JOURNAL OF NATURAL PRODUCTS

ARTICLE

pubs.acs.org/jnp

Exceptionally Fast Uptake and Metabolism of Cyanidin 3-Glucoside by Rat Kidneys and Liver

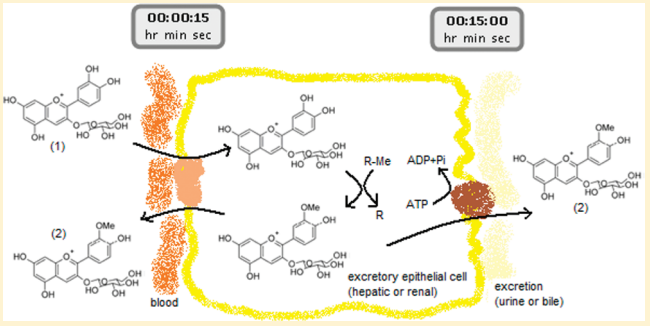
Andreja Vanzo,[†] Urska Vrhovsek,[‡] Federica Tramer,[§] Fulvio Mattivi,[‡] and Sabina Passamonti*[,§]

[†]Central Laboratory, Agricultural Institute of Slovenia, Hacquetova 17, 1000 Ljubljana, Slovenia

[‡]Food Quality and Nutrition Area, IASMA Research and Innovation Centre, Edmund Mach Foundation, Via E. Mach 1, 38010 San Michele all'Adige, Italy

[§]Department of Life Sciences, University of Trieste, Via L. Giorgieri 1, 34127 Trieste, Italy

ABSTRACT: To asses the hypothesis that anthocyanins are rapidly taken up from the blood into tissues, where they accumulate up to their bioactivity threshold, an intravenous dose of cyanidin 3-glucoside (**1**) was administered to anaesthetized rats. Cyanidin 3-glucoside (**1**) and its metabolites were analyzed in the plasma, kidneys, liver, urine, and bile, using last-generation mass spectrometry. Compound **1** was found to rapidly disappear from plasma ($t/2$ = 0.36 min). As soon as 15 s after its administration, both **1** and its methylation product, peonidin 3-glucoside (**2**), were detected in the plasma, kidneys, and liver. At 1 min, both **1** and **2** had almost disappeared from the plasma, but attained their peak concentrations in the kidneys and in the liver. Compound **2** was rapidly excreted both in the bile and in the urine. Three additional methylated metabolites were detected in traces, namely, delphinidin 3-glucoside (**3**), petunidin 3-glucoside (**4**), and malvidin 3-glucoside (**5**). These data contribute to solving the paradox of the high bioactivity of anthocyanins in spite of their apparent low bioavailability.



Anthocyanis are a subclass of flavonoids occurring in fruits, vegetables, and beverages. Though their intake may range from 12.5[1] to about 200 mg/day,[2] their bioavailability in humans is low, as reflected by postabsorption peak concentrations in plasma in the range 10^{-8}–10^{-7} M.[3] Nevertheless, numerous observations have suggested their remarkable bioactivity, as potential anti-inflammatory,[4] anticarcinogenic,[5] antineurodegenerative,[6] normolipidemic,[7] and normoglycemic agents.[8] Thus, it might be inferred that anthocyanins are rapidly taken up from the blood into tissues, where they might accumulate and attain their bioactivity threshold.

This study was aimed to directly assess uptake and metabolism of the dietary anthocyanin cyanidin 3-glucoside (**1**) into the kidneys and liver following intravenous administration to anaesthetized rats. Although compound **1** could be taken up in other organs, this study was limited to the main excretory organs, where an in-depth investigation was needed to give a reasonably clear description of its cellular fate. A distinctive feature of this work is the accurate characterization of the fate of **1** at so far unexplored time points less than 5 min after administration. There are indeed only a few studies of anthocyanin pharmacokinetics following intravenous injection,[9−13] and just one examined the plasma of rats injected with a similar dose of **1**.[14] None of these investigations have examined plasma anthocyanins at times as short as those employed in this study, i.e., only 15 s after administration.

The data obtained show that the kinetics of the transfer of **1** from the blood into the investigated tissues was challengingly rapid. Furthermore, **1** was rapidly methylated to peonidin 3-glucoside (**2**) and excreted in the urine and bile. Such a rapid uptake and methylation of **1**, followed by an active transport in the urine and in the bile, helps to solve the paradox of the observed high bioactivity of anthocyanins in spite of their apparent low bioavailability.

RESULTS AND DISCUSSION

Cyanidin 3-glucoside (**1**) dissolved in saline solution (0.67 μmol, 76 μM in plasma) was injected into the iliac vein of fasting anaesthetized rats. Rats were sacrificed at different times to analyze their plasma, bile, urine, kidneys, and liver. Tables 1 and 2 and Figure 1 show the amounts and concentrations of **1** and its metabolites.

