Intact Anthocyanins and Metabolites in Rat Urine and Plasma After 3 Months of Anthocyanin Supplementation

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Abstract: Anthocyanins are polyphenols responsible for most red to purple colors in plants. Human consumption of these pigments is increasing because of their potential health benefits and use as natural colorants. With more than 600 different anthocyanins found in nature, the impact of chemical structure on their absorption and metabolism needs to be investigated. Urine and plasma samples were collected from 32 rats receiving control diet or chokeberry-, bilberry-, and grape-enriched (3.85 g cyanidin 3-galatoside equivalent/kg) diet for 14 wk. Below 2 μmol/l of anthocyanins and relatively higher levels of presumable metabolites were detected by high-performance liquid chromatography-photodiode array in the plasma. In the urine the total concentration of intact anthocyanins and methylated derivatives ranged from 17.4 (bilberry) to 52.6 (chokeberry) nmol/l. The type and number of anthocyanin glycosylations affected the absorption remarkably. Detection of an acylated anthocyanin in plasma and urine suggests bioavailability of these anthocyanin derivatives that are commonly found in commercially available colorants.

Introduction

Anthocyanins are a group of polyphenols that comprise an important part of our diet. Human consumption has been estimated at more than 100 mg/day (1,2). They are widely distributed in fruits, vegetables, and processed foods or beverages such as juices and wines (3). Recently, as the concern about synthetic food dyes rises, demand and interest in anthocyanins as potential natural colorants are steadily increasing (3,4). Studies have revealed potential benefits of anthocyanins on human health (5), including cancer-prevention effects (6–13). Intact anthocyanin glycosides have been detected in rat and human plasma as well as in urine (14–16). However, low bioavailability of anthocyanins was also suggested by the low concentration observed in plasma and urine after anthocyanin ingestion in some short-term studies (15–19).

Our objective was to evaluate the absorption and potential metabolism of anthocyanins in rats fed anthocyanin-rich diet for a relatively longer period. This study was a part of a larger project to evaluate the potential chemopreventive effects of anthocyanins on colon cancer prevention. A variety of individual anthocyanins with distinctive chemical structure are included in this study. Chokeberry, bilberry, and grape anthocyanin-rich extracts (AREs) were chosen as the anthocyanin diet sources based on 1) the reports that they are good sources of antioxidants (20, 21), 2) their chemical composition (22), and 3) our previous findings that they inhibit colon cancer in vitro (10,23). A comparison of the concentration of total and individual anthocyanins in rat urine and plasma after feeding with the different extracts may provide valuable information for the impact of structural differences on anthocyanin absorption and excretion.

Materials and Methods

Chemicals and Materials

Commercially available AREs of bilberry (Vaccinium myrtillus L.) and chokeberry (Aronia melanocarpa E.) were supplied by Artemis International, Inc. (Fort Wayne, IN). Grape extract (Vitis vinifera) was supplied by Polyphenolics, Inc. (Madera, CA). High-performance liquid chromatography (HPLC) profiles of the AREs are shown in Fig. 1. Cyanidin 3-galatoside (cy-3-gal) standard for 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). Trifluoracetic acid (TFA) and azoxymethane (AOM), a potent colon carcinogen, were obtained from Sigma Chemical (St. Louis, MO). AIN-93 powdered diets were from Dyets, Inc. (Bethlehem, PA). Sep-Pak Vac (6 cc, 1 g; 12 cc, 2 g) C18 cartridges for solid-phase extraction 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 Lala...
et al. (24). Briefly, weaning male specific pathogen-free Fischer 344 rats (Harlan, Indianapolis, IN) were fed either the control AIN-93 powdered diet or a modified AIN-93 diet containing 3.85 g/kg monomeric anthocyanin from chokeberry, bilberry, or grape ARE based on their monomeric anthocyanin content at the expense of corn starch (26–50 g/kg). As anthocyanin extracts are highly colored and the AIN-93 diet is white, mixing was performed until a uniform color was obtained. Diets were prepared fresh on a weekly basis and stored at 4°C until use. After 1-wk feeding, all animals received a subcutaneous injection of AOM in saline solution (20 mg/kg body weight). Rats were fed the powdered diet in standard feeding cups for an additional 13 wk. 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%. Clinical signs for all the animals were recorded regularly. Body weight was recorded twice per week, and 3-day food intake was measured twice during the 14-wk study. The University of Maryland Institutional Animal Care and Use Committee approved all animal protocols.

