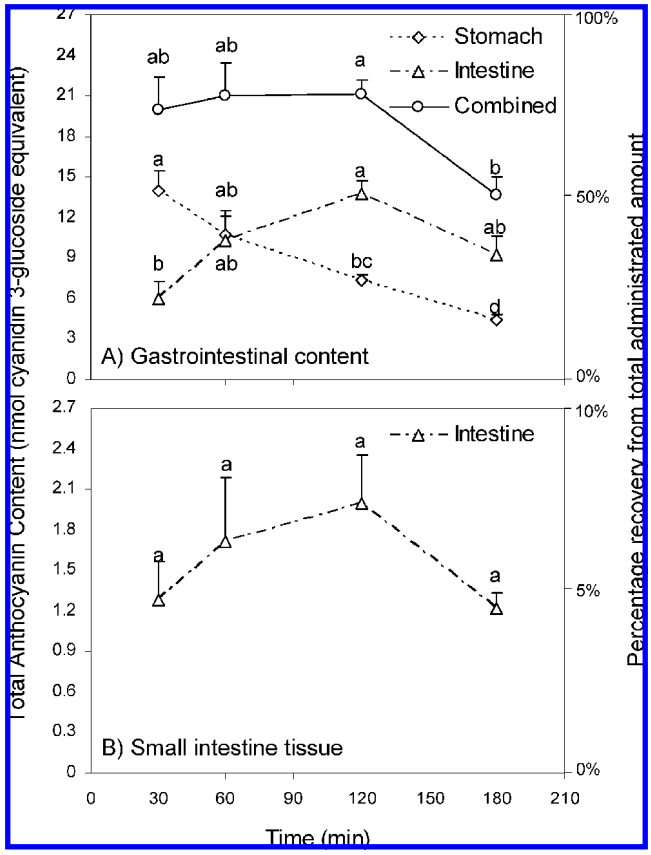

Figure 2. Black raspberry anthocyanins in (**A**) the gastric and small intestinal contents and (**B**) the small intestinal tissue. Rats were administered approximately 27 nmol (cyanidin 3-glucoside equivalent) of anthocyanins in acidic solution by stomach tube. Data are means ± SE for six rats. Within each line, means with different letters are significantly different ($P < 0.05$).

pseudobases, quinoidal bases, and chalcones that are subject to nucleophilic attack by water (*23*). However, we hypothesize that binding of anthocyanins to mucus, secretions, and food residues in vivo may increase stability. Knowing the stability of anthocyanins in the GI lumen is important not only because the accessibility affects absorption but also because some suspected health benefits depend on continuous exposure to anthocyanins. For example, Malik et al. (*24*) reported that exposure of colon cancer cells to anthocyanins caused cell cycle arrest at G1/G0 and G2/M phases, yet after the anthocyanins were removed the cells could recover from both the G1/G0 and G2/M blocks. By 180 min the anthocyanin content in the combined gastric and small intestinal contents decreased significantly as anthocyanins presumably entered the large intestine (**Figure 2A**).

Anthocyanins in GI Tissues. Intense red color was present in the acidic extract of all gastric and small intestine tissue samples. The intensity of the red color in the gastric tissue extract steadily decreased over time, coinciding with the linear decrease of anthocyanins in the gastric lumen. However, anthocyanins appeared to bind to unidentified protein in the stomach tissue and thus could not be quantified as free anthocyanins by HPLC. The presence of anthocyanins in the gastric tissue extract was confirmed by observing spectroscopic changes at pH 1.0, 4.5, and 10.0 (**Figure 3**). Such binding may be attributed to nonspecific binding or perhaps specific binding to transporter protein in the stomach (*25*). There was a parallel trend of anthocyanin concentrations in the small intestinal tissue and luminal contents (**Figure 2**). This correlation suggests that

