

Strawberry Anthocyanins Are Recovered in Urine as Glucuro- and Sulfoconjugates in Humans

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ABSTRACT Anthocyanins are phenolic compounds widely distributed in fruits and vegetables. Their consumption has been shown to prevent some chronic diseases. Anthocyanin metabolism, however, is still not fully understood. The aim of this work was to evaluate the bioavailability of anthocyanins in humans consuming a meal containing strawberries and to identify possible metabolites in urine. Six healthy volunteers (three women and three men) consumed a meal containing 200 g strawberries (providing 179 μmol pelargonidin-3-glucoside). Urine samples were collected before and after the meal and rapidly treated by solid-phase extraction. Identification and quantification of anthocyanin metabolites were carried out by HPLC-ESI-MS-MS and HPLC with UV-visible detection, respectively. In addition to pelargonidin-3-glucoside, five anthocyanin metabolites were identified in urine: three monoglucuronides of pelargonidin, one sulfoconjugate of pelargonidin and pelargonidin itself. Total urinary excretion of strawberry anthocyanin metabolites corresponded to $1.80 \pm 0.29\%$ (mean \pm SEM, $n = 6$) of pelargonidin-3-glucoside ingested. More than 80% of this excretion was related to a monoglucuronide. Four hours after the meal, more than two-thirds of anthocyanin metabolites had been excreted, although urinary excretion of the metabolites continued until the end of the 24-h experiment. This study demonstrated that anthocyanins were glucuro- and sulfo-conjugated in humans and that the main metabolite of strawberry anthocyanins in human urine was a monoglucuronide of pelargonidin. *J. Nutr.* 133: 1296–1301, 2003.

KEY WORDS: • anthocyanins • bioavailability • glucuronides • humans • strawberry

Anthocyanins are a group of naturally occurring phenolic compounds responsible for the color of many fruits and vegetables. They are glycosylated polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts (1). The daily intake of anthocyanins in humans is estimated as ≥ 180 –215 mg/d in the United States (2), a result of their widespread distribution and occurrence in fruits and vegetables. This value is much higher than the consumption of other flavonoids such as flavones and flavonols in the Dutch diet (23 mg/d) (3). Consumption of anthocyanins has been shown to reduce the risk of coronary heart disease and to prevent some chronic diseases (4,5). The positive effects of these pigments could be related to their potent antioxidant activity demonstrated in various *in vitro* and *in vivo* studies (6–10).

In view of these multiple biological effects, the bioavailability of anthocyanins is considered to be an important issue. Several studies have shown that anthocyanins are absorbed as glycosides in humans and rats (11–15). Indeed, the intact glycosidic forms have been recovered in plasma and urine after oral administration of anthocyanins. However, the bioavailability of anthocyanins is very low and their metabolism is still not fully understood. Only a few studies have reported metab-

olites such as the methylated forms (11,12,15,16) or, more recently, the glucuronide conjugated forms present in very low concentrations in urine (16). Previous human studies investigated the ingestion of anthocyanins in the form of an extract or beverage, without consumption of any other food. Thus, in the present work, we evaluated the bioavailability of anthocyanins in humans after they consumed a meal containing an anthocyanin-rich fruit to mimic what could happen daily. We chose strawberries as the anthocyanin-rich fruit because their consumption is common in our countries and they are characterized by one major pigment, pelargonidin-3-glucoside (Fig. 1) (17). By use of the HPLC-ESI-MS-MS technique, we investigated for possible metabolites as described for various flavonoids (18–20).

SUBJECTS AND METHODS

Chemicals. β -Glucuronidase type VII-A from *Escherichia coli* was purchased from Sigma Chemical (Saint-Quentin-Fallavier, France). All other chemicals were purchased from Extrasynthèse (Genay, France). Deep-frozen strawberries were from a supplier of deep-frozen food products (Szymczak-Nadreau, Romagnat, France).

