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Anthocyanin Glycosides from Berry Fruit Are Absorbed and Excreted Unmetabolized by Both Humans and Rats

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Anthocyanins, the red/blue pigments found in plants, are polyphenolic compounds consumed by humans and are part of a normal diet. Recent studies have shown that anthocyanins have substantial bioactivity including antioxidant activity and therefore may have beneficial effects on human health. Anthocyanins are a group of over 500 compounds of diverse structures containing different core phenolic aglycons and conjugated with sugars in a variety of glycosylation patterns. In this study, we have investigated the bioabsorption of 15 anthocyanins with structures containing different aglycons and conjugated sugars extracted from blueberry, boysenberry, black raspberry, and blackcurrant in both humans and rats. Intact and unmetabolized anthocyanins were detected in urine of rats and humans following dosing for all molecular structures investigated, thus demonstrating that anthocyanins with diverse molecular structure and from different dietary sources are bioavailable at diet relevant dosage rates. In addition, the relative concentrations of anthocyanins detected in urine following dosing varied, indicating that differences in bioavailability are due to variations in chemical structure. Our results suggest that the nature of the sugar conjugate and the phenolic aglycon are both important determinants of anthocyanin absorption and excretion in rats and humans.

KEYWORDS: Anthocyanins; bioabsorption; human; berry fruit; flavonoid glycosides

INTRODUCTION

Anthocyanins are the red and blue pigments present in a large number of plant tissues including leaves, flowers, and fruit and therefore are consumed as normal components of the diet. The main dietary sources of anthocyanins include red-colored fruit, some vegetables, and red wine. Dietary consumption has been estimated at up to 200 mg/day, which is higher than for other flavonoids such as quercetin (*1*−*3*). Berry fruit in particular accumulate substantial quantities of anthocyanins and are rich dietary sources; single servings of some berry fruit can comprise in the order of 100−300 mg of anthocyanin. Like other flavonoid compounds, anthocyanins have antioxidant activity (*4*, *5*). In addition, anthocyanins have also been reported to have anticancer (*6*, *7*) and antiinflammatory (*8*) activity. However, despite the relatively high amounts of anthocyanins consumed in the diet and the reported biological activities, little is known about the in vivo biological activity of anthocyanins including bioabsorption and subsequent metabolism.

To achieve a biological effect in a target organ or tissue (except for the gastrointestinal tract), bioactive components in the diet must be bioavailable, that is effectively absorbed, transported into the circulatory system, and delivered to the appropriate site. Until quite recently, it was thought that flavonoids, including anthocyanins, were not absorbed from the diet to any significant extent and were therefore biologically inactive. More recent research has now shown that flavonoids (e.g., quercetin glycosides and catechin) are indeed absorbed when consumed as part of the diet (*9*−*11*). There is still considerable uncertainty about the precise mechanisms of absorption of dietary phenolic compounds from the gastrointestinal tract. It appears that quercetin glycosides are deglycosylated prior to absorption or absorbed through direct interaction with the hexose transport pathway (*12*, *13*) although only glucuronyl quercetin conjugates are present in plasma (*14*). In contrast, catechin is absorbed directly and without chemical modification but is then extensively metabolized in vivo (*15*). Recently, a limited number of investigations have shown that anthocyanins, too, are absorbed by humans and rats, albeit apparently by a different mechanism and at lower rates. Absorption of intact anthocyanins has been reported in rats (*16*, *17*) and humans (*18*−*24*). Until recently, these investigations were restricted to cyanidin-containing anthocyanins; however, recent papers describe the absorption of both the cyanidin and the delphinidin glycosides of blackcurrant by rats and humans (*23*) and anthocyanins found in blueberry (*24*). The mechanism for the absorption of anthocyanins has not been studied but could

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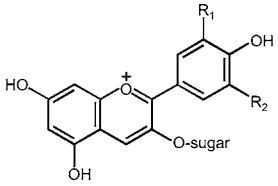

Peak #	MW	Name	R1	R2
1	611	Cyanidin 3-*O*-sophorose	OH	H
2	757	Cyanidin 3-*O*-2^G glucosylrutinose	OH	H
3	449	Cyanidin 3-*O*-glucose	OH	H
4	639	Cyanidin 3-*O*-2^G xylosylrutinose	OH	H
5	595	Cyanidin 3-*O*-rutinose	OH	H
6	465	Delphinidin 3-*O*-galactose	OH	OH
7	465	Delphinidin 3-*O*-glucose	OH	OH
8	611	Delphinidin 3-*O*-rutinose	OH	OH
9	435	Delphinidin 3-*O*-arabinose	OH	OH
10	479	Petunidin 3-*O*-galactose	OCH_3	OH
11	479	Petunidin 3-*O*-glucose	OCH_3	OH
12	449	Petunidin 3-*O*-arabinose	OCH_3	OH
13	493	Malvidin 3-*O*-galactose	OCH_3	OCH_3
14	493	Malvidin 3-*O*-glucose	OCH_3	OCH_3
15	463	Malvidin 3-*O*-arabinose	OCH_3	OCH_3

Figure 1. Chemical structures of the anthocyanins used in this study.

involve the hexose transport pathway as for quercetin (*12*) or an anion translocator such as bilitranslocase (*25*).

Although cyanidin glycosides are the anthocyanins most commonly found in fruit, berry fruit also contain anthocyanin glycosides with the aglycons: delphinidin, petunidin, peonidin, and malvidin (**Figure 1**). For example, blackcurrant (*Ribes nigrum* L.) contains delphinidin- and cyanidin-based anthocyanins and blueberry and grape contain glycosides of five different aglycons. There is additional diversity in that different sugar types are attached to the phenolic aglycon of anthocyanins. Thus, the structural diversity of anthocyanins present in the diet is considerable. The current study was undertaken to investigate the absorption of anthocyanins with a wide range of types of aglycons and glycosidic groups obtained from concentrates of a variety of berry fruit including blackcurrant, boysenberry, black raspberry, and blueberry.

MATERIALS AND METHODS

The sources of berry fruit anthocyanins were blackcurrant juice liquid concentrate (Barkers Fruit Processors, Geraldine, NZ), and boysenberry, black raspberry, and blueberry dried extracts provided by NutraColor (Palmerston North, NZ). Boysenberry extract was prepared from a commercial supply of boysenberry (*Rubus loganbaccus* x *baileyanus* Britt.) fruit that contained a mixture of cultivars whereas the blueberry extract was prepared from a single cultivar Reka (*Vaccunium corymbosum* L.). Black raspberry (*Rubus occidentalis* L.) fruit were provided by Harvey Hall, Nelson Research Centre, HortResearch, Motueka, New Zealand.

