JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of Dose Size on Bioavailability of Acylated and Nonacylated Anthocyanins from Red Cabbage (*Brassica oleracea* L. Var. *capitata*)

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Recent studies indicate that anthocyanin intake conveys a variety of health benefits, which depend on absorption and metabolic mechanisms that deliver anthocyanins and their bioactive metabolites to responsive tissues. The anthocyanin bioavailability of red cabbage (*Brassica oleracea* L. var. *capitata*) was evaluated as reflected by urinary excretion of anthocyanins and anthocyanin metabolites. Twelve volunteers consumed 100, 200, and 300 g of steamed red cabbage (containing 1.38 μmol of anthocyanins/g of cabbage) in a crossover design. Anthocyanin concentration in cabbage extract and urine was measured by HPLC-MS/MS. Six nonacylated and 30 acylated anthocyanins were detected in red cabbage, and 3 nonacylated anthocyanins, 8 acylated anthocyanins, and 4 metabolites were present in urine. Mean 24 h excretion of intact anthocyanins increased linearly from 45 (100 g dose) to 65 nmol (300 g dose) for acylated anthocyanins and from 52 (100 g dose) to 79 nmol (300 g dose) for nonacylated anthocyanins. Urinary recovery of intact anthocyanins (percent of anthocyanin intake) decreased linearly from 0.041% (100 g dose) to 0.020% (300 g dose) for acylated anthocyanins and from 0.18% (100 g dose) to 0.09% (300 g dose) for nonacylated anthocyanins. Anthocyanin metabolites consisted of glucuronidated and methylated anthocyanins. The results show that red cabbage anthocyanins were excreted in both intact and metabolized forms and that recovery of nonacylated anthocyanins in urine was >4-fold that of acylated anthocyanins.

KEYWORDS: Anthocyanin; cyanidin; acylated; bioavailability; cabbage (*Brassica oleracea* L. var. *capitata*)

INTRODUCTION

Anthocyanins are secondary metabolites responsible for many of the colors found in leaves, fruits, vegetables, flowers, and grains. They serve a number of functions in plants including photoprotection, scavenging of free radicals, and attraction of animals for pollination and seed dispersal (*1*, *2*). Anthocyanins are a subgroup of the flavonoids and are glycosylated forms of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts (*3*). The sugar residues of many anthocyanins are further modified by one or more acylations with aliphatic or aromatic acids (*4*).

The human health benefits of anthocyanin intake are broad and have become increasingly apparent with recent investigations. Emerging evidence suggests that anthocyanin intake may reverse age-related neurodegenerative declines (*5*, *6*), provide cancer protection and suppression (*7*−*13*), improve cardiovascular health (*14*, *15*), stimulate insulin secretion (*16*, *17*), and ameliorate oxidative stress (*18*−*20*). These highly desirable health benefits depend on absorption and metabolic mechanisms that deliver anthocyanins and their bioactive metabolites to responsive tissues.

Anthocyanin absorption is affected by other dietary components. Nielsen et al. (*21*) reported that the peak concentration of black currant (*Ribes nigrum* L.) anthocyanins in plasma of human subjects was delayed about 2 h when a rice cake was ingested along with black currant juice. Anthocyanin levels in plasma of pigs reached a maximum earlier when black currant powder was ingested with sugar and water compared to when the same dose of black currant powder was provided either with a meal of cereal, milk, and sugar or with a meal of cereal, milk, sugar, and the flavonol rutin (*22*). Because other dietary components influence anthocyanin absorption, it follows that the food matrix in which anthocyanins are contained may also affect bioavailability. The possibility of food matrix effects underscores the value of investigating anthocyanin bioavailability using foods that are commonly consumed, particularly because anthocyanins are not normally consumed as pure compounds.

In addition to the food matrix, anthocyanin absorption is influenced by anthocyanin structure. Recent studies have demonstrated that acylated and nonacylated anthocyanins can

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10.1021/jf0710736
Published on Web 06/02/2007

be absorbed in their intact forms (*23*−*26*). Anthocyanin absorption may be mediated by the sodium-dependent glucose transporter (SGLT1), which is involved in the transport of the flavonoid quercetin (*27*), or the organic anion membrane carrier bilitranslocase (*28*). The efficiency of carrier proteins in anthocyanin transport would likely be related to anthocyanin structure. A study using Caco-2 human intestinal cell monolayers showed that cyanidin-3-glucoside and peonidin-3-glucoside had higher transport efficiencies than cyanidin-3-galactoside and peonidin-3-galactoside, respectively, indicating the higher bioavailability of glucose-based anthocyanins (*29*). In the same study, it was found that the presence of more free hydroxyl groups and fewer OCH_3 groups on the aglycone was generally associated with decreased anthocyanin bioavailability. Acylation of anthocyanins has a significant effect on absorption. In a study evaluating the absorption of purple carrot (*Daucus carota* L.) anthocyanins, acylation resulted in an 11−14-fold decrease in anthocyanin recovery in urine and an 8−10-fold decrease in anthocyanin recovery in plasma (*30*).

Daily intake of anthocyanins has been estimated to be 12.53 mg, primarily as vegetables, fruits, and beverages (*31*). Red cabbage (*Brassica oleracea* L. var. *capitata*), which has become popular in fresh-cut salad mixes, is distinct from many cruciferous vegetables in that it has a substantial anthocyanin content (*31*). Cabbage consumption has risen in recent years due to increased marketing of fresh-cut products, away-from-home eating (an important factor for cole slaw consumption), and research linking the consumption of cruciferous vegetables with protection against cancer (*32*, *33*). Therefore, red cabbage has significant potential as a health-promoting food. Moreover, the predominance in red cabbage of cyanidin 3-diglucoside-5-glucoside and acylated anthocyanins derived therefrom allows for side-by-side comparisons of absorption of nonacylated and acylated anthocyanins. Our objective was to determine the effect of dose size on bioavailability of acylated and nonacylated anthocyanins from red cabbage.

