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Absorption and Excretion of Black Currant Anthocyanins in Humans and Watanabe Heritable Hyperlipidemic Rabbits

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Anthocyanins are thought to protect against cardiovascular diseases. Watanabe heritable hyperlipidemic (WHHL) rabbits are hypercholesterolemic and used as a model of the development of atherosclerosis. To compare the uptake and excretion of anthocyanins in humans and WHHL rabbits, single-dose black currant anthocyanin studies were performed. Procedures for workup and analyses of urine and plasma samples containing anthocyanins were developed with high recoveries (99 and 81%, respectively) and low limits of quantification (≥6.6 and ≥1.1 nM, respectively). The excretion and absorption of anthocyanins from black currant juice were found to be within the same order of magnitude in the two species regarding urinary excretion within the first 4 h (rabbits, 0.035%; humans, 0.072%) and t_{max} (rabbits, ∼30 min; humans, ∼45 min). A food matrix effect was detected in rabbits, resulting in the absorption of a higher proportion of the anthocyanins from black currant juice than from an aqueous citric acid matrix. In humans the absorption and urinary excretion of anthocyanins from black currant juice were found to be proportional with dose and not influenced by the ingestion of a rice cake. In both species a larger proportion of the anthocyanin rutinosides than of the glucosides was absorbed, whereas the structure of the aglycon had no influence on the absorption and excretion. The anthocyanins had no effect in rabbits on the antioxidant capacity of plasma measured as Trolox equivalent antioxidant capacity and ferruc reducing ability of plasma.

KEYWORDS: WHHL rabbits; humans; anthocyanins; black currant; *Ribes nigrum* L.; urine; plasma; HPLC

INTRODUCTION

Anthocyanins, which belong to the group of flavonoids, are responsible for the red, violet, and blue color of most berries and fruits. They have been reported to have many and diverse protective effects on health, for example, improvement of dark vision (*1*), antiulcer effect in rats (*2*), antitrombus and vasoprotective effects in humans and rats (*2*−*4*), and also antiinflammatory and antimutagenic effects in vitro (*5*, *6*). Anthocyanins have furthermore been found to protect the low-density lipoproteins (LDL) against oxidative damage in vitro (*7*). LDL oxidation is recognized as an important step in the formation of atherosclerotic plaques and subsequent cardiovascular disease (*8*). Watanabe heritable hyperlipidemic (WHHL) rabbits are LDL receptor deficient and therefore a good model of human homozygous familial hypercholesterolemia. Before this animal model is used to investigate the effect of anthocyanins on the development of atherosclerosis, the uptake and excretion of anthocyanins must be investigated. Anthocyanin bioavailability has not previously been studied in rabbits in comparison with humans.

Among rich dietary sources of anthocyanins are red grapes, red wine, strawberries, elderberries, and black currants. Black currants (*Ribes nigrum* L.) are one of the most important sources of anthocyanins due to their dietary prevalence and because they have ∼250 mg of anthocyanins/100 g of fresh fruit (*9*). We have therefore chosen to use black currant anthocyanins for investigating the uptake and excretion of anthocyanins in the WHHL rabbit. The berries contain ∼15 different anthocyanins, of which the four major ones, cyanidin 3-*O*-glucoside (Cy-3-glc), cyanidin 3-*O*-rutinoside (Cy-3-rut), delphinidin 3-*O*-glucoside (Dp-3-glc), and delphinidin 3-*O*-rutinoside (Dp-3-rut), are responsible for ∼98% of the total anthocyanin content (*10*). In the present study we focused on these four major black currant anthocyanins and used two other structurally related anthocyanins not present in black currants, cyanidin 3,5-di-*O*-glucoside (Cy-3,5-diglc) and pelargonidin 3-*O*-glucoside (Pg-3-glc), as internal standards. All structures are shown in **Figure 1**. Detection and precise quantification of anthocyanins in biological samples are challenging due to their low concentration

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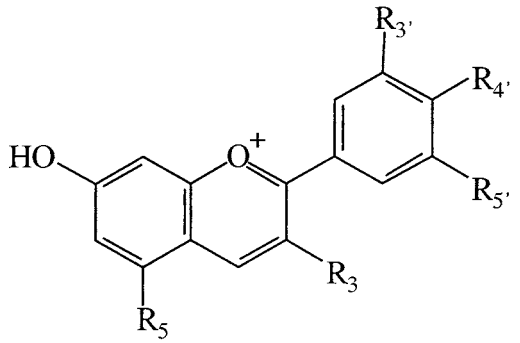

	$R_{3'}$	$R_{4'}$	$R_{5'}$	R_3	R_5
Cy-3-glc	OH	OH	H	O-Glc	OH
Cy-3-rut	OH	OH	H	O-Rut	OH
Cy-3,5-diglc	OH	OH	H	O-Glc	O-Glc
Dp-3-glc	OH	OH	OH	O-Glc	OH
Dp-3-rut	OH	OH	OH	O-Rut	OH
Pg-3-glc	H	OH	H	O-Glc	OH

Figure 1. Structures of the four major black currant anthocyanins and the two internal standards used. Cy, cyanidin; Dp, delphinidin; Pg, pelargonidin; Glc, glucoside; Rut, rutinoside.

