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Bioavailability and Tissue Distribution of Anthocyanins in Bilberry (*Vaccinium myrtillus* L.) Extract in Rats

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To clarify how structural diversity of anthocyanins relates to their in vivo function, bioavailability was precisely studied in rats using bilberry (*Vaccinium myrtillus* L.) extract (Bilberon 25) as an anthocyanin source that contains 15 different anthocyanins. The bilberry extract was orally or intravenously administered to rats, and the plasma levels of each anthocyanin were determined by high-performance liquid chromatography. As the result, all anthocyanins except peonidin 3-*O*-α-L-arabinoside were detectable in the blood plasma. The plasma concentration of anthocyanins as a whole reached the maximum level of 1.2 μM at 15 min after oral administration of 400 mg/kg bilberry extract (153.2 mg/kg as anthocyanins) and then decreased with time. Uptake and decay profiles of each anthocyanin in the plasma were almost the same for all anthocyanins except a few with their maximum after 30 min. Among the anthocyanins carrying the same aglycone, the plasma level after 15 min of oral administration was as follows: galactoside > glucoside > arabinoside. Plasma clearance of anthocyanins after intravenous administration clearly showed that arabinoside disappeared more rapidly than glucoside and galactoside. On the other hand, when anthocyanins carrying the same sugar moiety were compared, the half disappearance time of plasma anthocyanins was in the following order: delphinidin > cyanidin > petunidin = peonidin > malvidin. The bioavailability of anthocyanins was in the range of 0.61−1.82% and was 0.93% as the anthocyanin mixture. The bioavailability of anthocyanins carrying the same aglycone was in the following order: Galactoside showed the highest followed by glucoside and arabinoside for cyanidin and delphinidin, but arabinoside and galactoside showed a higher bioavailability than glucoside for petunidin and malvidin. Anthocyanins recovered in urine and bile during the first 4 h after intravenous administration were only 30.8 and 13.4%, respectively. Anthocyanin profiles in tissues were quite different from those in blood plasma. The major anthocyanins distributed in liver and kidney were the *O*-methyl anthocyanins such as peonidin, malvidin, and other *O*-methyl anthocyanins derived from delphinidin, cyanidin, and petunidin-glycosides.

KEYWORDS: Anthocyanin; bilberry; bioavailability; absorption; tissue distribution; excretion; metabolism; delphinidin; cyanidin; petunidin; peonidin; malvidin; aglycone; sugar moiety

INTRODUCTION

Anthocyanins are reddish pigments widely distributed in colored fruits (*1*−*4*) and vegetables (*5*−*9*). The importance of anthocyanins as nutraceuticals has been extensively discussed because they are one of the major flavonoids ingested from the daily meal, and moreover, they have a variety of physiological activities such as improvement of vision (*10*, *11*), α-glucosidase inhibition (*12*, *13*), and antioxidant activity (*7*, *14*−*16*). The anticancer activity of anthocyanins has also been reported in vitro (*17*−*22*).

We previously studied the reactivity of anthocyanins from bilberry (*Vaccinium myrtillus* L.) toward reactive oxygen and nitrogen species determined simultaneously by capillary zone electrophoresis under the same reaction condition and discussed their structure−reactivity relationship (*23*−*25*). However, for further discussion on in vivo function of anthocyanins, it is critical to know the physiological uptake and distribution of each anthocyanin in plasma and tissues together with their metabolic fate.

Studies on the anthocyanin absorption and metabolism were reported elsewhere in both human and experimental animals mainly using fruit extracts as anthocyanin sources (*26*−*31*). However, such studies using fruit extracts as anthocyanin sources are not suitable for precise discussion of anthocyanin

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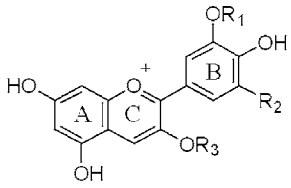

Anthocyanin	R_1	R_2	R_3	Peak
Delphinidin 3-*O*-*β*-D-galactopyranoside	H	OH	galactopyranose	1
Delphinidin 3-*O*-*β*-D-glucopyranoside	H	OH	glucopyranose	2
Delphinidin 3-*O*-*α*-L-arabinopyranoside	H	OH	arabinopyranose	4
Cyanidin 3-*O*-*β*-D-galactopyranoside	H	H	galactopyranose	3
Cyanidin 3-*O*-*β*-D-glucopyranoside	H	H	glucopyranose	5
Cyanidin 3-*O*-*α*-L-arabinopyranoside	H	H	arabinopyranose	7
Petunidin 3-*O*-*β*-D-galactopyranoside	H	CH_3O	galactopyranose	6
Petunidin 3-*O*-*β*-D-glucopyranoside	H	CH_3O	glucopyranose	8
Petunidin 3-*O*-*α*-L-arabinopyranoside	H	CH_3O	arabinopyranose	10
Peonidin 3-*O*-*β*-D-galactopyranoside	CH_3	H	galactopyranose	9
Peonidin 3-*O*-*β*-D-glucopyranoside	CH_3	H	glucopyranose	11
Peonidin 3-*O*-*α*-L-arabinopyranoside	CH_3	H	arabinopyranose	13
Malvidin 3-*O*-*β*-D-galactopyranoside	CH_3	CH_3O	galactopyranose	12
Malvidin 3-*O*-*β*-D-glucopyranoside	CH_3	CH_3O	glucopyranose	14
Malvidin 3-*O*-*α*-L-arabinopyranoside	CH_3	CH_3O	arabinopyranose	15

Figure 1. Chemical structures of bilberry anthocyanins.

metabolism. We thus studied the absorption and metabolism of anthocyanins in experimental animals using purified anthocyanins (*32*−*35*). In those studies, we showed that the uptake and metabolism of anthocyanins are variable depending on their B ring structures. However, quantitative and systematic comparison of bioavailabilities of anthocyanins has not been carried out because the numbers of available authentic anthocyanin were limited.

Bilberry, a wild-type blueberry, is one of the best anthocyanin sources because it contains 15 types of anthocyanins comprised of five types of anthocyanidin (anthocyanin aglycone) and three types of attached sugars, as we showed using capillary zone electrophoresis and high-performance liquid chromatography (HPLC) (**Figure 1**) (*1*, *2*). In the present study, we precisely examined the absorption and excretion of anthocyanins from bilberry extract in rats together with tissue distribution using high sensitive semimicro-HPLC that we developed previously for the study of anthocyanins in biological samples (*34*, *35*) and discussed the relationship between the structural diversity and the biological behavior of bilberry anthocyanins in vivo.

