Absorption and Metabolism of Anthocyanins in Elderly Women after Consumption of Elderberry or Blueberry

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ABSTRACT The absorption and metabolism of anthocyanins (ACN) in humans was studied in four elderly women given 12 g elderberry extract (EBX) (720 mg total ACN), and six elderly women given 189 g lowbush blueberry (BB) (690 mg total ACN). The two major ACN in EBX, cyanidin-3-glucoside and cyanidin-3-sambubioside, as well as four metabolites: 1) peonidin 3-glucoside, 2) peonidin 3-sambubioside, 3) peonidin monoglucuronide, and 4) cyanidin-3-glucoside monoglucuronide were identified in urine within 4 h of consumption using HPLC-MS/MS with diode-array detector detection and retention time. Total EBX ACN excretion was 554 ± 90 μg (mean ± SD, $n = 4$) (0.077% of intake/4 h, wt/wt). In 5 of 6 women fed BB, urine samples contained ACN, which were identified as the original forms based upon comparisons to the BB food sample, which contained 24 ACN, 22 of which were identified by HPLC-MS/MS. Reasonable correlations between BB and urine proportions of the different ACN were obtained except for ACN arabinosides. Total urinary excretion during the first 6 h was 23.2 ± 10.9 μg (mean ± SD, $n = 5$) (0.004% of intake/6 h, wt/wt). Plasma ACN levels were below detection limits using 2 mL plasma in women that consumed BB. This study demonstrates for the first time that in vivo methylation of cyanidin to peonidin and glucuronide conjugate formation occurs after people consume ACN and demonstrates the low absorption and excretion of ACN compared with other flavonoids. J. Nutr. 132: 1865–1871, 2002.

KEY WORDS: • *anthocyanin* • *metabolite* • *elderberry (Sambucus* nigra*)* • *blueberry (Vaccinium* angustifolium*)*

Anthocyanins (ACN) are part of a large and widespread group of plant constituents known collectively as flavonoids. They are mainly distributed among flowers, fruits and vegetables and are responsible for the bright colors that occur in flower petals, fruits and other plant organelles (1,2). ACN can be used as natural colorants (3), but their use has been limited because of their poor stability. As a potential major component of our daily diet (4), more and more research has concentrated on their biological activities and possible health benefits in protecting against some chronic diseases, including cancer, cardio- and cerebrovascular, atherosclerosis, and diabetes. Some of these biological activities and protective functions may be attributable to their high antioxidant capacities (5). Studies on antioxidant capacities of ACN revealed that they could act in scavenging free radicals (6–8), metal chelation (4), protein binding (9), and other mechanisms.

To evaluate the health benefits of ACN in humans, the bioavailibility including absorption, distribution, metabolism, and excretion must be known. It is also necessary to consider the metabolites (such as ring-fission products produced by microorganisms; conjugated and methylated derivatives, etc.) instead of considering only the original form of ACN, as the concentration of the original forms in human body is much less than what was consumed (10). Metabolites may contribute, to some extent, to the positive health effects. However, information about metabolism of ACN is quite limited.

As early as 1933, Horwitt (11) observed that the urine of rabbits became highly pigmented after feeding ACN from grapes. He concluded that small quantities of the grape ACN or anthocyanidins were absorbed and passed through to the circulation. ACN, which are present in plants as glycosides, were long assumed not to be absorbed in the human body unless they were hydrolyzed by intestinal microorganisms. Recently, several papers on the absorption and metabolism of dietary ACN have shown that ACN can be absorbed in experimental animals and humans as glycosides (12–23).

We have previously detected the intact glycoside forms in blood and urine of humans after consuming elderberry ACN (16–17). Murkovic and coworkers (20–22) studied the absorption and metabolism of ACN in elderberry juice, and showed that ACN, though in low concentration, could be detected in blood and urine as intact glycosides. This report documents that additional ACN metabolites are present in humans after consumption of ACN from an elderberry extract or whole blueberry.

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[2] Abbreviations used: ACN, anthocyanins; BB, lowbush blueberry; EBX, elderberry extract; MS, mass spectrometer; *m/z*: mass to charge ratio; SPE, solid-phase extraction; TFA, trifluoroacetic acid.

[3] Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

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MATERIALS AND METHODS

Chemicals and materials. The 3-*O*-β-glucoside of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (six mixed ACN standard, HPLC grade), and cyanidin-3-*O*-β-glucoside (HPLC grade) were obtained from Polyphenols Laboratories (Sandnes, Norway); methanol from Fisher Scientific (Fair Lawn, NJ); formic acid from Aldrich Chemical Company (Milwaukee, WI); trifluoroacetic acid (TFA) and β-glucuronidase from Sigma Chemical (St. Louis, MO); Sep-Pak Vac RC (500 mg) C_{18} Cartridges for solid-phase extraction (SPE) were purchased from Waters (Milford, MA).

Subjects and study design. The study protocol was approved by the Human Investigation Review Committee of Tufts University and the New England Medical Center, and written, informed consent was obtained from each study participant.

For the elderberry study, four healthy women, 60–70 y old, consumed 12 g elderberry extract that contained a total of 720 mg of ACN blended in 500 mL water after fasting overnight as described previously (17). Urine samples were collected from these subjects before the consumption of elderberry extract and between 0 and 2, 2 and 4, 4 and 6, 6 and 8, 8 and 12, and 12 and 24 h after consumption of the elderberry extract. The urine samples were treated with 0.44 mol/L TFA (1:0.2, v/v) and then stored at −70°C before analysis.

For the blueberry study, six healthy elderly women, 60–70 y old, were given 189 g frozen lowbush blueberry (BB) (containing a total of 690 mg of ACN) blended in 315 mL water after fasting overnight. The urine samples were collected and treated as described above. Blood samples were collected before and at 10, 20, 30, 45 min, and 1, 2, 4, 6, and 24 h after consumption of blueberry. The blood samples were centrifuged at 500 × *g* for 10 min at 4°C, and then plasma samples were quickly removed and immediately treated with an aqueous solution of 0.44 mol/L TFA (1:0.2; v/v). These samples were stored in −70°C until analysis.