Sample Collection

Urine samples were collected from 32 rats (8 animals per diet group) 1 wk before rats were euthanized. 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. Urine was collected from 6 am to 12 pm and immediately stored at −80°C after adding 20% TFA (16,25,26). At the end of Week 14, all rats were anesthetized in a carbon dioxide chamber early in the morning without feeding. Rats were immediately decapitated to collect blood using heparinized tubes. Plasma samples were immediately prepared according to the method of Tsuda et al. (19) with slight modification. Collected blood was centrifuged at 3,000 rpm for 15 min at room temperature, and collected plasma samples were quickly removed and immediately treated with an aqueous solution of 0.44 mol/l TFA (1:0.2; vol/vol) (16). Proteins in the plasma were flocculated by TFA and precipitated by centrifugation for 5 min at 3,000 rpm at 4°C (17). All treated samples were stored at −80°C prior to anthocyanin analysis.

HPLC Analysis

All samples were semipurified with a C18 cartridge, and the phenolic fraction (containing anthocyanins) was eluted with 1% TFA-acidified methanol, dried with a rotary evaporator, taken to a known volume, and filtered through a 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), and an autosampler (Waters 717 plus). Separation of anthocyanins was accomplished on a Symmetry C18 column (5 µm; 4.6 × 50 mm) (flow rate: 1 ml/min; mobile phase: A, 10% acetic acid and 1% phosphoric acid in deionized water; B, acetonitrile; 0–5 min, 0% B; 5–40 min, 0–35% B). Spectral data (260–650 nm) were 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, 150 µl for urine and 300 µl for plasma samples. 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, published literature (20–22, 27), and data from our laboratory (28).

HPLC–Mass Spectrometry (MS)/MS Analysis of the Anthocyanin Metabolites

Separation of anthocyanins was conducted on a Waters Symmetry C18 column (4.6 × 75 mm, 3.5 µm) using an HPLC system coupled with a 996 photodiode array (PDA) detector (Waters) and a triple quadrupole ion-tunnel mass spectrometer (Quattro Ultima, Micromass UK Ltd., Manchester, UK). The HPLC flow rate was set at 1 ml/min (mobile phase: A, 10% formic acid; B, acetonitrile; 0–20 min, 100–85% A; 20–25 min, 85–100% A). Absorption spectra of anthocyanins were recorded from 200 to 600 nm. Mass spectra were obtained using selective ion monitoring. Approximately 100 µl of the HPLC eluate separated by a microsplitter valve (Upchurch Scientific, Oak Harbor, WA) was delivered to the electrospray ionization (ESI) source. The quadrupole instrument was operated at the following setting: capillary voltage, 3.2 kV; cone voltage, 35 V; RF lens 1, 50 V; desolvation gas temperature, 500°C at a flow rate of 269 l/h; and source temperature, 105°C; m/z 419 (cy-pentosides), m/z 449 (cy-hexosides), m/z 433 (pn-pentosides), and m/z 463 (pn-hexosides) were monitored.

Standards and Calibration Curves

Commercially available cy-3-gal standard was dissolved in deionized-distilled water containing 1% TFA to 223 µmol/l and thereafter diluted (1.74–111 µg/l) to make a standard curve ($R^2 = 0.99$). All anthocyanins analyzed fell within the standard curve range and were expressed as cy-3-gal equivalent in moles. Total anthocyanins recovered from urine samples were calculated by adding the area under the curve of individual anthocyanin peaks together and using a calibration curve.