MATERIALS AND METHODS

Chemicals. All reagents including trifluoroacetic acid (TFA) and methanol (MeOH) were purchased from Wako Pure Chemical Industries Co. Ltd. (Japan) and used without further purification. Bilberon 25, a concentrated extract of wild-type blueberry (*V. myrtillus* L.), was generously donated by Tokiwa Phytochemical Co. Ltd. (Japan). The structures of bilberry anthocyanins are shown in **Figure 1**. The anthocyanin contents in the Bilberon sample were determined by HPLC using authentic anthocyanins as standards, and the results are given in **Table 1**. Fifteen types of authentic anthocyanins were purified from bilberry extract by the method described in our previous paper (*1*). Briefly, Bilberon 25 (10 g) was dissolved in 1% TFA aqueous solution (10 mL) and subjected to low-pressure liquid chromatography (Waters, United States). Anthocyanins were recovered in the fraction eluted with 30% MeOH containing 1% TFA (yield, 3.3 g). The anthocyanin fraction was further chromatographed over MCI-gel CHP-20 (45 cm × 4.5 cm) (Mitsubishi, Japan) with H_2O containing increased amounts of MeOH (0:1 → 1:0) to give 11 fractions. Anthocyanin-containing fractions were further separated by Sephadex LH-20 chromatography (26 cm × 2.5 cm) (Amarsham Biosciences Co., United States). Each anthocyanin was purified by HPLC (HITACHI L-7000) equipped with a Develosil ODS-HG5 column (250 mm × 20 mm, Nomura Chemical Co. Ltd., Japan), with 20% MeOH containing 0.1% TFA as the elution solvent. The anthocyanin structures were assigned by extensive one- and two-dimensional NMR as well as by tandem MS analysis. NMR spectra were recorded on a JEOL ECA500 spectrometer, and chemical shifts are reported as δ (ppm) with tetramethylsilane (TMS) as the internal standard. Identification of anthocyanin was performed by tandem MS.

Table 1. Contents and Type of Anthocyanins in Bilberon 25

	contents		
peak	mg/kg	mmol/kg	molecular weight
1	19.6	0.0422	465
2	18.7	0.0401	465
4	16.5	0.0379	435
3	16.9	0.0376	449
5	16.3	0.0363	449
7	12.4	0.0296	419
6	6.1	0.0126	479
8	11.6	0.0241	479
10	3.8	0.0085	449
9	1.6	0.0034	463
11	7.9	0.0171	463
13	1.0	0.0022	433
12	6.7	0.0136	493
14	12.1	0.0245	493
15	2.1	0.0045	463
total	153.2	0.3343	–

Animals and Diets. SPF male Wistar ST rats (6 weeks old, 170 g of average body weight) purchased from Japan SLC Inc. (Hamamatsu, Japan) were individually housed in stainless steel wire mesh cages at 23 ± 1 °C for conditioning under a 12 h light/dark cycle. The rats were allowed free access to tap water and control diet for 7 days before the experiment. Rats were treated in accordance with the Guidelines of Niigata University of Pharmacy and Applied Life Sciences.

Experimental Design and Plasma Preparation. After a 7 day conditioning period, carotid vein cannulation was carried out according to our previous method (*34*, *35*). Briefly, eight rats were cannulated with a polyethylene tube (PE-50) into a neck vein under anesthesia with diethyl ether. The neck vein was isolated, and a small hole was made by the use of scissors to insert the polyethylene tube. After insertion of the tube, the vein and tube were occluded, and the tube was guided out from the back of the rat. After starvation for 24 h, the rats were randomly assigned into two groups, and Bilberon 25 (400 mg/kg body weight) dissolved in 0.1% citric acid was orally administered. In the case of intravenous administration, Bilberon 25 (5 mg/kg body weight) dissolved in physiological saline was injected into the neck vein. Blood samples were collected via the cannulated tube using a heparinized syringe at 15, 30, 60, 120, 240, and 480 min after the oral administration and 1, 15, 30, 60, 120, and 240 min after intravenous administration of bilberry extract. After withdrawal of each blood sample (600 μL), the same volume of donor blood was injected through the tube. The donor blood was obtained from the inferior vena cava of other healthy rats using sodium citrate (500 μL of 10% sodium citrate for 8 mL of blood). Each blood sample was immediately centrifuged at 3000*g* for 5 min at 4 °C for HPLC analysis. On the basis of the detection limit of the present HPLC method, the dose administered was determined for both orally and intravenously administered experiments as described above. The urine, which flew down through the wire mesh floor, was collected into a plate set under the floor and then immediately acidified with 1% TFA aqueous solution. The urine samples recovered during each time period (0−2, 2−4, and 4−8 h) were combined and kept in the dark for further analysis.

Studies on Urinary and Bile Excretion of Anthocyanins. Urinary and bile excretion of anthocyanins were studied after the intravenous administration (5 mg/kg body weight) according to our previous report (*35*). Briefly, four rats cannulated to neck vein as described above were starved for 24 h and then bile cannulation was carried out using a polyethylene tube (PE-50) under anesthesia with diethyl ether. From a hole made by scissors in the abdomen, the bile duct was isolated, a small hole was made, and the polyethylene tube was inserted and occluded. For the urine collection, a plastic tube was attached to the penis of each animal. After the surgery, the rats were set in a Boleman

cages. Then, anthocyanin sample (Bilberon 25, 5 mg/kg body weight) was injected into the neck vein through the tube, and then, urine and bile were continuously recovered into glass tubes containing 1 mL of 3% TFA aqueous solution for 4 h.

Determination of Anthocyanins in Plasma, Urine, and Bile. Sample preparation for HPLC analysis of anthocyanins from biological samples was followed using our previously reported method (*34*, *35*). Briefly, the plasma samples (300 μL) were applied to Sep-Pak C_{18} cartridges light (Waters) preconditioned with MeOH (3 mL) and 3% TFA aqueous solution (3 mL). Then, the cartridges were washed successively with 3 mL of 3% TFA aqueous solution, dichloromethane, and benzene (3 mL each). Anthocyanins were eluted with 50% acetonitrile containing 1% TFA aqueous solution. The recovered anthocyanins were evaporated to dryness in vacuo at a temperature below 40 °C and dissolved in 150 μL of distilled water containing 0.5% TFA, and then, an aliquot (100 μL) of the solutions was injected into an HPLC system (Hitachi 7200). In the case of intravenous administered plasma samples, further dilution was carried out depending on the color of the samples. Urine and bile samples were prepared as described above using Sep-Pak C_{18} cartridges Environment (Waters). All of the washing and elution procedures were carried out using 10 mL of the solvent. HPLC was carried out on a Develosil ODS HG-5 column (Nomura Chemical Co. Ltd., Japan, 150 mm × 1.0 mm) using 18% MeOH containing 0.5% TFA as the elution solvent at a flow rate of 0.1 mL/min, and the elution peaks were monitored at 520 nm with a UV−visible detector (Hitachi, Japan). The recovery of anthocyanins through the above method was checked using Bilberon 25, and it was determined as 87.5%. A calibration curve for each anthocyanin was made using purified anthocyanidin-glycoside (3-*O*-glycopyranoside of delphinidin, cyanidin, petunidin, peonidin, and malvidin) as the reference. The anthocyanin metabolites were quantified as cyanidin 3-*O*-β-D-glucoside equivalent.