Sample preparation. The urine samples were treated with the Sep-Pak C_{18} SPE cartridge as follows. The cartridge was washed with 5 mL methanol, and then equilibrated with 5 mL 5% formic acid/aqueous solution. Urine samples (5 mL) were loaded onto the cartridge. The cartridge was washed with 5 mL 5% formic acid/aqueous solution. ACN were recovered with 5 mL 5% formic acid/methanol solution. The methanol solution was evaporated completely with a SpeedVac (SC210A, ThermoSavant, Holbrook, NY) and redissolved in 200 μL 5% formic acid methanol solution. After filtration with a syringe filter (0.45 μm, Phenomenex, Torrance, CA), the solution was injected into the HPLC/MS system for analysis of ACN.

Blood samples were also treated with the Sep-Pak C_{18} SPE cartridge as previously described (16). After SPE treatment, water-soluble compounds, polar lipids, and neutral lipids were removed from the plasma samples. ACN were eluted finally with 5 mL of 0.44 mol/L TFA in methanol. The methanol phase was evaporated completely with a SpeedVac (SC210A, ThermoSavant) and redissolved in 200 μL 0.44 mol/L TFA in methanol. After filtration with a syringe filter (0.45 μm, Phenomenex), the solution was injected into the HPLC/MS system for the analysis of ACN.

Before experimental samples were prepared, blank urine and blood samples spiked with cyanidin-3-*O*-β-glucoside were prepared using the same procedures to test the recovery of the sample preparation method.

Analysis of ACN in urine and plasma. The analysis of ACN in urine was carried out on an HP series 1100 HPLC system including an autosampler, a binary pump, Zorbax SB-C_{18} column (4.6 × 250 mm), and a diode array detector (Agilent Technologies, Palo Alto, CA). Elution was performed using mobile phase A (5% formic acid aqueous solution) and mobile phase B (pure methanol). The flow rate was 1 mL/min and detection was at 520 nm. The gradient system used was as follows: 0–2 min, 5% B; 2–10 min, 5–20% B; 10–15 min, 20% B; 15–30 min, 20–30% B; 30–35 min, 30% B; 35–50 min, 30–45% B; 50–55 min, 45% B; 55–65 min, 45–5% B; and 65–68 min, 5% B. Low-resolution electrospray mass spectrometry was performed with an Esquire-LC Mass Spectrometer (MS) (Bruker Daltoniks, Billerica, MA), an ion trap instrument equipped with an electrospray interface. Column effluent was monitored in positive mode of the MS. Major MS parameters were: capillary exit, 3500 V; capillary offset, 500 V; skim 1, 25.4 V; dry gas, 8 L/min; temperature, 300°C. Values are reported as means ± SD.

RESULTS

Recovery tests. Results of recovery tests on SPE of cyanidin-3-glucoside from urine was 90.0 ± 1.5% (n = 3); the recovery of cyanidin-3-glucoside on SPE from plasma was 91.4 ± 0.9% (n = 3).

Elderberry study. No ACN peaks were detected in urine samples collected before consumption of the elderberry extract (**Fig. 1***A*). In all samples collected from the four women after they consumed the elderberry extract, a relatively large peak at 520 nm was observed in the chromatogram with a retention time of 27 min (Fig. 1*B*). Compared with the profile of the mixed standard of six ACN (Fig. 1C), it appeared that this peak contained cyanidin-3-glucoside. The enlarged chromatogram indicated that it contained two peaks (**Fig. 2***A*, Peaks 1 and 2). From the mass spectral data (Fig. 2B), we found one of them, which was eluted a little bit earlier than the other, has a molecular weight of 581.0 (mass-to-charge ratio, or m/z: 581.0). Its daughter fragment ion had an m/z of 287.2 from the MS/MS data (Fig. 2*B*). The other coeluting peak had an m/z of 449.1, with a daughter fragment peak with an m/z of 287.2. Comparing their elution times, spectra, and data from the previous paper (17), we identified these two peaks as cyanidin-3-sambubioside and cyanidin-3-glucoside. They are the major ACN that account for >90% of the total ACN in elderberry (24) and cyanidin is the only ACN aglycone present in elderberry (25).

There were some additional small peaks with retention times of 33–36 min. Their spectra indicated that they were also ACN. Their structures were further confirmed by MS

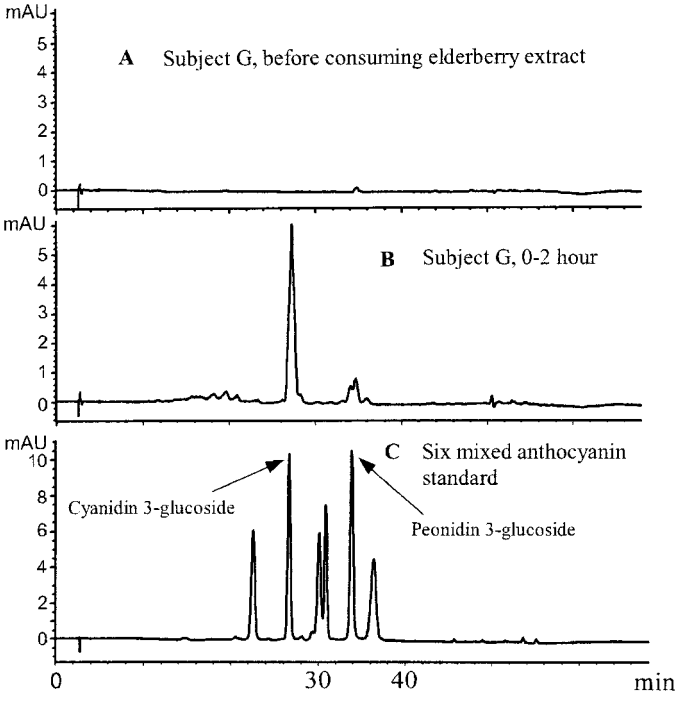

FIGURE 1 RP-HPLC chromatograms of urine samples from subject G before (*A*) and 0–2 h after consuming the elderberry extract (*B*). Chromatogram *C* is of six mixed anthocyanins: delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, pelargonidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside from left to right on the chromatogram.