Statistical Analysis

One-way analysis of variance was conducted using SPSS (version 13, 2004, SPSS, Inc., Chicago, IL) with log transformation, and values were given as mean ± SE. When appropriate, significance of differences between values was determined by Tukey’s Honestly Significant Differences (HSD) post hoc test. Differences of $P < 0.05$ were considered signif-
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Results and Discussion

Urinary Anthocyanin Concentration

No significant differences in either body weight or food consumption were found among diet groups (data not shown). At the time of collection urine samples showed no red coloration but immediately turned red after acidification with 20% volume (vol/vol) of 0.44 mol/l TFA (16). This color change was attributed to the re-equilibration of the chemical forms of anthocyanin in acidic pH. Under neutral pH condition anthocyanins are predominantly in colorless pseudobase form, but under acidic pH condition most anthocyanins convert to the red colored flavylum form. Considering the approximate volume of urines (~1–1.5 ml), and the average anthocyanin daily intake of rats (64–72 mg), less than 0.05% of the ingested anthocyanin was excreted through urine within 6 h. These results agree with numerous previous reports that anthocyanin concentration in rat urine was low (14,17,19,25). Compared with the diet (Fig. 1), anthocyanin profiles in urine changed remarkably (Fig. 2) due to the preferential absorption and metabolism. Dietary supplementation of different AREs with equal amounts of total anthocyanins resulted in significantly different ($P < 0.001$) total anthocyanin concentrations in the urine (Fig. 3), suggesting an impact of anthocyanin composition on their bioavailability. The highest concentration of anthocyanin in the urine was detected from rats in the chokeberry treatment, whereas the lowest level was de-

![Figure 1](https://example.com/fig1.png)

Figure 1. High-performance liquid chromatography chromatograms of anthocyanins from (A) chokeberry, (B) bilberry, and (C) grape extracts. Detection wavelength: 520 nm. Peak identities: 1, dp-3,5-diglu; 2, dp-3-gal; 3, cy-3,5-diglu; 4, dp-3-glu; 5, cy-3-gal; 6, dp-3-arab; 7, pt-3,5-diglu; 8, cy-3-glu; 9, pt-3-gal; 10, cy-3-arab; 11, pm-3,5-diglu; 12, pt-3-glu; 13, pt-3-gal; 14, mv-3,5-diglu; 15, pm-3-gal; 16, pt-3-arab; 17, pm-3-glu; 18, mv-3-gal; 19, pm-3-arab; 20, cy-3-xyl; 21, mv-3-glu; 22, mv-3-arab; 23–25, unknown; 26, dp-3-(coum-glu); 27, unknown; 28, pt-3-(coum-glu); 29, unknown; and 30, mv-3-(coum-glu).
detected in the bilberry treatment. However, the concentration difference has to be attributed to both anthocyanin excretion and the urinary volume. We observed that, among all the 31 urine samples collected (including controls), 8 samples had less than 1-ml volume, and 5 of those were from chokeberry treatment. One rat from the chokeberry treatment did not produce any urine excretion at all, representing the only missing sample in this study. Supplementation of chokeberry ARE appeared to reduce the urine volume in rats, although we did not measure consumption of water. One urine sample in the chokeberry treatment and one in the grape treatment have unusual ratios of some major peaks and extremely low total anthocyanin concentrations. These two data points represented outliers and were not included in the statistical analysis.

Effect of Sugar Moiety on Urinary Anthocyanin Content

The type of sugar moiety attached to the anthocyanin played an important role in anthocyanin absorption. The percentage of individual anthocyanins excreted in the urine was compared with the ARE consumed in the diet, and results obtained are presented in Tables 1–3. The proportion of anthocyanin monoglucosides generally decreased compared with their proportions in AREs (Tables 1 and 2). A possible reason is a vast and selective degradation of anthocyanin glucosides over other anthocyanin glycosides in the small intestine, as previously reported (29). Another interesting observation was that all anthocyanin 3,5-diglucosides (diglu) in grape were consistently excreted in the urine in higher pro-