Determination of Anthocyanin Distributed in Tissues. Rats were sacrificed 15 min after oral or intravenous administration of Bilberon 25. The tissues (liver and kidney) obtained were well washed and further perfused with physiological saline to remove residual blood contaminants and then homogenized in 3% TFA aqueous solution (0.25 g/mL). After centrifugation at 3000*g* for 5 min, the supernatant was treated with the same method as plasma for HPLC analysis. Each peak on the chromatogram was assigned by coelution with authentic anthocyanins and tandem MS analysis. The anthocyanin metabolites were quantified as cyanidin 3-*O*-β-D-glucoside equivalent.

Determination of Anthocyanin Metabolites in Tissues. The HPLC peaks detected in tissues (liver and kidneys), bile, and urine samples were isolated by HPLC, and tandem MS analysis was carried out according to the method described above to obtain structural data of these metabolites. Each metabolite sample was dissolved in MeOH and was subjected to mass spectrometry performed with a quantitative time-of-flight (Q-TOF) Ultima (Micromass, Manchester, United Kingdom). The conditions for TOF MS-MS were as follows: A syringe pump (KD Science Inc., United States) was used to provide a constant infusion (300 μL/h) of the sample into the MS ion source. MS parameters used were as follows: 3.2 kV for capillary and 9.1 kV for reflection. Argon gas was used for collision at a pressure of 11 psi, and the applied voltage was 13 V.

Statistical Analysis. Data were analyzed by one-way analysis of variance, and differences were considered statistically significant at $P < 0.05$.

RESULTS

Absorption of Anthocyanins. Figure 2A shows a typical HPLC chromatogram of anthocyanins in Bilberon 25. Fifteen anthocyanins were detectable in Bilberon 25 together with minor two anthocyanidins (aglycone). The content of each anthocyanin in the Bilberon 25 is summarized in **Table 1**. The HPLC profile of anthocyanins in the plasma after 15 min of oral administration of Bilberon 25 was also given in **Figure 2B** in which 14 anthocyanins were detectable. The difference of the HPLC profile of anthocyanins in the plasma from that of original

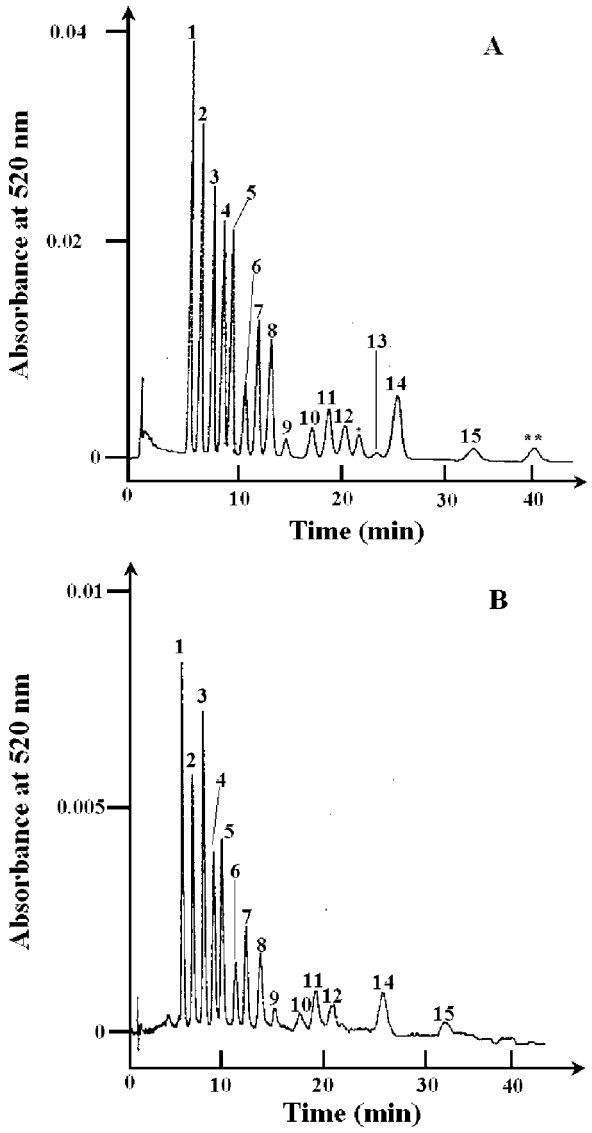

Figure 2. Typical HPLC chromatograms of bilberry anthocyanins: (**A**) bilberry extract and (**B**) rat plasma after 15 min of oral administration of Bilberon 25. Peak numbers correspond to **Table 1**. * and ** are cyanidin and petunidin, respectively.

Bilberon 25 indicates that the absorption, excretion, and probably the metabolic fate are different among the anthocyanins. For more precise discussion, each concentration of anthocyanins determined by HPLC in plasma after 15 min of oral administration was divided by the dose (mmol/kg) (C_{15min}/dose) in original Bilberon 25 and the results are given in **Figure 3**. When the C_{15min}/dose of anthocyanins in the plasma was compared, galactosides gave the highest value, then glucosides and arabinosides followed in any anthocyanins with the same aglycone except malvidin (significant difference between galactoside and arabinoside anthocyanins). The difference in C_{15min}/dose was also significant among the anthocyanins with different aglycones (significant difference between galactoside and arabinoside anthocyanins; see **Figure 3**). C_{15min}/Dose of glucosides and arabinosides showed the same tendency in the order such that delphinidin and cyanidin showed a higher level than petunidin and peonidin. Again malvidin behaved differently.

The plasma concentration profiles of the major six anthocyanins are shown in **Figure 4** after the plasma concentrations were normalized by the orally administered dose (mmol/kg) of each anthocyanin. The maximum plasma concentration (C_{max}),

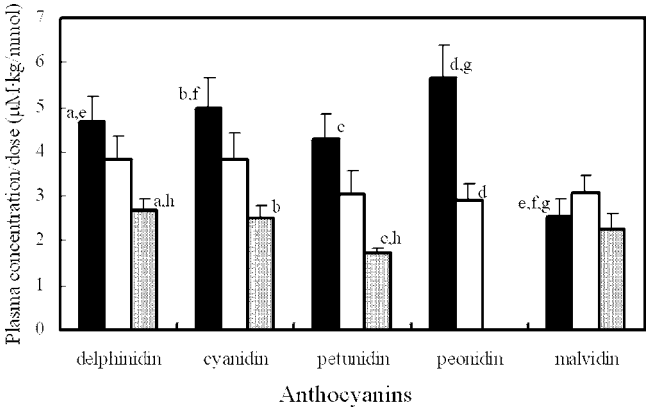

Figure 3. Plasma levels of anthocyanins at 15 min after oral administration. Values are given as C_{15min}/administered dose and are means ± SEM of four rats. Key: galactoside, black bars; glucoside, white bars; and arabinoside, gray bars. Letters a–g, significantly different between the same alphabet.

