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Blackberry Anthocyanins Are Mainly Recovered from Urine as Methylated and Glucuronidated Conjugates in Humans

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The consumption of anthocyanins has been shown to prevent certain chronic diseases. However, anthocyanin metabolism has not yet been fully elucidated. The aim of this study was to evaluate anthocyanin urinary excretion in humans receiving a meal containing blackberries and to identify possible metabolites in urine. Five healthy volunteers were fed 200 g of blackberries (960 μmol of anthocyanins). Urine samples were collected and rapidly treated by solid-phase extraction. Anthocyanin metabolites were identified and quantified by HPLC-ESI-MS-MS and HPLC with UV−vis detection, respectively. In addition to native cyanidin 3-glucoside, several other anthocyanin metabolites were identified in the urine: methylated glycosides, glucuronides of anthocyanidins and anthocyanins, a sulfoconjugate of cyanidin, and anthocyanidins. Total urinary excretion of blackberry anthocyanin metabolites was 0.160 ± 0.020% ($n = 5$) of the amount of anthocyanins ingested. Monoglucuronides of anthocyanidins represented >60% of this excretion. Urinary excretion of anthocyanins was maximal between 2 and 4 h after the meal, but continued during the 24 h of the experiment. This study highlighted the influence of aglycon structure on anthocyanin urinary excretion. It demonstrated that anthocyanins are not only methylated but also glucuroconjugated and sulfoconjugated in humans and that the main metabolites of blackberry anthocyanins in human urine were anthocyanidin monoglucuronides.

KEYWORDS: Humans; anthocyanins; blackberry; glucuronides; urinary excretion

INTRODUCTION

Anthocyanins are a group of naturally occurring phenolic compounds responsible for the color of many fruits and vegetables. They are glycosylated polyhydroxyl or polymethoxyl derivatives of 2-phenylbenzopyrylium or flavylium cation (*1*). Daily intake of anthocyanins in humans has been estimated as ≈200 mg in the United States due to their widespread distribution and occurrence in fruits and vegetables (*2*). Consumption of anthocyanins has been shown to reduce the risk of coronary heart disease and to prevent certain chronic diseases (*3*, *4*). Moreover, it has been reported that anthocyanins could exert beneficial neurological effects (*5*). In vitro experiments have also shown that anthocyanins can inhibit cellular growth and induce apoptosis in cancer cells (*6*, *7*). The positive effects of these pigments could be related to their potent antioxidant activity, as demonstrated in various in vitro and in vivo studies (*8*−*11*).

Given these multiple biological effects, anthocyanin bioavailability and metabolism are considered to be important issues. Recent studies in humans have reported that anthocyanins can be excreted in urine as intact glycosides as well as methylated and/or glucuronidated conjugates (*12*−*14*). We have recently demonstrated the presence of several anthocyanin metabolites, such as pelargonidin glucuronoconjugates and sulfoconjugates, in human urine following the consumption of strawberries (containing mainly pelargonidin 3-glucoside) (*15*). Moreover, the total urinary excretion of strawberry anthocyanin metabolites (≈1.80% of the ingested amount) was higher than the urinary anthocyanin excretion previously reported (≤0.1%) (*12*, *16*, *17*). In our previous study, anthocyanins were fed in the form of whole strawberries included in a meal, whereas other human studies were carried out by ingestion of anthocyanins in extract or beverage form, without the consumption of any other food (*13*, *14*, *16*, *17*). Hence, it was important to verify whether the higher urinary recovery of anthocyanins occurring after the ingestion of strawberries was therefore a special feature of pelargonidin 3-glucoside or due to the consumption of the anthocyanins in whole berry form.

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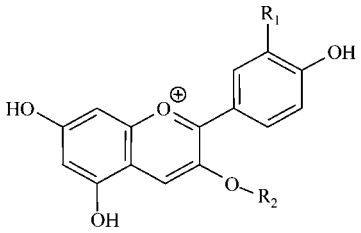

	R_1	R_2
Cyanidin	OH	H
Cyanidin 3-glucoside	OH	Glucose
Cyanidin 3-xyloside	OH	Xylose
Peonidin	OCH_3	H
Peonidin 3-glucoside	OCH_3	Glucose
Peonidin 3-xyloside	OCH_3	Xylose
Pelargonidin 3-glucoside (strawberry)	H	Glucose

Figure 1. Anthocyanin structures.

For this purpose, we evaluated the metabolism and urinary excretion of blackberry anthocyanins in humans having consumed whole blackberries as part of a meal. This fruit was chosen because it contains one major pigment, cyanidin 3-glucoside (**Figure 1**), widely distributed in various berries and also extensively studied in both rats and humans. We used the HPLC-ESI-MS-MS technique to investigate possible metabolites.

SUBJECTS AND METHODS

Chemicals. All anthocyanins were purchased from Extrasynthèse (Genay, France). Deep-frozen blackberries were obtained from a deep-frozen food product supplier (Szymczak-Nadreau, Romagnat, France).

Subjects and Study Design. Five healthy volunteers (three women and two men) aged 42 ± 5 years and with a mean body mass index of 21 ± 1 kg/m^2 participated in this study, which was carried out at our laboratory and performed in compliance with the Declaration of Helsinki. Volunteers did not consume any kind of polyphenol-rich product (vegetables, fruits, tea, etc.) at the dinner before the experiment or during the 24 h experiment period.

After an overnight fast, subjects consumed a breakfast consisting of 200 g of blackberries (containing 960 μmol of anthocyanins expressed as cyanidin 3-glucoside equivalents) with 15 g of sugar, 60 g of bread, and 10 g of butter. Two hundred grams of blackberries corresponds to a portion of fruits usually ingested. Water was the only beverage consumed during the experiment.

Urine samples were collected from these subjects before the experiment meal and between 0 and 2, 2 and 4, 4 and 6, 6 and 8, 8 and 12, and 12 and 24 h after the breakfast had been eaten. The samples were immediately acidified with 0.2 mol/L HCl and treated as described below.