Figure 2. Typical high-performance liquid chromatography chromatograms of urinary anthocyanins detected in (A) chokeberry, (B) bilberry, and (C) grape diet groups. Detection wavelength: 520 nm. Peak identities: 1, dp-3,5-diglu; 2, dp-3-gal; 3, cy-3,5-diglu; 4, dp-3-glu; 5, cy-3-gal; 6, dp-3-arab; 7, pt-3,5-diglu; 8, cy-3-glu; 9, pt-3-gal; 10, cy-3-arab; 11, pn-3,5-diglu; 12, pt-3-glu; 13, mv-3,5-diglu; 14, pn-3-gal; 15, pt-3-arab; 16, pn-3-glu; 17, mv-3-gal; 18, pn-3-arab; 19, mv-3-glu; 20, mv-3-arab; and 21, pt-3-(coum-glu).
portion than their corresponding monoglucosides (3-glu), as evidenced by a comparison with their proportion in the AREs. The second glucose moiety seemed to improve the absorption of anthocyanins, although partial hydrolysis of diglu would favor an increased ratio of mono-glu/diglu. This observation is in agreement with our finding that a second sugar moiety helped to protect the aglycone better than the monoglycoside from decomposing in the gastrointestinal tract (GIT) (29). Two glucosides close to each other sterically might block the access of β-glucosidase in the GIT and stabilize the 3,5-diglu. The distinctly different decomposing tendency of 3,5-diglu and 3-glu may partially explain why the bilberry treatment group has only half of the total urinary anthocyanin concentration as the grape treatment group (Fig. 3). In grape ARE approximately 65% of anthocyanins was 3,5-diglu, whereas, in bilberry ARE, approximately 50% was less-stable 3-glu. Prior (2) suggested that cy-3-glu might be more susceptible to degradation than cy-3-sambubioside in GIT and thus result in reduced excretion. This explanation is in agreement with our findings.

Influence of Acylation on Urinary Anthocyanin Content

Acylated anthocyanins were found in urine samples (and in plasma samples as described later) of rats fed the grape diet. As stated by Prior in 2004 (2), only one study, by Mazza et al. (18), had reported a possible intact acylated anthocyanin in serum. To our knowledge, no other literature has reported the detection of acylated anthocyanins in either plasma or urine. In most studies, isolated nonacylated anthocyanins or berries that contain no acylated anthocyanins have been used. Also, methodology available in the past was not sensitive enough to detect these compounds. In the grape ARE we used, acylated anthocyanins accounted for ~22% of the total anthocyanins, and pt-3-(coum-glu) in particular accounted for ~14%. The acylated pt-derivative was clearly present (~2% of total anthocyanins) in the urine (Table 3). With the same aglycones, the percentage area of acylated anthocyanin pt-3-(coum-glu) dropped to 16% of that in the diet, whereas the percentage area of nonacylated anthocyanin pt-3,5-glu increased to 110% of that in the diet. The t-test suggests that the absorption of pt-3-(coum-glu) was significantly lower than that of pt-3,5-glu ($P < 0.0001; n = 7$). The limited absorption of acylated anthocyanins was not attributed to the hydrolysis in the stomach and gut because we reported previously that only a slight decrease of acylated anthocyanins in cecal content and feces compared with that in the grape ARE was found (29). We suspect that the acyl group greatly impairs its ability to transport through the intestinal mucosal cells. Despite the low absorption, the finding of acylated anthocyanins in urine (and plasma) may be important because acylated anthocyanins are used in more and more applications in the food industry due to their good stability (4), and, therefore, they may become more abundant in the human diet.

Anthocyanin Metabolites in Urine

Three anthocyanin-like major metabolites (derived from cyanidin glycosides) were detected in rat urine from the chokeberry treatment (Fig. 2A, Table 1). The three metabolites were identified as methylated cy-glycosides, likely be-