Subjects and study design. Six healthy volunteers (three women and three men) aged 43 ± 5 y with a mean body mass index of 22 ± 1.3 kg/m² participated in this study, which was carried out at our lab and was performed according to the Helsinki Declaration. Vol-

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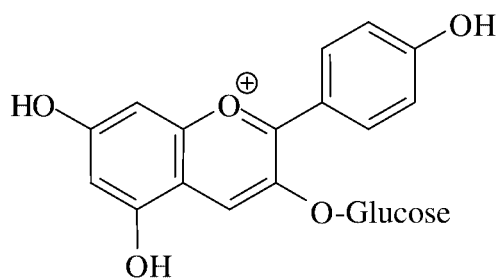


FIGURE 1 Structure of pelargonidin-3-glucoside.

unteers did not consume any kind of product rich in polyphenols (vegetables, fruits, tea, etc.) at the dinner before the experiment and during the 24 h of the experiment.

After an overnight fast, subjects consumed a breakfast consisting of 200 g strawberries (containing 179 μmol of pelargonidin-3-glucoside) with 15 g sugar, 60 g bread and 10 g butter. Water was the only beverage consumed during the experiment.

Urine samples were collected from these subjects before the experimental meal and between 0 and 2, 2 and 4, 4 and 6, 6 and 8, 8 and 12 and 12 and 24 h after eating breakfast. The samples were immediately acidified with 1/60 volume of 12 mol/L HCl and treated as described below. A portion of the samples was acidified to pH 5.0 with 1 mol/L acetic acid (10 $\mu\text{L}/\text{mL}$ urine) to be treated by β -glucuronidase.

Quantification of strawberry anthocyanins. Deep-frozen strawberries (100 g) were thawed and ground with a domestic mixer to obtain a homogeneous mixture. To extract the strawberry anthocyanins, 5 g of this mixture was stirred for 30 min with 90 mL of 0.12 mol/L HCl in methanol. After filtration, the volume of solution was adjusted to 100 mL, and this solution was diluted fivefold with 0.12 mol/L HCl in water. This latter dilution (20 μL) was analyzed by HPLC as described below.

Sample preparation. Anthocyanins exist under four different structures in equilibrium (1). The proportion of each structure depends on the pH. In acidic conditions (pH below 2), anthocyanins exist primarily in the form of a flavylium-colored cation detectable at 520 nm (1). Thus, urine samples acidified with 1/60 volume of 12 mol/L HCl were maintained for 1 h at room temperature before treatment to obtain the maximal yield of the colored flavylium cations. Anthocyanins present in urine samples were then extracted with a solid-phase extraction (SPE) cartridge (Sep-Pak C₁₈ Plus; Waters, Milford, MA) as follows. The cartridge was washed with 10 mL of methanol and equilibrated with 10 mL of 12 mmol/L aqueous HCl before use. Urine samples (5 mL) diluted with an equal volume of deionized water and spiked with 3.66 nmol cyanidin-3-glucoside as an internal standard were loaded onto the cartridge. Use of this internal standard corrected for the possible loss of anthocyanins during the sample preparation. The cartridge was then washed with 10 mL of 12 mmol/L aqueous HCl, and anthocyanins were eluted with 3 mL of 12 mmol/L HCl in methanol. The methanolic extract was evaporated to dryness by use of a rotary evaporator at 35°C. The dried extract was dissolved with 300 μL of 0.12 mol/L aqueous HCl. This solution was analyzed by HPLC-ESI-MS-MS and HPLC with UV-visible detection to identify and quantify anthocyanin metabolites, respectively. We found that SPE allowed good recovery of anthocyanin metabolites by comparison of the HPLC profile of a urine sample before and after SPE (data not shown). Recovery of cyanidin-3-glucoside (internal standard) was between 80 and 90%.

To search for glucuroconjugates, urine samples acidified with acetic acid were incubated for 5 min at 37°C with or without 10⁶ u/L β -glucuronidase (from *E. coli*). Samples were then acidified with 1/30 volume of 12 mol/L HCl and treated by adding 2.8 volumes of acetone. The resulting mixtures were centrifuged for 5 min at 12,000 \times g at room temperature. Supernatants were evaporated under a nitrogen stream to the initial volume of urine. A 100- μL aliquot was immediately analyzed by HPLC as described below.