in vivo and poor stability. Anthocyanins exist in a pH-dependent equilibrium among four different structural conformations, of which the red flavylium cation present at pH ~1 is the most stable form (*11*). Elevation of pH leads to changes in the conformation and color of the anthocyanins and to a faster breakdown of the compounds. Furthermore, anthocyanins are sensitive to light and elevated temperatures (*12*). Most previous papers on methods for the detection and quantification of anthocyanins in plasma and/or urine do not contain information on the recoveries and limits of quantification of the methodologies. Among the validated methods none are applicable for both urine and plasma samples with sufficient sensitivity and recovery and, furthermore, they do not include the use of internal standards for the correction of loss of the labile anthocyanins during collection, storage, workup, and analysis.

In the present study we investigated the absorption and excretion of anthocyanins in humans and WHHL rabbits by single-dose studies with black currant anthocyanins to evaluate the WHHL rabbits as a model for studying the influence of anthocyanins on the development of atherosclerosis. We looked into the influence of the food matrix, the anthocyanin dose, and the structure of the anthocyanin aglycon and glycoside on the plasma and urinary anthocyanin levels in both species. Furthermore, the effect of anthocyanins on the antioxidant capacity of plasma monitored as TEAC and FRAP was investigated in rabbits to study this possible mechanism of the proposed protective effect of anthocyanins against atherosclerosis. For the investigations new, sensitive, and reproducible methodologies including internal standards were developed to determine anthocyanins in plasma and urine.

MATERIALS AND METHODS

Chemicals. Concentrates of mixed black currant anthocyanins and anthocyanin standards delphinidin 3-*O*-glucoside (Dp-3-glc), delphidin 3-*O*-rutinoside (Dp-3-rut), cyanidin 3-*O*-glucoside (Cy-3-glc), cyanidin 3-*O*-rutinoside (Cy-3-rut), cyanidin 3,5-*O*-diglucoside (Cy-3,5-diglc), and pelargonidin 3-*O*-glucoside (Pg-3-glc) were obtained from Polyphenols Laboratories (Sandnes, Norway). A black currant juice concentrate was obtained from a pilot plant at BioCentrum (DTU, Lyngby, Denmark), and Altromin 2113, a standard pelleted rabbit diet, was obtained from Altromin International (Large, Germany).

Animals, Housing, and Clinical Observations. Animal experiments were performed in accordance with the Danish Animal Experimentation Act on a license granted by the Ministry of Legal Affairs. All housing procedures were performed according to the European convention for the protection of vertebrate animals used for experimental and other scientific purposes, No. 123, Strasbourg, 18. III. 1986.

Twenty male WHHL rabbits at 6 weeks of age [plasma cholesterol = 22.4 ± 1.9 mM (mean ± SD), body weight (bw) = 1.244 ± 0.139 kg] were obtained from our own breeding colony. The animals were housed individually under controlled environmental conditions with temperature at 20 ± 1 °C, relative humidity at 55 ± 5%, a 12/12 h light/dark cycle (8:00 a.m.−8:00 p.m.), and air exchanged 10 times/h. The animals had every day access to 100 g of Altromin 2113 and tap water ad libitum, and they were observed at least twice a day for any abnormalities in clinical appearance.

Experimental Design of Animal Study. At 9 weeks of age the animals were randomly assigned to four treatment groups of five animals each according to their plasma cholesterol and body weight and fasted for 24 h prior to dosage. Group I (control) was intubated with 6 mL of aqueous citric acid pH 3.5/kg of bw and group II with a black currant juice concentrate pH 3.5 (6 mL, containing 21 mg of Dp-3-glc, 58 mg of Dp-3-rut, 10 mg of Cy-3-glc, and 28 mg of Cy-3-rut per kg of bw, mean anthocyanin intake = 182 mg/animal). Group III was intubated with a 63% pure anthocyanin fraction isolated from black currants dissolved in aqueous citric acid pH 3.5 (6 mL containing 63 mg of Dp-3-glc, 45 mg of Dp-3-rut, 22 mg of Cy-3-glc, and 34 mg of Cy-3-rut per kg of bw, mean anthocyanin intake = 256 mg/animal) and group IV with a 79% pure anthocyanin fraction containing two black currant Dp-3-gly in aqueous citric acid pH 3.5 (6 mL containing 8 mg of Dp-3-glc and 45 mg of Dp-3-rut per kg of bw, mean anthocyanin intake = 81 mg/animal). The remaining 37 and 21% of the anthocyanin fractions used for groups III and IV, respectively, were found by LC-MS and NMR mainly to consist of monosaccharide derivates and other similar low molecular weight compounds originating from the black currant source. The test compounds were administered as a single intubation after which the animals had access to Altromin 2113 again. At all times the animals had access to water ad libitum.

Human Study. The human study was carried out at the Department of Applied Chemistry and Microbiology, University of Helsinki. The Ethics Committee of the Faculty of Agriculture and Forestry, University of Helsinki, accepted the study plan (22.2.2001), and the volunteers gave their informed consent.