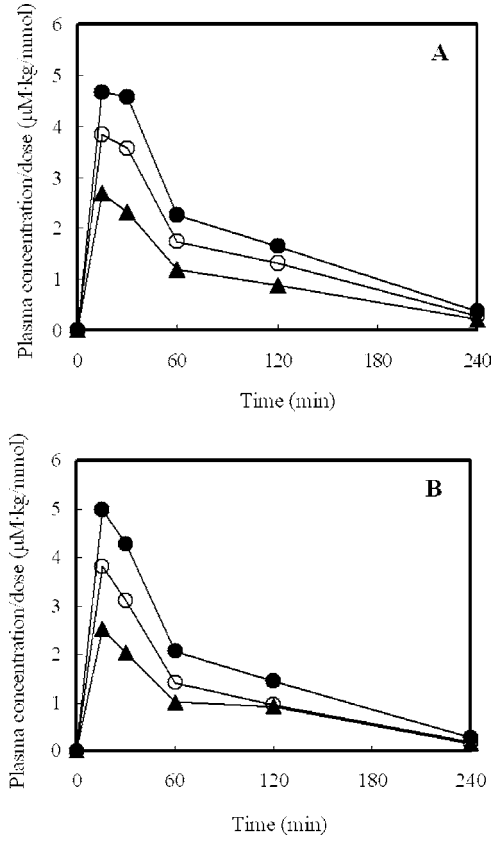

Figure 4. Plasma anthocyanin concentration profiles after oral administration. Plasma concentration was normalized by administered dose: (**A**) delphinidin and (**B**) cyanidin. Symbols: galactoside, ●; glucoside, ○; and arabinoside, ▲. Values are means of four rats.

normalized plasma concentration (C_{max}/dose), and time to reach the maximum plasma concentration (t_{max}) are summarized in **Table 2**. The maximum anthocyanin level as the whole in the plasma reached approximately 1.2 μM at 15 min after the administration of 400 mg Bilberon 25/kg body weight. Most anthocyanins rapidly appeared in the plasma and reached their maximum level at 15 min except galactosides of petunidin, peonidin, and malvidin that reached their maximum at 30 min (**Table 2**).

Comparison of Bioavailability of Anthocyanins. From the plasma profiles obtained after oral and intravenous administration of bilberry extract (Bilberon 25), both the area under the plasma concentration curve (AUC) and the bioavailability of anthocyanins were calculated and the results are given in **Table 2**. The bioavailability calculated was in the range from 0.6 to 1.8%. When the bioavailability of anthocyanins carrying the same aglycone was compared, galactoside tended to show a higher value than glucoside. On the other hand, when the bioavailability of anthocyanins with the same sugar moiety was compared, peonidin tended to show the highest bioavailability; however, the order of other anthocyanins was variable dependent on the attached sugar type. The bioavailability of anthocyanin as a mixture was 0.93%.

Excretion and Disappearance of Anthocyanins from Blood Plasma. For precise discussion of anthocyanin behavior in vivo, anthocyanin excretion was also examined. First, the half plasma disappearance time ($t_{1/2}$) of each anthocyanin was determined from the plasma concentration curve obtained after intravenous administration of bilberry extract (**Figure 5**) and the results are summarized in **Table 3**. When the anthocyanins having the same sugar moiety were compared, delphinidin and cyanidin showed longer $t_{1/2}$ times than their *O*-methyl analogues. On the other hand, when the anthocyanins with the same aglycone were compared, $t_{1/2}$ was as follows: galactoside > glucoside > arabinoside except for malvidin.

Anthocyanins excreted in bile and urine after intravenous administration were examined next. As shown in **Figure 6 A**,**B**, HPLC profiles of anthocyanins in urine and bile were completely different from that of the original Bilberon 25. The major anthocyanins in bile and urine were *O*-methyl anthocyanins such as peonidin and malvidin-glycosides. The newly produced peaks assumed to be the anthocyanin metabolites were also detected in bile and urine. Recovery of anthocyanins in the urine and bile during the first 4 h of intravenous administration was only 30.8 and 13.4%, respectively.

Metabolism and Tissue Distribution of Anthocyanins. When the tissue distributions of anthocyanins after oral administration of Bilberon 25 were further examined by HPLC as shown in **Figure 7**, it was revealed that the major anthocyanins detected in liver and kidney at 15 min after oral administration of bilberry extract (Bilberon 25) were glycosides of peonidin and malvidin. The tissue distribution of anthocyanins after intravenous administration was also determined as shown in **Figure 8**. To elucidate the structures of anthocyanins in the tissues, HPLC peaks were isolated and analyzed by tandem MS, and the results are summarized in **Table 4**. It is clear that the anthocyanins detected in tissues were glucosides and galactosides of petunidin, peonidin and malvidin, and 4′-*O*-methyl metabolites of cyanidin, delphinidin, and petunidin 3-*O*-β-D-glucoside or galactoside, whereas no arabinosides were detected except 4′-*O*-methyl delphinidin 3-*O*-α-L-arabinoside.

DISCUSSION

Anthocyanins are a major group of polyphenols taken from daily food and thus attracting much attention to its phytoceutical properties useful for preventing diseases (*10*−*22*). Anthocyanins carry a positive charge as a stable flavilium cation at acidic conditions, but they become quite unstable at neutral to alkaline pH to form quinoidal or carbinol base structures, which easily decompose (*36*). Because of this chemical property, only a limited number of purified anthocyanins were commercially available, and moreover, the difficulty in the quantitative handling has restricted basic studies on the biological behavior of anthocyanins in experimental animals and humans as compared with other stable flavonoids such as epicatechin and