MATERIALS AND METHODS

Chemicals and Materials. High-performance liquid chromatography (HPLC) grade ethyl acetate, methanol, water and reagent grade trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Norcross, GA). Formic acid (reagent grade) was purchased from Sigma Chemical Co. (St. Louis, MO). Cyanidin-3-glucoside was purchased from Indofine Chemical Co. (Somerville, NJ). Pelargonidin-3-glucoside was purchased from Chromadex (Santa Ana, CA). Sep-Pak Vac RC (500 mg) C18 cartridges for solid-phase extraction (SPE) were obtained from Waters Corp. (Milford, MA). Nylon 0.2 μm centrifuge filter tubes were purchased from Alltech Associates (Deerfield, IL).

Subjects and Study Design. The study protocol was approved by the MedStar Research Institute (Hyattsville, MD), and written, informed consent was obtained from each study subject. The 12 subjects were healthy, nonsmoking volunteers (6 males, 6 females) averaging 46 years of age with a mean body weight of 74.6 kg and had a mean body mass index of 25.4.

A crossover design was used in which each subject received each of three treatments, which consisted of 100, 200, and 300 g of steamed red cabbage. Subjects were randomly assigned to one of three groups ($n = 4$/group), and each group had a different treatment order. There were three 2-day treatment periods separated by a minimum of a 5-day washout. On the first day of each treatment period, subjects consumed only anthocyanin-free meals provided by the Beltsville Human Nutrition Research Center (BHNRC). Following an overnight fast, subjects consumed one of the three red cabbage treatments in the morning. Subjects were provided anthocyanin-free meals and snacks starting 4 h after their dose. Urine was collected from subjects every 2 h for 12 h, and urine collected from 12 to 24 h postdose was pooled. Subjects were asked to abstain from mineral and supplement intake beginning 2 weeks prior to the study. Coffee and all teas except chamomile were disallowed during the treatment periods, but caffeine pills (200 g) were provided as needed to alleviate caffeine-withdrawal headaches.

Preparation of Red Cabbage. Heads of fresh red cabbage were purchased from local grocery stores on the same day, cut into wedges, and steamed in one batch for 15 min. The steamed cabbage was mixed for batch uniformity, and all red cabbage used in this study was removed from this batch. One hundred gram portions of red cabbage and 6.24 g of red cabbage juice (the drippings resulting from the steaming process) per portion were combined into plastic containers and frozen at −20 °C until used. The red cabbage juice was recombined with the red cabbage to restore anthocyanins leached out during the steaming process and did not add substantively to the dry matter of the portions. Twenty-two hours prior to consumption, red cabbage portions were thawed at 4 °C. Just prior to consumption, the red cabbage was microwave-cooked (Sharp 900 W microwave ovens) for 30 s to achieve an internal temperature of 60 °C. Subjects received one, two, or three 100 g portions of cabbage depending on whether they were scheduled for the 100, 200, or 300 g dose, respectively. Salt (0.75 g) and margarine (7.1 g) were consumed with each 100 g portion of red cabbage.

Extraction of Anthocyanins from Red Cabbage and Urine. Three 100 g portions of red cabbage from the batch prepared for consumption were randomly selected and lyophilized. Each portion represented a replication. A 0.10 g sample of lyophilized powder from each replication was combined with 1.90 mL of 10% formic acid in methanol (v/v) and 0.10 mL of pelargonidin-3-glucoside stock solution (200 nmol/mL of 0.1% HCl in methanol) as an internal standard to estimate extraction losses, vortex-mixed for 10 s, sonicated for 10 min, and centrifuged (3000*g*, 20 °C, 10 min). The supernatant was decanted to a collection vial, and the pellet was extracted three more times with 2.0 mL of 10% formic acid in methanol. The combined extracts were brought to volume with 10% formic acid in methanol in a 10 mL volumetric flask, and a 0.8 mL aliquot was filtered through a 0.2 μm nylon centrifuge filter tube prior to analysis.

Upon collection, 10 mL of each urine sample was combined with 2 mL of 0.44 M TFA and 50 μL of pelargonidin-3-glucoside (29.4 nmol/mL of 0.1% HCl in methanol) as an internal standard and was frozen at −80 °C until analyzed. Anthocyanins were extracted from urine as previously described except that pelargonidin-3-glucoside was used as the internal standard and was added prior to the samples being frozen (*30*).

HPLC-DAD-ESI/MS/MS Analysis of Anthocyanins. Anthocyanin analysis was performed with an Agilent Technologies (Palo Alto, CA) series 1100 HPLC followed by a model SL ion trap mass spectrometer (MS) with electrospray ionization. A 150 × 4.6 mm i.d., 3.5 μm Zorbax StableBond C18 column (Agilent) was used for anthocyanin separation and was held at 30 °C. The flow rate was 0.8 mL/min, and detection was at 520 nm. Mobile phase A was 10% formic acid in water (v/v), and mobile phase B was methanol. The gradient used was as follows: 0−20 min, 5−15% B; 20−40 min, 15−22% B; 40−55 min, 22−30% B; 55−80 min, 30−35% B; 80−85 min, 35−100% B; 85−90 min, 100% B; 90−95 min, 100−5% B; 95−100 min, 5% B. Because no anthocyanins or anthocyanin metabolites were detected in urine after 58 min, the run time was reduced to save solvent as follows: 0−20 min, 5−15% B; 20−40 min, 15−22% B; 40−55 min, 22−30% B; 55−65 min, 30−32% B; 65−70 min, 32−100% B; 70−75 min, 100% B; 75−80 min, 100−5% B; 80−85 min, 5% B. The conditions for mass spectrometry analysis were as follows: ESI interface, nebulizer, 60.0 psi; dry gas, 11.00 L/min; dry temperature, 327 °C; MS/MS, scan from *m*/*z* 200 to 1500; maximum accrual time, 200.0 ms; smart parameter setting (SPS), on; compound stability, 75%; trap drive level, 100%.

Calculations and Statistics. A standard curve was created using cyanidin-3-glucoside to calculate molar concentrations of individual anthocyanins expressed as cyanidin-3-glucoside equivalents. The concentration of acylated anthocyanins was calculated as the sum of individual acylated anthocyanins. The concentration of nonacylated anthocyanins was calculated as the sum of individual nonacylated anthocyanins. Molar concentrations were corrected for extraction losses of the internal standard, pelargonidin-3-glucoside, which averaged 34%. The interday coefficient of variation of HPLC analyses was 8.30%.