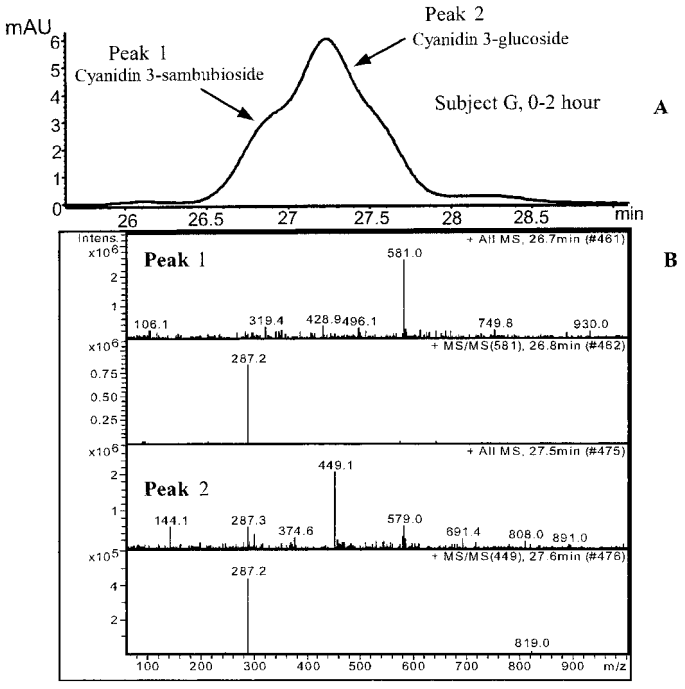

FIGURE 2 Liquid chromatography-mass spectrometry analysis of anthocyanins (ACN) in the urine sample from subject G (0–2 h). *A* is an enlarged chromatogram during 25–29 min of Figure 1 (*B*), which showed the high peak at approximately 27 min contained two peaks (Peaks 1 and 2). They are two major ACN cyanidin-3-glucoside and cyanidin-3-sambubioside in elderberry. Their MS spectral data are presented below (*B*).

spectral data and by comparison with authentic standards. On the representative chromatogram of subject G (0–2 h), one peak appeared with a retention time of 34 min (**Fig. 3***A*, Peak 3) and with an *m/z* of 463.1 and a daughter fragmentary peak from MS/MS with an *m/z* of 301.2 (Fig. 3*B*). Based upon the *m/z* of 301.2, we assumed this peak to be peonidin glycoside or another methylated form of cyanidin glycoside. We compared the chromatogram of the mixed ACN standards under the same experimental conditions (Fig. 1C) and found that the retention time of this peak was identical to that of peonidin-3-glucoside, confirming this peak to be peonidin-3-glucoside, a methylated form of cyanidin-3-glucoside. This observation also supports the conclusion from other studies, which demonstrated that for several flavonoids with 3′, 4′-dihydroxylation of ring B, methylation tends to occur at the 3′-*O*-position (26). Another small peak appeared at 33.1 min (Fig. 3*A*, Peak 4) with an *m/z* of 595.9, and a fragment ion MS/MS of 301.2 (Fig. 3*B*). From the results above, we have shown that cyanidin can be transformed to peonidin, so we contend that this peak may also contain peonidin as its aglycone. The molecular weight of a sambubiosidose residue is 294.2, which is the difference between the *m/z* of the parent and daughter ion. Thus, this peak at 33.1 min appears to be the methylated form of cyanidin-3-sambubioside, the other major ACN in elderberry. Another two peaks (Peaks 5 and 6) were not identified because they do not have typical ACN MS spectral data.

From the chromatogram and MS data of subject B (2–4 h), a small peak with a retention time at 35.5 min is notable (**Fig. 4***A*, Peak 7). It had an *m/z* of 477.1 and a fragment ion with an *m/z* of 301.2 (Fig. 4*A*). The fragment peak indicated that this peak was also an ACN containing peonidin aglycone. An *m/z* of 176 for the substitution group is indicative of a glucuronide residue, which is a very common conjugated form of flavonoids in humans (26). This peak may be peonidin monoglucuronide. However, we could not determine the exact site of glucuronidation on the peonidin from the available data. Two other small peaks (Peaks 8 and 9) which eluted earlier than Peak 7 were not identified because of their low concentrations. There was another glucuronide conjugated form with a retention time of 19.8 min on the subject G chromatogram (0–2 h) (Fig. 4*B*, Peak 10). MS spectral results indicated an *m/z* of 625.3 and MS/MS data gave two fragments with an *m/z* of 463.1 and 287.2. (Fig. 4*B*). An *m/z* of 287.2 is typical for cyanidin aglycone, and 463.1 minus 287.2 is 176, which is indicative of a glucuronide residue. The difference between 463.1 and the *m/z* of the parent compound (625.3) is 162.2, which matches that of a hexose residue. Because only cyanidin-3-glucoside is present in elderberry, this peak should be the monoglucuronide form of cyanidin-3-glucoside, a compound which has four free hydroxyl groups. The exact glucuronidation site on cyanidin is not known. Two other peaks in the same chromatogram (Peaks 11 and 12) were not identified. They were not typical ACN based on their MS spectral data. Not all metabolites were detected in all subjects. A summary of the MS data and total urinary excretion is shown in **Table 1**.