Anthocyanin analysis. Analysis of anthocyanins was carried out by HPLC by use of a photodiode array detector (991, Waters) and a

UV-visible detector (785A, Perkin Elmer, Courtabœuf, France) at 524 nm. Samples were loaded onto a Hypersil C18 5- μm column (150 \times 4.6 mm) protected by a guard column (Hypersil C18 5- μm , 10 \times 4 mm; Interchim, Montluçon, France). Elution was performed by use of water:H₃PO₄ (99:1) as solvent A and acetonitrile as solvent B at a flow rate of 1.0 mL/min. Analyses were carried out with linear gradient conditions from 100% A to 90% A for 10 min and then to 75% A for 30 min.

Identification of anthocyanin metabolites was made by HPLC-ESI-MS-MS analysis of urine samples. These analyses were performed on a Hewlett-Packard HPLC system equipped with MS-MS detection (API 2000; Applied Biosystems, Les Ulis, France). The column was a Hypersil BDS C18 5- μm (150 \times 2.1 mm) (Touzart & Matignon, Les Ulis, France) and the mobile phases consisted of acetonitrile/formic acid/water (5/2/93) (solvent A') and acetonitrile/formic acid/water (40/2/58) (solvent B'). A linear gradient from 0% B' to 100% B' in 40 min was applied. The flow rate was 0.2 mL/min. Urine samples, prepared as previously described, were diluted fourfold in mobile phase A' before analysis. Detection was carried out by use of electrospray ionization conducted at 450°C in the positive mode, with a nebulizer pressure of 90 psi, a drying nitrogen gas flow of 11 L/min, a fragmentor voltage of 20 V and a capillary voltage of 4000 V as previously described (21). The MS data were collected in the MRM mode by monitoring the transition of parent and product ions specific for each compound at a dwell time of 0.5 s. Cyanidin-3-glucoside (internal standard) and anthocyanin metabolites were detected according to the respective m/z values of their parent and product ions: cyanidin-3-glucoside (449/287), pelargonidin (271/121), pelargonidin-3-glucoside (433/271), pelargonidin glucuronide (447/271) and pelargonidin sulfate (351/271).

Data analysis. Values were given as means \pm SEM and, when appropriate, significance of differences between values was determined by one-way ANOVA followed by Student-Newman-Keuls test (GraphPad; Instat, San Diego, CA). Values of $P < 0.05$ were considered significant.

RESULTS

Pelargonidin-3-glucoside (retention time [RT] = 24.5 min) was the major anthocyanin present in strawberries consumed in this study (Fig. 2A). Its concentration in strawberries was 895 $\mu\text{mol}/\text{kg}$. Three other minor anthocyanins were detected, although their amounts were too low to allow identification

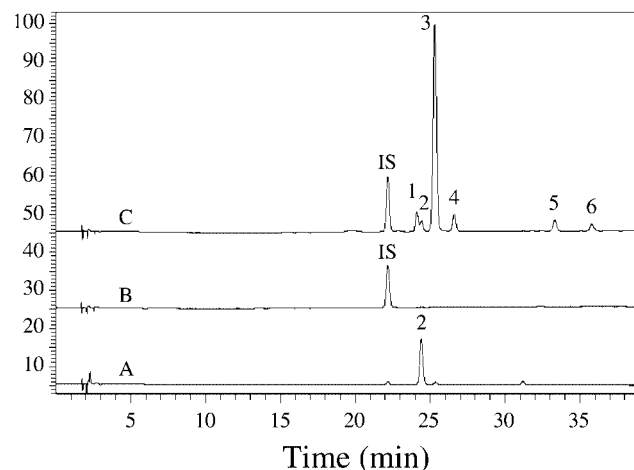


FIGURE 2 Representative HPLC chromatograms of strawberry anthocyanins (A), and of human urine (one subject) collected before (B) and 2 h (C) after the consumption of a meal containing 200 g strawberries. Detection was performed at 524 nm. IS, internal standard (cyanidin-3-glucoside). Urine was treated by solid-phase extraction. Peaks are as follows: 1, 3, 4: pelargonidin monoglucuronides; 2: pelargonidin-3-glucoside; 5: pelargonidin sulfate; 6: pelargonidin.