Seventeen healthy normolipidemic female volunteers participated in the study. Their average age was 28.9 years (range = 19−48 years) and BMI was 23.0 kg/m^2 (range = 19.8−29.5 kg/m^2). A black currant juice similar to the one used in the rabbit study was used with sugar added to 16.7 w/w %. The dose of the undiluted juice was 4.4 g containing 7.5 mg of Dp-3-glc, 6.4 mg of Dp-3-rut, 0.61 mg of Cy-3-glc, and 5.2 mg of Cy-3-rut per kg of bw in group 1 (n = 5, mean anthocyanin intake = 1239 mg). Group 2 received 2.7 g of juice containing 4.6 mg of Dp-3-glc , 3.4 mg of Dp-3-rut, 0.38 mg of Cy-3-glc, and 3.2 mg of Cy-3-rut per kg of bw (n = 5, mean anthocyanin intake = 716 mg), and group 3 received the same dose as group 2 and in addition a rice cake consisting of 79% complex carbohydrates, 9% protein, 3.5% fiber, and 3% fat (Wasa, Filipstad, Sweden) (n = 7, mean anthocyanin intake = 746 mg). The study was conducted as a partial crossover study, where the subjects received either a control drink consisting of sugar water (16.7 w/w %) or one of the three juice treatments in randomized order. The two study days were two weeks apart. The subjects were asked to refrain from berries and berry products as well as alcohol during the last 24 h before the study days. On the preceding evenings, the intake of tea, coffee, cocoa, cola drinks and other soft drinks, fruits, and fruit products was prohibited. During the overnight fasting only water was allowed. When they came in the morning after overnight fasting, the volunteers gave baseline urine and blood samples and received 8 g/kg of bw of either diluted sweetened

juice or control drink in randomized order. No food was allowed during this 4-h period.

Sample Collection and Preparation. Rabbit blood samples were collected from the marginal ear vein in vacuum heparin tubes (Vacuette, Greiner Labortechnik, Kremsmünster, Austria) before and at 15, 30, and 60 min and 2, 4, 6, 24, and 48 h after intubation. The tubes were placed on ice after sampling and centrifuged for 12 min at 2600*g* and 4 °C. Plasma for antioxidant analyses was stored until analyses at −80 °C. Human blood samples were taken from the antecubital vein with minimal stasis in vacuum EDTA tubes (Venoject II, Terumo Europe, Leuven, Belgium) before and at 45, 90, 150, and 240 min after dosage. The tubes were placed in ice after sampling and centrifuged for 15 min at 1100*g* and 4 °C. Plasma from both species was prepared for anthocyanin analyses according to the method of Miyazawa et al. (*13*) with small modifications. Each milliliter of plasma was added to 200 μL of 0.44 M TFA containing 8.6 μg of Cy-3,5-diglc as an internal standard and stored at −80 °C until workup and analysis. Plasma samples for anthocyanin analyses were worked up by precipitating the plasma proteins with 2 mL of 10% TFA/mL of sample. After 15 min at −20 °C, the samples were centrifuged for 5 min at 4100*g* and 4 °C, and the protein pellet was washed twice with 2 mL of 10% TFA. The three supernatants were combined, and the anthocyanins were extracted by solid phase extraction (SPE) on Bond Elut-C18 columns (500 mg from Varian, Middelburg, The Netherlands) as follows. The column was preconditioned with two portions of 4 mL of acetonitrile (ACN) and two portions of 4 mL of 10% aqueous formic acid. The plasma extract was applied on the cartridge, and the column was washed with two portions of 4 mL of 10% aqueous formic acid before elution with 800 μL of 40% aqueous formic acid containing 40% ACN (pH 0.9). The eluate was evaporated to dryness, dissolved in 250 μL of 10% aqueous formic acid, and centrifuged for 10 min at 5000 rpm prior to injection of the in entire supernatant onto the HPLC system.

Urine samples from rabbits were collected before and in the intervals 0−2, 2−4, 4−6, 6−24, and 24−48 h after intubation. Aqueous citric acid (0.5 M) containing 0.7 mg of Cy-3,5-diglc/L as an internal standard was initially added to the urine collection trays, using 10 mL for each hour of collection. After collection, concentrated HCl was added to the urine samples to pH 1.5. Samples were stored at −80 °C until workup and analysis. Urine samples from the humans were collected before dosing when they came in after overnight fasting and during the 4-h followup. All samples collected before the end of the 4 h were refrigerated and pooled as one sample at the end of the 4-h collection interval. Concentrated HCl was immediately added to the samples to pH 1.5, and 5 μg of Cy-3,5-diglc in 10% aqueous formic acid was added to the samples as an internal standard. The samples were stored at −80 °C until workup and analysis. All urine samples were worked up by SPE using the same methodology as for the plasma extracts with one modification. After elution from the Bond-Elut columns, the eluates were added to 10 μg of Pg-3-glc in 10% aqueous formic acid containing 10% ACN, as an additional internal standard for volume correction. An aliquot of 40 μL of the eluate was diluted with 200 μL of 10% aqueous formic acid to reduce the ACN content, and the resulting 240 μL was analyzed by HPLC.