Blueberry study. Urine samples from the five women who consumed blueberry had ACN in their urine. However, the ACN concentrations were much lower than those in the women who consumed elderberry. When we tried to identify ACN with MS, we were unable to detect the ACN signal separate from the background signals. We used the blueberry

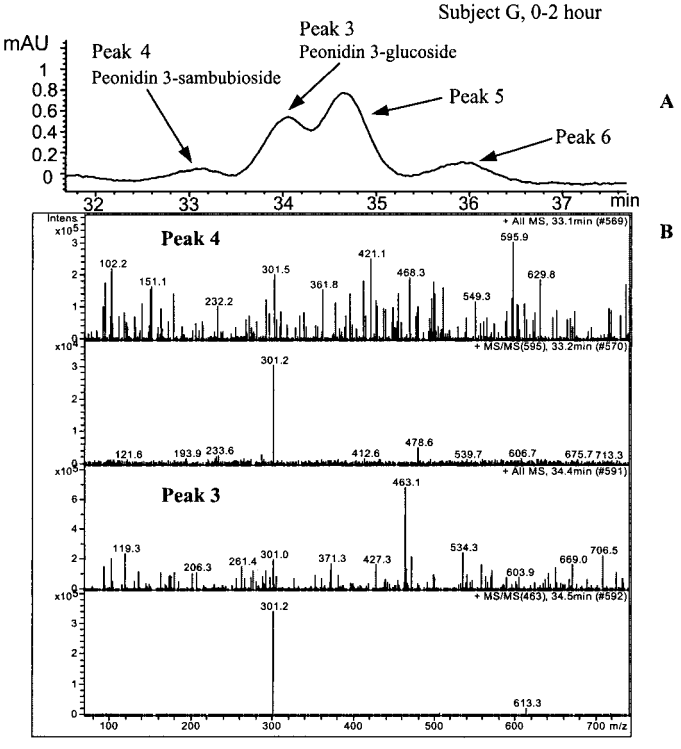

FIGURE 3 Liquid chromatography-mass spectrometry (MS) analysis of anthocyanins (ACN) metabolites in the urine sample from subject G (0–2 h). *A* is enlarged chromatogram during 32–38 min of Figure 1 (*B*), which showed that peaks of peonidin-3-glucoside (Peak 3) and peonidin-3-sambubioside (Peak 4). Their MS spectral data are presented below (*B*). Peaks 5 and 6 have not been identified as they do not have typical MS spectra for ACN.

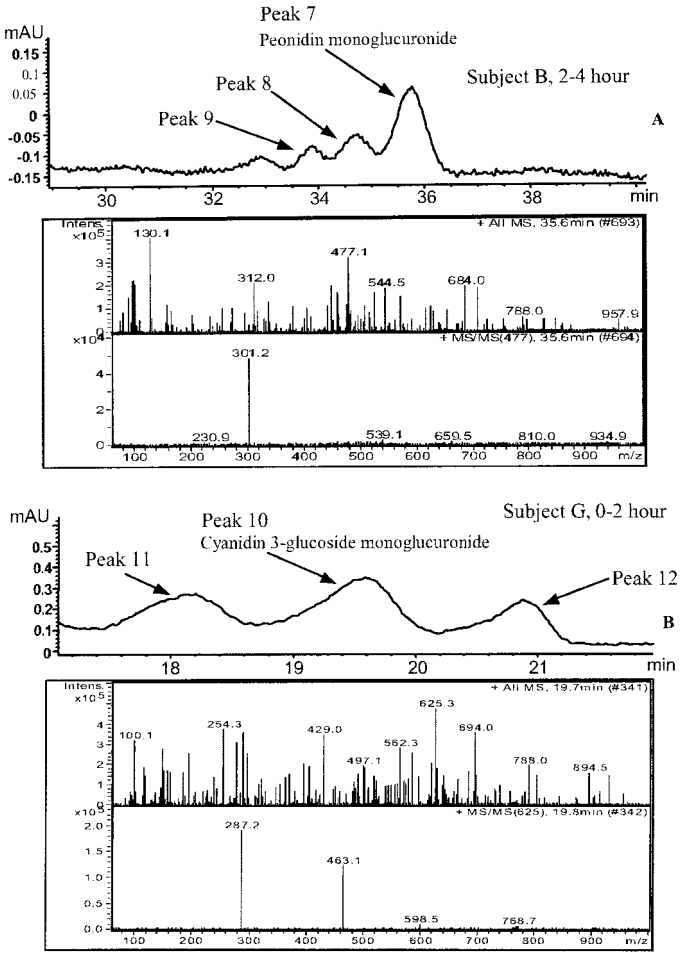

FIGURE 4 Liquid chromatography-mass spectrometry (MS) analysis of anthocyanin glucuronide conjugated forms in urine samples from women that consumed elderberry. *A* is the enlarged portion during 30–39 min of the original chromatogram of subject B (2–4 h), which showed the peak of peonidin monoglucuronide in the urine sample (Peak 7). Its MS spectral data are presented below the chromatogram. Peaks 8 and 9 have not been identified. *B* is the enlarged chromatogram during 17–22 min of original chromatogram of subject G (0–2 h), which showed the peak of cyanidin-3-glucoside monoglucuronide (Peak 10). The MS spectral data are presented below the chromatogram. Peaks 11 and 12 have not been identified.