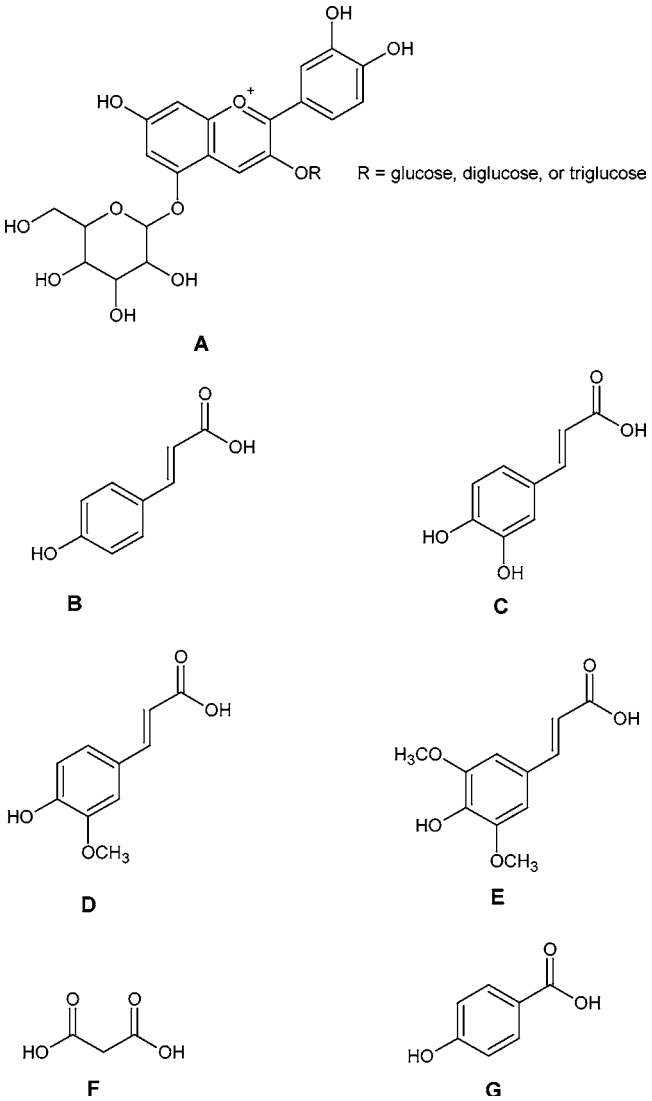

Figure 1. Chemical structures of predominant anthocyanins and acylated groups in red cabbage and in urine after red cabbage consumption: (**A**) cyaninidin 3-glucoside-5-glucoside, cyanidin 3-diglucoside-5-glucoside, or cyanidin 3-triglucoside-5-glucoside; (**B**) *p*-coumaric acid; (**C**) caffeic acid; (**D**) ferulic acid; (**E**) sinapic acid; (**F**) malonic acid; (**G**) *p*-hydroxybenzoic acid.

Incremental polynomial regression analysis was performed to characterize anthocyanin accumulation in urine with respect to the red cabbage dose ingested. Spearman rank order correlation was conducted to evaluate the relationship between the urinary recoveries of individual anthocyanins (as percent of anthocyanin dose ingested) and their HPLC elution orders. SigmaStat software (version 3.0, SPSS Inc., Chicago, IL) was used for all statistical analyses.

RESULTS

Anthocyanins in Red Cabbage. Thirty-six anthocyanins were identified, 8 of which were found in red cabbage for the first time (**Figures 1** and **2**; **Table 1**). Peak numbers refer to the red cabbage anthocyanins listed in **Table 1**. Acylated anthocyanin concentration was 1088 nmol/g of cooked red cabbage (78.7% of total), and nonacylated anthocyanin concentration was 294 nmol/g (21.3% of total). Total anthocyanins in a 100, 200, and 300 g doses were 138.2, 276.5, and 414.7 μmol, respectively. Details of the identification of previously reported anthocyanins were described by investigators using a methodology similar to the one we used and will not be repeated here (*4*). Identification of newly found anthocyanins was based on mass spectra and the prevalence in red cabbage of acylated anthocyanins derived from cyanidin 3-diglucoside-5-glucoside (*4*, *34*−*38*). Interpretation of mass spectra was based on previous observations that fragmentation of anthocyanins occurs almost exclusively at the glycosidic bonds between the flavylium ring and adjacent glucosides and that cleavage of ester linkages between glucosides and acylated groups was not observed (*39*). Acylated groups were determined by calculating possible combinations of aliphatic and aromatic acids found in acylated anthocyanins (*4*). Peak 6 was identified as peonidin 3-diglucoside-5-glucoside on the basis of its mass spectrum ($[M]^+$ *m*/*z* 787, MS/MS *m*/*z* 625, $[M - \text{glucose}]^+$, 463 $[M - \text{diglucose}]^+$, and 301 $[\text{peonidin}]^+$). Peak 9 was identified as peonidin 3,5-diglucoside ($[M]^+$ *m*/*z* 625, MS/MS *m*/*z* 463 $[M - \text{glucose}]^+$, and 301 $[\text{peonidin}]^+$). These peonidin-based compounds constituted 0.06% of total anthocyanins.

Peaks 16, 17, 19, 21, 23, and 30 were identified as diacylated tetraglucosides of cyanidin. Anthocyanins in this group had a single glucose substitution at position 5 and a triglucoside and two acylated groups attached at the 3-position of cyanidin. Peaks 16 and 21 were isomers with $[M]^+$ *m*/*z* 1287 yielding MS/MS fragment ions of *m*/*z* 1125 $[M - \text{glucose}]^+$ and 449 $[M - 838]^+$. The loss of 838 mu corresponds to the loss of triglucose and acylated groups of *p*-coumaric acid and sinapic acid, so this peak was identified as cyanidin 3-(*p*-coumaroyl)(sinapoyl)-triglucoside-5-glucoside. There were two isomers of cyanidin 3-(feruloyl)(sinapoyl)triglucoside-5-glucoside (peaks 17 and 23; $[M]^+$ *m*/*z* 1317, MS/MS *m*/*z* 1155 $[M - \text{glucose}]^+$, and 449 $[M - 868]^+$); the loss of 868 mu is consistent with the loss of triglucoside, ferulic acid, and sinapic acid. The mass spectrum of peak 30 indicated that the molecular mass of the acylated group(s) bonded to a triglucoside was 236. Therefore, this anthocyanin was identified as cyanidin 3-(*p*-hydroxybenzoyl)-(malonoyl)triglucoside-5-glucoside ($[M]^+$ *m*/*z* 1171, MS/MS *m*/*z* 1009 $[M - \text{glucose}]^+$, and 449 $[M - 722]^+$). The anthocyanin with the largest molecular ion ($[M]^+$ *m*/*z* 1347) was peak 19 and was tentatively identified as cyanidin 3-(sinapoyl)(sinapoyl)-triglucoside-5-glucoside.