The anthocyanin content in the black currant juices and the anthocyanin dosages in aqueous citric acid was determined in triplicate by the same HPLC methodology as for the urine and plasma samples without preceding workup.

Anthocyanin Analyses. The identification and quantification of the four major black currant anthocyanins and the two internal standards were performed using an HP 1090 series HPLC system with an HP 1100 UV−vis detector. The system was equipped with a 250 μL injection loop, a Zorbax SB-C3 column (4.6 × 12.5 mm, 5 μm) with a guard cartridge (C-3, 4 × 4 mm, 5 μm) as column 1, and a Zorbax SB-C18 column (4.6 × 150 mm, 5 μm) as column 2, all from Agilent Technologies (Palo Alto, CA). The columns were maintained at 40 °C during analyses using a thermostatically controlled column compartment. Detection was carried out simultaneously at 520, 350, 290, and 254 nm, with peak scanning between 190 and 800 nm in 2 nm steps and reference at 650 ± 50 nm. The chromatographic conditions are shown in **Table 1** and **Figure 2**, using mobile phases A [10% (v/v) aqueous formic acid], B (100% methanol), and C (100% ACN). The

Table 1. Chromatographic Conditions for the HPLC System

time (min)	% mobile phase[a] (v/v) B	C	flow (mL/min)	column switch (min/position of valve)
0.0	1.0	0.0	1.0	0.0/1
1.0	1.0	0.0	1.0	1.5/2
2.4	8.0	0.0	0.9	
8.0	8.0	0.0	0.9	8.0/1
10.0	100.0	0.0	1.2	
13.0	100.0	0.0	1.2	
13.2	0.0	1.0	1.2	
17.0	0.0	1.0	1.0	17.0/2
18.0	0.0	1.0	0.64	
19.0	0.0	7.5	0.64	
24.0	0.0	7.5	0.64	
34.0	0.0	18.0	0.64	
35.5	0.0	100.0	0.9	
38.0	0.0	100.0	0.9	
38.5	0.0	1.0	0.9	
43.0	0.0	1.0	0.9	

[a] The remaining mobile phase up to 100% consists of mobile phase A. Mobile phase A was 10% aqueous formic acid, mobile phase B was methanol, and mobile phase C was ACN.

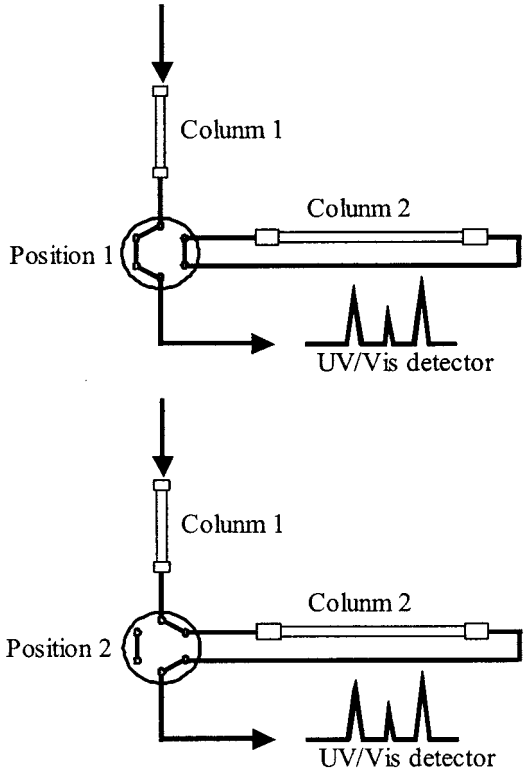

Figure 2. Schematic diagram of the HPLC system used for the determination of anthocyanins in plasma and urine from humans and rabbits.

column-switching system illustrated in **Figure 2** is similar to the one described by Nielsen et al. (*14*). Briefly, the samples were injected onto column 1 with the column-switching valve in position 1. At 1.5 min, when the anthocyanins were eluting from column 1, the automatic six-port valve switched to position 2, depositing the anthocyanins on column 2. At 8 min the valve switched back to position 1 and column 1 was washed with 100% B, removing more apolar impurities and, after a short conditioning of column 1 using 99% A, 1% C elution from column 2 was initiated at 17 min with the valve in position 2.

Quality Assurance of Sample Workup and HPLC Analyses. The calibration curves were prepared by spiking blank plasma and urine with the four major black currant anthocyanins and Cy-3,5-diglc at six different concentration levels in triplicate prior to sample workup and analyses. The spiked levels of each anthocyanin were 0, 0.5, 5, 50, 200, and 500 ng/mL of plasma and 0, 0.25, 2.5, 25, 250, and 600 ng/

mL of urine. To ensure reproducibility of the sample workup and analyses, a selected blank urine or blank plasma sample, respectively, was repeatedly spiked, worked up, and analyzed with each series of samples. Furthermore, an aqueous standard solution of the anthocyanin standards was included repeatedly in each HPLC sequence. The workup and analyses were validated with respect to recovery, intra- and interday variation, limit of detection (LOD), and limit of quantification (LOQ). In addition, correction for the breakdown of anthocyanins during storage, workup, and analyses was performed by the use of the internal standard.