Quantification of Blackberry Anthocyanins. Deep-frozen blackberries (100 g) were thawed and ground with a domestic mixer to obtain a homogeneous mixture. Blackberry anthocyanins were then extracted by stirring 5 g of this mixture for 30 min with 90 mL of 0.12 mol/L HCl in methanol. After filtration, the solution volume was adjusted to 100 mL and then diluted 5-fold with 0.12 mol/L HCl in water. The resulting solution (20 μL) was analyzed by HPLC as described below.

Sample Preparation. As we had previously observed that some peaks in HPLC urine chromatograms disappeared or decreased markedly when urine samples were frozen before analysis (*15*), we analyzed the urine samples immediately after collection. Anthocyanins exist in four different structures in equilibrium (*1*). The proportion of each structure depends on pH. In acidic conditions (pH <2), anthocyanins mainly exist in the form of a colored flavylium cation detectable at 500−550 nm, whereas colorless forms predominate at higher pH (such as urinary pH) (*1*). Therefore, the urine samples acidified with 0.2 mol/L HCl were maintained for 1 h at room temperature before treatment to obtain the maximal transformation of colorless forms into colored flavylium cations. Anthocyanins present in urine samples were then extracted using a Sep-Pak C_{18} Plus solid-phase extraction cartridge (Waters, Milford, MA) as previously described (*15*), using cyanidin 3,5-diglucoside as an internal standard. Briefly, 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) were loaded onto the cartridge. It 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 using a rotary evaporator at 35 °C. The dried extract was dissolved with 300 μL of 0.12 mol/L aqueous HCl. Samples were then analyzed by HPLC-ESI-MS-MS and HPLC with UV−vis detection to identify and quantify, respectively, any anthocyanin metabolites. Cyanidin 3,5-diglucoside (internal standard) was recovered at between 75 and 87%. Individual internal standard recoveries were taken into account to calculate anthocyanin concentrations in each urine sample.

Anthocyanin Analysis. Analysis of anthocyanins was carried out by HPLC using a DAD 200 photodiode array detector (Perkin-Elmer, Courtabœuf, France) and a 785A UV−vis detector (Perkin-Elmer) at 524 nm, as previously described (*15*). Samples were loaded onto a Hypersil C18-5 μm column (150 × 4.6 mm) protected by a guard column (Hypersil C18-5 μm, 10 × 4 mm) (Interchim, Montluçon, France). Elution was performed using water/H_3PO_4 (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 the compounds present in samples was performed by comparison with the authentic compounds based on retention time and the UV−vis spectrum and by spiking with individual compounds. Anthocyanin quantification was expressed as cyanidin 3-glucoside equivalents.

Identification of anthocyanin metabolites was carried out by HPLC-ESI-MS-MS analysis of urine samples. These analyses were performed on a Hewlett-Packard HPLC system equipped with API 2000 MS-MS detection (Applied Biosystem, Les Ulis, France) as previously described (*15*). The column was a Hypersil BDS C18-5 μm (150 × 2.1 mm) (Touzart and 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. Detection was carried out by using electrospray ionization conducted at 450 °C in 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. The MS data were collected in multiple-reaction monitoring mode by monitoring the transition of parent and product ions specific for each compound at a dwell time of 0.5 s. Cyanidin 3,5-diglucoside (internal standard) and anthocyanin metabolites were detected according to the respective *m*/*z* values of their parent and product ions: cyanidin 3,5-diglucoside (611/287), cyanidin 3-glucoside (449/287), cyanidin glucuronide (463/287), cyanidin diglucuronide (639/287), cyanidin 3-xyloside (419/287), cyanidin sulfate (367/287), cyanidin (287/137), peonidin 3-glucoside glucuronide (639/301), peonidin 3-glucoside (463/301), peonidin glucuronide (477/301), peonidin 3-xyloside (433/301), and peonidin (301/201).

Data Analysis. Values are given as means ± SEM and, when appropriate, significance of differences between values was determined by one-way ANOVA followed by a Student−Newman−Keuls test (GraphPad, Instat, San Diego, CA). Values of $P < 0.05$ were considered to be significant.

RESULTS

As shown in **Figure 2A**, cyanidin 3-glucoside (peak 3), at a concentration of 4.28 mmol/kg, was the major anthocyanin (89.2%) in blackberries consumed in this study. Three other minor anthocyanins were detected and identified by HPLC-ESI-MS-MS. Peak a was identified as a cyanidin pentose by detection of the respective parent and product ion pairs (*m*/*z*

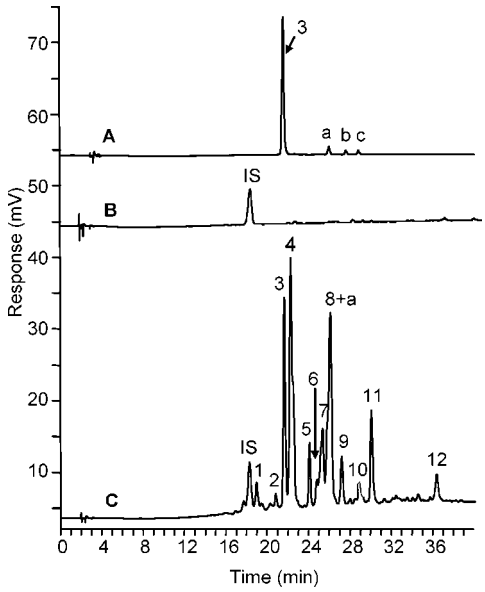

Figure 2. Representative HPLC chromatograms of blackberry anthocyanins (**A**) and of human urine (one subject) collected before (**B**) and 2 h after (**C**) the consumption of a meal containing 200 g of blackberries. Detection was performed at 524 nm. IS, internal standard (cyanidin 3,5-diglucoside). Urine was treated by solid-phase extraction. Peaks: 1 and 10, unknown anthocyanin metabolites; 2, cyanidin diglucuronide; 3, cyanidin 3-glucoside; 4 and 6, cyanidin monoglucuronides; 5, 8, and 9, peonidin monoglucuronides; 7, peonidin 3-glucoside; 11, cyanidin; 12, peonidin; a, cyanidin 3-xyloside; b, cyanidin malonylglucoside; c, cyanidin dioxalylglucoside.