and quantification. Whereas no anthocyanins were observed in urine collected before the experimental meal (except cyanidin-3-glucoside used as internal standard, RT = 22.3 min) (Fig. 2B), six principal peaks appeared in urine collected after consumption of strawberries (Fig. 2C). Peaks 2 (RT = 24.5 min) and 6 (RT = 35.9 min) were identified as pelargonidin-3-glucoside and pelargonidin, respectively, by comparison with the authentic compounds based on the retention time in the HPLC analysis and UV-visible spectrum, and by spiking with individual compounds. It should be pointed out that several peaks (1, 4, 5 and 6) nearly disappeared and peak 3 markedly decreased when urine samples were frozen before SPE. Thus, analyses were carried out immediately after the collection of urine samples.

HPLC-ESI-MS-MS was used to identify anthocyanin metabolites (Fig. 3). The presence of small amounts of pelargonidin-3-glucoside and pelargonidin was confirmed by detection of the respective parent and product ion pairs (m/z values: 433/271 and 271/121, respectively). The major peak (peak 3) as well as peak 1, which eluted just before pelargonidin-3-glucoside, and peak 4 had m/z values of 447 and 271 for their parent and product ions, respectively. An m/z value of 176 for the substitution group is indicative of a glucuronide residue. These peaks were thus identified as pelargonidin monoglucuronides. However, we could not specify the exact site of glucuronidation for each of these compounds. Peak 5 was identified as pelargonidin monosulfate, given that the parent and product ion m/z values were 351 and 271, respectively. No response was obtained by MS-MS when searching for glucuro- or sulfoconjugates of pelargonidin-3-glucoside or for combined glucuro- and sulfoconjugates of pelargonidin. Moreover, such conjugates would be more polar and eluted earlier than the glucoside and the aglycone monoglucuronides, respectively. No peak was detected before peak 1.

Urine acidified to pH 5.0 was treated by β -glucuronidase (Fig. 4). This enzyme acts at a pH at which anthocyanins and particularly aglycones are unstable. However, it acted very quickly, in that 5 min after its addition, peaks 1 and 4 disappeared and peak 3 markedly decreased, whereas a small amount of pelargonidin (peak 6) appeared. This result indicated that peaks 1, 3 and 4 corresponded to glucuroconjugates that liberated pelargonidin by hydrolysis. Neither pelargonidin-3-glucoside (peak 2) nor pelargonidin sulfate (peak 5) was affected by this treatment. Longer incubation periods led to complete disappearance of monoglucuronides but failed to detect aglycone because of its high instability at this pH.

Urinary excretion of each anthocyanin metabolite was calculated relative to pelargonidin-3-glucoside and expressed as nmol pelargonidin-3-glucoside/24 h (Table 1). Urinary excretion of pelargonidin-3-glucoside was low, whereas the major portion of strawberry anthocyanins was excreted as a pelargonidin monoglucuronide (peak 3 \approx 83% of total metabolite excretion). Total urinary excretion of strawberry pelargonidin-3-glucoside metabolites corresponded to $1.80 \pm 0.29\%$ of pelargonidin-3-glucoside consumed. More than two-thirds of pelargonidin-3-glucoside metabolites were excreted during the first 4 h after the meal (Fig. 5). Although urinary excretion of pelargonidin-3-glucoside was maximal during the first 2 h, excretion of the main glucuronide (peak 3) was slightly delayed (maximal excretion between 2 and 4 h after the meal) (data not shown). However, urinary excretion of the metabolites (mainly the monoglucuronide of pelargonidin, peak 3) continued until the end of the experiment.