Cyanidin 3-Glucoside (1) and Peonidin 3-Glucoside (2) in the Plasma. Figure 1A shows the amounts of **1** and **2** found in the plasma of rats 15 s and 1, 5, and 15 min after intravenous administration of cyanidin 3-glucoside. The first-order rate constant of the elimination of **1** was 1.9 ± 0.0 min^{-1}. The time zero dose calculated by curve extrapolation was 0.25 ± 0.00 μmol, i.e., ca. 37% of the administered dose (0.67 μmol). The most critical factor in the inability to obtain an extrapolated value

Received: December 26, 2010
Published: April 21, 2011

Table 1. Amounts and Concentrations of Cyanidin 3-Glucoside (1) and Its Major Metabolite Peonidin 3-Glucoside (2) Detected in Plasma, Kidneys, Liver, Urine, and Bile of Rats at Different Times after Intravenous Administration of 1

		cyanidin 3-glucoside (**1**)		peonidin 3-glucoside (**2**)	
time (min)	rats[b]	(nmol)[a]	(μM)	(nmol)[a]	(μM)
plasma					
0.25	4	160.1 ± 22.9	18.2 ± 2.6	10.4 ± 1.4	1.2 ± 0.1
1	4	50.2 ± 7.8	5.7 ± 0.9	4.2 ± 0.5	0.5 ± 0.0
5	8	14.2 ± 1.1	1.6 ± 0.1	2.6 ± 0.3	0.3 ± 0.0
15	9	14.2 ± 3.4	1.6 ± 0.4	4.9 ± 0.2	0.6 ± 0.2
kidneys					
0.25	4	27.1 ± 3.4	11.3 ± 1.4	46.0 ± 8.6	19.2 ± 3.7
1	4	29.8 ± 2.9	12.4 ± 1.2	99.5 ± 5.3	41.5 ± 2.2
5	8	10.7 ± 4.0	4.5 ± 1.6	38.8 ± 11.5	16.1 ± 4.8
15	9	10.6 ± 2.9	4.4 ± 1.2	49.2 ± 13.1	20.5 ± 5.5
liver					
0.25	4	2.5 ± 0.3	0.4 ± 0.0	41.1 ± 9.5	5.9 ± 1.4
1	4	2.5 ± 0.6	0.4 ± 0.1	80.4 ± 20.4	11.5 ± 2.9
5	8	0.1 ± 0.1	0.02 ± 0.00	34.5 ± 9.9	4.9 ± 1.4
15	9	0.4 ± 0.2	0.1 ± 0.0	31.9 ± 8.0	4.6 ± 1.1
urine					
15	9	41.3 ± 13.5	316.6 ± 119.2	98.7 ± 29.5	706.9 ± 136.4
bile					
15	4	0.4 ± 0.1	2.3 ± 0.8	58.5 ± 20.9	397.9 ± 105.8

[a] Amounts in tissues are calculated by assuming that rat plasma volume was 8.8 mL (http://www.ratbehavior.org/Stats.htm), urine and bile volume were measured, and the kidney and liver masses were 2.4 and 7.0 g, respectively. [b] Number of investigated rats.

more similar to the administered dose seemed to be the very high rate of the plasma elimination of **1**. The estimated steady state amount of **1** calculated by the disappearance curve was 14.2 ± 0.0 nmol, resulting in about 1.5 μM plasma concentration. Even though the gastrointestinal epithelium barrier was bypassed, this plasma concentration was very similar to what could be observed following oral administration.[3] Peonidin 3-glucoside (**2**) was detected in plasma as soon as 15 s after intravenous administration of **1**. Its amount was 10.4 ± 1.4 nmol, i.e., 6.5% of its precursor **1** (160.1 ± 22.9 nmol) (Table 1). At 1 min, compound **2** in plasma declined to an estimated steady-state amount of 3.8 ± 1.1 nmol, when calculated as above.

Cyanidin 3-Glucoside (1) and Peonidin 3-Glucoside (2) in the Kidneys and Liver. Figure 1B shows the amounts of **1** and **2** found in the kidneys and liver of rats 15 s and 1, 5, and 15 min after intravenous administration of **1**. Compound **1** appeared in the kidneys and the liver as soon as at 15 s, peaked at 1 min, and then decreased to the steady state at about 5 min. The steady-state amounts of cyanidin 3-glucoside in the kidneys and in the liver, calculated by averaging data obtained at 5 min (n = 8) and 15 min (n = 9), were 10.7 ± 2.4 and 0.3 ± 0.1 nmol, respectively. Both at the peak and at the steady state, the amounts in the kidneys were significantly (p < 0.05) higher than in the liver (12- and 37-fold, respectively). In both organs, **2** also appeared as soon as at 15 s. It peaked at 1 min, with similar amounts in both the kidneys and the liver (99.5 ± 5.3 and 80.4 ± 20.4 nmol, respectively; p = 0.40). At 1 min, the **2**/**1** ratios were 3.3 in the kidneys and 32.2 in the liver. At 5 min, compound **2** in the kidneys and the liver declined to an estimated steady-state amount of 44.3 ± 8.6 and 33.1 ± 6.1 nmol, respectively (calculated as above).

