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Urinary Excretion of Black Raspberry (*Rubus occidentalis*) Anthocyanins and Their Metabolites

QINGGUO TIAN,† M. MONICA GIUSTI,† GARY D. STONER,‡ AND STEVEN J. SCHWARTZ*,†

Department of Food Science and Technology, The Ohio State University, 2015 Fyffe Road, Columbus, Ohio 43210, and Division of Environmental Health Sciences, School of Public Health and Ohio State University Comprehensive Cancer Center, Suite 1148, 300 West 10th Avenue, Columbus, Ohio 43210

Anthocyanins are the most abundant phenolic compounds, widely distributed in fruits and vegetables, and exhibit potent antioxidant capacity. Humans ingest a significant amount of anthocyanins in the daily diet. The objective of the current study was to examine human absorption and metabolism of black raspberry anthocyanins when administered at high doses (2.69 ± 0.085 g/day). Ten healthy men consumed 45 g of freeze-dried black raspberries daily for 1 week. Urine samples were collected over a 12 h period in 4 h intervals at day 1 and day 7. Urinary anthocyanins were analyzed by high-performance liquid chromatography coupled to a photodiode array detector and a tandem mass spectrometer using precursor ion and product ion analyses. Anthocyanins were excreted in intact forms and metabolized into methylated derivatives in human urine. The urinary excretion of anthocyanins reached a maximum concentration (1091.8 ± 1081.3 pmol/L, $n = 10$) during the 4−8 h period after black raspberry ingestion. As compared to the anthocyanin distribution in black raspberries, urinary cyanidin 3-xylosylrutinoside was detected at a higher concentration than that of cyanidin-3-rutinoside.

KEYWORDS: Anthocyanins; black raspberries; anthocyanin methylated derivatives; absorption; metabolism

INTRODUCTION

Epidemiological studies have shown that polyphenols may contribute to the protective effects of fruits and vegetables against degenerative conditions such as cardiovascular diseases and cancers (*1*−*4*). Anthocyanins are one of the most abundant phenolics in nature and are responsible for the red, purple, and blue colors of most fruits and vegetables. Humans ingest a significant amount of anthocyanins by consumption of anthocyanin-rich foods, such as berries, grapes, radishes, red cabbage, and red wine, etc. Anthocyanin intake through the diet has been estimated at 180−215 mg/day in the United States (*5*). Numerous studies have demonstrated that anthocyanins possess antioxidant activity (*6*), antiinflammatory effects (*7*), and antimutagenic action against various mutagens (*8*). In addition, anthocyanins protect against DNA damage (*9*), inhibit skin tumorigenesis in rats (*10*), prevent low-density lipoprotein oxidation (*11*), and inhibit platelet aggregation (*12*). However, information on the absorption and metabolism of anthocyanins in humans is limited. Several studies have reported that anthocyanins are absorbed and circulate in human biological fluids as intact glycoside forms (*13*−*16*). Some researchers (*17*, *18*) have detected glucuronide and sulfate derivatives of anthocyanins in human urine after consumption of berries or berry extracts. However, previous human dietary interventions only investigated mono- and diglycosylated anthocyanins, while information on the absorption of higher molecular weight anthocyanins, such as anthocyanin triglycosides, is not available. In addition, the highest dosage of anthocyanins administered in previous studies has been less than 1.2 g (*13*, *19*).

In this study, we investigated human absorption and metabolism of black raspberry anthocyanins at high doses of ingestion (~2.7 g anthocyanins/day). We also evaluated the effect of anthocyanin glycosylation on absorption by comparing anthocyanin diglycosides and triglycosides from black raspberries, which contain a high amount of cyanidin 3-rutinoside and cyanidin 3-xylosylrutinoside (**Figure 1**).