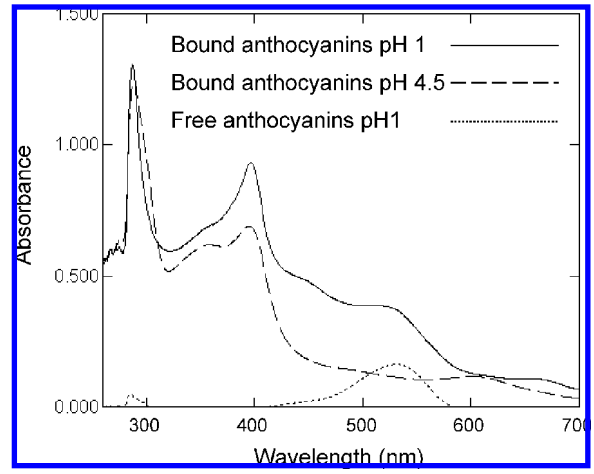

Figure 3. UV−vis spectra of the anthocyanin−protein complex extracted from the stomach tissue. Reversible decrease of absorbance near 520 nm is unique for anthocyanins when the pH changes from 1 to 4.5.

some portion of the newly acquired anthocyanins within GI mucosa was quickly degraded and/or effluxed across the apical or basolateral membranes. Otherwise, anthocyanins would be expected to continue to accumulate in the tissues with increasing time.

The total amount of anthocyanins in the small intestine tissue reached 7.5% of the administered dose (120 min), a much higher percentage than the reported anthocyanin absorption (normally <0.1%), which was based on plasma and urine anthocyanin concentrations (*2*). Considering the fact that anthocyanins would degrade or exit GI tissue rapidly, the actual uptake by the small intestine tissue may be much larger than 7.5%. In the current study when 27 nmol of cyanidin 3-glucoside equivalent of black raspberry anthocyanins was administered as a bolus, the highest concentration in the small intestine tissue (0.282 nmol of cyanidin 3-glucoside equivalent/g, $n = 6$) was observed at 120 min. This observation agrees with a recent paper by Talavera et al. (*4*) in which 0.605 nmol of cyanidin 3-glucoside equivalent/g of jejunum tissue was found in rats ($n = 6$) fed ~370 nmol of cyanidin 3-glucoside equivalent of blackberry anthocyanins for 15 days. The exceptionally high anthocyanin concentration in GI tissues as compared to the much lower concentrations in blood or other tissues (*4, 26*) suggests that intact anthocyanins may be taken up by GI tissues efficiently but not effectively transported into circulation. Our speculation is supported by a recent study evaluating the absorption of black currant anthocyanins by monolayers of human intestinal epithelial Caco-2 cells (*27*). This in vitro model revealed that transport across the apical membrane occurs to a much larger extent than further translocation across the basolateral membrane. Despite inefficient transfer across the basolateral membrane into plasma, anthocyanins in GI tissues may have protective effects in situ. Bruce et al. (*10*) suggested that colon cancer development might be inhibited by agents that prevent epithelial barrier damage, inhibit inflammation, or quench reactive oxygen species in local epithelial cells. Anthocyanins have been shown to be potent antioxidant and anti-inflammatory agents in vitro (*28*). In vivo studies examining the ability of anthocyanins to protect GI against oxidative stress are merited.

Anthocyanin Transformation in the GI. Besides the four major anthocyanins identified in the black raspberry diet, a minor peak of cyanidin (anthocyanidin) was observed in both stomach and intestinal contents (peak 5 in **Figure 1C**,**D**). Anthocyanidins are degradation intermediates of glycosylated anthocyanins when