Cyanidin 3-Glucoside (1) and Peonidin 3-Glucoside (2) in Urine and Bile. Compounds **1** and **2** were detected in the urine and bile collected at 15 min (Table 1). Compound **1** was more abundant in the urine than in the bile (41.3 ± 13.5 and 0.4 ± 0.1 nmol, respectively; p < 0.05). Compound **2** was similarly abundant in both the urine and bile (98.7 ± 29.5 and 58.5 ± 20.9 nmol, respectively; p = 0.39). The **2**/**1** ratios in the urine and bile were 2.4 and 146.2, respectively. The ratio in the urine reflects that in the kidneys (3.3), but the ratio in the bile was 5-fold higher than in the liver (32.2).

Minor Metabolites of Cyanidin 3-Glucoside (1). The superior sensitivity obtained with tandem MS detection also allowed us to detect and quantify minor metabolites, present at trace levels (pmol). Like major metabolite **2**, minor metabolites of **1** were also products of B-ring methylation (Table 2, Figure 2). Delphinidin 3-glucoside (**3**) appeared transiently in the plasma, kidneys, and liver at 1 min at very low concentrations. Petunidin 3-glucoside (**4**) was detected in only trace amounts in the plasma. It was found in higher amounts in the kidneys at 5 and 15 min, probably following the appearance of **3**, from which it was presumably derived. The liver, urine, and bile contained no **4**. Malvidin 3-glucoside (**5**) was detected in only trace amounts in the plasma from 1 min on. In the kidneys, it was detected only at 5 and 15 min, whereas in the liver it was found only at 1 min. Again, the amounts were higher in the kidneys than in the liver, and it was detected in the urine, but not in the bile.

Rapid Uptake and Efflux from the Kidneys and Liver. The timeline of these tests is justified by the fact that the rat heart rate is about 120 beats/min. This ensures that the injected dose was diluted homogeneously in the blood even 15 s after intravenous administration. Since peonidin 3-glucoside (**2**) was detected in the plasma as early as at 15 s, it must be concluded that the following events occurred in 15 s: (a) cellular uptake, (b) methylation, and (c) transport of **2** back to the plasma. These events took place to cause the rapid plasma disappearance of cyanidin 3-glucoside (**1**). Given their physicochemical features, membrane transport of both compounds **1** and **2** would require carrier-mediated mechanisms.[15] The anthocyanin-specific membrane transporter bilitranslocase (T.C. 2.A.65.1.1)[16] is expressed at the basolateral (vascular) domain of both liver parenchymal cells and kidney tubular cells[17] and has, therefore, the intrinsic capacity to mediate both influx of **1** into cells and efflux of its metabolites back to the blood.[18] At 15 s, the plasma/tissue ratio of **1** was strikingly different in the kidneys (ca. 1) as compared to the liver (ca. 0.02). This might be the effect of the dissimilar kinetic properties of bilitranslocase isoforms in the kidneys and the liver, with the former having lower affinity than the latter.[17] As a consequence, kidney bilitranslocase might increase its rate of transport over a wider plasma concentration range than liver bilitranslocase. The involvement of other membrane transporters in the cellular transport of anthocyanins cannot be ruled out, though they have not yet been identified.

Rapid Methylation in Kidneys and Liver. Methylation of **1** to **2** was the major biotransformation step in both the kidneys and the liver, as is documented elsewhere.[3,13,19] It was both fast and thorough, being detected already after 15 s and accounting for 63% and 94% of the total anthocyanins found in the kidneys and the liver, respectively. The enzyme probably involved is catechol *O*-methyl transferase (COMT, EC 2.1.1.6), known to be active