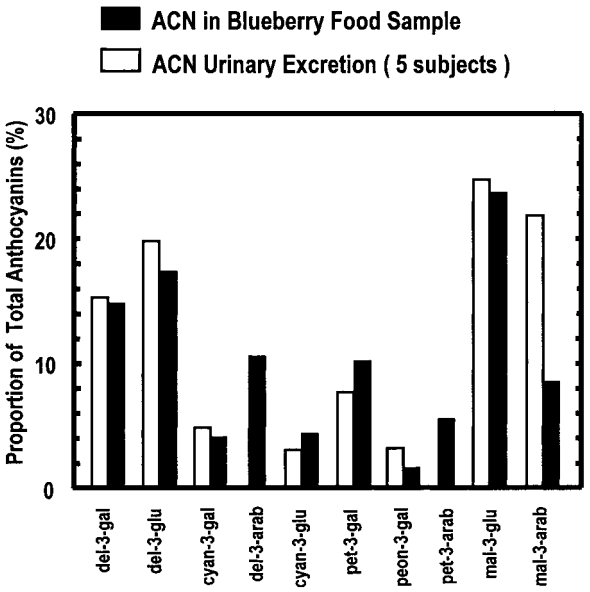

FIGURE 5 the percentage of individual anthocyanins (ACN) excreted in the urine of women who consumed blueberry compared with the total ACN consumed in the meal. Except for arabinosides, the amounts excreted were consistent with that in the meal.

food sample as a standard to identify ACN in urine. ACN in blueberry have been studied thoroughly, with at least 25 ACN being detected in BB (27). By means of HPLC-MS/MS and comparing with previous studies, we identified 22 ACN in the blueberry food sample. We found several ACN in urine samples when comparing to the blueberry sample. Total urinary excretions were 7.62 ± 5.00, 10.07 ± 3.28, 5.56 ± 5.07, and 23.25 ± 10.88 μg for 0–2 h, 2–4h, 4–6 h, and 0–6 h, respectively (n = 5). The total excreted accounted for 0.004% (wt/wt) of the amount consumed. We also compared the urinary ACN excretion to ACN in the meal. The results are shown in **Figure 5**. The relative proportions of the different ACN were similar except for the arabinose forms of delphinidin, petunidin, and malvidin. We did not detect any ACN peaks in plasma at 520 nm from 2 mL of sample in any of the subjects. The

TABLE 1

Identification and excretion (0–4 h) of anthocyanins in the urine of elderly women consuming elderberry extract[1]

Compound	MS (*m/z*)	MS/MS (*m/z*)	Subject B	Subject G	Subject J	Subject R
				(μg/4 h)		
Cyanidin–3-sambubioside	581.2	287.2	417.12	498.21	388.70	360.02[2]
Cyanidin–3-glucoside	449.1	287.2				
Peonidin–3-glucoside	463.3	301.2	64.22	66.98	26.00	31.62
Peonidin–3-sambubioside	595.9	301.2	ND[3]	29.53	ND	ND
Peonidin monoglucuronide	477.1	301.2	142.25	ND	ND	95.10
Cyanidin–3-glucoside monoglucuronide	625.3	463.1/287.2	ND	40.27	50.77	ND
Total (μg)			627.89	634.99	465.47	486.74
Mean ± SD (μg)					553.77 ± 90.15	
Total excretion (% intake)			0.087	0.088	0.065	0.068
Mean ± SD (% intake)					0.077 ± 0.013	

[1] Quantities calculated based upon either cyanidin- or peonidin-3-glucoside standards.
[2] Cyanidin–3-sambubioside and cyanidin–3-glucoside appeared as one peak; the values of this line include both of them and calculated as cyanidin–3-glucoside equivalent.
[3] Not detected.

lower detection limit under our current conditions would be 0.005 mg/L (based on cyanidin-3-glucoside).

DISCUSSION

ACN have been considered to not be absorbed in the human body, unless they were first hydrolyzed to the aglycone in the gastrointestinal tract. The poor lipophilic properties of ACN have led to the conviction that they are underabsorbed. Horwitt (11) was perhaps the first to observe the ACN pigments in the urine of rabbits. Recently, however, several investigators have concluded that ACN can be absorbed in both human and animal bodies as intact forms (12–23). Some investigators have shown that quercetin glucosides may interact with the intestinal glucose transporter and be absorbed in that manner (28–30). Whether ACN could be absorbed by a similar mechanism remains unknown. Mülleder et al. (22) compared the urinary excretion when elderberry ACN were consumed with and without simultaneous ingestion of sugar. The total excretion of ACN with sugar was lower than that without sugar, indicating that sugar carriers might play a role in flavonoid absorption. The presence of the sambubioside form gives strong credence to this as it is unlikely that the sambubioside form could be reformed from the aglycone after its absorption.

Previous studies have also indicated that methylation is an established pathway in the metabolism of flavonoids (26). For several flavonoids with 3′, 4′-dihydroxylation of ring B, conjugates of their 3′-O-methyl ethers were excreted (31–32). This transformation occurred in the liver and was catalyzed by catechol-O-methyl transferase (14,33). Cyanidin is an anthocyanidin with a 3′, 4′-dihydroxylation pattern of ring B. We detected peonidin 3-glucoside and peonidin 3-sambubioside in the urine from humans. Miyazawa et al. (15) found a methylated form of an ACN in liver of rats and the concentration of the methylated form was much higher than the original form in the liver, but they did not detect it in plasma and urine. They (15) assumed that these methylated compounds were excreted from the liver into bile directly. However, we detected the methylated form of ACN in the urine of humans. Thus, they must appear in the blood and be excreted by the kidney.

Conjugations with glucuronic acid or with sulfate are considered the major detoxication pathways of many drugs and xenobiotic compounds. They are also the common final steps in the metabolic pathway of flavonoids in mammalian systems (26,34). These modifications could start in the small intestine, liver, or kidney (35–36). UDP-glucuronosyl transferase and sulfotransferase in the liver would likely be responsible for this conjugation (26,37). So far, many different subclasses of flavonoids have been found to be absorbed and excreted as glucuronide forms in both humans and experimental animals (26,35,37,38). But for ACN, no glucuronide or sulfate conjugated forms have been found in urine or plasma of either humans or experimental animals. This observation is completely different from other flavonoids, such as catechin and epicatechin, or isoflavones such as genistein and daidzein, whose basic structures are similar to ACN. Miyazawa et al. (15) assumed that the flavylium cation structure of ACN may impart resistance to such enzymatic conversion into conjugates.