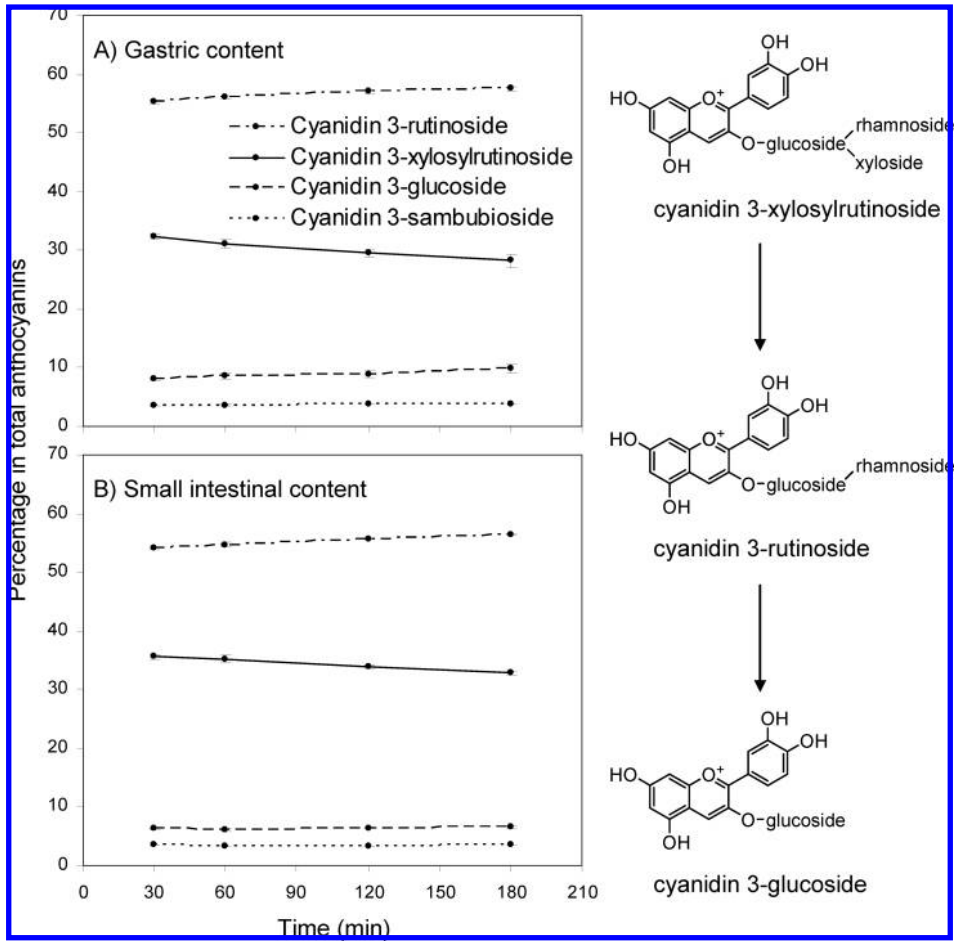

Figure 4. Profile of black raspberry anthocyanins ($n = 6$) in the gastric (**A**) and small intestinal lumens (**B**). ANOVA for linear regression model indicated significant ($P < 0.05$) decrease in the relative amount of cyanidin 3-xylosylrutinoside and a significant increase in the relative amounts of cyanidin 3-rutinoside and cyanidin 3-glucoside in gastric lumen. The pattern of anthocyanin profile in the small intestine is similar to that in the stomach, indicating no further metabolism with the exception of the decrease in cyanidin 3-glucoside.

deglycosylation occurs. As anthocyanidins degrade quickly at room temperature, to preserve them we shortened sample preparation to avoid using rotary evaporation or a stream of N_2 to remove organic solvents. A slight change of the anthocyanin profile over time occurred in the gastric content, probably due to acid hydrolysis. Anthocyanidins are usually produced from glycosylated anthocyanins via acid hydrolysis in the laboratory (*14*). Boiling anthocyanins in 2 N HCl ensures nearly complete cleavage of glycosidic bonds within 30 min. The pH in the gastric lumen after fasting can be as low as 1, and this acidic environment may cause limited hydrolysis. It has to be noted that the vehicle for delivery of the black raspberry extract to rat stomach was slightly acidic and thus expected to stabilize anthocyanins. In contrast, introduction of food matrix into the gastric lumen leads to rapid elevation of gastric pH, resulting in perhaps no acid hydrolysis and decreased overall stability of anthocyanins. The presence of cyanidin in the gastric content (**Figure 1C**) suggested cleavage of glycosidic bond between anthocyanidin and sugar moieties. As cleavage may also occur at glycosidic bonds within a multiglycoside moiety, hydrolysis of triglycoside (e.g., between rutinose and xylose) or diglycoside (e.g., between glucose and rhamnose) to the simpler glycoside was also observed (**Figure 4**). Such hydrolysis explained the slight but steady decrease of cyanidin 3-rutinoside with proportional increases in cyanidin3-rutinoside and cyanidin 3-glucoside in the stomach over time. The small intestinal contents are neutral, so further acid hydrolysis is not expected. Changes in the anthocyanin profile in the GI are likely to influence the