MATERIALS AND METHODS

Subjects. Ten healthy nonsmoking male subjects aged 29.6 ± 10.6 years and with a mean (±SD) body mass index of 25.5 ± 2.8 kg/m^2 were recruited in Columbus, OH. To be eligible for participation, individuals had to (i) have not participated in any other clinical trials within 30 days prior to this study; (ii) have not used any medications or recreational drugs within 5 months; (iii) have no history of any

* To whom correspondence should be addressed. Tel: 614-292-2934. Fax: 614-292-4233. E-mail: schwartz.177@osu.edu.
† Department of Food Science and Technology.
‡ Division of Environmental Health Sciences.

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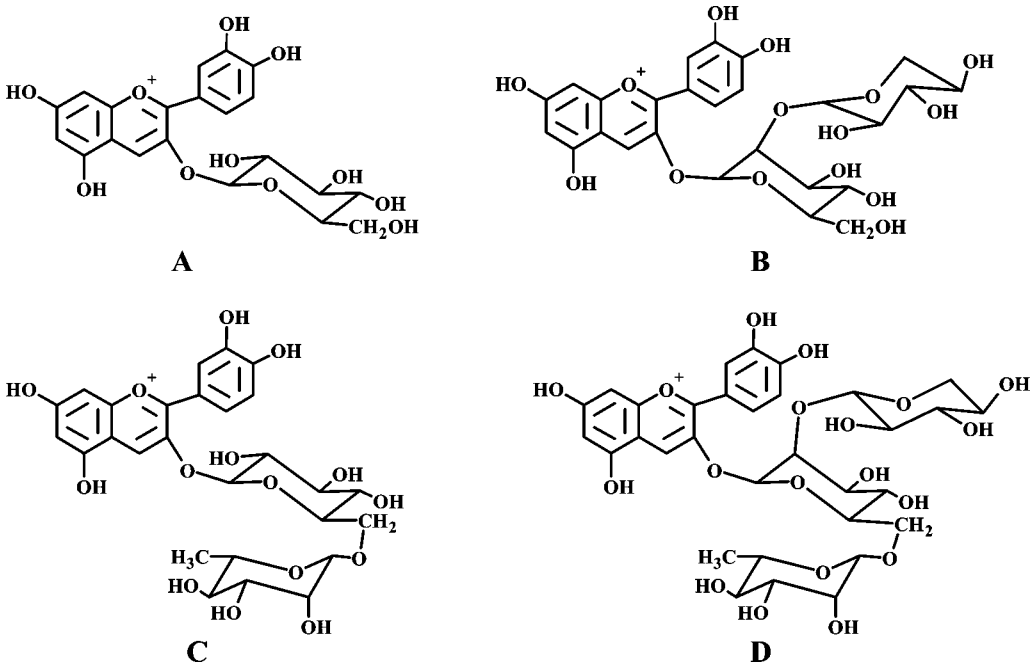

Figure 1. Chemical structures of anthocyanins in black raspberries. (**A**) Cyanidin 3-glucoside, (**B**) cyanidin 3-sambubioside, (**C**) cyanidin 3-rutinoside, and (**D**) cyanidin 3-xylosylrutinoside.

cancers; (iv) have no antibiotic or supplemental vitamins or minerals in the past 4 weeks; and (v) have no known or suspected allergy to any type of berries. The experimental protocol was approved by the Ohio State University (OSU) Comprehensive Cancer Center's Clinical Scientific Review Committee (CSRC) and the Institutional Review Board (IRB) of OSU. All subjects provided written informed consent prior to participation.

Experimental Protocol. Eligible subjects were provided instructions for a strict low-phenolic diet for 48 h prior to the morning of the first dose on day 1 and last dose on day 7. For the other days (days 2−6), subjects were instructed to follow a less strict, reduced phenolic diet, which limits anthocyanin-containing fruits, vegetables, and red wine. Subjects were admitted to the Clinical Pharmacology Unit at OSU from the evening prior to days 1 and 7 (beginning at least 12 h prior to dosing until completion of a 12 h postdose urine collection).