In our studies we detected the glucuronide conjugate forms for the first time. These results indicate that ACN might share some of the same metabolic pathways with other flavonoid compounds. Perhaps, others did not detect them because of the low concentrations of these metabolites. We detected and tentatively identified them using diode–array detector and MS. We were unsuccessful in hydrolyzing the conjugate with the enzyme β-glucuronidase (1 U/L for 30 min). Hydrolysis of the conjugated forms of ACN presents particular challenges in that ACN are unstable at neutral pH. The ACN disappeared completely when we incubated the plasma samples for 3 h. Because previous investigators have relied on detecting the conjugated forms using the difference in ACN concentrations before and after incubation with β-glucuronidase, small amounts of degradation during the incubation might mask any conjugates that were present.

In this study, we still do not know how the peonidin monoglucuronide is formed. Two possible pathways could describe the formation of this metabolite (**Fig. 6**). Cyanidin-3-glucoside could be absorbed intact and some methylated to form peonidin-3-glucoside in the liver (Fig. 6; Pathway 1). Either cyanidin-3-glucoside or peonidin-3-glucoside could serve as a substrate for UDP-glucose dehydrogenase to form the corresponding glucuronide from the glucose form. For this to occur, cyanidin-3-glucoside or peonidin-3-glucoside would have to serve as a substrate for the enzyme UDP-glucose dehydrogenase, which converts UDP-glucose into UDP-glucuronic acid. UDP-glucose dehydrogenase (GDH) is a unique enzyme pathway which furnishes in vertebrates the UDP-glucuronic acid for numerous transferases, including those of glycosaminoglycan synthesis and xenobiotics elimination (39). GDH is present in both the liver and the small intestine of the rats, guinea pigs, and mice (40,41). This hypothesis would explain the presence of peonidin-glucuronide. This pathway seems likely since we have found all of these compounds in urine.

A second possibility (Fig. 6, Pathway 2) is that cyanidin-3-glucoside is hydrolyzed to the aglycone in the intestine and absorbed. In the liver, the cyanidin could be methylated and conjugated to form peonidin monoglucuronide. However, cyanidin is unstable in plasma and may form protocatechuic acid (14). No cyanidin aglycone has been detected in the plasma of humans (15) or rats (14), but it has been demonstrated in rat jejunum (14). If these reactions occurred, we would expect to find peonidin aglycone and cyanidin glucuronide, which we have not detected. However, a challenge in all of this work is that we are generally working near the limits of detection of

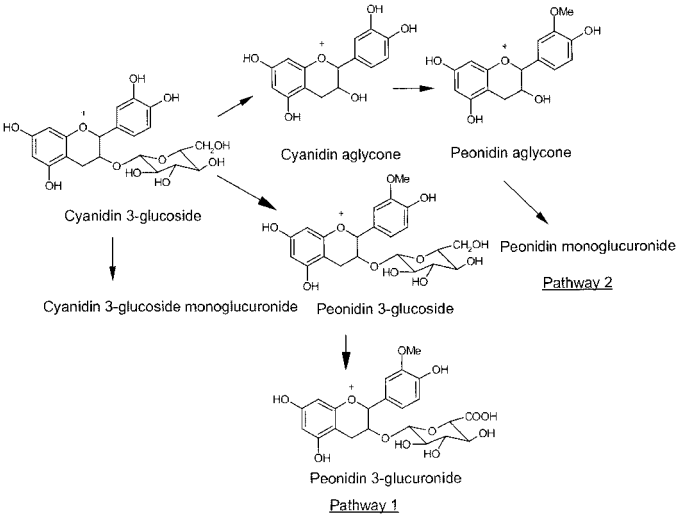

FIGURE 6 Glucuronide conjugated forms from cyanidin-3-glucoside. Two possible pathways of metabolism of peonidin monoglucuronide are shown.

TABLE 2

Comparison of published data on anthocyanins (ACN) in plasma and urinary excretion in humans after consuming food ACN

Source of ACN	Gender of subjects	Dosage	Plasma (max)	Urine recovery (%, w/w)	References
Elderberry and black currant (Juice)	F[1], M[2]	2.95 mg/kg	29 ± 10 nmol/L[3]	NA[4]	15
Black currant (Concentrate)	M	3.58 mg/kg	115 nmol/L	0.11%/8 h	18
Elderberry (Extract)	F	10.9 mg/kg	97.4 nmol/L	0.055%/8 h 0.077%/4 h	17 this paper
Blueberry (Whole berry)	F	10.0 mg/kg	ND[5]	0.004%/6 h	this paper
Red wine	M	68 mg (total)	1.4 nmol/L	0.024%/6 h	19
Dealcoholized red wine	M	58 mg (total)	1.5 nmol/L	0.0224%/6 h	19
Red grape juice	M	117 mg (total)	2.8 nmol/L	0.023%/6 h	19
Wine	F, M	218 mg (total)	NA	1.5–5.1%/12 h	13
Elderberry (Juice)	F, M	500 mg (total)	NA	0.05% (total)	22

[1] Female, [2] Male, [3] Mean ± SD, [4] Not analyzed, [5] Not detected.

many of these compounds with current instrumentation and methods, and the concentrations of some of these intermediates may be so low that they are undetectable.

We found a glucuronide of cyanidin-3-glucoside in the urine of 2 of the 4 women who consumed elderberry (Table 1). Cyanidin has four free hydroxyl groups. According to Boutin et al. (37), different hydroxyl positions on the flavonoid diosmetin have different capacities for glucuronidation. Based on these results, we would expect that each hydroxyl group on cyanidin, likewise, would have a different glucuronidation capacity. Based on the work by Boutin et al. (37), for 5, 7-dihydroxy compounds, the main site for glucuronidation seems to be the 7-position; for the trihydroxy compound, the main conjugation site was the 3′-position. We speculate that glucuronidation of cyanidin-3-glucoside may occur most readily at the 3′-position, but this needs to be verified. Even though there are differences in the activities of the different hydroxyl groups, we might expect to see more than the one major glucuronide conjugate form. Again, it may be that concentrations are so low that we are unable to detect them.