All chemicals were obtained from Merck New Zealand Ltd (Palmerston North, NZ). Solvents were of high-performance liquid chromatography (HPLC) grade, whereas other chemicals were Analar grade.

Animal Study Design. Male Sprague−Dawley rats were bred and raised at the Small Animals Production Unit, Massey University, Palmerston North, NZ, until they reached a body weight of approximately 300 g. The night before the experiment, food was withdrawn but water was continued ad libitum. The rats were divided into four groups with each group comprising four rats. Group A received Raspenol S (black raspberry); group B received Boyzenol S (boysenberry); group C received blueberry extract; and group D was treated as the control and was not treated. Anthocyanin extracts were dissolved in tap water and administered to each rat by stomach intubation. The amount of extract introduced to the stomach of each rat was determined by recording the weight of the syringe and intubation tube before and after the intubation procedure. After they were intubated, the rats were returned to their cages and provided with water but were not fed.

Exactly 60 min after intubation, each rat was anaesthetized with Halothane and a blood sample was collected by heart puncture. Plasma was immediately separated by centrifugation (2000*g*, 4 °C, 10 min) and acidified with a 20% volume of 5% trifluoroacetic acid (TFA). Urine samples were collected directly by bladder puncture and also acidified with a 20% volume of 5% TFA. All urine and plasma samples were frozen and then stored at −20 °C until analyzed. All animal procedures were approved in advance by the Animal Ethics Committee of Massey University.

Human Study Design. Five males (20−45 years) took part in the study. All were healthy and were not taking regular medication. The participation of all individuals was voluntary, and prior to the commencement of the experiment, all were informed of the requirements and experimental protocols and gave written informed consent. Experiments with each berry fruit extract occurred sequentially, with first blackcurrant, then boysenberry, and finally blueberry. The five participants were the same in all experiments, and a wash-out period of 7 days separated each berry fruit. Diet was not regulated between experiments.

Twenty-four hours prior to consuming berry fruit extract, each participant restricted their diet by avoiding foods containing phenolic compounds. All fruit and vegetables were avoided, as were beverages such as tea and coffee. On the morning of the experiment, each participant consumed 300 mL of water at 7:00 am and then an additional 300 mL of water every hour for the next 10 h. Blackcurrant or boysenberry concentrate or blueberry extract (total volume of 300 mL) were consumed at 10:00 am instead of 300 mL of water. Each participant voided their bladder at 1 h intervals commencing at 10:00 am until 4:00 pm, and urine samples were collected. For each urine

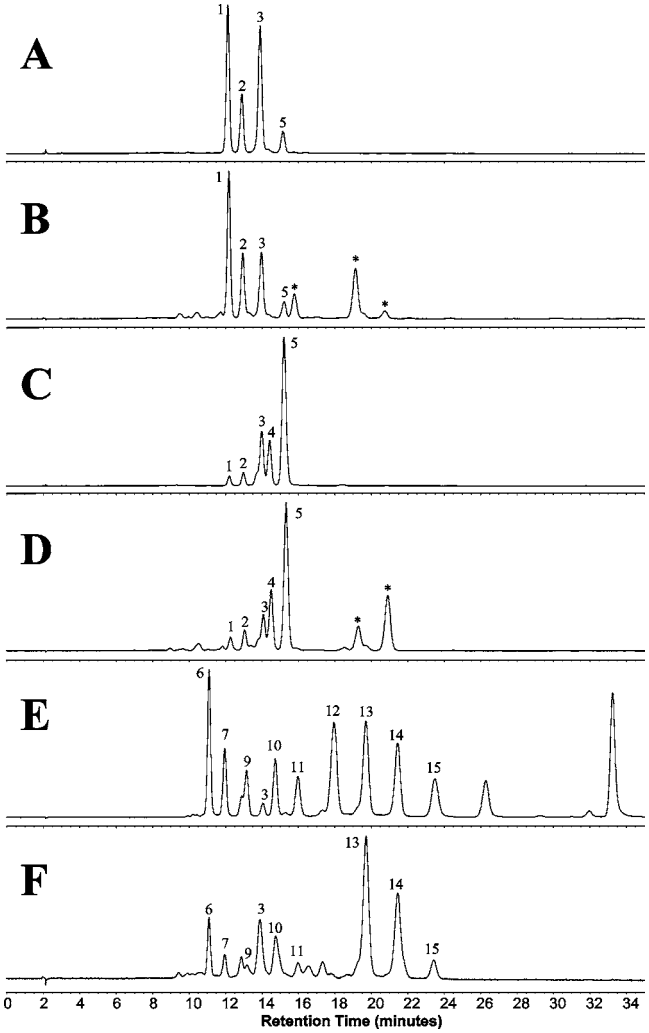

Figure 2. Chromatogram traces of anthocyanins present in berry extracts and rat urine following direct dosing. Boysenberry anthocyanins in (**A**) extract and (**B**) urine; black raspberry anthocyanins in (**C**) extract and (**D**) urine; blueberry anthocyanins in (**E**) extract and (**F**) urine. Peak identities are listed in **Figure 1**. Berry extracts were analyzed directly by HPLC; urine samples were first extracted by SPE as described under the Materials and Methods. Detection is at 530 nm.