Table 2. Pharmacokinetic Parameters of Bilberry Anthocyanin

peak	C_{max} (μM)	C_{max}/dose (μM kg/mmol)	t_{max} (min)	AUC_{oral}/dose (μM min kg/mmol)	$AUC_{intravenous}$/dose (μM min kg/mmol)	bioavailability (%)
1	0.197 ± 0.024	4.664 ± 0.575 a	15	1.3159 ± 0.15432	158.5632 ± 4.2309	0.8299 ± 0.0973 d
2	0.154 ± 0.021	3.830 ± 0.510	15	1.0378 ± 0.1173	134.3623 ± 2.0947	0.7724 ± 0.0873
4	0.101 ± 0.001	2.677 ± 0.253 a,f	15	0.7486 ± 0.0791	105.3326 ± 1.7194	0.7107 ± 0.0751 g
3	0.188 ± 0.025	4.994 ± 0.625 b	15	1.2853 ± 0.1459	130.7163 ± 1.4442	0.9833 ± 0.1116 e
5	0.139 ± 0.022	3.828 ± 0.592	15	0.9157 ± 0.1067	115.6034 ± 3.6972	0.7921 ± 0.0923
7	0.075 ± 0.008	2.522 ± 0.282 b	15	0.7008 ± 0.0725	93.6637 ± 6.2983	0.7482 ± 0.0774 h
6	0.055 ± 0.013	4.338 ± 1.031 c	30	1.2825 ± 0.1450	105.9207 ± 4.2858	1.2108 ± 0.1369 a
8	0.074 ± 0.013	3.055 ± 0.518	15	0.7718 ± 0.1013	101.6546 ± 4.2467	0.7592 ± 0.0996 a
10	0.015 ± 0.001	1.735 ± 0.104 c,f	15	1.9794 ± 0.3804	108.8818 ± 6.2200	1.8179 ± 0.3493 g–i
9	0.025 ± 0.008	7.288 ± 2.495d	30	0.4997 ± 0.0830	35.2244 ± 1.8086	1.4185 ± 0.2357 d–f
11	0.050 ± 0.007	2.903 ± 0.396	15	0.7052 ± 0.0878	83.9669 ± 4.7673	0.8399 ± 0.1045
13						
12	0.039 ± 0.006	2.827 ± 0.454 d	30	0.7401 ± 0.0620	81.5674 ± 5.5401	0.9074 ± 0.076 b,f
14	0.075 ± 0.009	3.069 ± 0.385	15	0.7228 ± 0.0691	118.8704 ± 5.9463	0.608 ± 0.0581b,c
15	0.010 ± 0.002	2.260 ± 0.358	15	0.6127 ± 0.0726	60.4707 ± 15.7764	1.0132 ± 0.1201 c,i
anthocyanin as mixture	1.207 ± 0.131	54.925 ± 4.833	15	13.3182 ± 1.4999	1434.7984 ± 46.2708	0.9282 ± 0.1045

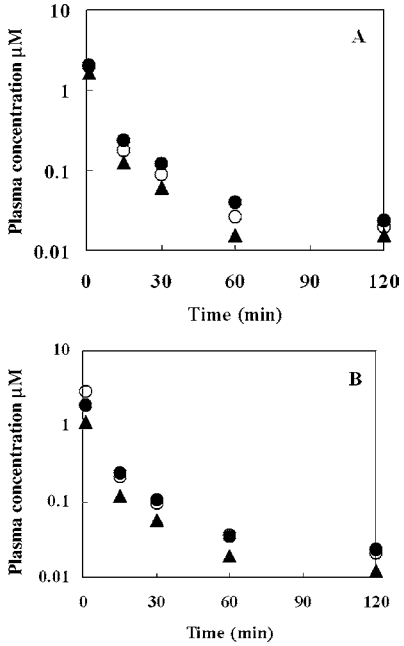

Figure 5. Plasma anthocyanin profiles after intravenous administration: (**A**) delphinidin and (**B**) cyanidin. Symbols: galactoside, ●; glucoside, ○; and arabinoside, ▲. Values are means of four rats.

Table 3. Plasma Disappearance of Anthocyanins $t_{1/2}$ (min) after Intravenous Administration of Bilberon 25

	galactoside	glucoside	arabinoside
delphinidin	13.31 ± 0.82 a,g–j	12.10 ± 0.59 b,l–o	9.98 ± 0.52 a,b,q,r
cyanidin	10.82 ± 0.44 g,k	10.35 ± 0.47 l,p	9.39 ± 0.71 s,t
petunidin	8.95 ± 0.13 c,h	8.91 ± 0.55 d,m	5.19 ± 0.25 c,d,q,s
peonidin	9.07 ± 1.41 i	8.49 ± 0.48 n,p	
malvidin	7.61 ± 0.68 e,j,k	8.91 ± 0.45 f,o	5.11 ± 0.58 e,f,r,t

quercetin (*37*, *38*). Moreover, it was reported that gastrointestinal absorption of anthocyanins in mammals was considerably less than other flavonoids such as epicatechin (*26*−*35*, *37*, *38*). Thus, first, we established the highly sensitive semimicro-HPLC method to determine the plasma concentration of anthocyanins after oral administration (*34*, *35*). Using this method, we have studied the uptake and metabolism of several purified anthocyanins including cyanidin 3-*O*-β-D-glucoside and delphinidin 3-*O*-β-D-glucoside in rat (*32*−*35*). In those studies, we revealed that *O*-methylation is one of the major metabolic paths of

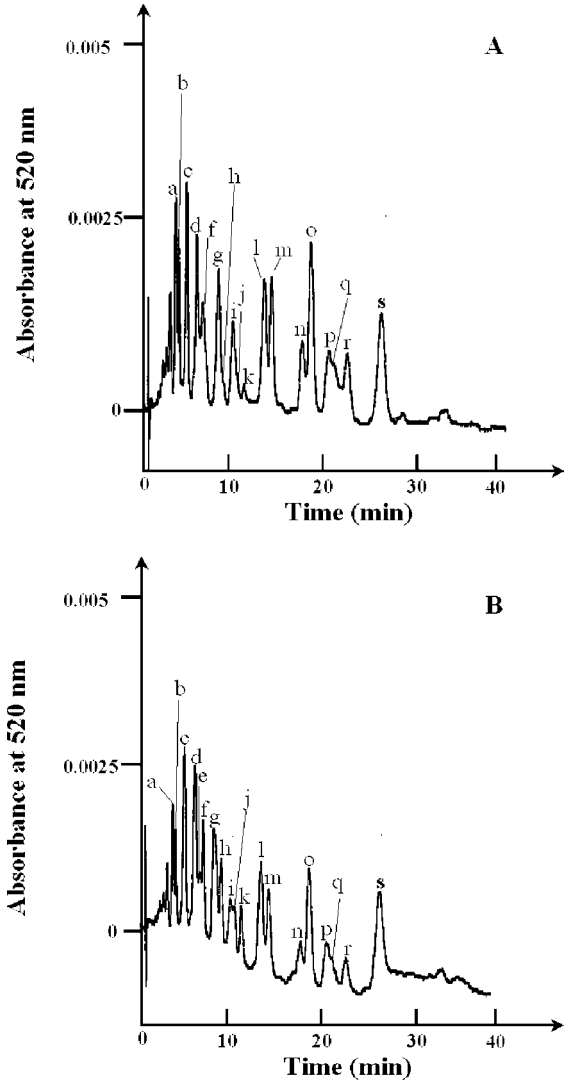

Figure 6. Typical HPLC chromatograms of anthocyanins and metabolites in rat bile and urine recovered during the first 2 h of oral administration of Bilberon 25: (**A**) bile and (**B**) urine. Peak numbers correspond to **Table 4**.