Although we used nearly the same amount of total ACN from blueberry as from elderberry, the amount of ACN in the urine samples from the women who consumed the blueberry was much lower. That may be for two reasons. First, the content of any individual ACN in blueberry is lower by a factor of five- to 10-fold compared with that in elderberry, thus making detection of any single ACN much more difficult. There are >25 individual ACN in blueberry and no single ACN is dominant. However, in elderberry, two ACN account for >90% of the total ACN. The second reason for the lower absorption from blueberry may lie in the form in which the ACN were given. In the elderberry study, the ACN were in an extract and thus in a concentrated form. In the blueberry study, the ACN were given as part of the whole berry. Absorption from a plant extract will likely be much better than from the raw plant where the ACN have not been extracted from the plant cell. When we compared the urinary excretion of the individual ACN to the relative amounts of ACN in the blueberry meal (Fig. 5), the proportion of most ACN excreted in urine was consistent with that in the meal. The exceptions were with the arabinosides of delphinidin, petunidin, and malvidin. This may indicate that the arabinoside forms may be absorbed or metabolized in a different manner.

In **Table 2**, we compared the concentrations of ACN in plasma and the total urinary ACN excretion from different papers that we are aware of that report this information in humans. It is clear from all but one of these studies that the recovery of ACN or ACN metabolites in urine is quite low (0.004–0.11% of dose). The one study in which urinary excretion was much higher (13) involved subjects who consumed wine and the "ACN-like" compounds which absorbed at 520 nm and were not well characterized. It may be that ACN in wine are absorbed to a much greater extent than from berries or berry juices or extracts.

In summary, we observed for the first time the presence of glucuronide forms of ACN, namely peonidin monoglucuronide and cyanidin-3-glucoside monoglucuronide, in the urine of humans after the consumption of elderberry ACN. We observed the apparent methylation of both cyanidin-3-glucoside and cyanidin-3-sambubioside to peonidin-3-glucoside and peonidin-3-sambubioside. We were unable to detect ACN in plasma from women who consumed a single meal of blueberries; however, they were detectable in the urine but the quantities were quite low, with the total amount excreted during the first 6 h being only 0.004% of the quantity consumed.

LITERATURE CITED

1. Mazza, G. & Miniati, E. (1993) Anthocyanins in Fruits, Vegetables, and Grains. Boca Raton, FL: CRC Press. 1–6.
2. Strack, D. & Wray, V. (1993) The anthocyanins. In: Harborne, J. B., editor. The Flavonoids: Advances in Research since 1986. London: Chapman & Hall. 1–22.
3. Timberlake, C. F. & Henry, B. S. (1988) Anthocyanins as natural food colorants. Prog. Clin. Biol. Res. 280: 107–121.
4. Kühnau, J. (1976) The flavonoids. A class of semi-essential food components: their role in human nutrition. World Rev. Nutr. Diet. 24: 117–191.
5. Prior, R. L. & Cao, G. (2000) Flavonoids: diet and health relationships. Nutr. Clin. Care. 3: 279–288.
6. Chimi, H., Cillard, J., Cillard, P. & Rahmani, M. (1991) Peroxyl and hydroxyl radical scavenging activity of some natural phenolic antioxidants. J. Am. Oil Chem. Soc. 68: 307–312.
7. Tsuda, T., Shiga, K., Ohshima, K., Kawakishi, S. & Osawa, T. (1996) Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from *Phaseolus vulgaris L*. Biochem. Pharmacol. 52: 1033–1039.
8. Wang, H., Cao, G. & Prior, R. L. (1997) The oxygen radical absorbing capacity of anthocyanins. J. Agric. Food Chem. 45: 304–309.
9. Teissedre, P. L., Frankel, E. N., Waterhouse, A. L., Peleg, H. & German, J. B. (1996) Inhibition of in vitro human LDL oxidation by phenolic antioxidants from grapes and wines. J. Sci. Food Agric. 70: 55–61.
10. Manach, C., Morand, C., Demigné C., Texier, O., Régérat, F. & Rémésy, C. (1997) Bioavailability of rutin and quercetin in rats. FEBS Lett. 409:12–16.

11. Horwitt, M. K. (1933) Observation on behavior of the anthocyanins in pigment from concord grapes in the animal body. Proc. Soc. Exp. Biol. Med. 30: 949–951.

12. Morazzoni, P., Livio, S., Scilingo, A. & Malandrino, S. (1991) *Vaccinium myrtillus* anthocyanosides pharmacokinetics in rats Arzneimittelforschung. 41: 128–131.

13. Lapidot, T., Harel, S., Granit, R. & Kanner, J. (1998) Bioavailability of red wine anthocyanins as detected in human urine. J. Agric. Food Chem. 46: 4297–4302.

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

15. Miyazawa, T., Nakagawa, K., Kudo, M., Muraishi, K. & Someya, K. (1999) Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3,5-diglucoside, into rats and humans J. Agric. Food Chem. 47: 1083–1091.

16. Cao, G. & Prior, R. L. (1999) Anthocyanins are detected in human plasma after oral administration of an elderberry extract. Clin. Chem. 45: 574–576.

17. Cao, G., Muccitelli, H. U., Sanchez-Moreno, C. & Prior, R. L. (2000) Anthocyanins are absorbed in glycated forms in elder women: a pharmacokinetic study. Am. J. Clin. Nutr. 73: 920–926.

18. Matsumoto, H., Inaba, H., Kishi, M., Tominaga, S., Hirayama, M. & Tsuda, T. (2001) Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. J. Agric. Food Chem. 49: 1546–1551.