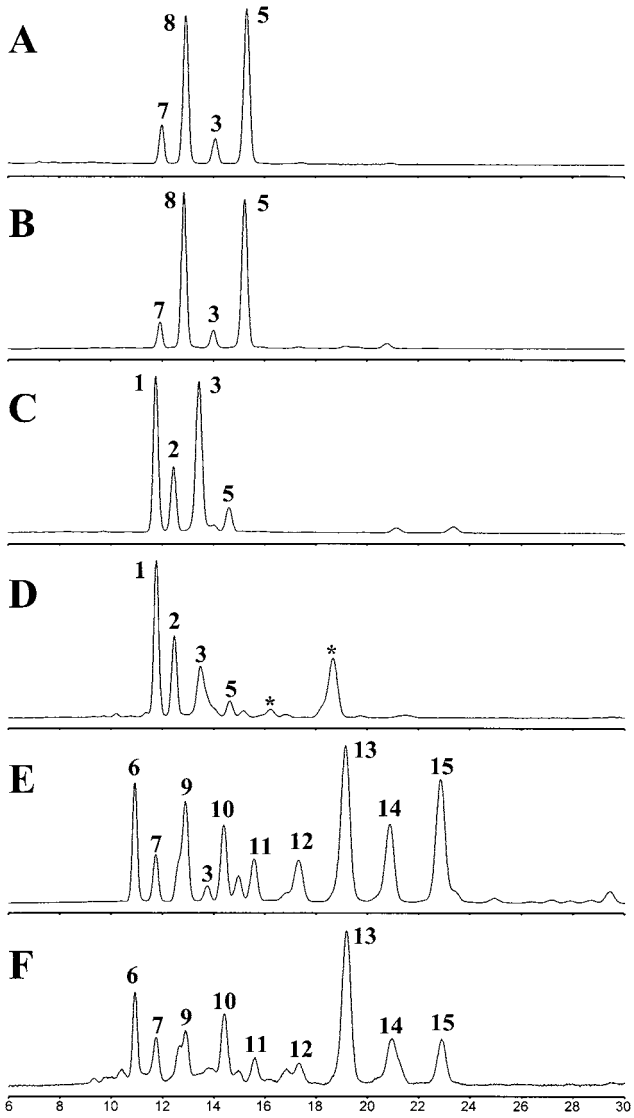

Figure 3. Chromatogram traces of anthocyanins present in berry extracts and human urine following direct dosing. Blackcurrant anthocyanins in (**A**) extract and (**B**) urine; boysenberry anthocyanins in (**C**) extract and (**D**) urine; blueberry anthocyanins in (**E**) extract and (**F**) urine. Peak identities are listed in **Figure 1**. Berry extracts were analyzed directly by HPLC, whereas urine samples were first extracted by SPE as described under the Materials and Methods. Detection is at 530 nm.

sample, total volume and time of collection were recorded. Aliquots of urine for creatinine and 8-hydroxy-deoxyguanosine analysis were stored at −20 °C. The rest of the sample was acidified with a 0.2 volume of 5% TFA and stored at 4 °C until analyzed.

HPLC Analysis of Anthocyanins. Anthocyanins concentrations in the urine samples were determined by HPLC following solid phase extraction (SPE). SPE cartridges (500 mg of C18 endcapped cat no. 221-0050H, ISI Chem) were conditioned with methanol (5 mL) and 5% formic acid (10 mL). Acidified urine (10 mL for the human study or total volume recovered from rat bladders) was applied, and the cartridge was washed with 5% formic acid (15 mL) and then ethyl acetate (5 mL). After ethyl acetate was removed, the bound anthocyanins were eluted with 5% formic acid in methanol (2 mL). The extract was dried under a stream of N_2 at 30 °C, and the residue was dissolved in 5% formic acid (300 μL). Samples were analyzed using a HPLC JASCO (LG-980-02 ternary gradient controller, AS-950 autosampler) equipped with a JASCO UV-975 UV/vis detector. The analytical column employed was a LiChrosphere 100 RP-18 endcapped column 5 μm 250 mm × 4 mm i.d. (Merck, Darmstadt, Germany) maintained at 35 °C. Separation of the anthocyanins was achieved using the following mobile phases: (A) 1.5% v/v H_3PO_4 and (B) formic acid: acetonitrile:H_3PO_4:water (20:26:1.5:56). The solvent gradient was as follows: initial composition 80% A 20% B; changing to 30% A 70% B at 25 min; then 10% A 90% B at 30 min; 10% A 90% B was held until 35 min, and then, the composition returned to 80% A 20% B (initial conditions) at 40 min before another sample injection at 45 min. All gradient curves were linear. The sample injection volume was 100 μL, and the detection wavelength was 530 nm. Chromatography data were collected and processed using a Water Millennium Chromatography Manager V4.0. Anthocyanin concentrations were calculated using a calibration curve for pure cyanidin 3-*O*-galactoside obtained from Polyphenols (Norway).

Liquid Chromatography−Mass Spectrometry (LC-MS) Analysis. LC-MS employed an LCQ Deca ion trap mass spectrometer fitted with an ESI interface (ThermoQuest, Finnigan, San Jose, CA) and coupled to a Surveyor HPLC and PDA detector. The analytical column employed was a LiChroCart Superpher^R 100 RP-18 endcapped column (Merck KgaA), 250 mm × 2 mm i.d. A 0.2 μm in-line filter (Alltech, Deerfield, IL) was installed before the analytical column, and the temperature of the column oven was maintained at 35 °C. Gradient elution was performed using 5%:95% formic acid:water v/v (solvent A) and 100% methanol (solvent B) as follows: the gradient was programmed using a linear solvent gradient from the initial mobile phase, 75% A/25% B to 40% A/60% B over 40 min, then to 100% B

Table 1. Amounts of Boysenberry, Black Raspberry, and Blueberry Anthocyanins Delivered by Gastric Intubation in Rats ($n = 3$) and the Anthocyanins Present in Urine 60 min after Dosing by Intubation[a]

	anthocyanin			
	consumed		excreted in urine	
	mg	normalized	μg/mL	normalized
	boysenberry			
cyanidin 3-*O*-sophoroside	2.28	100	0.80	100 (0)
cyanidin 3-*O*-2^G glucosylrutinoside	0.96	42.1	0.37	43.7 (6.6)
cyanidin 3-*O*-glucoside	2.35	103.2	0.36	40.8 (8.6)
cyanidin 3-*O*-rutinoside	0.41	17.9	0.12	15.9 (2.8)
total	6.0		1.65	
	black raspberry			
cyanidin 3-*O*-sophoroside	0.34	5.0	0.14	8.1 (0.1)
cyanidin 3-*O*-2^G glucosylrutinoside	0.48	7.1	0.21	12.2 (0.6)
cyanidin 3-*O*-glucoside	2.62	38.7	0.40	22.5 (1.5)
cyanidin 3-*O*-2^G xylosylrutinoside	1.87	27.7	0.65	37.7 (3.1)
cyanidin 3-*O*-rutinoside	6.77	100	1.76	100 (0)
total	12.08		3.25	
	blueberry			
delphinidin 3-*O*-galactoside	1.20	81.0	0.19	22.6 (1.9)
delphinidin 3-*O*-glucoside	0.63	42.3	0.08	10.5 (1.6)
delphinidin 3-*O*-arabinoside	0.48	32.1	0.03	5.1 (3.9)
cyanidin 3-*O*-galactoside	0.16	10.9	0.07	8.2 (0.6)
cyanidin 3-*O*-glucoside	0.14	9.6	0.25	30.4 (3.6)
cyanidin 3-*O*-arabinoside	0.05	3.5	n.d.	0.4 (0.6)
petunidin 3-*O*-galactoside	0.60	40.8	0.21	25.5 (3.0)
petunidin 3-*O*-glucoside	0.50	33.9	0.07	8.5 (1.0)
petunidin 3-*O*-arabinoside	1.52	102.8	0.02	3.6 (1.5)
malvidin 3-*O*-galactoside	1.48	100	0.85	100 (0)
malvidin 3-*O*-glucoside	1.06	71.9	0.57	67.0 (1.9)
malvidin 3-*O*-arabinoside	0.64	43.0	0.11	12.7 (0.6)
total	8.5		2.45	