Table 2. Amounts and Concentrations of Minor Metabolites Detected in Plasma, Kidneys, Liver, Urine, and Bile of Rats at Different Times after Intravenous Administration of Cyanidin 3-Glucoside (1)

		minor metabolites					
		delphinidin 3-glucoside (3)		petunidin 3-glucoside (4)		malvidin 3-glucoside (5)	
time (min)	rats[b]	(pmol)[a]	(nM)	(pmol)[a]	(nM)	(pmol)[a]	(nM)
plasma							
0.25	4	nd[c]	nd[c]	15 ± 15	2 ± 2	nd[c]	nd[c]
1	4	265 ± 75	30 ± 9	25 ± 17	2 ± 2	83 ± 83	10 ± 10
5	8	nd[c]	nd[c]	nd[c]	nd[c]	47 ± 26	6 ± 4
15	9	nd[c]	nd[c]	nd[c]	nd[c]	245 ± 172	28 ± 20
kidneys							
0.25	4	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]
1	4	138 ± 34	58 ± 15	nd[c]	nd[c]	nd[c]	nd[c]
5	8	nd[c]	nd[c]	2210 ± 720	879 ± 298	122 ± 114	81 ± 45
15	9	nd[c]	nd[c]	2317 ± 816	966 ± 340	546 ± 373	227 ± 156
liver							
0.25	4	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]
1	4	641 ± 537	92 ± 77	nd[c]	nd[c]	370 ± 370	53 ± 53
5	8	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]
15	9	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]
urine							
15	9	6 ± 4	172 ± 125	nd[c]	nd[c]	280 ± 152	1539 ± 732
bile							
15	4	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]	nd[c]

[a] Amounts in tissues are calculated as described in Table 1. [b] Number of investigated rats. [c] Not detected.

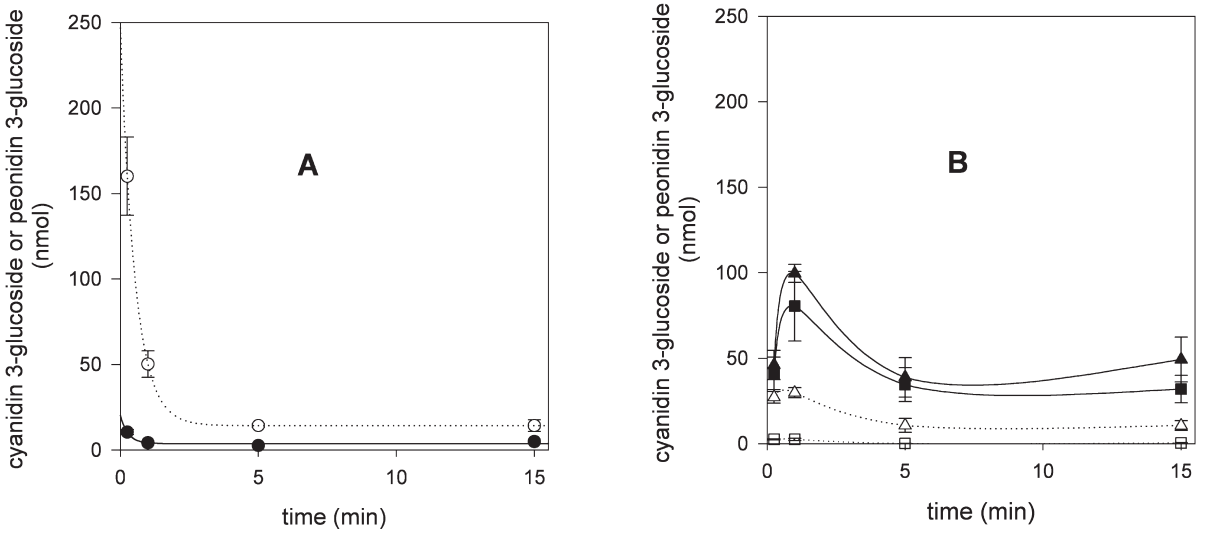


Figure 1. Anthocyanins recovered in rat tissues at various times following intravenous administration of cyanidin 3-glucoside (**1**). (A) Disappearance kinetics of **1** and peonidin 3-glucoside (**2**) in the plasma. Data were fitted to the equation $y = y_0 + ae^{-kt}$, where y is the amount (nmol) of either **1** (○) or **2** (●), y_0 is the amount at the steady state, a is the calculated amount at t_0 minus the calculated amount at the steady state, $e = 2.7183$, t = time, and k is the first-order inhibition rate constant. The parameters of the curves were as follows: dotted line: $y_0 = 14.2 \pm 0.01$ nmol, $a = 232.6 \pm 0.04$ nmol, $k = 1.9 \pm 0.0$ min^{-1}, $r^2 = 1.00$; continuous line: $y_0 = 3.75 \pm 1.1$ nmol, $a = 16.3 \pm 22.7$ nmol, $k = 3.6 \pm 5.8$ min^{-1}, $r^2 = 0.92$. (B) Time profile of total amounts of **1** (open symbols) and **2** (solid symbols) in the kidneys (triangles) and the liver (squares).

on flavonoids.[20] This is the first time that such a transformation has been detected so early after intravenous administration and points to the high catalytic efficiency of this enzyme, coupled with the availability of the methyl group donor, *S*-adenosylmethionine.