419/287), accounting for 5.5% of total anthocyanins. Its chromatographic profile strongly suggested that it could have been cyanidin 3-xyloside, as previously reported in blackberry and chokeberry (*18*−*20*). The two other minor components (peak b, 2.6%; and peak c, 2.7%) were identified as acylated derivatives of cyanidin 3-glucoside: they responded to the specific cyanidin malonylglucoside transition (*m*/*z* 535/287) and cyanidin dioxalylglucoside transition (*m*/*z* 593/287), respectively, according to previous studies on various blackberry genotypes (*20*, *21*). Therefore, blackberries contained only cyanidin derivatives.

Excluding the cyanidin 3,5-diglucoside used as internal standard, no anthocyanins were observed in urine collected before the experimental meal (**Figure 2B**). However, 12 quantifiable peaks appeared in urine collected after the consumption of blackberries (**Figure 2C**). Peaks 3, 7, 11, and 12 were identified as cyanidin 3-glucoside, peonidin 3-glucoside, cyanidin, and peonidin, respectively, by comparison with the authentic compounds based on retention time in the HPLC analysis and UV−vis spectrum and by spiking with individual compounds.

HPLC-ESI-MS-MS was used to identify anthocyanin metabolites. The presence of cyanidin 3-glucoside and peonidin 3-glucoside was confirmed by detection of their respective parent and product ion pairs (*m*/*z* 449/287 and 463/301, respectively) (**Figure 3C**,**D**). Two peaks had *m*/*z* values of 463 and 287 for their parent and product ions, respectively (**Figure 3E**). An *m*/*z* value of 176 for the substitution group is indicative of a glucuronide residue. These peaks were thus identified as cyanidin monoglucuronides and are shown as peaks 4 and 6 in **Figure 2C**. However, the exact site of glucuronidation could not be specified for each of these compounds. Similarly, three peaks had *m*/*z* values of 477 and 301 for their parent and product

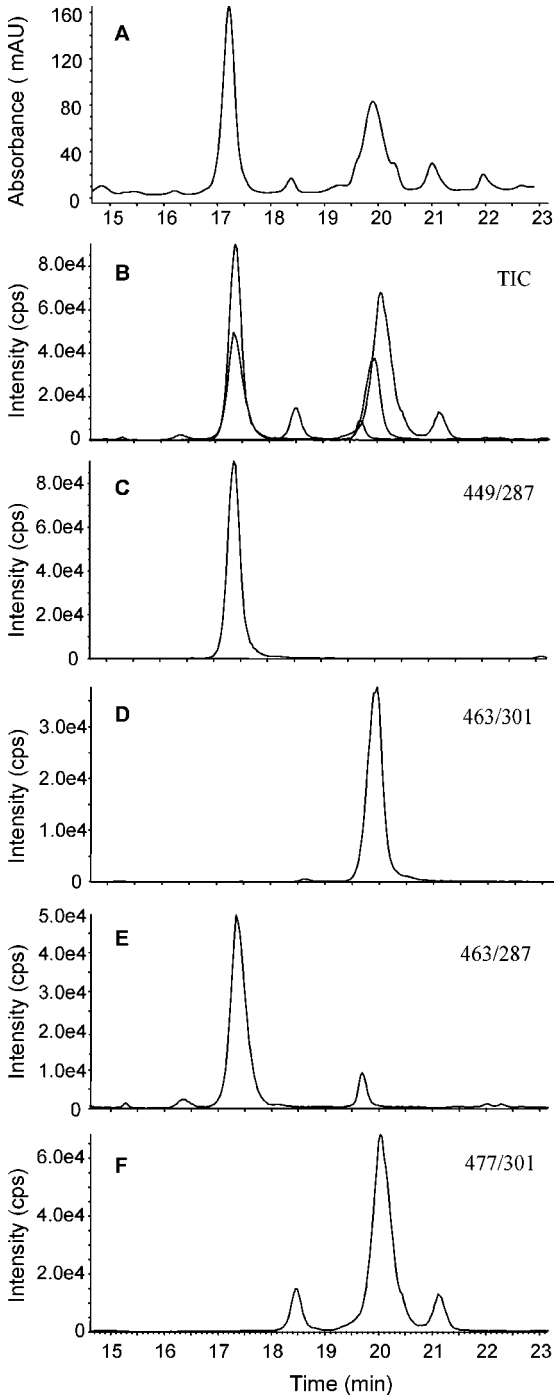

Figure 3. HPLC-ESI-MS-MS analysis of anthocyanin metabolites in human urine (one subject) collected 2 h after the consumption of a meal containing 200 g of blackberries: (**A**) detection at 524 nm. Detection of the respective *m*/*z* values of parent and product ions: (**B**) total ionic current (TIC); (**C**) cyanidin 3-glucoside; (**D**) peonidin 3-glucoside; (**E**) cyanidin glucuronide; (**F**) peonidin glucuronide. Urine was treated by solid-phase extraction.

ions, respectively (**Figure 3F**). They were thus identified as peonidin monoglucuronides and are shown as peaks 5, 8, and 9 in **Figure 2C**.