[a] Values in brackets are standard deviations.

over 5 min, and held for 5 min before resetting to the original conditions. The flow rate was 250 μL/min, and the injection volume was 10 μL. The UV−vis detection wavelength was 530 nm.

Full scan MS data were acquired in the positive mode from *m*/*z* 250−2000. The ESI voltage, capillary temperature, sheath gas pressure, and auxiliary gas were set at 39 V, 300 °C, 65 psi, and 20 psi, respectively.

RESULTS

Animal Experiments. Representative HPLC chromatogram traces of the original berry fruit extract and urine extracts are provided in **Figure 2**. Each of the berry fruit extracts contained a different composition of anthocyanins that produced a distinctive HPLC chromatogram based on the retention times of the individual anthocyanins and the relative amounts. Urine from rats receiving each of the berry extracts contained anthocyanins compounds with similar retention times and relative concentrations (except as discussed below) as the extract used for dosing. The similarity of the chromatogram profiles between extracts and urine samples shows that anthocyanins containing different core aglycons and conjugated with a variety of mono-, di-, and trisaccharides and derived from different fruit are absorbed from the rat stomach and excreted in the urine without modification or metabolism. No anthocyanin was detected in plasma from any rat suggesting that the concentration of anthocyanins in plasma was below the detection limit of the method used.

This experiment was designed to confirm absorption of anthocyanins with different chemical structures rather than measure the portion of the dose that is bioavailable; therefore, the total amount of anthocyanin excreted was not determined. However, the relative concentrations of each anthocyanin in urine were determined and compared with those present in the berry extracts (**Table 1**). For boysenberry, the anthocyanin concentrations of cyanidin 3-*O*-2^G glucosylrutinoside and cyanidin 3-*O*-rutinoside, relative to cyanidin 3-*O*-sophoroside, were similar in both the berry fruit extract and the urine. In contrast, the relative concentration of cyanidin 3-*O*-glucoside was 60% less in the urine than in the berry fruit extract. This suggests that absorption and excretion of cyanidin 3-*O*-sophoroside, cyanidin 3-*O*-2^G glucosylrutinoside, and cyanidin 3-*O*-rutinoside differ from that of cyanidin 3-*O*-glucoside. A similar observation was made with black raspberry anthocyanins, although the effect was not as marked. Different anthocyanins are present in blueberry as compared with boysenberry and black raspberry. In particular, blueberry contains monoglycosylated anthocyanins conjugated to five different aglycons; however, cyanidin-based anthocyanins are minor components. For blueberry anthocyanins, concentrations were calculated relative to malvidin 3-*O*-galactoside. The relative concentration of malvidin 3-*O*-glucoside was similar in both the urine and the berry extract suggesting that the determinants of absorption and excretion for malvidin 3-*O*-glucoside and malvidin 3-*O*-galactoside are similar. In contrast, the relative concentrations of anthocyanins based on the aglycons of delphinidin, cyanidin, and petunidin and conjugated with either galactose or glucose were decreased in the urine as compared with the extracts. All four arabinose-conjugated anthocyanins measured demonstrated substantially lower relative concentrations in urine than in the berry extracts. This was particularly evident for petunidin 3-*O*-arabinoside where the relative concentration was 102.8% in the extract but was only 3.6% in the urine. This suggests that malvidin 3-*O*-galactoside and petunidin 3-*O*-arabinoside are absorbed and excreted in substantially different ways. This comparison of

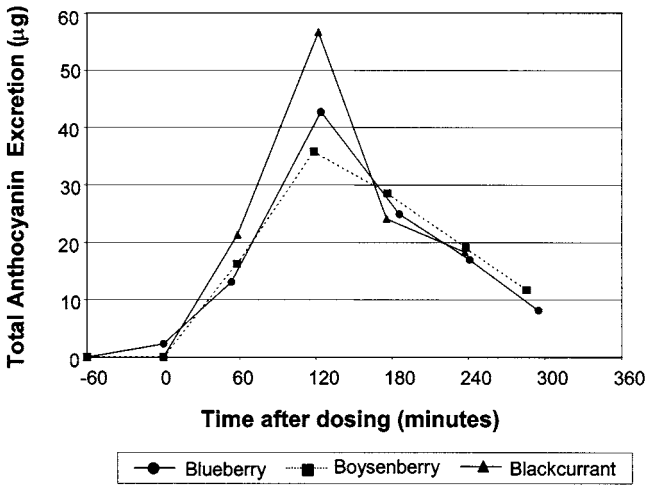

Figure 4. Excretion profiles of berry fruit anthocyanins in human urine following dosing with blueberry (439 mg), boysenberry (344 mg), and blackcurrant (188 mg) anthocyanins. The results represent the mean of five individuals.