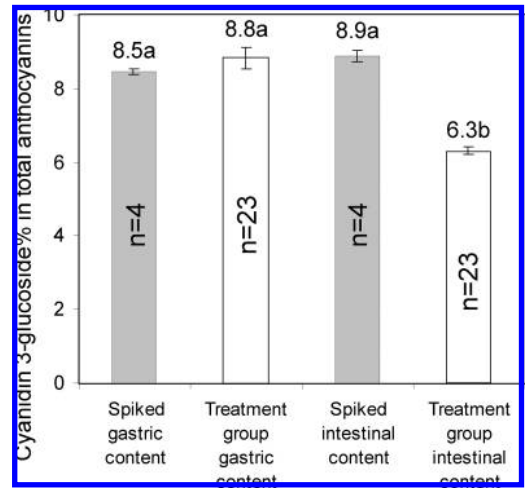

Figure 5. Relative share of cyanidin 3-glucoside from the total anthocyanins is decreased in the small intestinal content as compared to that in the administered material and the gastric content. Due to negligible change with time, all data from the four treatment groups are pooled. Means with different letters are significantly different ($P < 0.05$) as determined by Dunnett's T3 test following a Kruskal−Wallis test.

profile observed in plasma, tissues, and urine (*29*). Failure to consider this factor may overestimate the absorption of monoglycosides and underestimate the absorption of multiglycosides present in foods, beverages, and formulations.

Table 1. Black Raspberry Anthocyanins in Urine Collected from Rat Bladder[a]

	30 min ($n = 3$)	60 min ($n = 5$)	120 min ($n = 5$)	180 min ($n = 2$)
recovery of administered anthocyanins (%)	0.007 (0.003−0.010)[b]	0.024 (0.007−0.042)	0.045 (0.011−0.10)	0.013 (0.010−0.017)
% of coeluted[c] cyanidin 3-glucoside and cyanidin 3-sambuoside in total anthocyanins	9.3 (8.9−9.7)	8.6 (7.3−9.7)	7.4 (6.7−8.1)	6.4 (6.3−6.5)

[a] Data presented as mean values of all available samples in each group. [b] Numbers in parentheses indicate the range for each group. [c] The mobile phase gradient employed for urine samples was not able to separate all of the black raspberry anthocyanin peaks completely.

The anthocyanin profile of small intestinal contents was similar to that in the stomach (**Figure 4**) with one exception. The relative share of cyanidin 3-glucoside in total anthocyanins was significantly ($p < 0.001$) decreased in the small intestinal contents as compared to that in the administered extract and gastric contents (**Figure 5**). Such selective degradation of this anthocyanin glucoside was reported in our previous animal study using rats (*8*) and by Wu et al. using a pig model (*29, 30*). We observed additional supporting evidence for the hydrolysis of anthocyanins as cyanidin appeared in the intestinal contents (**Figure 1D**). Lactase−phlorizin hydrolase (LPH), which is located in the brush border membrane of mature small intestine enterocytes (*31*), and β-glycosidase activity in microorganisms residing in the small intestine are likely the basis for this hydrolysis. It is unlikely that the observed cyanidin was carried over from the stomach, because the aglycone is highly unstable.