A cyanidin diglucuronide was detected in urine on the basis of parent and product ion *m*/*z* values of 639 and 287, respectively (**Figure 4C**). It eluted just before cyanidin 3-glucoside and could correspond to peak 2 in **Figure 2C**. Two small peaks responded to the specific transition of peonidin 3-glucoside monoglucuronide (*m*/*z* 639/301) (**Figure 4D**). These conjugates eluted just before and after the internal standard

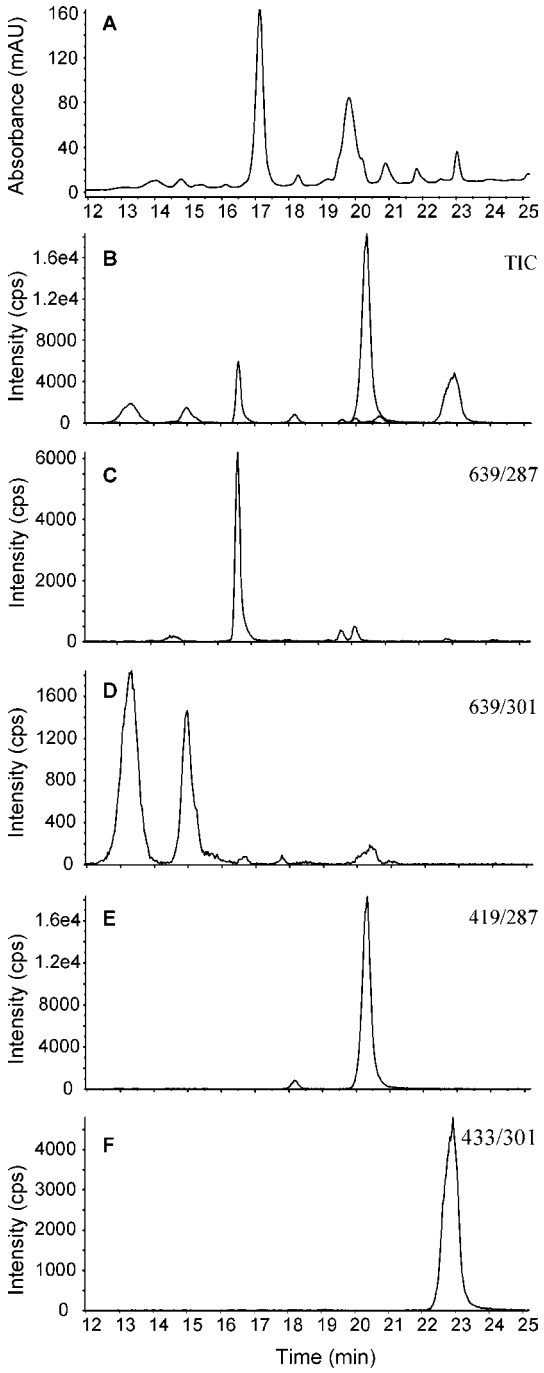

Figure 4. HPLC-ESI-MS-MS analysis of anthocyanin metabolites in human urine (one subject) collected 2 h after the consumption of a meal containing 200 g of blackberries: (**A**) detection at 524 nm. Detection of the respective *m*/*z* values of parent and product ions: (**B**) total ionic current (TIC); (**C**) cyanidin diglucuronide; (**D**) peonidin 3-glucoside glucuronide; (**E**) cyanidin 3-xyloside; (**F**) peonidin 3-xyloside. Urine was treated by solid-phase extraction.

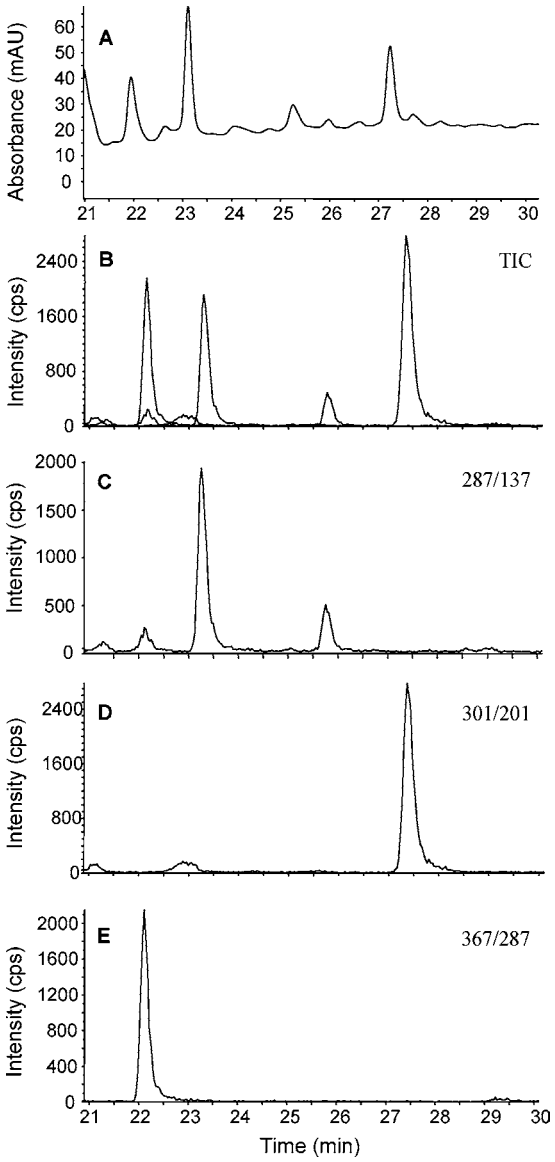

Figure 5. HPLC-ESI-MS-MS analysis of anthocyanin metabolites in human urine (one subject) collected 2 h after the consumption of a meal containing 200 g of blackberries: (**A**) detection at 524 nm. Detection of the respective *m*/*z* values of parent and product ions: (**B**) total ionic current (TIC); (**C**) cyanidin; (**D**) peonidin; (**E**) cyanidin sulfate. Urine was treated by solid-phase extraction.

cyanidin 3,5-diglucoside and probably corresponded to some of the small peaks observed near the internal standard on **Figure 2C**. No cyanidin 3-glucoside monoglucuronide (*m*/*z* 625/287) was detected. Cyanidin 3-xyloside was present in urine (*m*/*z* 419/287) (**Figure 4E**). This compound coeluted with a peonidin monoglucuronide (peak 8 in **Figure 2C**). Peonidin 3-xyloside was also detected in urine (*m*/*z* 433/301) (**Figure 4F**).