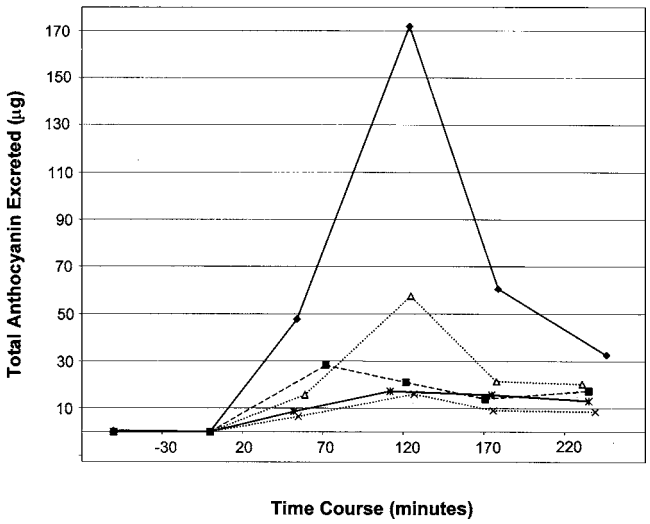

Figure 5. Excretion profiles of boysenberry anthocyanins in human urine following dosing showing the variation between the five individual participants.

differences in relative concentrations of anthocyanins in berry extracts and excreted in the urine suggests that the determinants of absorption and excretion of anthocyanins in rat are dependent on both the nature of the aglycon and the sugar conjugate.

Human Experiments. Similar to the results observed for rats, the anthocyanin profile of the berry extract ingested resembled those detected in urine samples after 1 h and then in subsequent samples. Example chromatograms of the anthocyanin profiles are shown in **Figure 3**. For each berry extract, the maximal excretion occurred approximately 120 min after dosing and then declined during the following 3 h. Anthocyanins were still readily detected in the urine 5 h after dosing. The time course for anthocyanin excretion is shown in **Figure 4**. The results presented in **Figure 4** are the mean values for the five subjects. To improve clarity, the errors are not included because there appears to be substantial variation between individuals in the amounts of anthocyanins excreted. This variation among the five subjects is shown for boysenberry in **Figure 5**.

The total amount of anthocyanin excreted as a percentage of the amount consumed is less than 0.1% **(Table 2)** for all anthocyanins. However, despite the high level of intersubject variation, the percentage of dose excreted for each of the anthocyanins appears to differ suggesting that the bioavailability of anthocyanins in humans varies according to their chemical structure. To compare absorption and excretion parameters for individual anthocyanins, concentrations for each sample were normalized within each sample and mean values are presented in **Table 2**. Similar to the rat experiment, for the boysenberry extract, the relative concentration of cyanidin 3-*O*-2^G glucosylrutinoside and cyanidin 3-*O*-rutinoside in urine was similar to the berry extract. This suggests that cyanidin 3-*O*-2^G glucosylrutinoside, cyanidin 3-*O*-rutinoside, and cyanidin 3-*O*-sophoroside all behave similarly with respect to absorption and excretion. In contrast, the relative concentration of cyanidin 3-*O*-glucoside was less in the urine as compared with the berry extract, an effect also observed in the rats above. For blackcurrant, anthocyanins concentrations (relative to cyanidin 3-*O*-rutinoside) were similar in the urine and berry extract for delphinidin 3-*O*-rutinoside but were less in the urine for cyanidin 3-*O*-glucoside and delphinidin 3-*O*-glucoside. Additionally, bioavailability, defined as the percentage excreted in the urine, was apparently lower for delphinidin 3-*O*-glucoside (0.042%) and cyanidin 3-*O*-glucoside (0.040%) as compared with delphinidin 3-*O*-rutinoside (0.067%) and cyanidin 3-*O*-rutinoside (0.63%), although intersubject variation is high (**Table 2**). Overall, these results for boysenberry and blackcurrant anthocyanins suggest that the determinants of absorption and excretion are influenced by structure of the conjugated sugar in particular conjugation with a single glucose. These data suggest that the nature of the sugar conjugate may largely determine differences in bioavailability between anthocyanins.

The anthocyanins present in the blueberry extract allow comparison of bioavailability for anthocyanins with different aglycons and monosaccharide combinations. As in the rat experiment, anthocyanin concentrations are presented relative to malvidin 3-*O*-galactoside. The relative concentration of malvidin 3-*O*-glucoside in urine was similar to that in the berry extract, whereas for malvidin 3-*O*-arabinoside the concentration was markedly reduced. For the delphinidin-based anthocyanins, relative concentrations of the galactoside and arabinoside conjugates decreased but increased for the glucoside, whereas for the petunidin-based anthocyanins, the galactoside remained constant but the glucoside and arabinoside decreased. Overall, changes in the relative concentrations of galactose and glucose vary for different aglycons. However, for arabinose conjugates, the relative concentrations were decreased in urine as compared with berry extract for all aglycons, suggesting that arabinose has a marked effect on bioavailability. The effect of aglycon on absorption and excretion is shown by the galactosides of malvidin, petunidin, and delphinidin. The relative concentrations of malvidin 3-*O*-galactoside and petunidin 3-*O*-galactoside were similar whereas the relative concentration of delphinidin 3-*O*-galactoside in urine decreased. This suggests that the increasing hydroxylation present in delphinidin is a determinant of bioavailability. Taken together, these data from the human study suggest that the determinants of absorption and excretion of anthocyanins are influenced by the nature of the sugar conjugate but further modulated by the nature of the aglycon.

HPLC Peak Identification. The absorption and excretion of anthocyanins differ from that of other phenolics as they appear to be largely excreted unmetabolized in the glycosylated form in which they were administered. To validate this interpretation, positive identification of the anthocyanins represented by the HPLC peaks detected in urine following dosing with dietary anthocyanins is necessary. Initial peak identity of

Table 2. Amounts of Blackcurrant, Boysenberry, and Blueberry Anthocyanins Consumed by Human Volunteers ($n = 5$) and the Total Amounts Excreted in the Urine over the Subsequent 7 h Period[a]