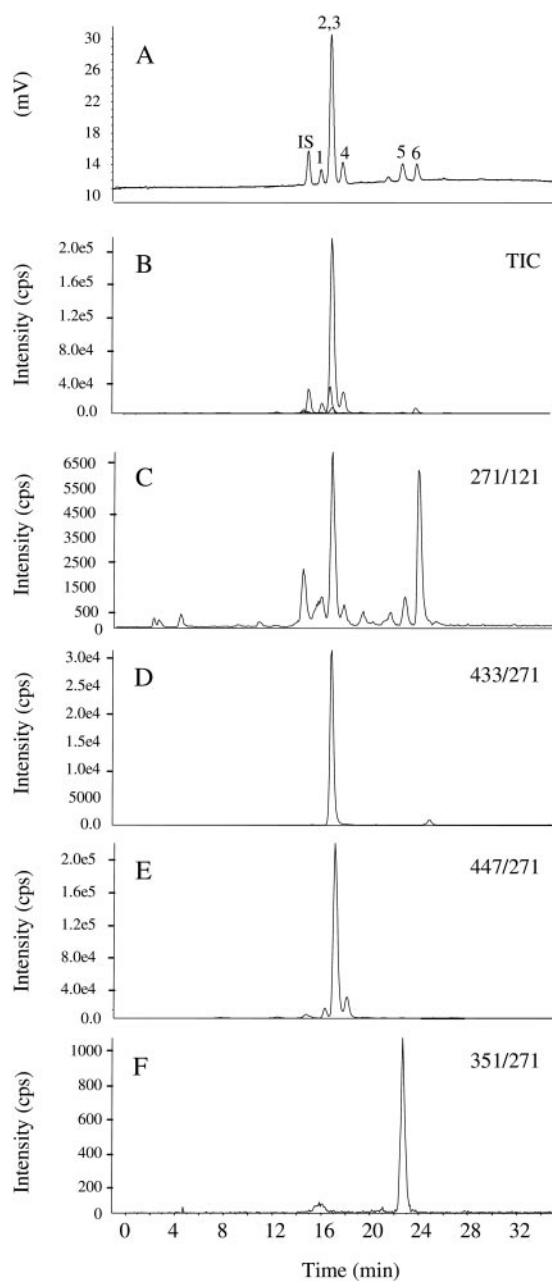


FIGURE 3 HPLC-ESI-MS-MS analysis of anthocyanin metabolites in human urine (one subject) collected 2 h after the consumption of a meal containing 200 g strawberries. Urine was treated by solid-phase extraction. (A) Detection at 524 nm. IS, internal standard (cyanidin-3-glucoside). Peaks are as follows: 1, 3, 4: pelargonidin monoglucuronides; 2: pelargonidin-3-glucoside; 5: pelargonidin sulfate; 6: pelargonidin. Detection of the respective m/z values of parent and product ions: (B) Total ionic current (TIC), (C) pelargonidin, (D) pelargonidin-3-glucoside, (E) pelargonidin glucuronide, (F) pelargonidin sulfate.

DISCUSSION

The purpose of this work was to evaluate the bioavailability of anthocyanins in humans after they consumed a strawberry-containing meal and to search for possible metabolites. In the past few years, several human studies have reported that anthocyanins are recovered in urine as the intact glycosidic forms, whereas neither anthocyanidin (aglycone) nor glucuro- or sulfoconjugates are detected (12,13,22). Recently, Wu et al.