The presence of the aglycons cyanidin (*m*/*z* 287/137) and peonidin (*m*/*z* 301/201) was confirmed by HPLC-ESI-MS-MS (**Figure 5C**,**D**). Moreover, a cyanidin monosulfate was identified on the basis of parent and product ion *m*/*z* values of 367 and 287, respectively (**Figure 5E**). This compound, as well as peonidin 3-xyloside, eluted between peaks 9 and 11 in **Figure 2C**. Either or both of these compounds could have eluted into peak 10, but their low concentrations made it difficult to locate them precisely in **Figure 2C**.

Table 1 gives the urinary excretion of each anthocyanin metabolite that was quantifiable, expressed as nanomoles of cyanidin 3-glucoside equivalents per 24 h. Urinary excretion of cyanidin 3-glucoside as native form was low (12.5% of total anthocyanin metabolites), whereas the major portion of blackberry anthocyanins was excreted as monoglucuronides of cyanidin and peonidin (≈64% of total metabolite excretion). Total urinary excretion of blackberry anthocyanin metabolites corresponded to 0.160 ± 0.020% of the amount of anthocyanins consumed. Fifty-five percent of anthocyanin metabolites were excreted during the first 4 h following the meal (**Figure 6**), whereas >70% of cyanidin 3-glucoside was excreted during

Table 1. Urinary Excretion of Anthocyanin Metabolites in Humans Following Consumption of 200 g of Blackberries Containing 960 μmol of Cyanidin Glycosides[a]

compound[b]	urinary excretion (nmol of cyanidin 3-glucoside equiv/24 h)
(1) unknown	36.8 ± 6.0
(2) cyanidin diglucuronide	19.3 ± 4.4
(3) cyanidin 3-glucoside	193 ± 29
(4) cyanidin glucuronide	355 ± 68
(5) peonidin glucuronide	30.7 ± 7.3
(6) cyanidin glucuronide	32.9 ± 3.3
(7) peonidin 3-glucoside	94.1 ± 5.1
(8) peonidin glucuronide (+ cyanidin 3-xyloside)	529 ± 132
(9) peonidin glucuronide	40.8 ± 8.7
(10) unknown	49.8 ± 11.4
(11) cyanidin	112 ± 57
(12) peonidin	43.5 ± 9.5
total metabolites	1537 ± 197
(i.e., 0.160 ± 0.020% of the ingested amount)	

[a] Values are expressed as means ± SEM, $n = 5$. [b] Numbers refer to the order of elution after HPLC (see **Figure 2C**).

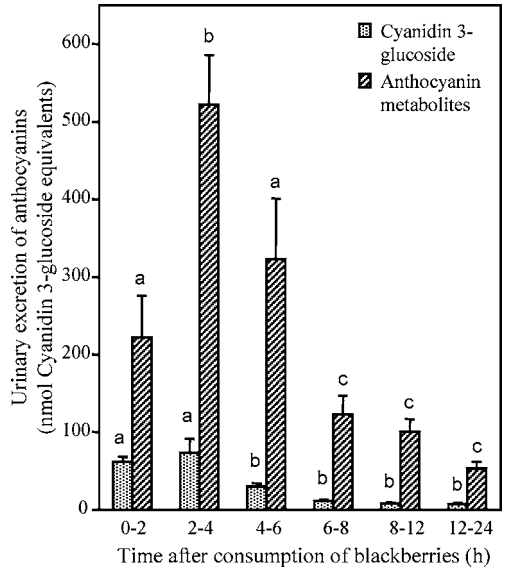

Figure 6. Urinary excretion of cyanidin 3-glucoside and total anthocyanin metabolites in humans after consumption of a meal containing 200 g of blackberries providing 960 μmol of anthocyanins expressed as cyanidin 3-glucoside. Results are expressed as cyanidin 3-glucoside equivalents. Values are means ± SEM, $n = 5$. Means without a common letter for the same compound differ significantly, $P < 0.05$. Cy 3-glc, cyanidin 3-glucoside.

this same period. Urinary excretion of all anthocyanins was maximal between 2 and 4 h after the meal. However, urinary excretion of the metabolites (mainly the monoglucuronides) continued until the end of the experiment.

DISCUSSION

Several studies in recent years have focused attention on anthocyanin bioavailability both in humans and in experimental animals. Most of these studies reported that anthocyanins were slightly absorbed and were excreted unmetabolized (*16*, *17*, *22*−*25*). Recently, we and others have demonstrated that anthocyanins can be metabolized into methylated and/or glucuronidated conjugates both in rats (*26*−*28*) and in humans (*12*−*15*). Moreover, it seems that the metabolic fate of anthocyanins may differ according to their aglycon structure (*15*, *29*, *30*).