Subjects ingested 45 g of freeze-dried black raspberries (FDBR) daily for a week. The FDBR had been pulverized into a fine power of 40 mesh and was mixed with half cup of water for ingestion. The anthocyanin content (mg/g) in FDBR was as follows: cyanidin-3-glucoside, 4.7 ± 0.1; cyanidin-3-sambubioside, 3.0 ± 0.1; cyanidin-3-xylosylrutinoside, 15.8 ± 1.5; and cyanidin-3-rutinoside, 36.3 ± 0.2.

Black Raspberry Anthocyanin Analysis. Using an analytical balance, 0.100 g of FDBR powders was accurately weighed. The berry powders were extracted with 60 mL of 95% methanol containing 1% formic acid in a capped Erlenmeyer flask and sonicated for 30 min. After centrifugation at 500*g* for 10 min, the residues were extracted repeatedly until no color was visible on the residue. The combined supernatants were transferred to a 250 mL volumetric flask, and the solution was adjusted to 250 mL using acidified 95% methanol. An aliquot of the solution was filtered through a 0.2 μm Nylon filter, and 20 μL was injected onto a high-performance liquid chromatograph (HPLC) for quantitative analyses. Four individual anthocyanins (*20*) were quantified and reported as cyanidin 3-glucoside (Polyphenols Laboratories, Hanaveien, Norway) equivalents. An absorption coefficient of 26900 (*21*) was used for calculation of standard cyanidin 3-glucoside concentration.

Urinary Anthocyanin and Metabolite Analyses. Urine samples collected at the OSU Clinical Pharmacology Unit were immediately acidified with 1% formic acid and stored at −80 °C until solid phase extraction was conducted. Sep-Pak C_{18} cartridges (Waters Corp., Milford, MA) were conditioned by washing with 6 mL of acidified methanol (0.1% formic acid), followed by 6 mL of acidified deionized water. Urine (6 mL) was loaded on the cartridge and washed with 10 mL of acidified deionized water. Anthocyanins and metabolites were eluted with 6 mL of acidified methanol (0.1% formic acid). The methanol eluate was dried under nitrogen gas and redissolved in 1 mL of 5% methanol in water (containing 1% formic acid). After the eluate was filtered through a 0.2 μm nylon filter (Waters Corp.), 50 μL was injected onto an HPLC for qualitative and quantitative analyses. Recovery of cyanidin 3-glucoside during the solid phase extraction procedure was between 90 and 95% ($n = 3$).

Identification of anthocyanins and their metabolites was conducted using a LC-MS system consisting of a Waters 2695 gradient HPLC separation module, an autoinjector, a 996 photodiode array (PDA) ultraviolet−visible (UV/vis) absorbance detector (Waters Corp.), and a triple quadrupole ion-tunnel mass spectrometer (Quattro Ultima, Micromass Limited, Manchester, United Kingdom) equipped with a Z-spray electrospray ionization (ESI) source. Calibration of the mass spectrometer was performed using sodium iodide and cesium iodide. HPLC analysis was performed on a 150 mm × 3.0 mm i.d., 3 μm Atlantis dC_{18} column (Waters Corp.) at 35 °C. The solvent system consisted of a gradient mobile phase from 5 to 20% B in 25 min and then to 5% B in 5 min. Solvent A was formic acid:water (5/95, v/v), and solvent B was acetonitrile:formic acid (95/5, v/v). The flow rate was set at 0.6 mL/min. UV/vis spectra of anthocyanins were recorded from 200 to 600 nm using the in-line PDA detector. Approximately 0.06 mL/min of the HPLC eluate separated by a microsplitter valve (Upchurch Scientific, Oak Harbor, WA) was delivered to the ESI source. Precursor ion and product ion analyses were conducted to identify urinary anthocyanins and metabolites. The settings of the quadrupole mass analyzer were as follows: capillary voltage, 3.0 kV; cone voltage, 35 V; radiofrequency (RF) lense 1, 50 V; desolvation gas temperature, 400 °C (flow rate, 17 L/min); source temperature, 105 °C; collision gas (argon) pressure, 7 psi; and the collision energy was in the range of 25−35 eV.