Antioxidant Capacity Analyses. The ferric reducing ability of plasma (FRAP) was determined as described by Benzie and Strain (*13*, *15*) and the Trolox equivalent antioxidant capacity (TEAC) by the use of a commercially available kit (catalog no. NX 2332, Randox).

Data Evaluation. Data with a normal distribution and homogeneity of variance were analyzed by ANOVA. Data that could not meet these criteria were analyzed by Kruskal−Wallis test. The values shown represent mean ± SD. p values <0.05 were considered to be significant. All statistical analyses were performed using Statistical Analysis System (SAS) software version 6.12 (SAS Institute Inc., Cary, NC).

Due to the different amounts of anthocyanins dosed to the groups, the anthocyanin concentration in plasma and urine was related to the anthocyanin dosage before the data were compared. The amount of anthocyanin in plasma was thus calculated as AUC_{dose} (area under the plasma/dose curve). Each point at the plasma dose curve was calculated as ng/mL(t)/(mg/kg of bw) as described by Matsumoto et al. (*16*). The urinary excretion was calculated as the percentage of the ingested dose excreted in urine during a certain time interval.

RESULTS

Anthocyanin Analyses. Figure 3 shows the HPLC chromatograms of blank and spiked samples of rabbit urine, a rabbit and a human urine sample, and a human plasma sample collected after dosage with black currant juice. The validation of the methodology for workup and analyses of anthocyanins in plasma and urine is seen in **Table 2**. Recoveries were higher than 81% in plasma and 99% in urine. The intra- and interday variations were below 10% in both sample types, and the LOQ was between 0.8 and 1.1 nM (0.5 ng/mL for each anthocyanin) in plasma and between 0.5 and 6.6 nM (0.22−2.95 ng/mL) in urine.

Plasma and Urine Levels of Total Anthocyanins in Rabbits and Humans. The total anthocyanin plasma concentrations in the rabbit and human studies are shown in **Figure 4**, panels A and B, respectively. The peak plasma concentrations of all anthocyanins were detected between 15 and 60 min ($t_{max} \sim 30$ min) in rabbits. In humans the peak occurred between 0 and 90 min ($t_{max} \sim 45$ min) for all anthocyanins in the two groups treated with black currant juice without a rice cake and between 45 and 150 min ($t_{max} \sim 90$ min) for the group treated with the additional rice cake. In both species the plasma samples collected at baseline prior to dosage contained anthocyanins. **Table 3** shows the total amount of anthocyanins found in rabbit plasma in relation to dose (AUC_{dose}) and the urinary excretion for the three anthocyanin-dosed groups. The AUC_{dose} for the rabbits dosed with black currant juice was significantly larger than for the two groups dosed with fractions of purified black currant anthocyanins or purified delphinidin glycosides dissolved in aqueous citric acid ($p = 0.016$ and $p = 0.048$, respectively). This difference was however, only reflected in urine between the juice and the black currant mix groups.

The dose-adjusted absorption (AUC_{dose}) and the urinary excretion in the three human treatment groups are shown in **Table 4**. No significant differences were detected between the groups. Values for AUC_{dose} were based on the plasma curves (**Figure 4B**), which had not reached the baseline level at 4 h. AUC_{dose} values for humans are thus only indicative.