Therefore, to assess the influence of the aglycon moiety on anthocyanin metabolism, we studied in humans the metabolism and urinary excretion of blackberry anthocyanins using a study design similar to the one we used previously to evaluate strawberry anthocyanin metabolism (*15*). Blackberries contained only cyanidin glycosides, with the majority (≈90%) attributed to cyanidin 3-glucoside, whereas pelargonidin 3-glucoside was the main anthocyanin in strawberries (**Figure 1**). After an overnight fast, subjects consumed a breakfast containing 200 g of blackberries as well as sugar, bread, and butter. The volunteers were the same as those included in our previous study (*15*), and in both experiments they ingested whole berries. The total amount of blackberry anthocyanin metabolites excreted in urine over 24 h accounted for 0.16% of the ingested amount. This value is >10-fold lower than that observed following the consumption of strawberries (*15*). Because the anthocyanin sugar moiety was the same (glucose), this thus emphasized the influence of the aglycon structure. Recently, Wu et al. (*30*) have also shown in weaning pigs gavaged with a freeze-dried powder of marionberry that pelargonidin 3-glucoside had a much higher total urinary excretion (0.583%) than cyanidin 3-glucoside (0.087%). This lower urinary excretion of cyanidin 3-glucoside compared to pelargonidin 3-glucoside could result from a lower stability at physiological pH or from a lower absorption rate throughout the digestive tract. However, we have previously demonstrated that cyanidin 3-glucoside remained virtually undegraded in a physiological buffer (pH 6.6) maintained for 45 min at 37 °C (*26*). Pelargonidin has only one hydroxyl group on the B ring. It thus cannot be methylated and may be more available for glucuronidation. On the other hand, anthocyanin urinary excretion was similar or slightly higher after blackberry consumption than after the ingestion of anthocyanin extracts or beverages containing cyanidin 3-glucoside (*12*, *16*, *17*, *22*, *23*). Hence, absorption from the raw plant is not worse than that from plant extract, contrary to what was previously suggested following blueberry consumption (*12*). Although a major part of anthocyanin metabolites was quickly excreted in urine, this excretion continued during 24 h. We could thus hypothesize that repeated meals could allow the maintenance of anthocyanin urinary excretion all day long.

The urinary excretion of cyanidin 3-glucoside as native form was very low (0.023% of the ingested amount) and in accordance with previous works that detected only anthocyanin glycosides (*16*, *17*, *25*). In most studies that have identified glucuronide conjugates as anthocyanin metabolites, these conjugates were detected in small amounts compared to native glycosides (*12*−*14*). Conversely, in the present study we found that only 12.5% of anthocyanins present in urine were in the native cyanidin 3-glucoside form. This result, drawn together with our previous study (*15*), clearly indicates that anthocyanin metabolism has many more similarities with flavonoid metabolism (*31*, *32*) than was expected until now. We should emphasize that analyses were carried out immediately after collection of the urine because we previously observed that anthocyanin metabolites decreased when samples were frozen (*15*). Moreover, according to the HPLC method used, peaks are more or less separated from each other and errors could arise concerning their identification by only UV−visible detection. On the other hand, this work focused on only anthocyanin metabolites having an anthocyanin skeleton and being thus detected at 524 nm. However, anthocyanins could be converted into colorless forms and/or degradation products due to their chemical instability at

physiological pH. They could also be metabolized into various phenolic acids by intestinal microflora (*33*, *34*).

Following blackberry consumption, urinary anthocyanins were mainly methylated and/or glucuronidated conjugates. Methylation of cyanidin 3-glucoside has been previously reported in human and animal studies (*12*, *27*, *30*, *35*−*37*). Whereas some authors identified two methylated derivatives of cyanidin 3-glucoside in rats or pigs (*27*, *30*, *35*) (as 3′-*O*-methyl and 4′-*O*-methyl esters of cyanidin), we found only one methylated derivative identified as peonidin 3-glucoside (3′-*O*-methyl ester). This is in accordance with previous studies that have shown that methylation tends to occur at the 3′-*O* position of flavonoids that possess a 3′,4′-dihydroxylation pattern in the B ring (*38*). We have previously suggested that this methylated form of cyanidin 3-glucoside was mainly formed in the liver and preferentially eliminated by bile (*26*, *39*). Recently, we have shown in anthocyanin-fed rats that methylated forms were the main anthocyanins recovered in the liver, but we also detected traces of methylated forms in the jejunum wall (*28*). Blackberries also contained a small amount (≈5%) of cyanidin 3-xyloside. This compound, as well as its methylated form (peonidin 3-xyloside), has been detected in urine.

Monoglucuronides of cyanidin and peonidin were the major urinary metabolites. However, the analytical techniques we used did not locate the exact sites of glucuronidation. Glucuronidation of flavonoids has been shown to occur at different hydroxyl groups within the structure (*40*, *41*), the major sites of which were the 7-, 3-, 3′-, or 4′-position of the polyphenol ring (*40*, *42*). Several possible pathways could explain the formation of cyanidin and peonidin monoglucuronides. The presence of cyanidin aglycon has been previously reported in rat jejunum following the ingestion of cyanidin 3-glucoside (*28*, *37*). Moreover, we have recently detected traces of glucurono-conjugated anthocyanidins (cyanidin and peonidin) in rat jejunum (*28*). Thus, as shown for various flavonoids (*40*), one possible pathway would be cyanidin 3-glucoside or peonidin 3-glucoside hydrolyzed to aglycon and then rapidly glucuronidated in the intestine. We could also hypothesize that cyanidin could be absorbed and later methylated and glucuronidated in the liver to form peonidin monoglucuronides. We have recently identified anthocyanin aglycons in the plasma of rats fed with a blackberry extract-enriched diet (*28*). On the other hand, cyanidin 3-glucoside and peonidin 3-glucoside could serve as substrates for UDP glucose dehydrogenase to form the corresponding glucuronides (cyanidin 3-glucuronide and peonidin 3-glucuronide, respectively) (*12*). Such an enzyme is present in both the small intestine and the liver in various species (*43*). This latter hypothesis does not require hydrolysis to aglycons, which are unstable at physiological pH. Therefore, it could be regarded as a principal glucuronidation pathway and could thus result in the formation of the major metabolites (peaks 4 and 8). Two monoglucuronides of peonidin 3-glucoside were also detected in the urine. We did not detect glucuronides of cyanidin 3-glucoside, but the concentrations of these metabolites may be too low to allow detection. Recently, Wu et al. (*30*) detected small amounts of monoglucuronides of cyanidin 3-glucoside and peonidin 3-glucoside in pig urine (0.0020 and 0.0083% of the ingested amounts, respectively) following administration of a high dose of anthocyanins.