Identification of Anthocyanins and Anthocyanin Metabolites in Urine. Eleven of the anthocyanins identified in urine were present in red cabbage (**Table 2**). Anthocyanins that were present in both red cabbage and urine are referred to by the peak numbers assigned in **Table 1**. Anthocyanin metabolites found only in urine were assigned peak numbers 37−40. Peak 14 was detected in urine but coeluted with a non-anthocyanin peak and therefore was not quantitated. Three glucuronides and an unknown compound were found. Peaks 37 and 39 were isomers with $[M]^+$ *m*/*z* 801, MS/MS *m*/*z* 639 $[M - \text{glucose}]^+$, 449 $[M - 352]^+$, and 287 $[\text{cyanidin}]^+$. The loss of 352 mu corresponds to the loss of two glucuronide fragments (176 mu each), so these peaks were identified as cyanidin 3-glucose diglucuronide, although there was insufficient information to determine the glucuronide attachment sites. Peonidin monoglucuronide (peak 40) had a molecular ion $[M]^+$ *m*/*z* 477 and a single fragment MS/MS *m*/*z* 301 $[M - \text{glucuronide}]^+$ representing peonidin. The fragment ions of peak 38 ($[M]^+$ *m*/*z* 801) were *m*/*z* 611, 477, and 287. The fragment ion *m*/*z* 611 corresponds to a loss of 190 mu, which we could not identify with the mass spectral information available. A loss of diglucoside or glucoside acylated with caffeic acid would explain the fragment ion of *m*/*z* 477, and *m*/*z* 287 represents cyanidin, but we could not further characterize peak 38 with the information available.

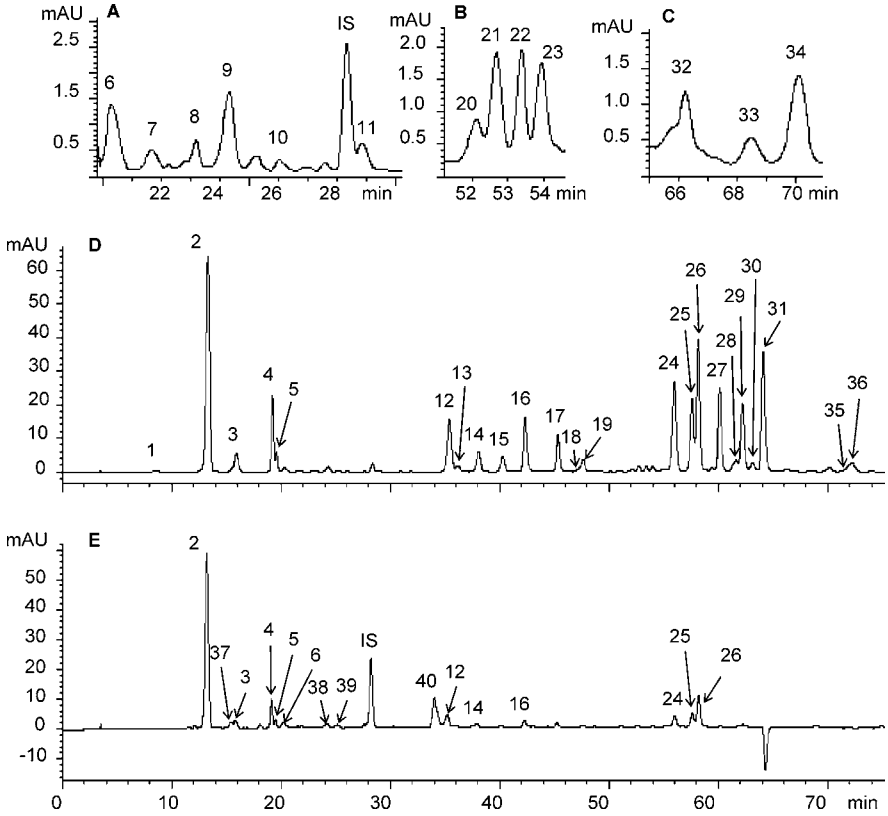

Figure 2. Reverse-phase HPLC chromatograms of anthocyanins in red cabbage (**A**–**D**) and anthocyanins and anthocyanin metabolites in urine (**E**). Peak identities are listed in **Tables 1** and **2**.

Urinary Anthocyanin Response. For unknown reasons, the urinary anthocyanin accumulation of one volunteer following consumption of 200 g of red cabbage was >3 standard deviations higher than the mean and, therefore, was excluded from calculations of accumulation and recovery. Urinary accumulation of total anthocyanins with time is shown in **Figure 3**. The pattern of accumulation was similar for each dose. Anthocyanins were detected in each subject's first urine sample after the consumption of red cabbage (at 2 h postdose), and the rate of anthocyanin accumulation was highest from 2 to 4 h. The amount of anthocyanins accumulated at 24 h was 7−12% higher than that at 12 h.