Quantification of anthocyanins and their metabolites was conducted using a standard curve generated from standard cyanidin 3-glucoside ($Y = 30.2x$; range, 0−255 pmol; $R^2 = 0.985$). The method detection limit (MDL) was determined to be 0.31 pmol according to the U.S. Environmental Protection Agency (EPA) approach that MDL is the concentration corresponding to 3σ of seven measurements of the analyte at very low concentrations (*22*). The lower limit of quantification (LLOQ) is 0.98 pmol according to an EPA approach representing the concentration corresponding to 3.18 times of MDL (*22*). Because cyanidin 3-glucoside and cyanidin 3-sambubioside coeluted and were present at relatively low concentrations, these two compounds were

quantified together. All anthocyanins are expressed as cyanidin 3-glucoside equivalents.

Statistics. A general linear multivariate model was designed with subjects and time as dependent factors and different anthocyanins as fixed factors. The data were tested for normality in $Q - Q$ plots. Following these analyses, post hoc test comparisons (Bonferroni) for time were conducted. A $P < 0.05$ (two-sided) was considered significant. The statistical analyses were performed using SPSS 13.0 (Chicago, IL).

RESULTS AND DISCUSSION

In the past few years, several human studies have been conducted to investigate the bioavailability of anthocyanins from fruit or fruit extracts (*13*, *15*, *16*, *18*, *19*, *23*−*25*). Most of these studies have focused their attention on anthocyanin monoglycosides or diglycosides such as cyanidin/peonidin 3-glucoside (*18*), cyanidin 3,5-diglucoside (*23*, *24*), and cyanidin 3-rutinoside (*23*). Little information is available on the bioavailability of higher molecular weight anthocyanins, such as anthocyanin triglycosides, which are widely present in our daily diet, including black and red raspberries, eggplant, and sweet potatoes, etc. Our current study investigated the absorption of high doses of black raspberry anthocyanins (~2.7 g/day) that contained high proportions of cyanidin 3-xylosylrutinoside (~26% of total anthocyanins) and cyanidin 3-rutinoside (~60% of total anthocyanins). The use of freeze-dried material facilitates ingestion (less bulk than berries) and standardizes the composition (all powders are homogeneous).

Our studies showed that 45 g of FDBR was well-tolerated. All black raspberry anthocyanins were detected in their intact glycosidic forms in human urine (**Figure 2**). Confirmation of each anthocyanins was performed by HPLC-ESI-MS/MS using precursor ion and product ion analyses (**Figure 3**). Compounds **1** (*m*/*z* 449), **2** (*m*/*z* 581), **3** (*m*/*z* 727), and **4** (*m*/*z* 595) in both black raspberries and urine were detected as precursors of *m*/*z* 287 (**Figure 3B**), indicating that they were cyanidin anthocyanins. On the basis of our previous characterization of black raspberry anthocyanins (*20*), we identified peaks 1−4 as cyanidin 3-glucoside, cyanidin 3-sambubioside, cyanidin 3-xylosylrutinoside, and cyanidin 3-rutinoside, respectively. Urinary excretion of total anthocyanins reached a maximum concentration during 4−8 h (**Figure 2C**). Human absorption of individual anthocyanins has been reported to be affected by the glycoside moieties rather than the aglycones, and anthocyanin glucosides were absorbed and excreted faster than anthocyanin rutinosides (*23*). Comparing the relative concentration of individual anthocyanins from black raspberries (**Figure 2A**) with those found in urine (**Figure 2B**−**D**), it was noted that in human urine cyanidin 3-xylosylrutinoside (~26% of total anthocyanins in black raspberries) was present at a considerably higher concentration than cyanidin 3-rutinoside (~60% of total anthocyanins in black raspberry) although it is the predominant anthocyanin in black raspberries (**Figure 2A**).