Table 1. Anthocyanin Composition of ARE and Urine in Chokeberry Treatment$^a$

<table>
<thead>
<tr>
<th>Anthocyanin Components</th>
<th>ARE ($n = 2$)</th>
<th>Urine ($n = 6$)</th>
<th>Proportion Urine/ARE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cy-3-gal</td>
<td>61.0</td>
<td>37.9 ± 1.6$^c$</td>
<td>62</td>
</tr>
<tr>
<td>cy-3-glu</td>
<td>3.40</td>
<td>0.95 ± 0.18$^c$</td>
<td>28</td>
</tr>
<tr>
<td>cy-3-arab</td>
<td>28.0</td>
<td>16.2 ± 0.56$^c$</td>
<td>58</td>
</tr>
<tr>
<td>pn-3-gal$^d$</td>
<td>ND</td>
<td>20.3 ± 0.53</td>
<td>NA</td>
</tr>
<tr>
<td>pn-3-glu$^e$</td>
<td>ND</td>
<td>4.8 ± 0.63</td>
<td>NA</td>
</tr>
<tr>
<td>pn-3-arab$^d$</td>
<td>ND</td>
<td>8.9 ± 0.57</td>
<td>NA</td>
</tr>
</tbody>
</table>

$^a$: Abbreviations are as follows: ARE, anthocyanin-rich extract; ND, not detectable; NA, not applicable.

$^b$: Percentage areas at 520 nm are expressed as mean ± SE.

$^c$: Newly generated anthocyanins result in decreased percentage of native anthocyanins.

$^d$: Tentative identification.
ing pn-3-gal, pn-3-glu, and pn-3-arab by comparing the retention time and spectrum with those peaks in bilberry ARE, and later confirmed by their molecular weight obtained from HPLC–tandem MS (Fig. 4). Interestingly, for the major compounds (Table 1), the ratio of pn-3-gal over cy-3-gal in urine samples (54%) was almost identical to the ratio of pn-3-arab over cy-3-arab (55%) suggesting that methylation was independent of the sugar moiety. In addition, the ratios of cy-3-gal/cy-3-arab in ARE, cy-3-gal/cy-3-arab in urine, and pn-3-gal/pn-3-arab in urine were almost constant (~2.3).

In the bilberry treatment (Fig. 2B, Table 2), a dramatic percentage increase of all the three pn-glycosides in urine strongly suggests the transformation of cy-glycosides to pn-glycosides. The percentage area of the pn-3-arab peak surprisingly increased ~12 times. Although the percentage area of pn-3-gal and pn-3-glu peaks only increased approximately two times, these two peaks co-eluted with other anthocyanin peaks, and the actual percentage increase of both pn-3-gal and pn-3-glu could be greater. Because cy-3-arab accounted for approximately 5% of total anthocyanin in bilberry ARE, which is 20 times higher than pn-3-arab (0.23%) in ARE, the transformation of cy-3-arab to pn-3-arab may explain the sharp increase of pn-3-arab percentage in the urine.

Methylation of cy-3-glu was earlier reported by Tsuda et al. (19). pn-3-glu and pn-3-sambubioside as methylated metabolites of cy-3-glu and cy-3-sambubioside were later reported (16,17). Methylation of chokeberry anthocyanins was recently reported by Kay et al. (30). However, in the previous rat studies where in vivo anthocyanin methylation was reported, only trace amounts of pn-glu were found (17). Even in human studies, the reported methylation extent is relatively lower than what we observed (16,30). These studies all employed short time adaptation (up to 8 days). In many other studies of urinary anthocyanins, the methylated metabolites