As previously reported after pelargonidin 3-glucoside consumption (*15*), a sulfoconjugate of anthocyanidin (cyanidin sulfate) was identified in urine by HPLC-MS-MS, but in too low amounts to allow it to be precisely localized on the HPLC-DAD chromatogram and quantified. Its retention time on the HPLC-ESI-MS-MS chromatogram was ≈1 min shorter than that for the corresponding aglycon cyanidin, as it was observed between pelargonidin sulfate and pelargonidin following strawberry consumption (*15*). Anthocyanidin sulfoconjugate formation requires hydrolysis of glucoside to the aglycon and then sulfoconjugation of the aglycon by sulfotransferases present in numerous tissues, including the intestine and liver (*44*).

Furthermore, significant amounts of the aglycons cyanidin and peonidin (≈10% of total urinary metabolites) were excreted in the urine. Given that aglycons are very unstable at physiological pH, it is unlikely that they all arise from the small intestine. Small amounts of aglycons could thus be released from conjugates by β-glucuronidases and sulfatases present in both kidney and urine (*45*, *46*). Thus, these results, taken together with our previous study (*15*), highlighted the influence of aglycon structure on anthocyanin metabolism and demonstrated that anthocyanins were mainly excreted in urine as glucuronidated and/or methylated derivatives following berry consumption in humans.

LITERATURE CITED

(1) Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables, and Grains*; CRC Press: Boca Raton, FL, 1993; p 362.

(2) Kühnau, J. The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev. Nutr. Diet.* **1976**, *24*, 117−191.

(3) Morazzoni, P.; Bombardelli, E. *Vaccinium myrtillus* L. *Fitoterapia* **1996**, *67*, 3−29.

(4) Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523−1526.

(5) Youdim, K. A.; Shukitt-Hale, B.; Martin, A.; Wang, H.; Denisova, N.; Bickford, P. C.; Joseph, J. A. Short-term dietary supplementation of blueberry polyphenolics: beneficial effects on aging brain performance and peripheral tissue function. *Nutr. Neurosci.* **2000**, *3*, 383−397.

(6) Katsube, N.; Iwashita, K.; Tsushida, T.; Yamaki, K.; Kobori, M. Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. *J. Agric. Food Chem.* **2003**, *51*, 68−75.

(7) Kang, S. Y.; Seeram, N. P.; Nair, M. G.; Bourquin, L. D. Tart cherry anthocyanins inhibit tumor development in Apc(Min) mice and reduce proliferation of human colon cancer cells. *Cancer Lett.* **2003**, *194*, 13−19.

(8) Wang, H.; Nair, M. G.; Strasburg, G. M.; Chang, Y. C.; Booren, A. M.; Gray, J. I.; DeWitt, D. L. Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *J. Nat. Prod.* **1999**, *62*, 294−296.

(9) Matsumoto, H.; Nakamura, Y.; Hirayama, M.; Yoshiki, Y.; Okubo, K. Antioxidant activity of black currant anthocyanin aglycons and their glycosides measured by chemiluminescence in a neutral pH region and in human plasma. *J. Agric. Food Chem.* **2002**, *50*, 5034−5037.

(10) Tsuda, T.; Horio, F.; Osawa, T. Dietary cyanidin 3-*O*-β-D-glucoside increases ex vivo oxidation resistance of serum in rats. *Lipids* **1998**, *33*, 583−588.

(11) Mazza, G.; Kay, C. D.; Cottrell, T.; Holub, B. J. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. *J. Agric. Food Chem.* **2002**, *50*, 7731−7737.

(12) Wu, X.; Cao, G.; Prior, R. L. Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry. *J. Nutr.* **2002**, *132*, 1865−1871.

(13) Kay, C. D.; Mazza, G.; Holub, B. J.; Wang, J. Anthocyanin metabolites in human urine and serum. *Br. J. Nutr.* **2004**, *91*, 933−942.

(14) Cooney, J. M.; Jensen, D. J.; McGhie, T. K. LC-MS identification of anthocyanins in boysenberry extract and anthocyanin metabolites in human urine following dosing. *J. Sci. Food Agric.* **2004**, *84*, 237−245.

(15) Felgines, C.; Talavéra, S.; Gonthier, M. P.; Texier, O.; Scalbert, A.; Lamaison, J. L.; Rémésy, C. Strawberry anthocyanins are recovered in urine as glucuro- and sulfoconjugates in humans. *J. Nutr.* **2003**, *133*, 1296−1301.

(16) McGhie, T. K.; Ainge, G. D.; Barnett, L. E.; Cooney, J. M.; Jensen, D. J. Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolized by both humans and rats. *J. Agric. Food Chem.* **2003**, *51*, 4539−4548.

(17) Bitsch, I.; Janssen, M.; Netzel, M.; Strass, G.; Frank, T. Bioavailability of anthocyanidin-3-glycosides following consumption of elderberry extract and blackcurrant juice. *Int. J. Clin. Pharmacol. Ther.* **2004**, *42*, 293−300.

(18) Maatta-Riihinen, K. R.; Kamal-Eldin, A.; Mattila, P. H.; Gonzalez-Paramas, A. M.; Torronen, A. R. Distribution and contents of phenolic compounds in eighteen Scandinavian berry species. *J. Agric. Food Chem.* **2004**, *52*, 4477−4486.

(19) Sapers, G.; Hicks, K.; Burgher, A.; Hargrave, D.; Sondey, S.; Bilyk, A. Anthocyanin patterns in ripening thornless blackberries. *J. Am. Soc. Hortic. Sci.* **1986**, *111*, 945−950.

(20) Cho, M. J.; Howard, L. R.; Prior, R. L.; Clark, J. R. Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *J. Sci. Food Agric.* **2004**, *84*, 1771−1782.

(21) Stintzing, F. C.; Stintzing, A. S.; Carle, R.; Wrolstad, R. E. A novel zwitterionic anthocyanin from evergreen blackberry (*Rubus laciniatus* Willd.). *J. Agric. Food Chem.* **2002**, *50*, 396−399.