Twenty-four hour urinary accumulation of total anthocyanins, acylated anthocyanins, nonacylated anthocyanins, anthocyanin metabolites, and most individual anthocyanins and anthocyanin metabolites increased linearly with increasing dose size (**Table 3**). Incremental polynomial regression analysis revealed that second (quadratic) and third (cubic) orders did not significantly improve the regression equations. Intact nonacylated anthocyanins constituted 48% of the sum of anthocyanins and anthocyanin metabolites for the 100 and 200 g doses and 49% for the 300 g dose. Intact acylated anthocyanins constituted 39, 37, and 36% of the sum of anthocyanins and anthocyanin metabolites for the 100, 200, and 300 g doses, respectively. Cyanidin 3-diglucoside-5-glucoside (peak 2), the predominant anthocyanin in urine for all doses, was at least 3-fold higher than the next most prevalent anthocyanin, cyanidin 3-(sinapoyl)diglucoside-5-glucoside (peak 26). Anthocyanin metabolites (peaks 37−40) comprised 12% of the sum of anthocyanins and anthocyanin metabolites for the 100 g dose and 15% of the sum of anthocyanins and anthocyanin metabolites for the 200 and 300 g doses.

Twenty-four hour urinary recoveries of total intact anthocyanins (percent of anthocyanin dose ingested) were 0.073, 0.042, and 0.036% for the 100, 200, and 300 g doses, respectively (**Table 4**). This decrease in urinary recovery reflects the fact that although urinary accumulation of anthocyanins increased linearly with increasing dose size, the increase in recovery did not match the increase in dose size on a percentage basis. For example, when dose size was increased by 100% (from 100 to 200 g), urinary accumulation increased by 20% (from 114.6 to 137.5 nmol). Similarly, when dose size was increased by 200% (from 100 to 300 g), urinary accumulation increased by 55% (from 114.6 to 177.7 nmol). Twenty-four hour recoveries of the sum of total anthocyanins and anthocyanin metabolites (percent of dose consumed) were 0.083, 0.050, and 0.043% for the 100, 200, and 300 g doses, respectively (data not shown). These values are higher than the recoveries of total anthocyanins due to the contribution of anthocyanin metabolites to the sum of total anthocyanins and anthocyanin metabolites.

Recovery of nonacylated anthocyanins was 4-fold higher than recovery of acylated anthocyanins at each dose level. A thorough investigation of extraction efficiencies of the different compounds showed that this was not related to differential retention on the SPE cartridges. The recovery of peonidin 3-diglucoside-5-glucoside (peak 6) was 3-fold higher than peak 2, which was the next most prevalent anthocyanin. Excluding peak 6, recovery of nonacylated anthocyanins ranged from 0.176 to 0.085% from the 100 to 300 g dose size, and recovery of acylated anthocyanins ranged from 0.041 to 0.020% from 100 to 300 g dose size. There was no difference in recovery for singly compared to doubly acylated anthocyanins (data not shown). For each dose size, anthocyanin recovery tended to decrease with increasing HPLC retention time. Spearman rank order correlation showed that the correlation coefficients (r_s) of anthocyanin urinary recovery and anthocyanin elution order were −0.717, −0.850, and −0.828 for the 100, 200, and 300 g dose sizes, respectively. Because in reverse phase HPLC (as was employed

Table 1. Anthocyanins in Red Cabbage Consumed in the Bioavailability Study[a]

peak	t_R (min)	$[M]^+$ (*m*/*z*)	MS/MS (*m*/*z*)	concentration (nmol g^{-1} of FW, mean ± SD)[b]	anthocyanin
1	8.6	611	449/287	2.3 ± 0.1	cyanidin 3-diglucoside
2	13.2	773	611/449/287	264.2 ± 34.3	cyanidin 3-diglucoside-5-glucoside
3	15.7	611	449/287	18.1 ± 0.8	cyanidin 3,5-diglucoside
4	19.0	979	817/449/287	58.1 ± 9.3	cyanidin 3-(sinapoyl)-diglucoside-5-glucoside
5	19.4	1141	979/449	15.1 ± 2.3	cyanidin 3-(glycopyranosyl-sinapoyl)diglucoside-5-glucoside
6[c]	**20.1**	**787**	**625/463/301**	**5.5 ± 0.8**	**peonidin 3-diglucoside-5-glucoside**
7	21.4	743	611/419/287	1.8 ± 0.3	cyanidin-3-diglucoside-5-xyloside
8	22.9	979	287	1.1 ± 0.0	cyanidin 3-(sinapoyl)-diglucoside-5-glucoside
9[c]	**24.1**	**625**	**463/301**	**2.5 ± 0.6**	**peonidin 3,5-diglucoside**
10	25.8	949	787/449/287	1.1 ± 0.2	cyanidin 3-(feruloyl) diglucoside-5-glucoside
11	28.6	979	817/449/287	1.8 ± 0.3	cyanidin 3-(sinapoyl) diglucoside-5-glucoside
12	35.0	1081	919/449	77.1 ± 7.3	cyanidin 3-(caffeoyl)(*p*-coumaroyl) diglucoside-5-glucoside
13	35.8	979	817/449/287	1.4 ± 0.3	cyanidin 3-(sinapoyl) diglucoside-5-glucoside
14	37.7	1111	949/787/449	28.4 ± 4.0	cyanidin 3-(glycopyranosyl-feruloyl) diglucoside-5-glucoside
15	40.0	1141	979/817/449	21.6 ± 3.8	cyanidin 3-(glycopyranosyl-sinapoyl) diglucoside-5-glucoside
16[c]	**42.0**	**1287**	**1125/449**	**62.3 ± 8.0**	**cyanidin 3-(*p*-coumaroyl)(sinapoyl) triglucoside-5-glucoside**
17[c]	**45.1**	**1317**	**1155/449**	**40.2 ± 7.3**	**cyanidin 3-(feruloyl)(sinapoyl) triglucoside-5-glucoside**
18	47.0	935	773/449/287	8.9 ± 0.9	cyanidin 3-(caffeoyl) diglucoside-5-glucoside
19[c]	**47.5**	**1347**	**1185/1023/449**	**9.4 ± 1.7**	**cyanidin 3-(sinapoyl)(sinapoyl) triglucoside-5-glucoside**
20	52.0	817	655/449/287	2.8 ± 0.4	cyanidin 3-(sinapoyl) glucoside-5-glucoside
21[c]	**52.6**	**1287**	**1125/449**	**6.5 ± 0.7**	**cyanidin 3-(*p*-coumaroyl) (sinapoyl) triglucoside-5-glucoside**
22	53.3	1141	979/449	5.3 ± 0.5	cyanidin 3-(glycopyranosyl-sinapoyl) diglucoside-5-glucoside
23[c]	**53.9**	**1317**	**1155/449**	**5.6 ± 0.5**	**cyanidin 3-(feruloyl)(sinapoyl) triglucoside-5-glucoside**
24	55.9	919	757/449/287	123.8 ± 12.2	cyanidin 3-(*p*-coumaroyl) diglucoside-5-glucoside
25	57.6	949	787/449/287	90.5 ± 11.6	cyanidin 3-(feruloyl) diglucoside-5-glucoside
26	58.1	979	817/449/287	151.5 ± 15.7	cyanidin 3-(sinapoyl) diglucoside-5-glucoside
27	60.1	1125	963/449	89.6 ± 7.1	cyanidin 3-(*p*-coumaroyl)(sinapoyl) diglucoside-5-glucoside
28	61.7	817	655/449/287	15.1 ± 0.7	cyanidin 3-(sinapoyl) glucoside-5-glucoside
29	62.3	1155	993/449	82.1 ± 10.4	cyanidin 3-(feruloyl)(sinapoyl) diglucoside-5-glucoside
30[c]	**63.2**	**1171**	**1009/449**	**9.9 ± 1.8**	**cyanidin 3-(*p*-hydroxybenzoyl) (malonoyl) triglucoside-5-glucoside**
31	64.2	1185	1023/449	146.4 ± 18.3	cyanidin 3-(sinapoyl)(sinapoyl) diglucoside-5-glucoside
32	66.4	1155	993/449	3.4 ± 0.5	cyanidin 3-(feruloyl)(sinapoyl) diglucoside-5-glucoside
33	68.7	949	817/419/287	2.3 ± 0.1	cyanidin 3-(sinapoyl)diglucoside-5-xyloside
34	70.3	1125	963/449	8.6 ± 1.0	cyanidin 3-(feruloyl)(feruloyl) diglucoside-5-glucoside
35	72.0	1155	993/449	2.2 ± 0.3	cyanidin 3-(feruloyl)(sinapoyl) diglucoside-5-glucoside
36	72.4	1185	1023/449	3.4 ± 0.5	cyanidin 3-(sinapoyl)(sinapoyl) diglucoside-5-glucoside
acylated anthocyanins				1088.1 ± 113.2	
nonacylated anthocyanins				294.3 ± 35.2	
total anthocyanins				1382.3 ± 148.0	