In addition, several peaks with maximum absorption close to 520 nm were detected in the urine (**Figure 2B**−**D**) that were not present in black raspberries (**Figure 2A**). These peaks also were not present in the urine samples collected from subjects before consumption of FDBR (data not shown) and were identified as anthocyanin metabolites. The anthocyanin metabolites were identified as cyanidin methylated derivatives, which were precursors of *m*/*z* 301 (**Figure 3C**). Two of the major metabolites, peaks 7 and 8 (**Figure 3A**,**C**), exhibited identical molecular mass (*m*/*z* 741) and fragmentation patterns (**Figure 3D**) during tandem mass spectrometry product ion scans. The fragment ion at *m*/*z* 595 was most likely produced by loss of a rhamnose unit $[M - 146]^+$ from *m*/*z* 741. The most abundant fragment (*m*/*z* 301), corresponding to peonidin or methylated derivative of cyanidin, was possibly formed by further loss of hexose and pentose substituents from *m*/*z* 595. These fragment ions as well as the molecular cation at *m*/*z* 741 indicated that compounds **7** and **8** are methylated derivatives of cyanidin 3-xylosylrutinoside (*m*/*z* 727). Because these two compounds were eluted at different times during HPLC, they are possibly isomers. Another major metabolite (peak 9) with a molecular mass of *m*/*z* 609 produced fragment ions at *m*/*z* 301 and 463 during product ion scans (**Figure 3E**). Similar to the fragmentation of *m*/*z* 741 (**Figure 3D**), *m*/*z* 463 was probably produced by loss of a rhamnose moiety from the molecular cation, indicating that this compound was a methylated derivative of cyanidin 3-rutinoside. Methylated derivatives of cyanidin 3-glucoside (peak 5) and cyanidin 3-sambubioside (peak 6) in **Figure 3A**,**C** were detected at very low concentrations due to the low content of these two compounds in black raspberries (spectra not shown).