anthocyanins with catechol and pyrogallol type B rings, and the B ring structure is the major determinant for the *O*-methylation reaction as was shown in the previous reports that

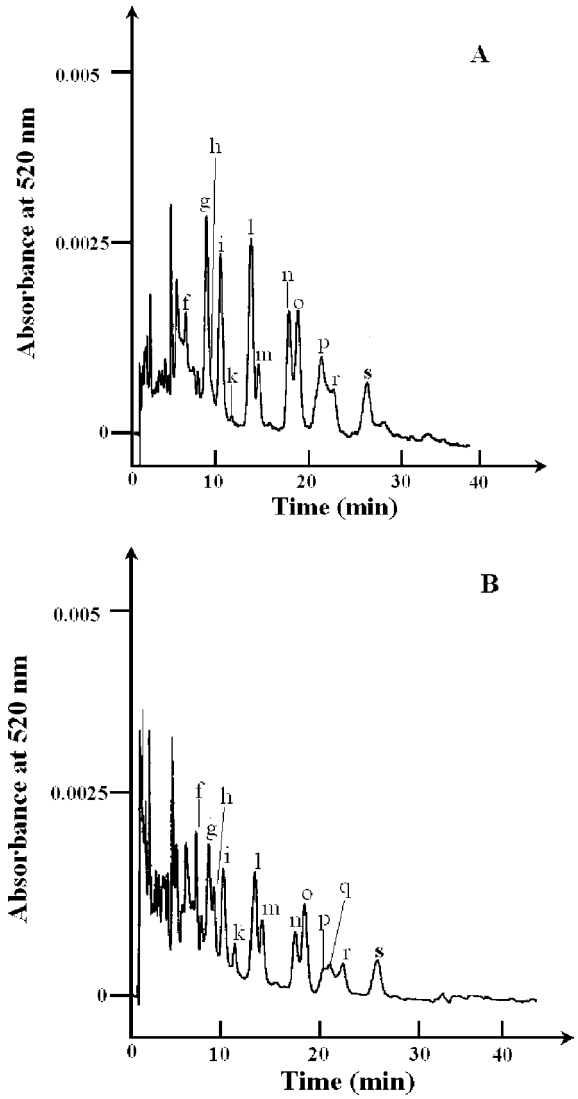

Figure 7. Typical HPLC chromatograms of anthocyanins and metabolites in rat tissues at 15 min of oral administration of Bilberon 25: (**A**) liver and (**B**) kidney. Peak numbers correspond to **Table 4**.

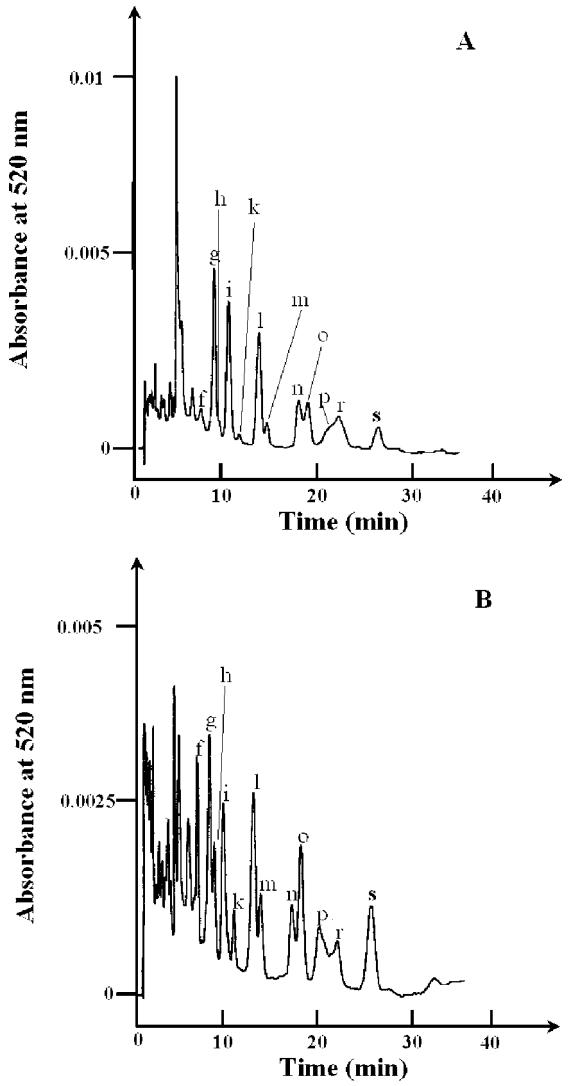

Figure 8. Typical HPLC chromatograms of anthocyanins and metabolites in rat tissues at 15 min of intravenous administration of Bilberon 25: (**A**) liver and (**B**) kidney. Peak numbers correspond to **Table 4**.

delphinidin 3-*O*-*β*-D-glucoside carrying pyrogallol structure was exclusively metabolized to 4′-*O*-methyl delphinidin 3-*O*-*β*-D-glucoside (*32*, *33*); on the other hand, both 3′- and 4′-*O*-methyl cyanidin 3-*O*-*β*-D-glucoside were formed from cyanidin 3-*O*-*β*-D-glucoside carrying the catechol structure (*34*). Furthermore, we revealed that extended glucuronidation products (glycoside-glucuronides) were formed as another major metabolite of cyanidin 3-*O*-*β*-D-glucoside (*35*). However, anthocyanidin (aglycone) glucuronides were seldom detected in contrast to the case of other flavonoids (*37*, *38*). This discrepancy is probably due to the difference in the stability of aglycones.

Several metabolic studies on anthocyanins in animal and human also have been reported from other laboratories using elder berry and black currant as anthocyanin sources (*26*−*31*). In these studies, urinary metabolites of human were also examined (*26*, *29*). However, we recently showed in rats that an excreted anthocyanin pattern in urine was quite different from that in plasma (*35*) and suggested that additional modification occurs in the kidney in anthocyanin metabolism. Therefore, analysis of urinary metabolite profile may not be sufficient to evaluate the functional role of anthocyanins.

Because anthocyanin comprises a variety of types of aglycone and attached sugars including acylated sugar, it is difficult to discuss comprehensively their structure−bioavailability relationships from limited experimental data. Moreover, the term of bioavailability was wrongly used in some reports; for example, the values were evaluated only from the amount of anthocyanins excreted in the urine (*26*, *29*).