[a] t_R, retention time; FW, fresh weight of cooked red cabbage; SD, standard deviation. [b] Mean of three replications. [c] Compounds in bold were identified in red cabbage for the first time.

in this study) hydrophobic compounds tend to be retained longer than relatively less hydrophobic compounds, this application of Spearman rank order correlation demonstrates that the urinary recovery and degree of hydrophobicity of individual anthocyanins were inversely related.

DISCUSSION

Red cabbage is a rich source of both acylated and nonacylated anthocyanins and therefore provides a unique opportunity for investigating the relationships of anthocyanin structure and bioavailability. Additionally studying different cabbage dose sizes allows the investigation of possible saturation of anthocyanin absorption and excretion.

Identification of Anthocyanins and Anthocyanin Metabolites. We found eight anthocyanins in red cabbage not previously reported. Peaks 6 and 9 were peonidin-based analogues of peaks 2 and 3. The other newly found peaks (numbers 16, 17, 19, 21, 23, and 30) also were structurally similar to previously reported anthocyanins, differing only by the addition of a glucoside. The isomeric peaks 16 and 21 had diacylations identical to peaks 27 and 34 but had an additional glucoside. Peaks 17 and 24, also diacylated isomers, had one more glucoside than their structural analogues, peaks 29, 32, and 35. As a result of the additional glucoside, the tetraglycosylated anthocyanins were among the anthocyanins of highest molecular mass.

Peak 19, tentatively identified as cyanidin 3-(sinapoyl)-(sinapoyl)triglucoside-5-glucoside, had fragment ions of *m*/*z* 1185, 1023, and 449. The *m*/*z* 1185 fragment corresponds to a loss of glucoside, most likely from the 5-position of the anthocyanin. The *m*/*z* 449 fragment corresponds to a loss of 898 mu, which can be explained by a fragment consisting of a triglucoside and two sinapoyl groups. The *m*/*z* 1023 fragment is more problematic in that it indicates a loss of 324 mu, which may represent some portion of the 898 mu fragment. There was insufficient information to clarify whether the loss of 324 mu corresponds to a loss of diglucose or represents some other fragment. Fragmentation of a polyglucoside moiety would be unusual (*39*). Interestingly, Wu and Prior (*4*) reported a loss of 324 from a similar red cabbage anthocyanin containing a single sinapoyl group, cyanidin 3-(sinapoyl)diglucoside-5-glucoside, which was peak 28 in our study.

Peak 38 was found in urine but not in red cabbage. On the basis of its UV−vis spectrum and MS data, we tentatively concluded that it is an anthocyanin metabolite. The product ion with *m*/*z* 611 corresponds to a loss of 190 mu from $[M]^+$ *m*/*z* 801, but we had insufficient data to characterize this fragment.

Table 2. Anthocyanins and Anthocyanin Metabolites in Urine[a]

peak	t_R (min)	$[M]^+$ (*m*/*z*)	MS/MS (*m*/*z*)	anthocyanin
2[b]	13.2	773	611/449/287	cyanidin 3-diglucoside-5-glucoside
37	15.4	801	639/449/287	cyanidin 3-glucose diglucuronide
3[b]	15.7	611	449/287	cyanidin 3,5-diglucoside
4[b]	19.0	979	817/449/287	cyanidin 3-(sinapoyl)-diglucoside-5-glucoside
5[b]	19.4	1141	979/449	cyanidin 3-(glycopyranosyl-sinapoyl)diglucoside-5-glucoside
6[b]	20.1	787	625/463/301	peonidin 3-diglucoside-5-glucoside
38	24.1	801	611/477/287	unknown
39	25.1	801	639/449/287	cyanidin 3-glucose diglucuronide
40	34.0	477	301	peonidin monoglucuronide
12[b]	35.0	1081	919/449	cyanidin 3-(caffeoyl)(*p*-coumaroyl)diglucoside-5-glucoside
14[b]	37.7	1111	949/787/449	cyanidin 3-(glycopyranosyl-feruloyl)diglucoside-5-glucoside
16[b]	42.0	1287	1125/449	cyanidin 3-(*p*-coumaroyl)-(sinapoyl)triglucoside-5-glucoside
24[b]	55.9	919	757/449/287	cyanidin 3-(*p*-coumaroyl)diglucoside-5-glucoside
25[b]	57.6	949	787/449/287	cyanidin 3-(feruloyl)diglucoside-5-glucoside
26[b]	58.1	979	817/449/287	cyanidin 3-(sinapoyl)diglucoside-5-glucoside

[a] t_R, retention time. [b] Also found in red cabbage as listed in **Table 1**; peak numbers assigned in **Table 1** are retained for compounds found in urine and listed in **Table 2**.