A preliminary test using the small intestinal mucosal layer of 8-month-old pigs has also provided supporting evidence of LPH activity by showing the selective hydrolysis of cyanidin 3-glucoside in black raspberry anthocyanins after incubation (unpublished data). An alternative explanation to the selective decrease of cyanidin 3-glucoside in the intestinal lumen is high uptake. However, the cyanidin 3-glucoside recovered from small intestinal tissue represented $7.5 \pm 0.16\%$ ($n = 23$) of the total anthocyanins extracted, which was significantly ($P < 0.001$) lower than that in the black raspberry extract and in the spiked control samples. Such evidence confirmed that the selective decrease was due to degradation rather than uptake. In a previous study using in situ intestine perfusion to evaluate anthocyanin uptake, Talavera et al. (*17*) stated that cyanidin 3-glucoside was preferably absorbed as compared to other cyanidin glycosides on the basis of the evidence that cyanidin 3-glucoside disappeared to a large extent in the intestinal efflux. We now suggest that their observation might instead be attributed to degradation of cyanidin 3-glucoside during intestine perfusion. In the present study, degradation of anthocyanin glucoside did not appear to be a major factor for overall anthocyanin stability. However, it is noteworthy that >90% of anthocyanins identified in nature contain glucoside moieties (*32*), and in weaning mammals or some adults (*33*) LPH activity remains remarkably high, potentially causing extensive biotransformation of ingested anthocyanins.

Hydrolysis of the glycosidic bond is considered to be critical for flavonoid digestion because their aglycones rather than the ingested glycosides are readily absorbed (*34*). Anthocyanins differ from most other flavonoids in that anthocyanidins (aglycones) are considered to be relatively unstable and rarely detected in plasma or urine in many studies (*6*). However, even if only present for a relatively short time, anthocyanidins may have the potential to be taken up by epithelial cells lining the gastric and small intestinal mucosa, where they are metabolized to become conjugated metabolites with some being absorbed (*35*). The anthocyanidins may also undergo spontaneous ring fission to generate smaller phenolic compounds that may be absorbed to have health-promoting benefits (*11, 12*). In the case of the cyanidin aglycone, protocatechuic acid is expected to be one of the ring fission products. Although we have observed the presence of protocatechuic acid when genuine cyanidin standard was degraded in buffer, it was not detected in either the gastric or small intestinal contents. In another study cyanidin and protocatechuic acid were particularly monitored but not found in rat plasma after gavage feeding of anthocyanin-rich extract from wild mulberry (*36*). Perhaps cyanidin and protocatechuic acid were promptly degraded during digestion in gastric and small intestinal lumens or only primarily produced in the large intestine, where absorption is much less efficient than the small intestine (*12*).

Metabolism and Excretion of Absorbed Anthocyanins. Black raspberry anthocyanins appeared in the urine within 30 min after stomach intubation (**Table 1**). At 120 min total anthocyanins in the urine collected directly from the bladder accounted for 0.045% of that administered. Although urine collection was incomplete, this low amount agrees with other studies indicating low absorption of anthocyanins. Anthocyanin content in the urine collected from the bladder at 180 min postadministration declined, suggesting excretion and further metabolism. Methylated anthocyanin metabolites (identified by comparing the retention time and *m*/*z* to known anthocyanin peaks), primarily from the most abundant black raspberry anthocyanin cyanidin 3-rutinoside, were identified in urine and accounted for 18.2% ($n = 15$) of total anthocyanins recovered. Nevertheless, such methylated metabolites were not detected in the GI contents. The enterohepatic cycle extends the plasma elimination half-life of many flavonoids due to reabsorption (*3*), but with such low concentrations, if any, of anthocyanin metabolites found in the GI lumen, the enterohepatic cycle (*35*) appeared to be incapable of influencing anthocyanin excretion half-life (*4*).