In the present paper, we investigated the uptake and excretion of anthocyanin in rats using Bilberon 25 as the composite anthocyanin source and evaluated the bioavailability of each anthocyanin in the mixture. The bilberry extract (Bilberon 25) contains 15 types of anthocyanins (**Figure 1**) comprising five aglycones (cyanidin, delphinidin, peonidin, petunidin, and malvidin) and three types of attached sugars (glucose, galactose, and arabinose). Therefore, it is a good target for the comparative study of uptake and excretion of anthocyanins with different structures under the same experimental condition and the discussion of their bioavailability. Although several studies on the anthocyanin absorption have been reported using bilberry extract as an anthocyanin source (*39*−*41*), the bioavailability and tissue distribution were not previously examined.

The advantage of using the bilberry extract (Bilberon 25) was clearly demonstrated in **Figure 2** when the plasma uptake was examined for the anthocyanins in the mixture after oral administration. All of the anthocyanins in the bilberry extract, except 3-*O*-α-L-arabinoside of peonidin, were detected in blood

Table 4. Structural Assignment and Quantification of Anthocyanins and Their Metabolites in Tissues by Tandem MS Spectrum and HPLC Retention Time Together with Their Distributed Amounts (pmol/g Tissue) in Liver and Kidneys

peak	peak no. in **Figure 2**	parent/production	anthocyanins	pmol/g tissue	
				liver	kidneys
a		463/287	cyandin 3-O-β-D-glycoside-glucuronide (*)[a]	ND	ND
b		463/287	cyandin 3-O-β-D-glycoside-glucuronide (*)	ND	ND
c		477/301	O-methylcyandin 3-O-β-D-glycoside-glucuronide (*)	ND	ND
d		477/301	O-methylcyandin 3-O-β-D-glycoside-glucuronide (*)	ND	ND
e			unidentified (*)	ND	ND
f	3	449/287	cyanidin 3-O-β-D-galactopyranoside	5.41	14.19
g		479/317	4′-O-methyl delphinidin 3-O-β-D-galactopyranoside	15.73	55.09
h	5	449/287	cyanidin-3-O-β-D-glycoside-glucuronide (*)	2.13	18.36
I			unidentified (*)	14.99	65.38
j			unidentified	ND	ND
k	6	479/317	petunidin 3-O-β-D-galactopyranoside	3.46	9.53
	8	479/317	petunidin 3-O-β-D-glucopyranoside		
l	8	449/317	4′-O-methyl delphinidin 3-O-α-L-arabinopyranoside	26	74.06
	8	463/301	4′-O-methyl cyanidin 3-O-β-D-galactopyranoside		
m	9	463/301	peonidin 3-O-β-D-galactopyranoside	7.24	54.07
n		463/301	4′-O-methyl cyanidin 3-O-β-D-glucopyranoside	19.18	46.12
o	11	493/331	peonidin 3-O-β-D-glucopyranoside	16.4	60.98
	11	493/331	4′-O-methyl petunidin 3-O-β-D-galactopyranoside		
p	12	493/331	malvidin 3-O-β-D-galactopyranoside	13.5	21.84
q			unidentified	ND	20.4
r		493/331	4′-methyl petunidin 3-O-β-D-glucopyranoside	11.11	21.6
s	14	493/331	malvidin 3-O-β-D-glucopyranoside	2.59	2.4

[a] Parent/product ion identification was carried out in urine and bile for symbol (*).

plasma by HPLC after 15 min of oral administration of bilberry extract (400 mg/kg as Bilberon 25). To evaluate the absorption efficiency of each anthocyanin, the anthocyanin concentrations in blood plasma after 15 min of oral administration were normalized by the orally administered dose of each anthocyanin in Bilberon 25 and the results (C_{15min}/dose) are shown in **Figure 3**. It was clear that the plasma appearance rate of each anthocyanin in the mixture was found to be remarkably different depending on the type of sugar attached and also on the aglycone structure, more correctly, the B ring structures of the aglycone (**Figure 3**). All of the anthocyanins reached the maximum plasma level at 15 or 30 min after oral administration suggesting that anthocyanins were effectively absorbed from the stomach as well as from the small intestine, as reported elsewhere (*32*, *42*−*44*). Because contents of each anthocyanin in original bilberry extract were different as shown in **Table 1**, the plasma concentration profile of each anthocyanin after normalization by the administered dose is given in **Figure 4**. When the values for the anthocyanins carrying the same aglycone were compared, galactosides clearly showed higher values than those of glucosides and arabinosides except malvidin (**Figure 3**). On the other hand, when the anthocyanins carrying the same sugar moiety were compared, differences among the aglycones were not marked, although cyanidin and delphinidin-glycosides showed rather higher values than the *O*-methyl analogues, malvidin and petunidin, but peonidin 3-*O*-β-D-galactoside was exceptional, which showed the highest values among all anthocyanins.

The plasma levels of anthocyanins will be governed by several factors such as gastrointestinal uptake process, tissue distribution rate, and excretion to urine and bile. To assess the plasma disappearance rates, Bilberon 25 was intravenously injected (**Figure 5)**. The plasma disappearance of each anthocyanin showed biphasic decay kinetics. From the initial phase of the disappearance curve, $t_{1/2}$ was obtained for each anthocyanin and the results are summarized in **Table 3**. When the $t_{1/2}$ of anthocyanins with the same attached sugar was compared, the values of delphinidin and cyanidin showed longer than those of *O*-methyl analogues (petunidin, peonidin, and malvidin). On the other hand, there was no significant difference in $t_{1/2}$ between galactosides and glucosides of the same aglycone, but arabinosides showed shorter values in all anthocyanins (significant difference between galactoside and arabinoside except cyanidin). The higher plasma level of galactoside was thus explained by its effective gastrointestinal uptake as compared with glucoside.

From the plasma concentration profiles of anthocyanins obtained by oral and intravenous administration of Bilberon 25, we could calculate the bioavailability of each bilberry anthocyanin as summarized in **Table 2**. The bioavailability values were in the range from 0.61 to 1.82%. The bioavailability of bilberry anthocyanins as a mixture was 0.93%. Although this value was several times lower than those reported for other polyphenols (*37*, *38*), several characteristics of anthocyanin bioavailability became apparent from this study. When the bioavailability of anthocyanins was compared, *O*-methyl analogues (petunidin, peonidin, and malvidin) showed a higher bioavailability than their original analogues, cyanidin and delphinidin-glycosides, especially in galactosides (significant difference between cyanidin and peonidin-galactoside and between delphinidin and petunidin-arabinoside). However, the bioavailabilities of petunidin-glycosides were higher than those of malvidin-glycosides although malvidin is the *O*-methyl analogue of petunidin-glycoside. This trend was not seen in the order of C_{max}/dose such that C_{max}/dose values of petunidin and malvidin-glycosides were smaller than those of delphinidin-glycosides (significant difference between delphinidin and petunidin-arabinoside). On the other hand, among the cyanidin family, the C_{max}/dose of peonidin 3-*O*-β-D-galactoside was higher than cyanidin 3-*O*-β-D-galactoside, but peonidin 3-*O*-β-D-glucoside showed a lower C_{max}/dose than cyanidin 3-*O*-β-D-glucoside, although the difference is not significant. Therefore, a rather high bioavailability of petunidin-glycosides could be explained by their prolonged gastrointestinal absorption due to the stable aglycone nature. The same happened for peonidin 3-*O*-β-D-galactoside, but the outstandingly high bioavailability indicates a more effective gastrointestinal absorption property of this analogue. When the bioavailability was compared on the basis of the attached sugar type, galactosides showed the highest value, then arabinosides followed (see **Table 3**), and

glucosides showed the lowest bioavailability among anthocyanins with the same aglycone except delphinidin and malvidin. From the C_{max}/dose values, it was suggested that the intestinal absorption step significantly contributed to the bioavailability of arabinosides that was higher than glucosides. Thus, it was concluded that both aglycone (*O*-methylation) and attached sugar types are the modulation factors for the bioavailability of anthocyanins.