<table>
<thead>
<tr>
<th>Anthocyanin Components</th>
<th>ARE (n = 2)</th>
<th>Urine (n = 8)</th>
<th>Proportion Urine/ARE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dp-3-gal</td>
<td>10.1</td>
<td>5.00 ± 0.33</td>
<td>49</td>
</tr>
<tr>
<td>dp-3-glu</td>
<td>15.8</td>
<td>5.48 ± 0.21</td>
<td>35</td>
</tr>
<tr>
<td>cy-3-gal + dp-3-arab</td>
<td>15.5</td>
<td>10.4 ± 0.37</td>
<td>67</td>
</tr>
<tr>
<td>cy-3-glu</td>
<td>11.2</td>
<td>12.1 ± 0.43</td>
<td>108</td>
</tr>
<tr>
<td>pt-3-gal + cy-3-arab</td>
<td>8.90</td>
<td>13.0 ± 0.26</td>
<td>146</td>
</tr>
<tr>
<td>pt-3-glu</td>
<td>11.4</td>
<td>9.32 ± 0.16</td>
<td>82</td>
</tr>
<tr>
<td>pn-3-gal + pt-3-arab</td>
<td>4.06</td>
<td>7.50 ± 0.19</td>
<td>185</td>
</tr>
<tr>
<td>pn-3-glu + mv-3-gal</td>
<td>8.96</td>
<td>17.1 ± 0.28</td>
<td>191</td>
</tr>
<tr>
<td>pn-3-arab</td>
<td>0.23</td>
<td>2.64 ± 0.17</td>
<td>1,167</td>
</tr>
<tr>
<td>mv-3-glu</td>
<td>11.6</td>
<td>14.8 ± 0.38</td>
<td>127</td>
</tr>
<tr>
<td>mv-3-arab</td>
<td>1.44</td>
<td>2.32 ± 0.15</td>
<td>161</td>
</tr>
</tbody>
</table>

a: Abbreviation is as follows: ARE, anthocyanin-rich extract.
b: Percentage areas at 520 nm are expressed as mean ± SE.

c: Abbreviation is as follows: ARE, anthocyanin-rich extract.
d: Percentage areas at 520 nm are expressed as mean ± SE.

Table 3. Anthocyanin Composition of ARE and Urine in Grape Treatment

<table>
<thead>
<tr>
<th>Anthocyanin Components</th>
<th>ARE (n = 2)</th>
<th>Urine (n = 7)</th>
<th>Proportion Urine/ARE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dp-3,5-diglu</td>
<td>2.91</td>
<td>2.00 ± 0.06</td>
<td>69</td>
</tr>
<tr>
<td>cy-3,5-diglu</td>
<td>0.85</td>
<td>1.18 ± 0.06</td>
<td>139</td>
</tr>
<tr>
<td>dp-3-glu</td>
<td>3.03</td>
<td>0.75 ± 0.10</td>
<td>25</td>
</tr>
<tr>
<td>pt-3,5-diglu</td>
<td>4.83</td>
<td>5.24 ± 0.13</td>
<td>108</td>
</tr>
<tr>
<td>cy-3-glu</td>
<td>1.71</td>
<td>0.43 ± 0.04</td>
<td>25</td>
</tr>
<tr>
<td>pn-3,5-diglu</td>
<td>21.2</td>
<td>32.2 ± 0.24</td>
<td>152</td>
</tr>
<tr>
<td>mv-3,5-diglu + pt-3-glu</td>
<td>35.5</td>
<td>46.2 ± 0.25</td>
<td>130</td>
</tr>
<tr>
<td>pn-3-glu</td>
<td>2.38</td>
<td>2.97 ± 0.09</td>
<td>125</td>
</tr>
<tr>
<td>mv-3-glu</td>
<td>4.79</td>
<td>5.31 ± 0.12</td>
<td>111</td>
</tr>
<tr>
<td>dp-3-(coum-glu)</td>
<td>2.61</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pt-3-(coum-glu)</td>
<td>14.1</td>
<td>2.2 ± 0.2</td>
<td>16</td>
</tr>
<tr>
<td>mv-3-(coum-glu)</td>
<td>1.50</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a: Abbreviations are as follows: ARE, anthocyanin-rich extract; ND, not detectable.
b: Percentage areas at 520 nm are expressed as mean ± SE.
c: pt-3-glu is a co-eluted minor peak.
were not reported at all. In contrast, our study involved 13 wk of anthocyanin-rich diet feeding before urine sample collection. It has to be noticed that pn-glycosides accounted for approximately one-third of the total anthocyanins recovered from urine samples in the chokeberry group (Table 1). This high percentage was greater than other reported studies, indicating the possible accumulation in tissues and later release of methylated cy-glycosides or induced methyltransferase activity. We did not measure the anthocyanin concentration in tissues, but, according to Tsuda et al. (19), methylated cy-3-glu was found to accumulate in liver as well as in kidney, although cy-3-glu was not even detected in liver. Some other minor anthocyanin-like peaks, possibly being metabolites, were detected in the urine (Fig. 2). Previous studies have reported other anthocyanin metabolism pathways such as glucuronidation, sulfoconjugation, and a secondary methylation (30–35). However, HPLC-MS/MS analysis of our samples did not find these derivatives under the conditions of our experiment.