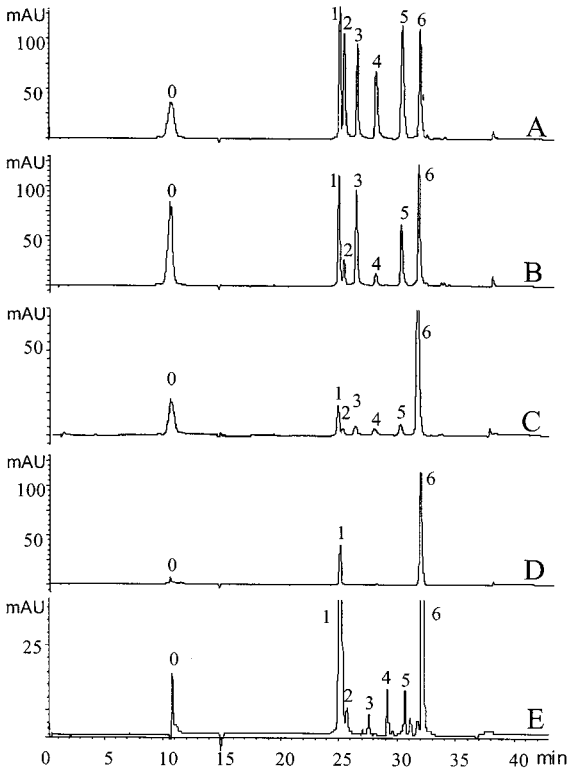

Figure 3. HPLC chromatograms detected at 520 nm of (A) a blank rabbit urine spiked with the four black currant anthocyanins and the internal standard. (0) apolar impurities from column 1, (1) cyanidine-3,5-di-*O*-glucoside (internal standard) and (6) pelargonidin-3-*O*-glucoside (internal standard); (B) blank rabbit urine spiked with the four black currant anthocyanins (peaks: 2, delphinidin 3-*O*-glucoside; 3, delphinidin 3-*O*-rutinoside; 4, cyanidin 3-*O*-glucoside; 5, cyanidin 3-*O*-rutinoside); (C) rabbit urine sample collected between 4 and 6 h after intubation with black currant juice (peak areas correspond to 2.2, 0.18, 0.28, 0.22, 0.19, and 10 μg/40 mL of urine, respectively); (D) human urine sample collected between 0 and 4 h after ingestion of black currant juice (peak areas correspond to 1.05, 0.29, 0.40, 0.36, 0.47, and 10 μg/40 mL of urine, respectively); (E) human plasma sample collected at 90 min after ingestion of black currant juice (peak areas correspond to 2600, 18.6, 21.0, 25.3, 32.9, and 10000 ng/0.5 mL of plasma, respectively).

Table 2. Validation of the Methodology for Anthocyanin Analyses

	%	CV%			
	recovery ($n = 10$)	intraday ($n = 9$)	interday ($n = 9$)	LOQ[c] (nM)	LOD[d] (nM)
plasma[a]					
Dp-3-glc	85	6.6	9.6	1.1	0.32
Dp-3-rut	86	6.4	9.7	0.8	0.25
Cy-3-glc	81	3.0	6.0	1.1	0.33
Cy-3-rut	81	3.5	6.9	0.8	0.25
Cy-3,5-diglc	83	3.5	7.6	0.8	0.25
urine[b]					
Dp-3-glc	99	6.3	6.3	0.5	0.1
Dp-3-rut	99	6.6	6.9	1.0	0.3
Cy-3-glc	99	7.6	10	6.6	2.0
Cy-3-rut	100	5.6	6.1	0.7	0.2
Cy-3,5-diglc	90	7.5	7.5	0.5	0.1

[a] $R^2 > 0.997$ (0–0.5 μg/mL). [b] $R^2 > 0.9998$ (0–0.6 μg/mL). [c] Determined as the lowest concentration on the standard curve for plasma and as the signal to noise ratio, S/N = 10, for urine. [d] Determined as S/N = 3.

Structure-Dependent Absorption and Excretion of Anthocyanins. Table 5 shows the dose-related absorption and urinary excretion of anthocyanins in the two species according

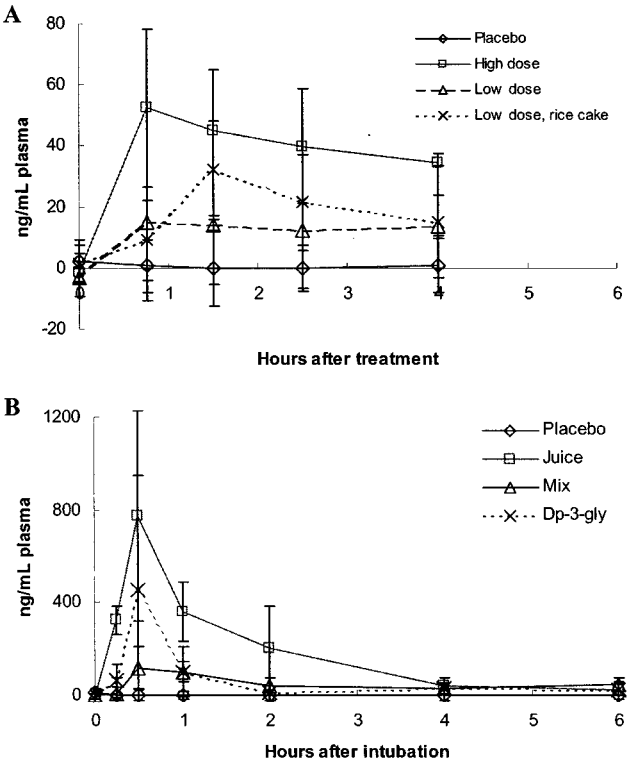

Figure 4. (A) Total plasma anthocyanin concentration in a fasting human subject during the first 4 h after dosage. Control: sugar water ($n = 17$). High dose ($n = 5$): black currant juice containing a total of 19.91 mg of anthocyanin/kg of bw. Low dose ($n = 5$): black currant juice containing a total of 12.22 mg of anthocyanin/kg of bw. Low dose, rice cake ($n = 7$): low dose ingested together with a rice cake. (B) Total plasma anthocyanin concentration in rabbits during the first 6 h after dosage. Placebo: 6 mL of aqueous citric acid/kg of bw. Juice: black currant juice containing a total of 117 mg of anthocyanin/kg of bw. Mix: All four major black currant anthocyanins dissolved in aqueous citric acid containing a total of 164 mg of anthocyanin/kg of bw. Dp-3-gly: black currant delphinidin glycosides dissolved in aqueous citric acid containing a total of 53 mg of anthocyanin/kg of bw.