The profile of anthocyanins in the urine was similar to that in the GI. Wu et al. (*29*) also reported a positive relationship between the anthocyanin profile in the GI and that in the urine of weaned pigs. Similarly, we found that hydrolysis of cyanidin 3-glucoside in the small intestine (~28% of initial cyanidin 3-glucoside, **Figure 5**) was associated with a relative decrease in urine at 180 min (~31% drop compared to 30 min, **Table 1**). At 180 min the stomach was almost empty; thus, gastric absorption was negligible and the small intestinal content profile determined the urine profile. At 30 min gastric absorption appeared to be predominant as the urinary profile of anthocyanins was quite similar to that in the gastric lumen (**Table 1**).

In conclusion, black raspberry anthocyanins were relatively stable in the gastric and small intestinal lumens of fasted rats, in contrast to reports from some in vitro studies. Total anthocyanins in the gastric lumen and tissue steadily decreased during the 180 min period following administration, whereas anthocyanin contents in the small intestinal lumen and tissue were highest at 120 min before decreasing. A significant portion of administered anthocyanins was taken up into the GI tissues, but neither extensively delivered into the blood nor cumulatively retained. The profile of anthocyanins changed in the GI with

some hydrolysis of glycosidic linkages occurring in the acidic gastric lumen, as well as selective hydrolysis of cyanidin 3-glucoside in the small intestinal content likely due to endogenous β-glucosidase activity. Unlike some other flavonoids, the stomach is an important site for absorption of anthocyanins. As anthocyanins transit the GI lumen, the predominant absorption site changed gradually from the stomach to the small intestine. The present study provides novel information about the kinetics of the gastrointestinal flux of anthocyanins in vivo and is expected to contribute to a better understanding of the metabolism, absorption, tissue uptake, and health-promoting activities of these water-soluble plant pigments.

ABBREVIATIONS USED

ESI, electron spray ionization; GI, gastrointestinal tract; LPH, lactase−phlorizin hydrolase; PBS, phosphate-buffered saline; PDA, photodiode array; SIM, selective ion monitoring; TFA, trifluoroacetic acid.

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LITERATURE CITED

(1) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *J. Agric. Food Chem.* **2006**, *54*, 4069–4075.

(2) Prior, R. L., Absorption and metabolism of anthocyanins: potential health effects. In *Phytochemicals: Mechanism of Action*; Meskin, M. S., Bidlack, W. R., Davies, A. J., D. S., L., R. K., R., Eds.; CRC Press: Boca Boton, FL, 2004; pp 1−19.

(3) Kay, C. D. Aspects of anthocyanin absorption, metabolism and pharmacokinetics in humans. *Nutr. Res. Rev.* **2006**, *19*, 137–146.

(4) Talavera, S.; Felgines, C.; Texier, O.; Besson, C.; Gil-Izquierdo, A.; Lamaison, J. L.; Remesy, C. Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. *J. Agric. Food Chem.* **2005**, *53*, 3902–3908.

(5) Kay, C. D.; Mazza, G. J.; Holub, B. J. Anthocyanins exist in the circulation primarily as metabolites in adult men. *J. Nutr.* **2005**, *135*, 2582–2588.

(6) McGhie, T. K.; Walton, M. C. The bioavailability and absorption of anthocyanins: towards a better understanding. *Mol. Nutr. Food Res.* **2007**, *51*, 702–713.

(7) Talavera, S.; Felgines, C.; Texier, O.; Besson, C.; Lamaison, J. L.; Remesy, C. Anthocyanins are efficiently absorbed from the stomach in anesthetized rats. *J. Nutr.* **2003**, *133*, 4178–4182.

(8) He, J.; Magnuson, B. A.; Giusti, M. M. Analysis of anthocyanins in rat intestinal contents—impact of anthocyanin chemical structure on fecal excretion. *J. Agric. Food Chem.* **2005**, *53*, 2859–2866.

(9) Lala, G.; Malik, M.; Zhao, C.; He, J.; Kwon, Y.; Giusti, M. M.; Magnuson, B. A. Anthocyanin-rich extracts inhibit multiple biomarkers of colon cancer in rats. *Nutr. Cancer* **2006**, *54*, 84–93.