(22) Netzel, M.; Strass, G.; Janssen, M.; Bitsch, I.; Bitsch, R. Bioactive anthocyanins detected in human urine after ingestion of blackcurrant juice. *J. Environ. Pathol. Toxicol. Oncol.* **2001**, *20*, 89−95.

(23) Frank, T.; Netzel, M.; Strass, G.; Bitsch, R.; Bitsch, I. Bioavailability of anthocyanidin-3-glucosides following consumption of red wine and red grape juice. *Can. J. Physiol. Pharmacol.* **2003**, *81*, 423−435.

(24) Nielsen, I. L.; Dragsted, L. O.; Ravn-Haren, G.; Freese, R.; Rasmussen, S. E. Absorption and excretion of black currant anthocyanins in humans and Watanabe heritable hyperlipidemic rabbits. *J. Agric. Food Chem.* **2003**, *51*, 2813−2820.

(25) Matsumoto, H.; Inaba, H.; Kishi, M.; Tominaga, S.; Hirayama, M.; Tsuda, T. 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.* **2001**, *49*, 1546−1551.

(26) Talavéra, S.; Felgines, C.; Texier, O.; Besson, C.; Manach, C.; Lamaison, J. L.; Rémésy, C. Anthocyanins are efficiently absorbed from the small intestine in rats. *J. Nutr.* **2004**, *134*, 2275−2279.

(27) Ichiyanagi, T.; Shida, Y.; Rahman, M. M.; Hatano, Y.; Matsumoto, H.; Hirayama, M.; Konishi, T. Metabolic pathway of cyanidin 3-*O*-*β*-D-glucopyranoside in rats. *J. Agric. Food Chem.* **2005**, *53*, 145−150.

(28) Talavéra, S.; Felgines, C.; Texier, O.; Besson, C.; Gil-Izquierdo, A.; Lamaison, J.-L.; Rémésy, C. Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. *J. Agric. Food Chem.* **2005**, *53*, 3902−3908.

(29) Talavéra, S.; Felgines, C.; Texier, O.; Besson, C.; Mazur, A.; Lamaison, J. L.; Rémésy, C. Bioavailability of a bilberry anthocyanin extract and its impact on plasma antioxidant capacity in rats. *J. Sci. Food. Agric.* **2005**, in press.

(30) Wu, X.; Pittman, H. E., 3rd; Prior, R. L. Pelargonidin is absorbed and metabolized differently than cyanidin after marionberry consumption in pigs. *J. Nutr.* **2004**, *134*, 2603−2610.

(31) Manach, C.; Texier, O.; Morand, C.; Crespy, V.; Régerat, F.; Demigné, C.; Rémésy, C. Comparison of the bioavailability of quercetin and catechin in rats. *Free Radical Biol. Med.* **1999**, *27*, 1259−1266.

(32) Rice-Evans, C.; Spencer, J. P.; Schroeter, H.; Rechner, A. R. Bioavailability of flavonoids and potential bioactive forms in vivo. *Drug Metabol. Drug Interact.* **2000**, *17*, 291−310.

(33) Aura, A. M.; Martin-Lopez, P.; O'Leary, K. A.; Williamson, G.; Oksman-Caldentey, K. M.; Poutanen, K.; Santos-Buelga, C. In vitro metabolism of anthocyanins by human gut microflora. *Eur. J. Nutr.* **2005**, *44*, 133−142.

(34) Fleschhut, J.; Kratzer, F.; Rechkemmer, G.; Kulling, S. E. Stability and biotransformation of various dietary anthocyanins in vitro. *Eur. J. Nutr.* **2005**, in press.

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

(36) Felgines, C.; Texier, O.; Besson, C.; Fraisse, D.; Lamaison, J. L.; Rémésy, C. Blackberry anthocyanins are slightly bioavailable in rats. *J. Nutr.* **2002**, *132*, 1249−1253.

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

(38) Hackett, A. M. The metabolism of flavonoid compounds in mammals. In *Plant Flavonoids in Biology and Medicine: Biological, Pharmacological, and Structure*−*Activity Relationships*; Cody, V., Middleton, E., Jr., Eds.; Harbourne: Buffalo, NY, 1986; pp 177−194.

(39) Talavéra, S.; Felgines, C.; Texier, O.; Besson, C.; Lamaison, J. L.; Rémésy, C. Anthocyanins are efficiently absorbed from the stomach in anesthetized rats. *J. Nutr.* **2003**, *133*, 4178−4182.

(40) Gee, J. M.; DuPont, M. S.; Day, A. J.; Plumb, G. W.; Williamson, G.; Johnson, I. T. Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. *J. Nutr.* **2000**, *130*, 2765−2771.

(41) Day, A. J.; Bao, Y.; Morgan, M. R.; Williamson, G. Conjugation position of quercetin glucuronides and effect on biological activity. *Free Radical Biol. Med.* **2000**, *29*, 1234−1243.

(42) Boersma, M. G.; van der Woude, H.; Bogaards, J.; Boeren, S.; Vervoort, J.; Cnubben, N. H.; van Iersel, M. L.; van Bladeren, P. J.; Rietjens, I. M. Regioselectivity of phase II metabolism of luteolin and quercetin by UDP-glucuronosyl transferases. *Chem. Res. Toxicol.* **2002**, *15*, 662−670.

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

(44) Runge-Morris, M. A. Regulation of expression of the rodent cytosolic sulfotransferases. *FASEB J.* **1997**, *11*, 109−117.

(45) Grompe, M.; Pieretti, M.; Caskey, C. T.; Ballabio, A. The sulfatase gene family: cross-species PCR cloning using the MOPAC technique. *Genomics* **1992**, *12*, 755−760.

(46) Borghoff, S. J.; Birnbaum, L. S. Age-related changes in glucuronidation and deglucuronidation in liver, small intestine, lung, and kidney of male Fischer rats. *Drug Metab. Dispos.* **1985**, *13*, 62−67.

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