With regard to minor metabolites, compound **3** derived from hydroxylation of C-5′ of the B-ring of **1** and was apparently rapidly methylated, forming **4** and **5** (Figure 2). This pathway was, however, of marginal importance.

dx.doi.org/10.1021/np100948a |*J. Nat. Prod.* 2011, 74, 1049–1054

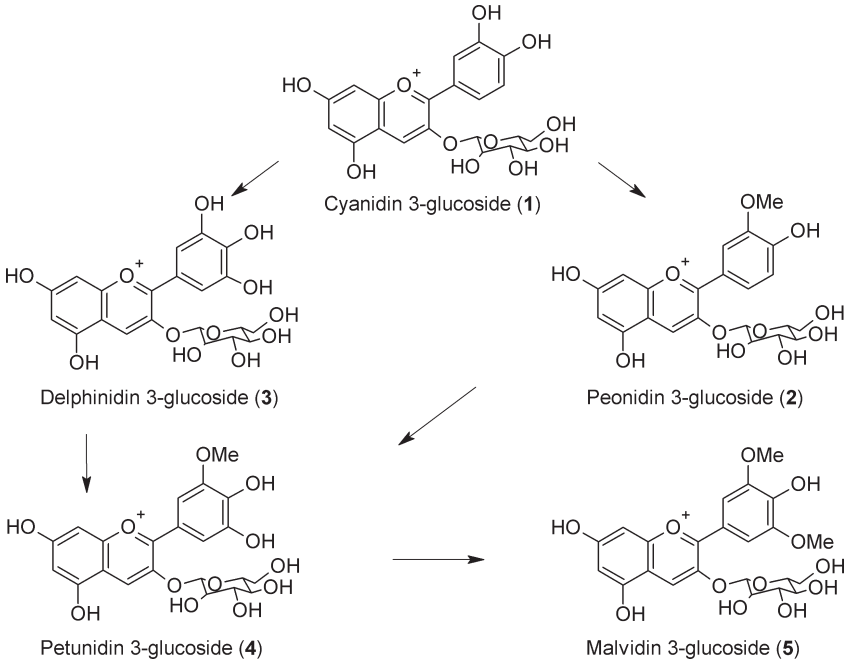


Figure 2. Conversion of cyanidin 3-glucoside (**1**) to its metabolites.

Table 3. Cyanidin 3-Glucoside (1) and Its Metabolites Excreted in the Rat Urine and Bile, 15 min after Intravenous Administration of 1

compound	urine (nmol)	urinary excretion (%)	bile (nmol)	biliary excretion (%)	urine/kidney (μM/μM)	bile/liver (μM/μM)
cyanidin 3-glucoside (**1**)	41.3	6.2	0.4	<0.1	71.9	34.2
peonidin 3-glucoside (**2**)	98.7	14.7	58.5	8.8	34.5	86.5
minor metabolites (**3**, **4**, **5**)	1.0	0.1	nd[a]	nd[a]	4.5	nd[a]
total	141.0	21.0	58.9	8.8	39.4	85.1

[a] Not detected.

Excretion in Urine and Bile. At 15 min after administration of **1**, the urine contained both **1** and major metabolite **2**, whereas the bile contained **2** and only trace amounts of **1**. Urinary and biliary excretions of anthocyanins (sum of **1**, **2**, **3**, **4**, and **5**) amounted to 21.0% and 8.8%, respectively, of the administered dose (Table 3). The fraction of the administered dose recovered in the bile (i.e., 8.8%) was very similar to that reported by Ichiyanagi and associates (10%).[14] These data show that the kidneys eliminate about twice as many anthocyanins as the liver. The most significant difference between these two organs was given by the presence of substantial amounts of **1** in the urine alone. Furthermore, compound **1** was more concentrated in the urine (71.9-fold) than **2** (34.5-fold) (Table 3), showing that it was pumped out of tubular cells into the urine by primary active efflux transporters more efficiently than **2**. In turn, this may have caused relative accumulation of **2** in renal tubular cells, shifting the equilibrium of the COMT reaction to the left, so that compound **1** increased. In contrast, efficient pumping of **2** from the liver into the bile apparently ensured that the COMT reaction could reach completion.