	anthocyanins				
	consumed		excreted in urine		
	mg	normalized	μg	percent total	normalized
		boysenberry			
cyanidin 3-*O*-sophoroside	120.8	100	31.6	0.026 (0.012)	100 (0)
cyanidin 3-*O*-2^G glucosylrutinoside	53.8	44.6	15.9	0.030 (0.018)	48.8 (8.66)
cyanidin 3-*O*-glucoside	147.0	121.6	17.7	0.012 (0.006)	56.0 (9.96)
cyanidin 3-*O*-rutinoside	22.9	18.9	3.7	0.013 (0.007)	12.2 (1.86)
total	344.5		99.0	0.029	
		blackcurrant			
delphinidin 3-*O*-glucoside	17.2	20.0	7.2	0.042 (0.041)	12.6 (2.03)
delphinidin 3-*O*-rutinoside	72.2	84.2	48.4	0.067 (0.067)	86.4 (8.35)
cyanidin 3-*O*-glucoside	13.0	15.2	5.2	0.040 (0.040)	8.5 (1.77)
cyanidin 3-*O*-rutinoside	85.8	100	58.2	0.063 (0.056)	100 (0)
total	188.5		120.4	0.064	
		blueberry			
delphinidin 3-*O*-galactoside	39.5	39.7	7.1	0.018 (0.007)	26.5 (1.87)
delphinidin 3-*O*-glucoside	17.3	17.4	5.2	0.030 (0.011)	20.0 (2.11)
delphinidin 3-*O*-arabinoside	53.8	54.0	7.2	0.013 (0.004)	28.2 (2.66)
cyanidin 3-*O*-glucoside	6.3	6.3	1.2	0.019 (0.007)	4.7 (2.70)
cyanidin 3-*O*-arabinoside	11.0	11.0	1.1	0.010 (0.003)	4.3 (0.87)
petunidin 3-*O*-galactoside	32.8	33.0	8.9	0.027 (0.011)	34.2 (2.61)
petunidin 3-*O*-glucoside	19.3	19.3	2.5	0.013 (0.004)	9.7 (0.91)
petunidin 3-*O*-arabinoside	25.6	25.7	3.2	0.012 (0.004)	12.5 (1.77)
malvidin 3-*O*-galactoside	99.6	100	25.8	0.026 (0.009)	100 (0)
malvidin 3-*O*-glucoside	49.5	49.7	9.9	0.020 (0.006)	38.5 (4.90)
malvidin 3-*O*-arabinoside	81.6	81.9	8.1	0.010 (0.004)	30.4 (2.45)
total	439.1		87.3	0.020	

[a] Values in brackets are standard deviations.

urinary anthocyanins was based on detection at 530 nm, the similarity of retention times by HPLC, and the fact that these compounds were retained during the sample cleanup procedure, which was designed to remove nonanthocyanin phenolic compounds. Additional peak identification by LC-PDA and LC-MS was performed on representative urine extracts resulting from the boysenberry and blackcurrant experiments. The LC-MS results are shown in **Figure 6** for boysenberry and **Figure 7** for blackcurrant. LC-MS analysis (using electrospray ionization) of anthocyanins provides information on both the molecular ion and a fragment ion representing the phenolic aglycon portion of the molecule (*26*). **Figure 6** shows clearly that the molecular ions of the four anthocyanins detected in the boysenberry extract were also detected in the urine extracts. Similar results were obtained for blackcurrant (**Figure 7**). The identities of the anthocyanins were also confirmed by LC-PDA (data not shown). These results demonstrate that the same glycosylated anthocyanins consumed by human subjects are present unmetabolized in the urine.

DISCUSSION

Although much evidence exists to support the role of fruit and vegetable consumption in reducing the incidence of degenerative diseases (*27*), as yet, there is a paucity of information relating to the bioabsorption and metabolism of flavonoids in humans and a general lack of understanding of the mechanisms involved. Recent studies have shown that some phenolics frequently found in the diet such as catechin and quercetin glycosides are absorbed (*9*, *10*). Studies, with mainly cyanidin-based anthocyanins conjugated with glucose, have shown that these anthocyanins are also absorbed. However, there appears to be several important differences in the absorption of anthocyanins as compared with other dietary phenolics. First, at dietary relevant doses, it seems that essentially all of the absorbed catechin or quercetin absorbed is present as metabolites, that is, conjugates of glucuronic acid or sulfate (*9*, *14*). In contrast, anthocyanins are also absorbed by both rats and humans but are excreted largely unmetabolized (*16*, *21*−*24*, *28*). The results presented here confirm previous studies and extend the observations to include an array of anthocyanins with a variety of sugar conjugates obtained from several berry sources. Second, we found that the bioavailablity of anthocyanins, expressed as the percentage of the ingested dose recovered from urine, is low, confirming results from previous studies that the apparent bioavailability of anthocyanins is substantially lower than for catechin or quercetin. We further show that anthocyanin absorption appears to be influenced by the chemical structure of both the phenolic aglycon and the sugar conjugate.

Anthocyanins (Glycosylated Anthocyanidins) Are Absorbed and Excreted Unmetabolized. As noted above, previous studies have shown that the original glycosylated anthocyanins appear in plasma and are excreted in the urine of either rats or humans. Chemical confirmation of peak identity varies with study but generally includes detection at 530 nm and similarity of retention time to authentic standards using reversed-phase HPLC. A few studies have confirmed the identity of the anthocyanins by determining the molecular weight and the nature of the aglycon for the bioabsorbed anthocyanin using LC-MS. The berry anthocyanins investigated in this study included compounds with the aglycon delphinidin, cyanidin, petunidin, peonidin, and malvidin and cyanidin anthocyanins conjugated with mono-, di-, or trisaccharides. Confirmation of absorption of all of these compounds was obtained using LC-MS. Several of these anthocyanins are relatively large and polar, particularly those conjugated with a di- or trisaccharide, and are unlikely to be passively absorbed from the gastrointes-