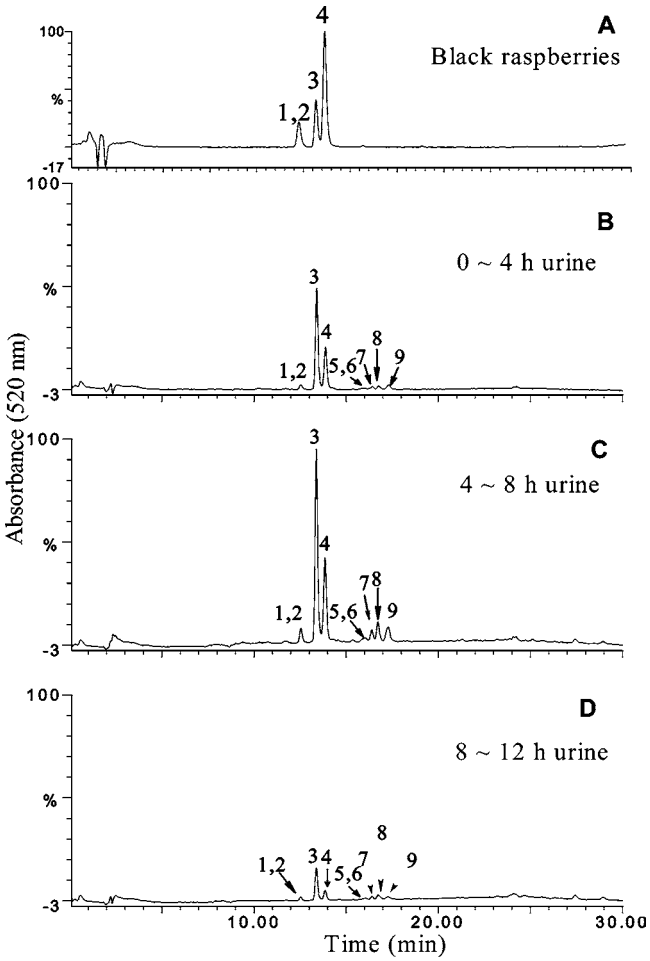

Figure 2. Representative HPLC chromatograms (520 nm) of anthocyanins in FDBR and urine of one subject after consumption of 45 g of FDBR. (**A**) Anthocyanins in FDBR. Peak labels: 1, cyanidin 3-glucoside; 2, cyanidin 3-sambubioside; 3, cyanidin 3-xylosylrutinoside; and 4, cyanidin 3-rutinoside. (**B**) Chromatogram of anthocyanins and metabolites in urine (0–4 h); peaks 5 and 6 are methylated derivatives of cyanidin 3-glucoside and cyanidin 3-sambubioside; peaks 7 and 8 are methylated derivatives of cyanidin 3-xylosylrutinoside; peak 9 is a methylated derivative of cyanidin 3-rutinoside. (**C**) Chromatogram of anthocyanins and metabolites in urine (4–8 h). (**D**) Chromatogram of anthocyanins and metabolites in urine (8–12 h).