Tian et al. (*40*) detected two cyanidin-based anthocyanins in a purple sweet potato cell line (*Ipomoea batatas* L. cv. Ayamurasaki) that exhibited a loss of 190 mu, but did not identify this fragment.

Urinary Anthocyanin Response. It has been well-established that anthocyanins can be absorbed and excreted in their intact forms (*23*, *24*, *41*, *42*). In line with a previous investigation, we found that recovery of intact anthocyanins was severalfold higher for nonacylated anthocyanins than for acylated anthocyanins (*30*). This study also showed that accumulation of acylated and nonacylated anthocyanins in urine is highly correlated to peak plasma response for each and that total anthocyanin accumulation in urine is highly correlated to area under the plasma concentration−time curve (*30*).

The presence of glucuronidated anthocyanins in urine has been previously reported for cyanidin-based and other anthocyanins (*43*, *44*). Because all red cabbage anthocyanins had at least two sugar residues, peonidin monoglucuronide (peak 40), which was found in urine, could be derived only following the deglycosylation and subsequent glucuronidation of one or more anthocyanins. Aura et al. (*45*) showed that human gut microflora deglycosylate cyanidin-3-glucose and cyanidin-3-rutinoside. The glucuronidation step would occur by attachment of the glucuronide to cyanidin or, less likely, by the action of UDP-glucose dehydrogenase on cyanidin-3-glucose (*46*). Methylation would be required to complete the synthesis of peak 40.

The apparently high recovery of peonidin 3-diglucoside-5-glucoside (peak 6) may be misleading. Peonidin-based anthocyanins are methylated forms of cyanidin-based anthocyanins. Previous studies have shown that for flavonoids such as cyanidin, which have 3′,4′-dihydroxylation of ring B, methylation occurs at the 3′-*O*-position by the action of catechol-*O*-methyltransferase (COMT) in the liver and kidneys (*47*). Cyanidin-3-glucose and cyanidin-3-rutinose were converted to methylated and glucuronidated forms in weanling pigs (*48*). Wu et al. (*24*) reported that cyanidin-3-glucoside, cyanidin-3-sambubioside, peonidin-3-glucoside, peonidin monoglucuronide, peonidin-3-sambubioside, and cyanidin-3-glucoside monoglucuorinide were detected in urine of women who had consumed elderberry extract containing cyanidin-3-glucoside and cyanidin-3-sambubioside. Therefore, it is plausible that some portion of cyanidin-3-diglucoside-5-glucoside (peak 2), which had the highest concentration in red cabbage of all anthocyanins, was methylated to peak 6. Therefore, peak 6 in urine may originate from the intact form in red cabbage and additionally from the methylation of peak 2. Consequently, peak 6 was excluded from calculations of nonacylated anthocyanin recovery.

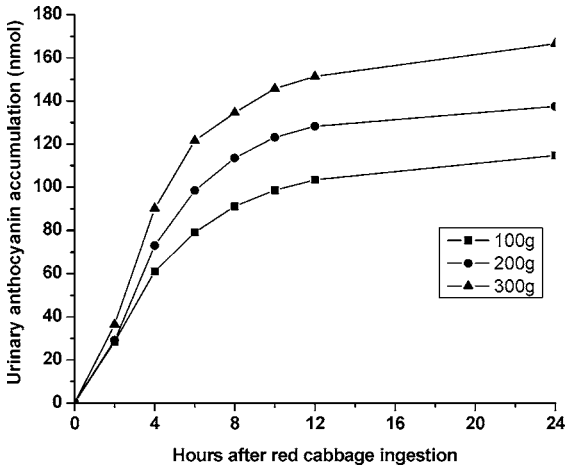

Figure 3. Total 24 h accumulation of urinary anthocyanins and anthocyanin metabolites after consumption of 100, 200, or 300 g of red cabbage ($n = 12$ for 100 and 300 g; $n = 11$ for 200 g).

We did not observe a dose response consistent with saturation of anthocyanin absorption mechanisms. The quantities of total, acylated, and nonacylated anthocyanins increased linearly with linear increases in red cabbage dose consumed. In a study in which cooked purple carrots were consumed containing anthocyanin dose sizes of 357.1 and 714.2 μmol, the masses of total, acylated, and nonacylated anthocyanins recovered in urine were not significantly different between the higher and lower doses, suggesting a saturation response occurring at or below 357.1 μmol (*30*). The highest dose of anthocyanins in red cabbage was 414.7 μmol and may not have been high enough for saturation to occur. Moreover, in contrast to red cabbage anthocyanins, which are predominantly cyanidin glucosides, purple carrot anthocyanins typically consist of cyanidin conjugated with xylose. A study using weanling pigs showed that anthocyanin rutinosides were excreted at higher levels than anthocyanin glucosides, demonstrating that the type of sugar moiety influences excretion in urine (*49*).