19. Bub, A., Watzl, B., Heeb, D., Rechkemmer, G. & Briviba, K. (2001) Malvidin-3-glucoside bioavailability in humans after ingestion of red wine, dealcoholized red wine and red grape juice. Eur. J. Nutr. 40: 113–120.

20. Murkovic, M., Adam, U. & Pfannhauser, W. (2000) Analysis of anthocyane glycosides in human serum. Fresenius J. Anal. Chem. 366: 379–381.

21. Murkovic, M., Mülleder, U., Adam, U. & Pfannhauser, W. (2001) Detection of anthocyanins from elderberry juice in human urine. J. Sci. Food Agric. 81: 934–937.

22. Mülleder, U., Murkovic, M. & Pfannhauser, W. (2001) Metabolism of cyanidin glucoside of elderberry. In: Pfannhauser, W., Fenwick, G. R. & Khokhar, S., editors. Biologically Active Phytochemicals in Food Analysis, Metabolism, Bioavailability and Function. London: RSC. 421–425.

23. Nielsen, I. L. F., Nielsen, S. E., Ravn-Haren, G. & Dragsted, L. O. (2001) Detection, stability and redox effects of black currant anthocyanin glycosides in vivo: positive identification by mass spectrometry. In: Pfannhauser, W., Fenwick, G. R. & Khokhar, S., editors. Biologically Active Phytochemicals in Food: Analysis, Metabolism, Bioavailability and Function. London: Royal Society of Chemistry. 389–393.

24. Prior, R. L. & Cao, G. (1999) Variability in dietary antioxidant related natural product supplements: the need for methods of standardization. J. Am. Nutraceut. Assoc. 2: 46–56.

25. Inami, O., Tamura, I., Kikuzaki, H. & Nakatani, N. (1996) Stability of anthocyanins of *Sambucus canadensis* and *Sambucus nigra.* J. Agric. Food Chem. 44: 3090–3096.

26. Hackett, A. M. (1986) The Metabolism of Flavonoid Compounds in Mammals Plant Flavonoids in Biology and Medicine: Biological, Pharmacological, and Structure-Activity Relationships. New York: Alan R. Liss, Inc. 177–194.

27. Gao, L. & Mazza, G. (1995) Characterization of acetylated anthocyanins in lowbush blueberries. J. Liquid Chromatogr. 18: 245–259.

28. Hollman, P. C. H., de Vries, J. H. M., van Leeuwen, S. D., Mengelers, M. J. B. & Katan, M. B. (1995) Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. Am. J. Clin. Nutr. 62: 1276–1282.

29. Walle, T., Otake, Y., Walle, U. K. & Wilson, F. A. (2000) Quercetin glucosides are completely hydrolyzed in ileostomy patients before absorption. J. Nutr. 130: 2658–2661.

30. Gee, J. M., DuPont, M. S., Rhodes, M. J. C. & Johnson, I. T. (1998) Quercetin glucosides interact with the intestinal glucose transport pathway. Free Radic. Biol. Med. 25: 19–25.

31. Shaw, I. C. & Griffiths, L. A. (1980) Identification of major biliary metabolite of (+)-catechin in the rat. Xenobiotica. 10: 905–911.

32. Griffiths, L. A. (1981) The metabolism of hydroxyethylrutoside: a review. In Pulvertaft, T. B., Lyons, J. S., Wink, C. A. S., editors. Hydroxyethylrutoside in Vascular Disease. Royal Society of Medicine. International Congress and Symposium Series No. 42: 3–7 London: Academic Press.

33. Griffiths, L. A. (1982) In The Flavonoids: Advances in Research. In: Harborne, J.B. & Mabry, T.J., editors. London: Chapman and Hall. 681–718.

34. Scheline, R. R. (1991) Metabolism of oxygen heterocyclic compounds. In: CRC Handbook of Mammalian Metabolism of Plant Compounds. Boca Raton, FL: CRC Press. 243–305.

35. Fuhr, U. & Kummert, A. L. (1995) The fate of naringin in humans: a key to grapefruit juice-drug interactions? Clin. Pharmacol. Ther. 58: 365–373.

36. Ameer, B., Weintraub, R. A., Johnson, J. V., Yost, R. A. & Rouseff, R. L. (1996) Flavanone absorption after naringin, hesperidin, and citrus administration. Clin. Pharmacol. Ther. 60: 34–40.

37. Boutin, J. A., Meunier, F., Lambert, P. H., Hennig, P., Bertin, D., Serkiz, B. & Volland, J. P. (1993) In vivo and in vitro glucuronidation of the flavonoid diosmetin in rats. Drug Metab. Dispos. 21: 1157–1166.

38. Cova, D., De Angelis, L., Giavarini, F., Palladini, G. & Perego, R. (1992) Pharmacokinetics and metabolism of oral diosmin in healthy volunteers. Int. J. Clin. Pharmacol. Ther. Toxicol. 30: 29–33.

39. Boontemps, Y., Maquartt, F. X. & Wegrowski, Y. (2000) Human UDP-glucose dehydrogenase gene: complete cloning and transcription start mapping. Biochem. Biophys. Res. Commun. 275: 981–985.

40. Reen, R. K., Jamwal, D. S., Taneja, S. C., Koul, J.L., Dubey, R. K., Wiebel, F. J. & Singh, J. (1993) Impairment of UDP-glucose dehydrogenase and glucuronidation activities in liver and small intestine of rat and guinea pig in vitro by piperine. Biochem. Pharmacol. 46: 229–238.

41. Hjelle, J. J., Hazelton, G. A. & Klaassen, C. C. (1985) Increased UDP-glucuronosyltransferase activity and UDP-glucuronic acid concentration in the small intestine of butylated hydroxyanisole-treated mice. Drug Metab. Dispos. 13: 68–70.