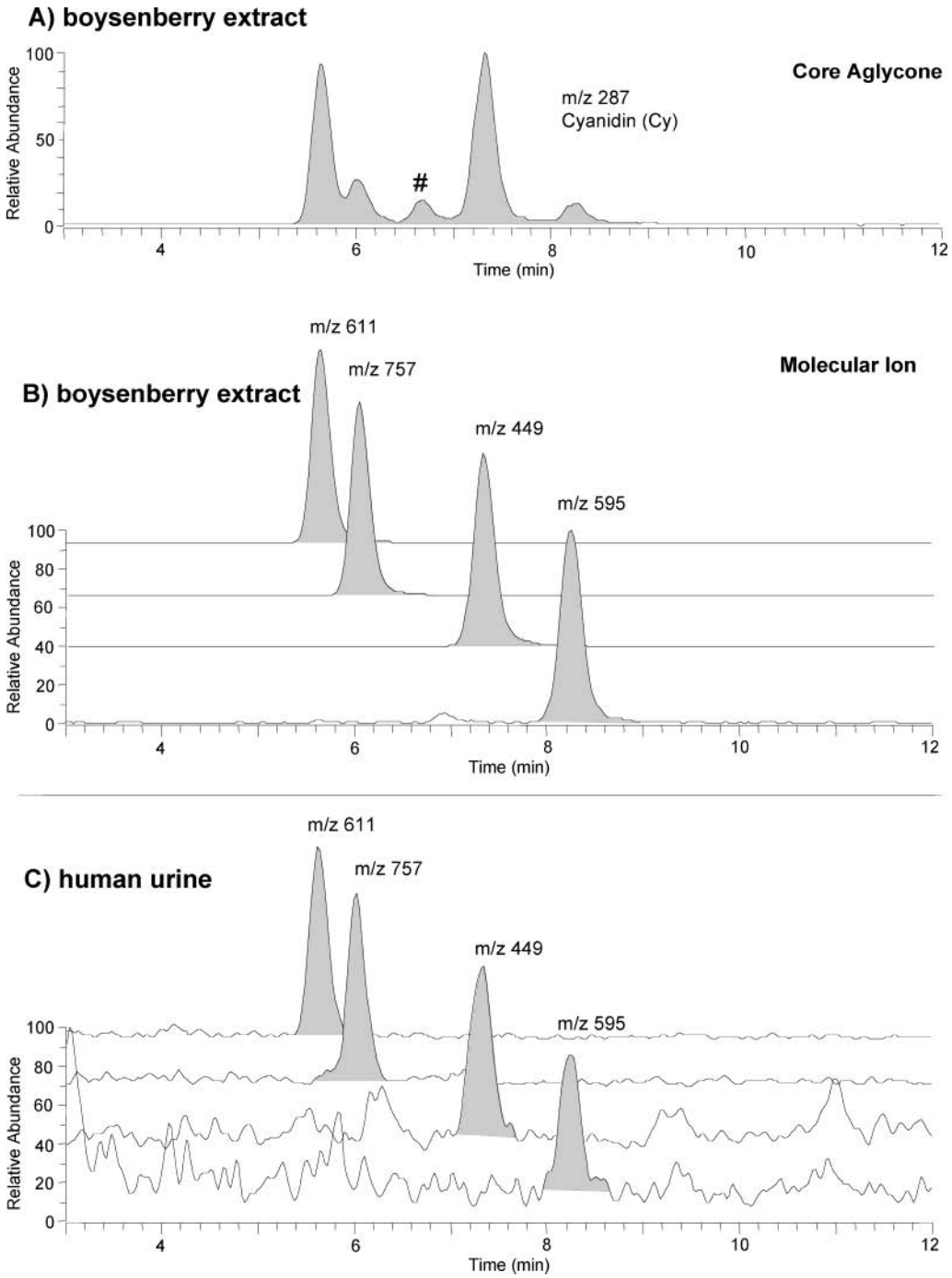

Figure 6. LC-MS confirmation of anthocyanin HPLC peaks detected in human urine following consumption of boysenberry extract. (**A**) Selected ion (*m*/*z* 287) trace of boysenberry extract confirming the presence of cyanidin-based anthocyanins in boysenberry (the peak marked # was later shown to be an artifact). (**B**) Selected ion traces of the molecular ions of the four major anthocyanins present in boysenberry. (**C**) Selected ion traces of a human urine sample.

tinal tract. For example, in the case of boysenberry extract, cyanidin 3-*O*-2^{G} glucosylrutinoside has a molecular weight of *m*/*z* 757 but the evidence presented here demonstrates that cyanidin 3-*O*-2^{G} glucosylrutinoside is apparently excreted in an unmetabolized form by both rats and humans after ingestion. In addition to the peaks representing unmetabolized anthocyanins, several additional peaks were observed in HPLC chromatograms at 530 nm. These peaks had longer retention times and were particularly evident in rats and humans receiving boysenberry, black raspberry, and blackcurrant extracts (marked with an "*" in **Figures 2B**,**D** and **3D**). These additional "anthocyanin-like" compounds have been reported by others and may be metabolites of anthocyanins. Recently, Wu et al. (*24*) reported that methylated and glucuronidated anthocyanin metabolites were present in urine from elderly women following ingestion of elderberry extract. Additional work is in progress to identify the peaks observed in the current study.

Chemical Structure Affects Bioavailibility. The utilization of a variety of fruits made it possible to investigate the affects of anthocyanin structure on absorption and excretion. All anthocyanins studied were absorbed and excreted to some extent by both rats and humans when dosed with dietary relevant amounts. However, variation in the relative amounts absorbed and excreted was observed in both the rat and the human experiments. In particular, cyanidin 3-*O*-glucoside in the rat study and cyanidin 3-*O*-glucoside and delphinidin 3-*O*-glucoside