(10) Bruce, W. R.; Giacca, A.; Medline, A. Possible mechanisms relating diet and risk of colon cancer. *Cancer Epidemiol. Biomarkers Prev.* **2000**, *9*, 1271–1279.

(11) Tsuda, T.; Horio, F.; Osawa, T. Absorption and metabolism of cyanidin 3-*O*-β-D-glucoside in rats. *FEBS Lett.* **1999**, *449*, 179–182.

(12) Fleschhut, J.; Kratzer, F.; Rechkemmer, G.; Kulling, S. E. Stability and biotransformation of various dietary anthocyanins in vitro. *Eur. J. Nutr.* **2006**, *45*, 7–18.

(13) Seeram, N. P.; Adams, L. S.; Zhang, Y.; Lee, R.; Sand, D.; Scheuller, H. S.; Heber, D. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* **2006**, *54*, 9329–9339.

(14) Giusti, M. M.; Wrolstad, R. E. Characterization of red radish anthocyanins. *J. Food Sci.* **1996**, *61*, 322–326.

(15) Jing, P.; Noriega, V.; Schwartz, S. J.; Giusti, M. M. Effects of growing conditions on purple corncob (*Zea mays* L.) anthocyanins. *J. Agric. Food Chem.* **2007**, *55*, 8625–8629.

(16) Giusti, M. M.; Wrolstad, R. E. Characterization and measurement of anthocyanins by UV-visible spectroscopy. In *Handbook of Food Analytical Chemistry, Pigments, Colorants, Flavors, Texture, And Bioactive Food Components*; Wrolstad, R. E., Acree, T. E., Decker, E. A., Penner, M. H., Reid, D. S., Schwartz, S. J., Shoemaker, C. F., Smith, D., Sporns, P., Eds.; Wiley-Interscience: Hoboken, NJ, 2004; pp 19−31.

(17) Talavera, S.; Felgines, C.; Texier, O.; Besson, C.; Manach, C.; Lamaison, J. L.; Remesy, C. Anthocyanins are efficiently absorbed from the small intestine in rats. *J. Nutr.* **2004**, *134*, 2275–2279.

(18) He, J.; Magnuson, B. A.; Lala, G.; Tian, Q.; Schwartz, S. J.; Giusti, M. M. Intact anthocyanins and metabolites in rat urine and plasma after 3 months of anthocyanin supplementation. *Nutr. Cancer* **2006**, *54*, 3–12.

(19) Passamonti, S.; Vrhovsek, U.; Vanzo, A.; Mattivi, F. The stomach as a site for anthocyanins absorption from food. *FEBS Lett.* **2003**, *544*, 210–213.

(20) Tian, Q.; Aziz, R. M.; Stoner, G. D.; Schwartz, S. J. Anthocyanin determination in black raspberry (*Rubus occidentalis*) and biological specimens using liquid chromatography−electrospray ionization tandem mass spectrometry. *J. Food Sci.* **2005**, *70*, C43−C47.

(21) Borges, G.; Roowi, S.; Rouanet, J. M.; Duthie, G. G.; Lean, M. E.; Crozier, A. The bioavailability of raspberry anthocyanins and ellagitannins in rats. *Mol. Nutr. Food Res.* **2007**, *51*, 714–725.

(22) Perez-Vicente, A.; Gil-Izquierdo, A.; Garcia-Viguera, C. In vitro gastrointestinal digestion study of pomegranate juice phenolic compounds, anthocyanins, and vitamin C. *J. Agric. Food Chem.* **2002**, *50*, 2308–2312.

(23) Bermudez-Soto, M. J.; Tomas-Barberan, F. A.; Garcia-Conesa, M. T. Stability of polyphenols in chokeberry (*Aronia melanocarpa*) subjected to in vitro gastric and pancreatic digestion. *Food Chem.* **2007**, *102*, 865–874.