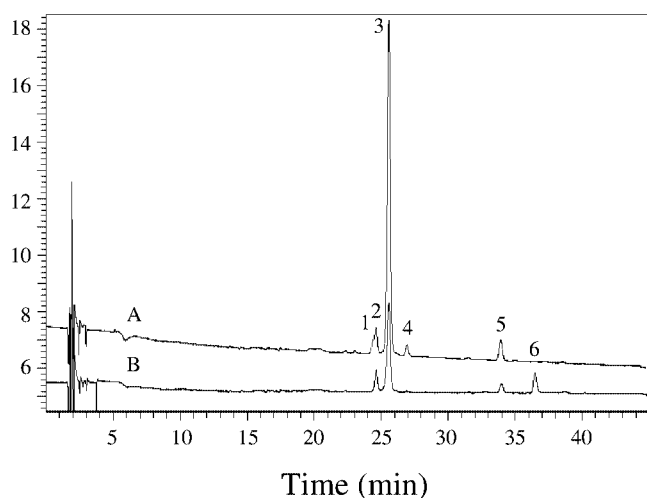


FIGURE 4 Representative HPLC chromatograms of human urine (one subject) collected after consumption of a meal containing 200 g strawberries and acidified to pH 5.0 after incubation without (A) or with (B) β -glucuronidase. Detection was performed at 524 nm. Peaks are as follows: 1, 3, 4: pelargonidin monoglucuronides; 2: pelargonidin-3-glucoside; 5: pelargonidin sulfate; 6: pelargonidin.

(16) have reported urinary excretion of cyanidin-3-glucoside monoglucuronide and peonidin monoglucuronide after ingestion of an elderberry extract. In the present work, we demonstrated for the first time the presence in urine of several anthocyanin metabolites such as three monoglucuroconjugates of pelargonidin, one sulfoconjugate of pelargonidin and pelargonidin itself. However, we should emphasize that analyses were carried out immediately after collection of urine. Indeed, we observed that all metabolites (except pelargonidin-3-glucoside) disappeared or markedly decreased when samples were frozen. A precipitate was formed in urine during freezing and metabolites could thus be retained. They could also be degraded during thawing. Given that previous works investigated frozen samples, labile and/or quantitatively minor metabolites could have escaped detection. Moreover, according to the HPLC method used (column, mobile phases, gradient elution, etc.), peaks are more or less separated from each other and errors could arise considering their identification by UV-visible detection. On the other hand, anthocyanins were consumed in the form of whole fruits included in a meal; thus,

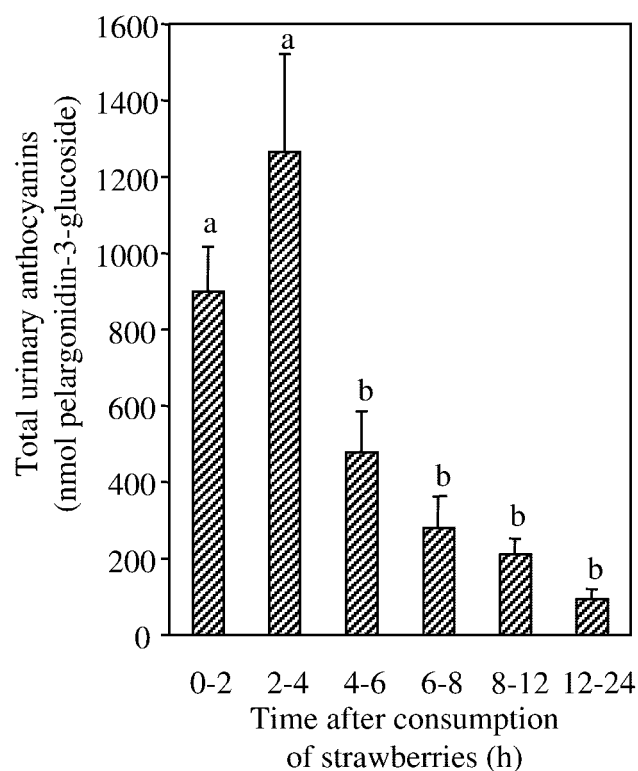


FIGURE 5 Urinary excretion of total anthocyanin metabolites in humans after consumption of a meal containing 200 g strawberries providing 179 μ mol pelargonidin-3-glucoside. Results are expressed as pelargonidin-3-glucoside equivalent. Values are means \pm SEM, $n = 6$. Means without a common letter differ, $P < 0.05$.

anthocyanin absorption was probably affected by the food matrix.