Table 3. Uptake and Excretion of Anthocyanins in Rabbits ($n = 20$)

	black currant juice[a]	black currant mix[b]	Dp-3-gly[c]
AUC_{dose},[d] 4 h	6.6 ± 2.6	0.76 ± 1.2*[e]	1.8 ± 2.2*
% excr,[f] 4 h	0.035 ± 0.015	0.0095 ± 0.0064*	0.023 ± 0.017
% excr,[f] 48 h	0.36 ± 0.06	0.21 ± 0.10*	0.36 ± 0.030

[a] Black currant juice giving a total of 117 mg of anthocyanin/kg of bw. [b] Mix of the four major black currant anthocyanins dissolved in aqueous citric acid giving a total of 164 mg of anthocyanin/kg of bw. [c] Dp-3-glc and Dp-3-rut dissolved in aqueous citric acid giving a total of 53 mg of anthocyanin/kg of bw. [d] AUC_{dose}, 4 h: area under the plasma/dose curve from 0 to 4 h. Each point at the plasma/dose curve was calculated as ng/mL(t)/(mg/kg of bw). [e] *, significantly different from the juice dosage ($p < 0.05$). [f] % excr: cumulative urinary excretion from 0 to 4 h or from 0 to 48 h as a percentage of dose.

to their aglycon structure (Dp and Cy) and according to their sugar moiety (Rut and Glc). No aglycon-dependent difference was detected between the absorption or excretion of anthocyanins in either of the species, whereas the absorption of the glucose-linked anthocyanins was significantly lower than the absorption of the anthocyanin rutinosides in both species. This difference was reflected only in the rabbit urine. **Figure 5** shows the cumulative urinary excretion as a percentage of doses of the anthocyanin glucosides or rutinosides in rabbits at all time points. After 6 h, a significantly higher proportion of the anthocyanin rutinoside dose than the anthocyanin glucoside dose was excreted through urine.

Table 4. Uptake and Excretion of Anthocyanins in Humans ($n = 14$)

	high dose[a]	low dose[b]	low dose, cake[c]
AUC_{dose},[d] 4 h	19 ± 16	19 ± 21	16 ± 26
% excr,[e] 4 h	0.072 ± 0.094	0.048 ± 0.059	0.045 ± 0.049

[a] High-dose black currant juice giving a total of 19.7 mg of anthocyanin/kg of bw. [b] Low-dose black currant juice giving a total of 11.6 mg of anthocyanin/kg of bw. [c] Low-dose black currant juice and a rice cake giving a total of 11.6 mg of anthocyanin/kg of bw. [d] AUC_{dose}, 4 h: area under the plasma/dose curve from 0 to 4 h. Each point at the plasma/dose curve was calculated as ng/mL(t)/(mg/kg of bw). [e] % excr, 4 h: cumulative urinary excretion from 0 to 4 h as a percentage of dose.

Antioxidant Markers. In the rabbits no difference was detected in TEAC, whereas FRAP was significantly increased in the juice group in comparison to the other groups between 0.25 and 2 h after dosage (data not shown).

DISCUSSION

Anthocyanin Analyses. According to recent findings the majority of the absorbed anthocyanins are found as the unchanged glycosides (*17*), and consequently the method was developed to investigate the excretion and absorption of the intact compounds. To stabilize the anthocyanins in the more robust red flavylium cation form, the samples were acidified immediately at collection and an internal standard was added to correct for any loss during freezing, storage, and workup. From our previous studies (*18*) we determined that a pH of 2.1 was optimal to stabilize the anthocyanins in urine samples. To gain a sufficiently low LOD, the plasma and urine samples had to be purified and concentrated. This was achieved by SPE, and a further purification step was included by column 1 in the HPLC column-switching method before detection. The use of detection within the visual range (vis) in addition to the ultraviolet (UV) increased the absorbance of the anthocyanins at 520 nm by 2-fold. The authenticity of the anthocyanin peaks was confirmed by retention time, UV−vis spectra, and spiking experiments. Furthermore, a positive identification of the peaks was performed by LC-APCI-MS as described previously (*18*). As shown in **Figure 3**, the HPLC method results in the separation of all four black currant anthocyanin peaks in urine from both species and plasma from humans dosed with black currant juice. The validation of the methodology showed high recoveries and low LOQs in plasma and urine. In the two in vivo studies, the recovery of the internal standard added at collection was 66% (SD = 17%) in plasma samples and 77% (SD = 34%) in urine samples when they were worked up and analyzed between 6 and 12 months after collection. This underscores the importance of the use of an internal standard to calculate for the loss of these very labile compounds during collection, storage, and workup. Most methodologies for the sample workup and detection of anthocyanins in urine and plasma reported in the literature include SPE prior to UV HPLC analyses (*13*, *16*, *19*−*23*). Among those, the validated methodologies have an LOD ranging from 1 to 13 nM (*16*, *20*), urine recoveries ranging from 85 to 90% (*16*, *20*, *24*), and plasma recoveries ranging from 70 to 88% (*16*, *22*). Although these values are all within the same range or poorer than for the present methodology, none of them are applicable in both urine and plasma samples with sufficient sensitivity, and they do not include an internal standard to improve the preciseness of the analyses as in the present methodology.