Bioavailability Implications. It is generally accepted that anthocyanin bioavailability is very low, due to limited intestinal absorption.[21,22] This study has shown clearly that an additional factor contributes to low anthocyanin bioavailability, namely, high rates of cellular uptake, metabolism, and excretion by the kidneys and the liver. The whole process is ensured by an extraordinarily high rate of transport of cyanidin 3-glucoside (**1**) at the basolateral side of both renal and hepatic cells, coupled with rapid and almost complete metabolism to the methylated derivative, peonidin 3-glucoside (**2**). For these features, the potential bioactivity of **1** and its metabolites seems to be at the limit of control. In this study, the uptake of **1** was assessed only in a limited number of tissues and fluids. However, this compound was surely taken up by other tissues and organs, such as the muscles, the adipose tissue, the brain,[23] the lungs, and, last but not least, the vascular endothelium, where bilitranslocase is expressed.[24] Indeed, only 40−50% of the injected dose of

Table 4. Total Amounts (nmol) of Anthocyanins Recovered in the Plasma, Kidneys, Liver, Urine, and Bile, at Different Times after Intravenous Administration of Cyanidin 3-Glucoside (1)

time (min)	plasma	kidneys	liver	urine	bile	SUM	recovery (%)
0.25	171.2	73.1	43.6	ns[a]	ns[a]	287.9	43.1
1	55.8	130.8	84.4	ns[a]	ns[a]	271.0	40.6
5	17.2	52.0	34.6	ns[a]	ns[a]	103.8	15.5
15	19.7	62.7	32.3	141.0	58.9	314.6	47.1

[a] Not sampled.

compound **1** was recovered at each of the investigated times (Table 4). It may be noted that these values were remarkably close to the calculated time zero amount of **1**, which was ca. 37% of the administered dose (see Figure 1A). Any interpretation of this observation would be very speculative at this stage.

EXPERIMENTAL SECTION

General Experimental Procedures. Cyanidin 3-glucoside (**1**), peonidin 3-glucoside (**2**), petunidin 3-glucoside (**3**), delphinidin 3-glucoside (**4**), and malvidin 3-glucoside (**5**) were purchased from Polyphenol Laboratories AS (Sandnes, Norway). Anesthetic: 2.5% solution of 2,2,2-tribromoethanol (Sigma-Aldrich, Steinheim, Germany) in ethanol−0.15 M NaCl (1:9, v/v). Phosphate saline buffer (PBS): 6.03 mM Na_2HPO_4, 3.91 mM NaH_2PO_4, and 139 mM NaCl (Carlo Erba, Milan, Italy) dissolved in deionized water (Millipore); pH was adjusted to 7.4 with HCl. Formic acid (LC-MS, Fluka), methanol (LC-MS, Chromasolv, Fluka), and Milli-Q deionized water were used for chromatography.

Intravenous Administration of Cyanidin 3-Glucoside (1) to Rats and Timeline of Sample Collection. Male rats (*Rattus norvegicus*, Wistar, weighing 250 g), bred at the Animal House of the University of Trieste, were housed in temperature-controlled rooms at 22−24 °C, in 50−60% humidity, and with 12 h light/dark cycles. Rats ($n = 30$) were fed standard laboratory chow (Harlan Teklad 2018). This study was approved by the Committee for Animal Studies at University of Trieste. Experiments were carried out at the Animal House of the University of Trieste in compliance with the provisions of the European Community Council Directive (n.86/609/CEE) of Italian law (D.L.vo 116/92 and D.Lvo 633/96, Ministry of Health) and preventing animal pain and distress.[25] Prior to the experiment, the rats ($n = 30$) were fasted overnight (with ad libitum water). The rats were anesthetized with an intraperitoneal injection of 2,2,2-tribromoethanol (0.25 g/kg body weight), and their vitality was assessed by monitoring their heart and ventilation rate for the entire duration of the tests (up to 15 min). Cyanidin 3-glucoside (**1**, 0.67 μmol, diluted in 0.3 mL of PBS just prior to administration) was injected into their iliac vein, following its exposure through a 1 cm cut on the skin of their iliac cavity. Before sacrifice, sodium heparin (0.1 mL, 500 IU) was injected into the contra lateral iliac vein, exposed in the same way. The animals were decapitated and held vertically to collect their blood from the trunk; their liver and both kidneys were excised and further processed in ice. A group of rats (group 15 min, see below) underwent laparatomy and bile duct cannulation prior to intravenous injection of **1**; in these rats, the urine was sampled at 15 min by bladder puncture. The rats were divided into groups, according to the time that elapsed after intravenous administration of the test compound **1**: 15 s ($n = 4$), 1 min ($n = 4$), 5 min ($n = 8$), 15 min ($n = 9$), control (solvent without **1**, 5 min) ($n = 5$). Tests were carried out to evaluate the best procedure to drain blood from the liver and the kidneys. The rats ($n = 8$) were injected with the test compound; after 5 min they were decapitated and held vertically, and the blood was exhaustively drained from the trunk. The liver and the kidneys were excised and either directly processed for analysis (group A, $n = 4$) or washed out by perfusion of ice-cold PBS into either the portal or the renal vein (group B, $n = 4$). No statistically significant difference of the measured parameters was found. Thus, data were pooled.