Conjugation of flavonoids with glucuronic acid or sulfate is the common final step in their metabolic pathway (23). Therefore, circulating metabolites of flavonoids are deglycosylated and glucuro- and/or sulfo-conjugated forms (18,19,23). Our results indicated that a similar metabolism could occur for anthocyanin glucosides. Glucuronidation of flavonoids occurred at different hydroxyl groups within the structure (24,25), the major sites of which were the 7-, 3-, 3'- or 4'-hydroxyl moiety (24,26). The analytical techniques we used did not allow us to determine the exact sites of glucuronida-

TABLE 1

Urinary excretion of anthocyanin metabolites in humans after consumption of 200 g strawberries containing 179 μ mol pelargonidin-3-glucoside¹

Compound ²	Urinary excretion	Total excretion
	nmol pel-3-glucoside/24 h	% of the ingested amount
(1) pel-glucuronide	112 \pm 32	
(2) pel-3-glucoside	118 \pm 34	
(3) pel-glucuronide	2675 \pm 412	
(4) pel-glucuronide	108 \pm 31	
(5) pel-sulfate	134 \pm 44	
(6) pelargonidin	79 \pm 25	
Total metabolites	3226 \pm 526	1.80 \pm 0.29

¹ Values are means \pm SEM, $n = 6$.

² Numbers refer to the order of elution after HPLC. pel, pelargonidin.

tion. However, because pelargonidin does not possess the 3'-hydroxyl group, we would suggest that the three glucuronides detected were 7-, 3- and 4'-monoglucuronides of pelargonidin.

Two possible pathways could explain the formation of monoglucuronides of pelargonidin. The presence of cyanidin aglycone has been previously reported in rat jejunum after ingestion of cyanidin-3-glucoside (11). Thus, as shown for various flavonoids (24), a possible pathway is that pelargonidin-3-glucoside was hydrolyzed to aglycone then rapidly glucuronidated in the intestine. On the other hand, as has been suggested by Wu et al. (16), another possible pathway is that pelargonidin-3-glucoside could serve as a substrate for UDP glucose dehydrogenase to form pelargonidin-3-glucuronide. Indeed, such an enzyme is present in both the small intestine and the liver in various species (27). This last hypothesis does not require hydrolysis to aglycone, which is unstable at physiological pH. Therefore, it could be regarded as a principal glucuronidation pathway and could thus result in the formation of the major metabolite (peak 3).

Sulfotransferases are present in numerous tissues such as intestine and liver (28,29). We detected a sulfoconjugate of pelargonidin in urine. Its formation requires hydrolysis of glucoside to aglycone then sulfoconjugation of the aglycone, more likely in the intestine than in the liver, given that no anthocyanin aglycone has yet been detected in the plasma (11,12). Because aglycones are very unstable at physiological pH, it is unlikely that pelargonidin found in urine arises from the small intestine. Both β -glucuronidases and sulfatases have been described in kidney and urine (30,31) and could thus release small amounts of aglycone from conjugates.

The total amount of anthocyanin metabolites excreted in urine in 24 h accounted for 1.80% of the ingested amount. This value was mainly related to the excretion of the major glucuronide (peak 3) and was in the same order of magnitude as those obtained with other classes of flavonoids that were excreted as glucuro- and/or sulfoconjugates (32–35). The urinary excretion of pelargonidin-3-glucoside in native form was very low (0.07% of the ingested amount) and in accordance with previous works that detected only anthocyanin glycosides (13,15,22). Urinary excretion of intact glucoside started very quickly, which reflected rapid absorption of glucosides that would take place mainly in the small intestine (12). However, a recent study has suggested that anthocyanins could also be absorbed from the stomach (36). Metabolite excretion occurred throughout the experimental day, thus reflecting their presence in the blood during this period. We could thus hypothesize that the presence of such metabolites in the body will be maintained by repeated anthocyanin-containing meals and that they could play a significant role in the antioxidant status. Indeed, Matsumoto et al. (9) have shown that the antioxidative activity of plasma lasted longer than the presence of anthocyanin glycosides in the plasma. They have thus assumed that anthocyanins were converted into some metabolites having antioxidant activity.

Thus, taken as a whole, our results suggest that anthocyanin metabolism presents more similarities with flavonoid metabolism than has been described to date. Future research should precisely determine the exact sites of conjugation and evaluate potent antioxidative activity of the metabolites.

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