**Determination of Anthocyanins and Metabolites in Plasma**

The major anthocyanins in each diet ARE were detected in plasma as intact forms (Fig. 5). The acylated anthocyanin pt-3-(coum-glu) found in the urine was also found in plasma samples from the grape treatment (Fig. 5D). cy-3-gal and cy-3-arab were the dominant plasma anthocyanins in both the chokeberry and bilberry groups (Fig. 5B and C). The major anthocyanin peaks in grape ARE, pn-3,5-diglu and mv-3,5-diglu, were also observed in the plasma as the dominant peaks (Fig. 5D). A major peak in bilberry ARE (dp-3-gal) was greatly reduced in the plasma. The anthocyanin concentration in plasma at the time of sample collection was too low for accurate quantification and clear identification of minor peaks with our analytical methodology. Total anthocyanin level in the plasma was estimated based on area under the curve to be in the range of 0.2–2 µmol/l plasma. Six anthocyanin-like compounds with maxi-
mum absorption close to 520 nm were detected in all three diet groups, but their retention times did not match any known anthocyanin in the corresponding AREs (Fig. 5B–D). No anthocyanidins were detected in any of the samples evaluated, likely due to their extremely low stability.

Two major non-anthocyanin peaks (compounds 1 and 2 in Fig. 6), with maximum absorption at ~310 nm and peak areas severalfold larger than plasma anthocyanins, were noticed in every plasma sample from ARE-fed groups. These two peaks were not observed in the plasma from the control group nor in any feces, cecal content (29), or AREs. Evidence suggests that they were anthocyanin metabolites, and the conversion of anthocyanins to such compounds occurred after absorption. Anthocyanin metabolites have been reported in several in vitro and in vivo studies in which anthocyanins were deconjugated to protocatechuic acid or other phenolics (19,36–38). The concentration of compound 1 found in the plasma was much higher in the grape treatment than in the bilberry and chokeberry treatments. Combining this observation with the evidence of extensive deglycosylation of grape ARE in the gut (29), we conjecture that it was the deglycosylated anthocyanins (aglycones) that further deconjugated to compound 1. Compounds 1 and 2 should receive further attention given their high concentration in the plasma.

**Summary**

After a 3-mo feeding trial, we evaluated the impact of chemical structure on the absorption and metabolism of anthocyanins in rats. The type of sugar substitution had significant impact on the absorption and excretion of individual anthocyanins in rats. Anthocyanin diglucosides were absorbed better than their corresponding monoglucosides. We demonstrated that intact acylated anthocyanins could be ab-
sorbed into plasma and excreted through urine. These findings provide information for screening better bioavailable anthocyanins for value-added foods. In this 3-mo study we observed larger urinary excretion of methylated anthocyanins than in several shorter (less than 8-day adaptation) studies reported, suggesting the possible accumulation of anthocyanins in tissues or induction of methyltransferase. Measuring tissue-bound anthocyanin may help to explain the apparent contradiction of observed health benefit and low plasma availability of anthocyanins. Besides anthocyanins, two possible anthocyanin metabolites were noticed in plasma, suggesting the potentially active form of anthocyanins in vivo aside from their intact forms. The current study of rat urine and plasma supported the finding by numerous researchers that anthocyanins have very low absorption, which in turn supports our hypothesis that anthocyanins in the gut content may influence GIT health even without being delivered by the blood circulation system. We hypothesize that this beneficial effect may be related to the direct interaction between anthocyanins and colon epithelial cells or through the prevention of lipid/protein oxidation in the GIT.

Acknowledgments and Notes

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