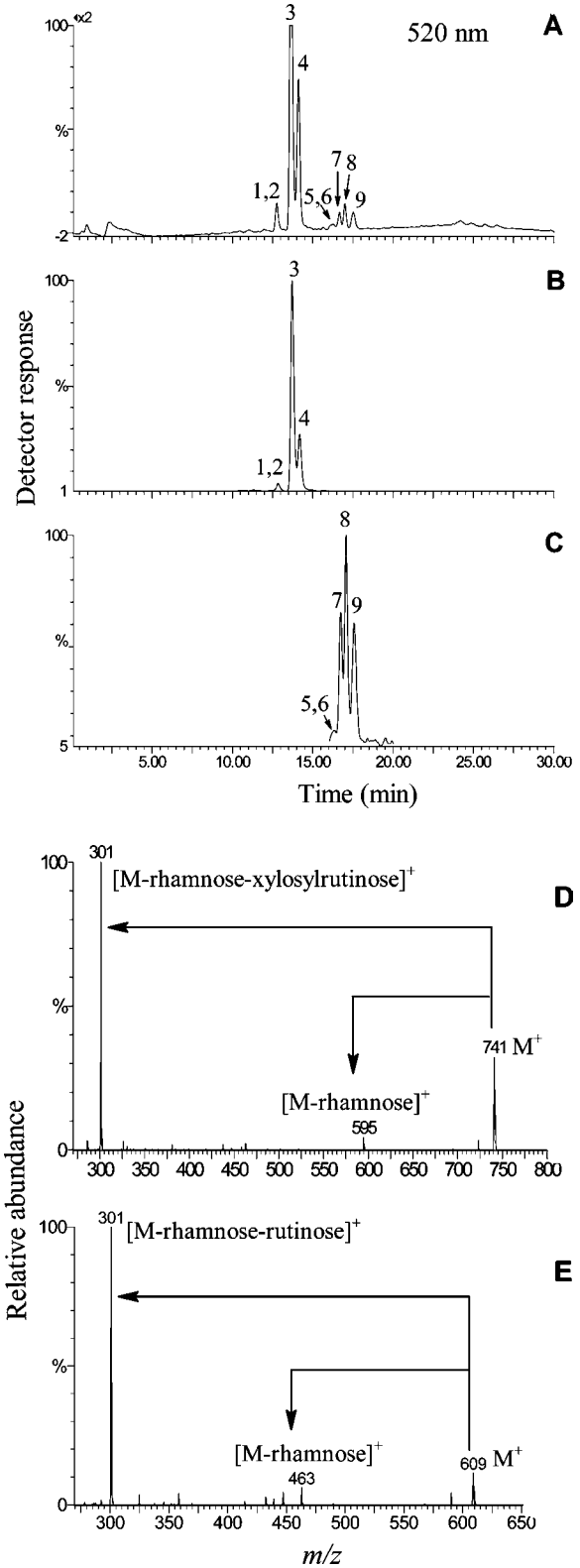

Figure 3. LC-ESI/MS/MS analysis of human urine samples after consumption of 45 g of FDBR. Peak labels are the same as in **Figure 1**. (**A**) HPLC chromatogram of 4–8 h urine from a subject after consumption of FDBR. (**B**) LC-ESI/MS/MS precursor ion analysis of *m*/*z* 287. (**C**) LC-ESI/MS/MS precursor ion analysis of *m*/*z* 301. (**D**) LC-ESI/MS/MS product ion analysis of *m*/*z* 741 (peaks 7 and 8). (**E**) LC-ESI/MS/MS product ion analysis of *m*/*z* 609 (peak 9).

Previous researchers reported the presence of methylated cyanidin 3-glucoside (*26*) and delphinidin 3-glucoside (*27*) in rats as well as methylated anthocyanin monoglycoside in human urine (*19*). Because of the low concentration of cyanidin-3-glucoside and cyanidin 3-sambubioside in black raspberries, we only detected the methylated derivatives of these two compounds at very low concentrations. However, our study is the first to report the methylation of an anthocyanin triglycoside in vivo. In addition, we have found that the methylation of cyanidin 3-xylosylrutinoside occurred at different positions of the aglycone because both compounds **7** and **8** were detected as methylated derivatives of cyanidin 3-xylosylrutinsoide (**Figure 3**) that exhibited identical fragmentation pattern during MS/MS analysis (**Figure 3D**) but different HPLC retention times. This methylation pattern was not observed for the other anthocyanins, such as the most abundant cyanidin 3-rutinoside in black raspberries, indicating that the structures of glycoside moieties may affect methylation. Recently, aglycones and sugar moieties have been reported to affect the absorption and metabolism of anthocyanins in weaning pigs (*28*). Methylation normally occurs at the 3′- or 4′-hydroxyl group of the aglycone of flavonoids (*29*); however, these cannot be differentiated by mass spectrometry alone since both derivatives exhibit identical fragmentation patterns.

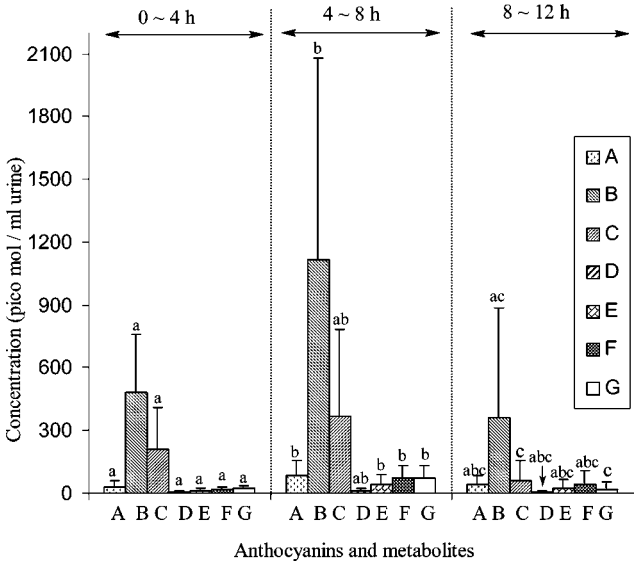

Figure 4. Twelve hour urine anthocyanin concentrations in human subjects after consumption of 45 g of FDBR. (**A**) Cyanidin 3-glucoside and cyanidin sambubioside, (**B**) cyanidin 3-xylosylrutinoside, (**C**) cyanidin 3-rutinoside, (**D**) methylated derivatives of cyanidin 3-glucoside and cyanidin 3-sambubioside, (**E**) methylated derivative of cyanidin 3-xylosylrutinoside, (**F**) methylated derivative of cyanidin 3-xylosylrutinoside, and (**G**) methylated derivative of cyanidin 3-rutinoside. Values are the mean ± SD of 10 subjects. (a–c) Values with different letters are significantly different at $P < 0.05$.