(24) Malik, M.; Zhao, C.; Schoene, N.; Guisti, M. M.; Moyer, M. P.; Magnuson, B. A. Anthocyanin-rich extract from *Aronia meloncarpa* induces a cell cycle block in colon cancer but not normal colonic cells. *Nutr. Cancer* **2003**, *46*, 186–196.

(25) Passamonti, S.; Vrhovsek, U.; Mattivi, F. The interaction of anthocyanins with bilitranslocase. *Biochem. Biophys. Res. Commun.* **2002**, *296*, 631–636.

(26) Kalt, W.; Blumberg, J. B.; McDonald, J. E.; Vinqvist-Tymchuk, M. R.; Fillmore, S. A. E.; Graf, B. A.; O'Leary, J. M.; Milbury, P. E. Identification of anthocyanins in the liver, eye, and brain of blueberry-fed pigs. *J. Agric. Food Chem.* **2008**, *56*, 705–712.

(27) Steinert, R. E.; Ditscheid, B.; Netzel, M.; Jahreis, G. Absorption of black currant anthocyanins by monolayers of human intestinal epithelial Caco-2 cells mounted in ussing type chambers. *J. Agric. Food Chem.* **2008**, *56*, 4995–5001.

(28) Seeram, N. P.; Momin, R. A.; Nair, M. G.; Bourquin, L. D. Cyclooxygenase inhibitory and antioxidant cyanidin glycosides in cherries and berries. *Phytomedicine* **2001**, *8*, 362–369.

(29) Wu, X.; Pittman, H. E., 3rd; Prior, R. L. Fate of anthocyanins and antioxidant capacity in contents of the gastrointestinal tract of weanling pigs following black raspberry consumption. *J. Agric. Food Chem.* **2006**, *54*, 583–589.

(30) Wu, X.; Pittman, H. E., 3rd; McKay, S.; Prior, R. L. Aglycones and sugar moieties alter anthocyanin absorption and metabolism after berry consumption in weanling pigs. *J. Nutr.* **2005**, *135*, 2417–2424.

(31) Nemeth, K.; Plumb, G. W.; Berrin, J. G.; Juge, N.; Jacob, R.; Naim, H. Y.; Williamson, G.; Swallow, D. M.; Kroon, P. A. Deglycosylation by small intestinal epithelial cell β-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. *Eur. J. Nutr.* **2003**, *42*, 29–42.

(32) Andersen, O. M.; Jordheim, M. The anthocyanins. In *Flavonoids: Chemistry, Biochemistry and Applications*; Andersen, O. M., Markham, K. R., Eds.; CRC Press: Boca Raton, FL, 2006; pp 471−552.

(33) Swallow, D. M. Genetics of lactase persistence and lactose intolerance. *Annu. Rev. Genet*. **2003**, *37*, 197–219.

(34) Wilkinson, A. P.; Gee, J. M.; Dupont, M. S.; Needs, P. W.; Mellon, F. A.; Williamson, G.; Johnson, I. T. Hydrolysis by lactase phlorizin hydrolase is the first step in the uptake of daidzein glucosides by rat small intestine in vitro. *Xenobiotica* **2003**, *33*, 255–264.

(35) Ichiyanagi, T.; Rahman, M. M.; Kashiwada, Y.; Ikeshiro, Y.; Shida, Y.; Hatano, Y.; Matsumoto, H.; Hirayama, M.; Tsuda, T.; Konishi, T. Absorption and metabolism of delphinidin 3-*O*-β-D-glucopyranoside in rats. *Free Radical Biol. Med*. **2004**, *36*, 930–937.

(36) Matsumoto, H.; Ichiyanagi, T.; Iida, H.; Ito, K.; Tsuda, T.; Hirayama, M.; Konishi, T. Ingested delphinidin-3-rutinoside is primarily excreted to urine as the intact form and to bile as the methylated form in rats. *J. Agric. Food Chem*. **2006**, *54*, 578–582.

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