One of the interesting observations in the present study is that the gastrointestinal uptake of anthocyanins in Bilberon 25 was several times higher than those observed for authentic anthocyanins examined independently (*28*, *32*−*35*). For example, in the present study, the maximum plasma level of delphinidin 3-*O*-*β*-D-glucoside reached 0.15 μM with the dose of 18.7 mg/kg in the mixture, whereas it was only 0.3 μM in the previous study even though 100 mg/kg of purified delphinidin 3-*O*-*β*-D-glucoside was administered (*32*, *33*). It was also shown that the maximum plasma concentration of cyanidin 3-*O*-*β*-D-glucoside reached 0.14 μM with the dose of 16.5 mg/kg in the present study but 0.18 μM when purified cyanidin 3-*O*-*β*-D-glucoside was given independently with a dose of 100 mg/kg (*34*, *35*). The same tendency that the anthocyanins given as fruit extract or the mixture showed a higher plasma concentration than the purified sample has been reported elsewhere (*27*, *28*, *39*); for example, Matsumoto et al. (*28*) reported that the maximum plasma concentrations were 0.85, 0.85, and 0.58 μM, respectively, for cyanidin 3-*O*-*β*-D-glucoside, cyanidin 3-*O*-rutinoside, and delphinidin 3-*O*-rutinoside with the dose of 400 mg/kg of purified samples in rats (*28*). However, the maximum plasma concentration of cyanidin 3-*O*-*β*-D-glucoside reached 3.2 μM with the dose of 320 mg/kg given in rats as the fruits extract (*27*). These studies strongly indicate that the synergism occurs among anthocyanins or between anthocyanin and other coexisted ingredients in the fruit extract to modulate anthocyanin absorption from the gastrointestinal tract. Recently, several examples were reported on the modulating effects of coexisting food components on flavonoid and polyphenol absorption (*45*−*50*). Thus, modulation of bioavailability of anthocyanins will be an important target for improving the beneficial use of anthocyanins as a neutraceutical.

To discuss the anthocyanin excretion in rats, the urine and bile were collected after intravenous administration of Bilberon 25. The recoveries of anthocyanin in urine and bile were only 30.8 and 13.4%, respectively. Similar values were obtained previously when purified cyanidin 3-*O*-*β*-D-glucoside was intravenously given to rats (*35*). Because the total recovery of intravenously administered anthocyanins in urine and bile was approximately 45% during the first 4 h, it was suggested that considerable decomposition and/or tissue accumulation of anthocyanins occurred. The anthocyanin profiles in urine and bile were completely different from those of original Bilberon 25 indicating that anthocyanins were metabolized in tissues such as liver and kidneys (**Figure 6**). Biphasic decay of plasma anthocyanins after intravenous administration supports the possibility of anthocyanin distribution in tissues (**Figure 5**).

Anthocyanin profiles in the liver and kidney extracts showed clear differences in the type and amounts of anthocyanins from that of Bilberon 25, the original anthocyanin mixture (**Figures 7** and **8**). It is obvious that the relative amounts of anthocyanin analogues having *O*-methyl group(s) in the B ring such as peonidin, petunidin, and malvidin-glycosides became more major than delphinidin and cyanidin-glycosides in original mixture. In addition, many new peaks were found that did not exist in the chromatogram of the original Bilberon 25, probably metabolite peaks. To obtain the structural information of these metabolites, major peaks were isolated and analyzed by tandem MS. Taking HPLC mobility data into consideration, we assigned these major metabolites in liver and kidney as shown in **Table 4**. All of which were *O*-methyl anthocyanins (petunidin, peonidin, and malvidin-glycosides) and the *O*-methyl metabolites of original anthocyanins (glycosides of delphinidin and cyanidin) that have been previously identified (*32*−*35*). It was thus strongly suggested that anthocyanins having free phenolic groups in B ring are metabolized into their *O*-methyl analogues in liver and then distributed in tissues as we have predicted elsewhere (*32*). Moreover, glycosides of peonidin, petunidin, and malvidin are also the original constituents of Bilberon 25, although peonidin 3-*O*-*β*-D-glucoside is produced as the metabolite of cyanidin 3-*O*-*β*-D-glucoside (*34*). It is thus indicated that *O*-methyl anthocyanins have a higher affinity to tissues and are more incorporated into tissues rather than the analogues having a free OH group in the B ring, such as delphinidin and cyanidin-glycosides; that is, the hydrophobicity of the anthocyanidin B ring is a critical factor determining tissue distribution properties of anthocyanins. This was supported from the anthocyanin pattern in tissues after intravenous administration, in which almost no cyanidin and delphinidin-glycosides were present (**Figure 8**). HPLC patterns of tissue extracts strongly suggested that the metabolites, which increased their water solubility, such as glucoside-glucuronides of cyanidin and delphinidin, were also present in tissue especially in kidney, although the conclusion has to be sustained until clear MS data are obtained (*35*).

When the role of the sugar moiety in tissue uptake was examined, glucosides and galactosides were detected in both liver and kidney although a quantitative difference was obscured in their tissue affinity. However, it was obvious that none of arabinosides was determined except 4′-*O*-methyl delphinidin 3-*O*-α-L-arabinoside in both tissues. Therefore, the sugar moiety was thought to be an additional factor affecting the tissue distribution of anthocyanins. It is not yet conclusive that this is special for anthocyanins or general for other flavonoids, but these facts strongly suggest that distribution of metabolites in tissues has to be considered when physiological functions of anthocyanins are elucidated.

In summary, gastrointestinal uptake of anthocyanins is mainly governed by the sugar moiety attached such that galactosides are absorbed more effectively than arabinosides when compared with the same aglycone. However, the bioavailability was manipulated by several factors such as metabolism, hydrophobicity of the aglycone B ring, and attached sugar type, all of which modulate the absorption and distribution behavior of anthocyanins. Therefore, the present studies are quite important for the future discussion of the physiological roles of anthocyanins in humans.

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