Preparation of Tissue Extracts. All procedures were carried out at 4 °C. Blood samples were centrifuged for 2 min at 10000*g*. Plasma was collected and added to 9 volumes of ice-cold methanol presaturated with nitrogen. Urine and bile samples were weighed, and 9 volumes of ice-cold methanol were immediately added. The liver and kidneys were trimmed and homogenized in 4 volumes of ice-cold PBS. Homogenates were then added to 9 volumes of ice-cold methanol. Methanol extracts were decanted in glass tubes under a stream of nitrogen after centrifugation at 3640*g* for 10 min. Samples were stored at −20 °C for no more than 4 days before analysis. Clean-up of tissue extracts in methanol was performed by solid phase extraction as previously described.[26]

HPLC-DAD-MS Analysis. Cyanidin 3-glucoside (**1**) and its metabolites were analyzed by mass spectrometry on a Xevo TQ MS (Micromass, Manchester, UK), equipped with an Acquity UPLC system, DAD eLambda 800 nm detector, and MassLynx software (Waters Corp., Milford, MA). Separation was performed using an Acquity UPLC BEH C_{18} (2.1 × 150 mm, 1.7 μm; Waters Corp.) coupled with a Van Guard BEH C_{18} precolumn (2.1 × 5 mm, 1.7 μm). The mobile phase consisted of 5% formic acid in water (A) and 5% formic acid in methanol (B). Separation was carried out for 10 min at 60 °C, under the following conditions: linear gradient from 5% B to 25% B in 10 min, to 90% B in 0.5 min, 90% B for 2 min, 5% B in 10 s. The column was equilibrated 4 min prior to each analysis. The flow rate was 0.45 mL/min, and injection volume was 10 μL. The UV−vis spectra were recorded from 220 to 600 nm, with the quantization at 520 nm. Samples in an autosampler were maintained at 4 °C. MS conditions: capillary voltage 500 V; source temperature 150 °C; desolvation temperature 500 °C; collision gas flow (N_2) 0.15 mL/min. Electrospray mass spectra ranging from m/z 20 to 800 were monitored in the positive mode. Compounds were identified on the basis of their retention times, DAD, MS spectra, molecular ion identifications, and accurate masses. Quantification and confirmation of the compounds were performed with tandem MS in the MRM mode using the external standard method. Quantification ions were cyanidin 3-glucoside (**1**), m/z 449 to >287; peonidin 3-glucoside (**2**), m/z 463 to >301; delphinidin 3-glucoside (**3**), m/z 465 to >303; petunidin-3-glucoside (**4**), m/z 479 to >317; and malvidin 3-glucoside (**5**), m/z 493 to >331. Confirmation ions were **1**, m/z 449 to >137; **2**, m/z 463 to >286; **3**, m/z 465 to >229; **4**, m/z 479 to >302; and **5**, m/z 493 to >315. For confirmation of the compounds two neutral loss scans were also performed for glucuronide (m/z 176) and glucoside (m/z 162).

Accurate masses were checked by injection on an Acquity-Synapt LC-Q-TOF system (Waters Corp.). LC conditions were the same as described above. The setting of Q-TOF was as follows: ESI positive mode, mass range 50−3000 Da, double W, capillary voltage 3 kV, sampling cone 25 V, extraction cone 3 V, source temp 150 °C, desolvation temp 500 °C, cone gas flow 50 L/h, desolvation gas flow 1000 L/h. The compound used as a reference for lock mass correction was leucine/enkephalin. The calibration of Q-TOF was performed with Na-formiate.

Determination of Anthocyanin Recovery from Tissues. Data obtained by MS analyses were corrected for the recovery factor, determined experimentally by spiking blood, kidney homogenates, and liver homogenates and urine of fasted rats. Pure anthocyanin standards were used at concentrations similar to those obtained after intravenous administration of cyanidin 3-glucoside (**1**). After spiking, tissues were extracted in methanol and cleaned up by SPE as described above. The recoveries of compounds **1**, **2**, **3**, **4**, and **5** were in the range 38.6−57.7% from the spiked blood ($n = 5$), 30.9−46.4% from the spiked kidney homogenates ($n = 5$), and 21.4−29.9% from the spiked liver homogenates ($n = 5$), respectively. The recovery from urine ($n = 3$) was in the range 29.0−59.7%.

Statistics. Data are expressed as means ± SEM. Differences between groups were evaluated by the Student's t test, with $p < 0.05$ considered statistically significant.

AUTHOR INFORMATION

Corresponding Author

*Tel: +39 040 558 3680. Fax: +39 040 558 3691. E-mail: spassamonti@units.it.

■ ACKNOWLEDGMENT

Thanks are due to D. Masuero for his excellent technical assistance in the MS analysis and to M. Gasperotti for his help in the sample preparation procedure. The authors gratefully acknowledge the following for financial support: the Ministry of Higher Education, Science and Technology, Slovenia and Slovenian Research Agency (projects: BI-IT/06-09-031 and Z4-2280), Fondazione Cassa di Risparmio di Trieste, Ministero degli Affari Esteri (Cooperazione scientifica e tecnologica Italia-Slovenia 2006−2009) and the Autonomous Province of Trento, Italy (project: ADP2009).