A few other studies have reported the possible metabolism of anthocyanins into glucuronide and sulfate derivatives in the human urine (*17*, *18*). However, after careful analyses of the urine samples using HPLC-MS/MS with neutral loss scan (monitoring loss of 176 and 80 for glucuronide and sulfate conjugates, respectively) and selected reaction monitoring, we did not detect the presence of glucurono- and sulfoconjugates of anthocyanins in the urine samples of any subjects in the current study. Similarly, a previous study has proposed that anthocyanins are a unique and rare group of flavonoids that undergo metabolism solely by methylation (*30*).

A significant concentration increase ($P < 0.05$) from 0−4 to 4−8 h was observed for most urinary anthocyanins and their metabolites (**Figure 4**). For all subjects, urinary anthocyanins

reached a maximum concentration (1091.8 ± 1081.3 pmol/mL, $n = 10$) in 4−8 h after ingestion of FDBR (**Figure 2C**) and decreased during the 8−12 h time period (**Figure 2D**). However, only a small increasing trend ($P = 0.34$) was observed for cyanidin 3-rutinoside over this period although this compound is the predominant anthocyanin in black raspberries. Previous studies reported that anthocyanin glucosides are absorbed and decreased more rapidly than anthocyanin rutinosides in human plasma (*23*). Because of the low concentration of cyanidin-3-glucoside and 3-sambubioside in FDBR and human urine, we are unable to confirm the earlier observation although the combined concentration of these two anthocyanins increased faster than cyanidin-3-rutinoside, which only showed a slight but not significant increase ($P = 0.34$). In contrast, we found that the concentration of cyanidin 3-rutinoside decreased significantly ($P < 0.05$) from 4−8 to 8−12 h. A similar trend was also observed for cyanidin 3-glucoside and cyanidin-3-sambubioside ($P = 0.09$) over this period of time. Furthermore, the concentration of cyanidin-3-xylosylrutinoside, a less abundant anthocyanin in black raspberries but predominant in urine, was found to increase ($P = 0.016$) and decrease ($P = 0.004$) more significantly than the rest of anthocyanins, indicating that cyanidin-3-xylosylrutinoside was possibly absorbed and excreted more quickly than the other anthocyanins.

The methylated derivative of cyanidin 3-rutinoside (**Figure 4G**) decreased significantly from 4−8 to 8−12 h ($P < 0.05$), while the two methylated derivatives of cyanidin 3-xylosylrutinoside (**Figure 4E**,**F**) only showed a trend of decrease ($P = 0.34$) over this period of time. In addition, we did not find any statistically significant concentration changes between day 1 and day 7 for all major anthocyanins and methylated derivatives (data not shown). Methylated cyanidin 3-glucoside and sambubioside were present at very low concentrations, and no significant changes were observed over this period of time.

Previous studies have proposed that 3′-hydroxyl of anthocyanins was selectively methylated by the catechol O-methyltransferase (*30*); however, we observed that methylation potentially occurred at both 3′- and 4′-hydroxyl positions of the triglycoside anthocyanins. Other investigators have postulated that methylation of high doses of anthocyanin associates with the conversion of S-adenosylmethionine to S-adenosylhomocysteine caused an increase of homocysteine in rodents, which may enhance the risk of vascular disease (*30*).

In conclusion, our study showed that methylation is the major metabolism pathway for anthocyanins and that the structures of the glycoside moieties affected the methylation pattern.

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