Anthocyanin structure is widely reported to influence anthocyanin bioavailability (*44*, *49*). Generally, there was no difference between the recovery of singly and doubly acylated anthocyanins. However, for three acylated anthocyanins having the cyanidin-3-diglucoside-5-glucoside skeleton, there was a striking difference. We observed that the singly acylated red cabbage anthocyanin peaks 24−26 were present in urine. Their doubly acylated counterparts, peaks 27, 29, and 31, although present in red cabbage at relatively high levels (≥82.1 nmol/g), were not detected in urine. Peaks 27, 29, and 31 each had an additional sinapoyl group compared to peaks 24−26, and

Table 3. Mean 24 h Urinary Accumulation (Nanomoles) of Anthocyanins and Anthocyanin Metabolites after Ingestion of 100, 200, or 300 g of Red Cabbage

dose (g)	total anthocyanins and metabolites[b]	acylated anthocyanins[b]	nonacylated anthocyanins[b]	anthocyanin metabolites[b]	peaks of individual anthocyanins and anthocyanin metabolites[a]													
					2	37	3	4	5	6	38	39	40	12	16	24	25	26
100	114.6 ± 47.3	45.1 ± 18.2	55.2 ± 25.6	14.2 ± 6.6	48.8	3.6	2.7	7.4	2.3	3.6	3.0	2.4	5.2	4.6	3.7	6.6	7.2	13.3
200	137.5 ± 48.2	50.7 ± 17.8	65.8 ± 24.5	21.0 ± 9.5	58.0	4.7	3.6	9.4	2.2	4.2	4.4	3.4	8.5	7.2	4.2	7.3	7.2	13.3
300	177.7 ± 62.1	64.7 ± 20.4	86.6 ± 34.0	26.3 ± 12.7	76.0	5.4	4.7	11.3	3.4	6.0	4.8	3.6	12.6	9.6	4.8	9.6	8.7	17.3
linear[c]	**	*	**	**	*	NS	**	*	*	**	*	NS	**	**	NS	*	NS	NS

[a] Anthocyanins are listed by elution order with the earliest eluting anthocyanin on the left. See **Tables 1** and **2** to reference identification of peak numbers. [b] Mean ± standard deviation. [c] Incremental polynomial regression indicated that adding second and third orders did not significantly improve the regression equation. The first order (linear) was the following: NS, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$.

Table 4. Twenty-four h Urinary Recovery of Anthocyanins Measured in both Red Cabbage and Urine (Percent of Anthocyanin Dose Consumed) after Ingestion of 100, 200, or 300 g of Red Cabbage

dose (g)	total intact antho-cyanins	acylated antho-cyanins	nonacylated antho-cyanins[b]	peaks and retention times (t_R, min) of individual anthocyanins[a]										Spearman rank order correlation	
				2 (13.2)	3 (15.7)	4 (19.0)	5 (19.4)	6 (20.1)	12 (35.0)	16 (42.0)	24 (55.9)	25 (57.6)	26 (58.1)	r_s[c]	P[d]
100	0.073	0.041	0.176	0.185	0.179	0.127	0.152	0.655	0.060	0.059	0.053	0.080	0.088	–0.717	*
200	0.042	0.023	0.105	0.110	0.119	0.081	0.073	0.382	0.047	0.034	0.029	0.040	0.044	–0.850	**
300	0.036	0.020	0.085	0.096	0.104	0.065	0.075	0.364	0.042	0.026	0.026	0.032	0.038	–0.828	**

[a] Retention times (t_R) of individual anthocyanins are listed in parentheses. See **Tables 1** and **2** to reference identification of peak numbers. [b] Peak 6 is not included in this calculation because it is likely that, in urine, peak 6 originates from the intact anthocyanin in red cabbage and the metabolism of other anthocyanins. [c] Within-row Spearman rank order correlation coefficient for percent recovery of individual anthocyanins and elution orders. [d] Probability value: *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$.

this sinapoyl group evidently prevented absorption and excretion. Due to the presence of a methyl group at each of two attachment sites on its benzyl ring, sinapic acid would tend to be more hydrophobic than caffeic, *p*-coumaric, ferulic, and *p*-hydroxybenzoic acids, which have one or no methyl groups; if this is the case, the higher hydrophobicity of sinapic acid compared to other phenolic acids could be particularly significant in affecting anthocyanin bioavailability.

We found a negative correlation between HPLC elution order of anthocyanins and their recovery in urine. Although there can be minor changes in elution order by changing HPLC columns and mobile phases, compounds of higher hydrophobicity characteristically elute later than less hydrophobic compounds. Thus, anthocyanin recovery in urine was inversely related to anthocyanin hydrophobicity. In addition to noting the inverse correlation of urinary recovery and HPLC elution order, we found that some isomers behaved very differently in terms of absorption and excretion. For example, although the MS data for peaks 4 and 26 were identical, urinary recovery of peak 4 was 44% (100 g dose) to 84% (200 g dose) higher than that of peak 26. Peak 26 eluted nearly 40 min after peak 4, suggesting a much stronger hydrophobic character for peak 26. The aforementioned peaks 27, 29, and 31, which were the latest eluting and therefore most hydrophobic of major red cabbage anthocyanins, were not detected at all in urine.

It remains to be clarified what role transport proteins have in the absorption of acylated anthocyanins. Bilitranslocase is an organic membrane carrier that exhibits differential sensitivity with regard to the hydrophobicity of anthocyanins. Passamonti et al. (*28*) reported that the first interaction of anthocyanins with bilitranslocase occurs with anthocyanin hydrophilic moieties and that acylation weakens the interaction between anthocyanins and the transport site. Malvidin-3-glucoside acylated with acetic acid was a noncompetitive inhibitor, whereas acylation with *p*-coumaric acid prevented interaction with the carrier. Molecular size is not the sole determinant of affinity, as it was shown that cyanidin and pelargonidin 3,5-diglucosides were actually better substrates than their respective aglycones. In our study, peak 16 ($[M]^+$ *m*/*z* 1287) was present in urine and had the second highest molecular mass of all red cabbage anthocyanins, confirming that factors besides molecular mass influence their absorption and subsequent excretion in urine.

In conclusion, we measured 36 anthocyanins in red cabbage, of which 8 are reported here for the first time. Eleven red cabbage anthocyanins and 4 anthocyanin metabolites were found in urine. We confirmed that acylation tends to decrease anthocyanin absorption and hypothesized that decreased anthocyanin bioavailability is linked to higher anthocyanin hydrophobicity. Urinary recovery of anthocyanins did not reflect saturation of absorption mechanisms up to the largest dose of red cabbage anthocyanins provided, 414.7 μmol.

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Received for review April 12, 2007. Accepted April 13, 2007.

JF0710736