■ REFERENCES

(1) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. *J. Agric. Food Chem.* **2006**, *54*, 4069–4075.
(2) Kuhnau, J. *World Rev. Nutr. Diet* **1976**, *24*, 117–191.
(3) McGhie, T. K.; Walton, M. C. *Mol. Nutr. Food Res.* **2007**, *51*, 702–713.
(4) Zafra-Stone, S.; Yasmin, T.; Bagchi, M.; Chatterjee, A.; Vinson, J. A.; Bagchi, D. *Mol. Nutr. Food Res.* **2007**, *51*, 675–683.
(5) Wang, L. S.; Stoner, G. D. *Cancer Lett.* **2008**, *269*, 281–290.
(6) Willis, L. M.; Shukitt-Hale, B.; Joseph, J. A. *Curr. Opin. Clin. Nutr. Metab. Care* **2009**, *12*, 91–94.
(7) Qin, Y.; Xia, M.; Ma, J.; Hao, Y.; Liu, J.; Mou, H.; Cao, L.; Ling, W. *Am. J. Clin. Nutr.* **2009**, *90*, 485–492.
(8) Sasaki, R.; Nishimura, N.; Hoshino, H.; Isa, Y.; Kadowaki, M.; Ichi, T.; Tanaka, A.; Nishiumi, S.; Fukuda, I.; Ashida, H.; Horio, F.; Tsuda, T. *Biochem. Pharmacol.* **2007**, *74*, 1619–1627.
(9) Matsumoto, H.; Ichiyanagi, T.; Iida, H.; Ito, K.; Tsuda, T.; Hirayama, M.; Konishi, T. *J. Agric. Food Chem.* **2006**, *54*, 578–582.
(10) Matsumoto, H.; Nakamura, Y.; Iida, H.; Ito, K.; Ohguro, H. *Exp. Eye Res.* **2006**, *83*, 348–356.
(11) Lietti, A.; Forni, G. *Arzneimittelforschung* **1976**, *26*, 832–835.
(12) Morazzoni, P.; Livio, S.; Scilingo, A.; Malandrino, S. *Arzneimittelforschung* **1991**, *41*, 128–131.
(13) Marczylo, T. H.; Cooke, D.; Brown, K.; Steward, W. P.; Gescher, A. J. *Cancer Chemother. Pharmacol.* **2009**, *64*, 1261–1268.
(14) Ichiyanagi, T.; Shida, Y.; Rahman, M. M.; Hatano, Y.; Matsumoto, H.; Hirayama, M.; Konishi, T. *J. Agric. Food Chem.* **2005**, *53*, 145–150.
(15) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug Delivery Rev.* **2001**, *46*, 3–26.
(16) Saier, M. H., Jr. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 354–411.
(17) Passamonti, S.; Terdoslavich, M.; Franca, R.; Vanzo, A.; Tramer, F.; Braidot, E.; Petrussa, E.; Vianello, A. *Curr. Drug Metab.* **2009**, *10*, 369–394.
(18) Passamonti, S.; Vrhovsek, U.; Mattivi, F. *Biochem. Biophys. Res. Commun.* **2002**, *296*, 631–636.
(19) Prior, R. L.; Wu, X. *Free Radical Res.* **2006**, *40*, 1014–1028.
(20) Zhu, B. T. *Curr. Drug Metab.* **2002**, *3*, 321–349.
(21) Steinert, R. E.; Ditscheid, B.; Netzel, M.; Jahreis, G. *J. Agric. Food Chem.* **2008**, *56*, 4995–5001.
(22) Yi, W.; Akoh, C. C.; Fischer, J.; Krewer, G. *J. Agric. Food Chem.* **2006**, *54*, 5651–5658.
(23) Passamonti, S.; Vrhovsek, U.; Vanzo, A.; Mattivi, F. *J. Agric. Food Chem.* **2005**, *53*, 7029–7034.
(24) Maestro, A.; Terdoslavich, M.; Vanzo, A.; Kuku, A.; Tramer, F.; Nicolin, V.; Micali, F.; Decorti, G.; Passamonti, S. *Cardiovasc. Res.* **2010**, *85*, 175–183.
(25) Demers, G.; Griffin, G.; De Vroey, G.; Haywood, J. R.; Zurlo, J.; Bedard, M. *Science* **2006**, *312*, 700–701.
(26) Passamonti, S.; Vrhovsek, U.; Vanzo, A.; Mattivi, F. *FEBS Lett.* **2003**, *544*, 210–213.