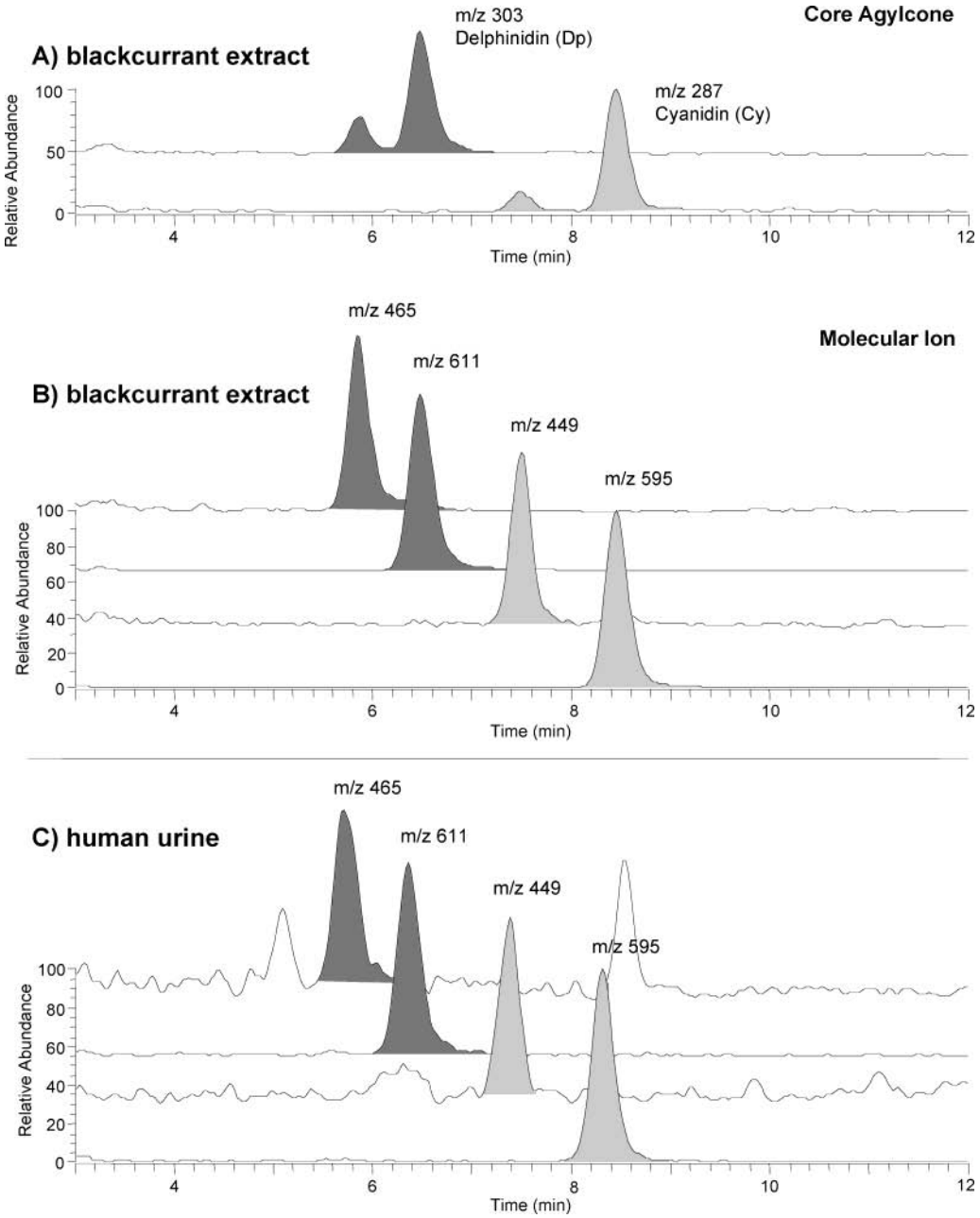

Figure 7. LC-MS confirmation of anthocyanin HPLC peaks detected in human urine following consumption of blackcurrant extract. (**A**) Selected ion (*m/z* 287 and 303) traces of blackcurrant extract confirming the presence of cyanidin and delphinidin-based anthocyanins in boysenberry. (**B**) Selected ion traces of the molecular ions of the four major anthocyanins present in boysenberry. (**C**) Selected ion traces of a human urine sample.

in the human study appear to be less bioavailable than other anthocyanins as indicated by lower concentrations relative to other anthocyanins detected in rat and human urine (**Tables 1** and **2**). There are a number of possible explanations for this. First, the absorption of glucose conjugates may be less than other anthocyanins, for example, those containing rutinose. Second, cyanidin 3-*O*-glucoside has a higher antioxidant capacity than other anthocyanins and therefore may be more readily oxidized in vivo. Third, various cells are known to contain specific transport mechanisms for glucose-containing compounds. Cyanidin 3-*O*-glucoside and delphinidin 3-*O*-glucoside might be transported into cells and tissues from the plasma more rapidly than other anthocyanins. These processes could each result in a decreased concentration in the urine relative to the other anthocyanins in the dose; however, the current data do not enable these alternatives to be distinguished. The relative concentrations of anthocyanin conjugates containing arabinose were also markedly decreased indicating either reduced bioavailability or greater chemical reactivity once absorbed.

The nature of aglycon of the anthocyanin also influenced the relative concentrations of excreted anthocyanin with a reduction in concentration of delphinidin-based as compared with malvidin-based anthocyanins. This may be a result of the greater number of hydroxyl groups in delphinidin (**Figure 1**) or the greater hydrophobic nature of malvidin facilitating increased partitioning into cells and tissues. Overall, the results of this study suggest that the bioavailability of anthocyanins is modulated by both the nature of the sugar conjugate and the phenolic aglycon. A greater understanding of the mechanisms associated with bioavailability should enable the development of tailored combinations of anthocyanins with improved bioavailability and enhanced in vivo function.

Limitations of Analytical Methods May Influence Estimates of Bioavailability and Biological Significance. Assess-

ments of anthocyanin bioavailability are consistently low as compared with other phenolic compounds such as quercetin and catechin. Anthocyanins are distinguished from other flavonoids by a number of chemicophysical properties. In particular, anthocyanins undergo molecular rearrangements in response to the pH of the chemical environment (*29*). Thus, they can be present as a number of interrelated forms such as the red flavylium cation at low pH (1−3) or the blue quinonoidal anion at pH 7−12. In an aqueous environment, the addition of a water molecule at position 2 produces the colorless hemiketal, which then undergoes molecular rearrangement to the cis and trans chalcone forms. These forms are all in equilibrium, and the relative amounts are dependent on the molecular structure of the anthocyanin and the pH. Anthocyanins are traditionally extracted and characterized as the red flavylium cation, as this is the most stable form. However, it is not known which molecular structure predominates in vivo. Robust analytical methods for the alternative forms do not exist, and consequently, these particular structures have been studied little and have not been studied in vivo.

In an effort to determine which anthocyanin form is present in vivo, the stomach and small intestines of several rats were dissected and opened with a longitudinal cut, 60 min after dosing with boysenberry extract. In the stomach, an intense red color indicated the presence of anthocyanins as the red flavylium cation. In contrast, no anthocyanins as the flavylium ion (red color) were visible in the intestine, as it was the same color as the intestine of an untreated rat. However, when a 5% trichloroacetic acid solution was added dropwise to the intestine, a red color appeared within 15 s indicating the conversion of a colorless form of anthocyanins into the red flavylium cation. We did not determine if the presumed anthocyanin observed had been absorbed into the epithelial tissue or was simply present as retained intestinal contents. Regardless, these observations raise several significant issues relating to the in vivo bioactivity of anthocyanins. At the neutral pH of the intestine and in the plasma, anthocyanins are probably not present as the flavylium cation but as the colorless hemiketal or chalcone forms. Importantly, the ability to detect anthocyanins by HPLC in vivo is based on the ability to transform colorless forms (or metabolites thereof) back into the flavylium cation. Metabolic conversion that prevents this from occurring will prevent these compounds from being detected by the current HPLC techniques. It is possible that anthocyanin bioavailability is currently underestimated due to an incomplete understanding of their metabolism and inadequate assessment of their in vivo forms. Further investigations are required to more fully understand the in vivo bioactivity of anthocyanins.

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