Table 5. Influence of Anthocyanin Aglycons and Glycosides on Uptake and Excretion

		Dp[a]	Cy[b]	Rut[c]	Glc[d]
rabbit[e]	AUC_{dose},[f] 4 h	6.99 ± 6.26	6.26 ± 2.79	8.94 ± 4.5	4.34 ± 3.89*[g]
	% excr,[h] 4 h	0.038 ± 0.022	0.038 ± 0.022	0.041 ± 0.026	0.035 ± 0.0091
	% excr,[h] 48 h	0.320 ± 0.096	0.380 ± 0.165	0.43 ± 0.0062	0.26 ± 0.13*
human[i]	AUC_{dose},[f] 4 h	16.3 ± 18.2	19.6 ± 27.0	24.9 ± 10.2	11.0 ± 29.3*
	% excr,[h] 4 h	0.044 ± 0.038	0.059 ± 0.038	0.058 ± 0.033	0.046 ± 0.043

[a] Dp: Dp-3-glc and Dp-3-rut. [b] Cy: Cy-3-glc and Cy-3-rut. [c] Rut: Dp-3-rut and Cy-3-rut. [d] Glc: Dp-3-glc and Cy-3-glc. [e] Rabbits dosed with black currant juice. [f] AUC_{dose}, 4 h: area under the plasma/dose curve from 0 to 4 h. Plasma/dose curve calculated as ng/mL(t)/(mg/kg of bw). [g] Significantly different from rutinosides ($p < 0.05$). [h] % excr: cumulative urinary excretion from 0 to 4 h or from 0 to 48 h as a percentage of dose. [i] All humans dosed with black currant juice.

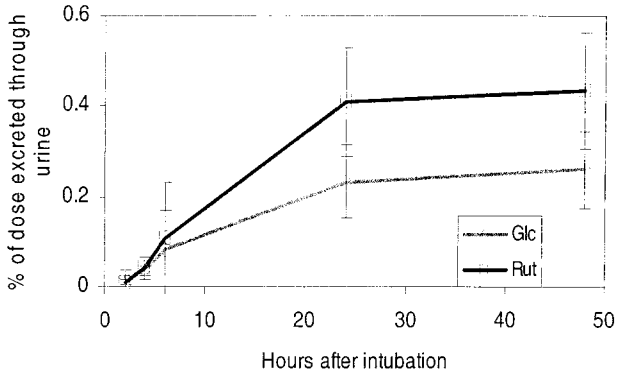

Figure 5. Urinary excretion of anthocyanin glucosides and rutinosides, respectively, in rabbits. Data are given as the cumulated urinary excretion at each time point after intubation. Glc, anthocyanin glucosides; Rut, anthocyanin rutinosides; *, significantly different ($p < 0.05$).

Arts et al. (*25*) have demonstrated that a number of flavonoids, which are structurally comparable with the anthocyanins, bind strongly to plasma proteins. Furthermore, Lietti and Forni (*26*) and Tsuda et al. (*22*) found that anthocyanins are distributed and deposited in organs, in particular, the kidneys, liver, heart, lungs, and the skin. The implication of an acidic extraction of the precipitated plasma proteins employed in the present study would release any hydrogen-bonded plasma anthocyanins. **Figure 5** shows that the urinary excretion of anthocyanins in rabbits continues until 48 h after dosage, even though the plasma concentration had declined to baseline already within 4 h. This supports the theory of plasma protein binding and/or tissue storage of anthocyanins, resulting in a slow release and eventually excretion of the anthocyanins.

Effect of Food Matrix. The total absorption and excretion of anthocyanins in the different dosage groups were compared in order to look for effects of the food matrix. In the rabbit study a juice matrix versus an aqueous citric acid matrix was investigated. In the human study the effect of the additional ingestion of a carbohydrate-rich meal (a rice cake) with the juice was investigated. As shown in **Figure 4B** a higher plasma level of anthocyanins was detected in the juice group than in the other anthocyanin-treated groups. This was in line with a significantly larger absorption of anthocyanins per milligram of dose (AUC_{dose}) for the rabbits dosed with black currant juice.

As seen in **Figure 4B**, t_{max} was equal for all of the dosed groups, and the anthocyanin plasma concentrations had declined to the baseline level already after 4 h, indicating that a difference in plasma kinetics cannot be responsible for the detected differences in AUC_{dose}. This suggests the presence of a food matrix effect giving a higher absorption of anthocyanins from juice than from an aqueous citric acid solution of purified anthocyanins. To our knowledge this is the first published report of the presence of a food matrix effect on the absorption of anthocyanins resulting in an increased bioavailability of the anthocyanins. According to Mülleder et al. (*27*), additional ingestion of sugar only slows and even decreases the absorption of Cy-3-glc. This suggests that the matrix effect detected presently is due to other potent components in the juice, which is even able to counteract any potential effects of the sugar content of the juice.

In the human study a delay was detected in the anthocyanin peak plasma concentration as an effect of the additional ingestion of a rice cake (**Figure 4A**). No other food matrix effects of the rice cake were detected on the total absorption or excretion of anthocyanins (**Table 4**). This indicates that the rice cake prolongs the stay in the stomach, giving a slower but equal uptake of anthocyanins from the intestine. Mülleder et al. (*27*) found a decrease in the absorption of Cy-3-glc but not of Cy-3-sambubioside with the additional ingestion of sugar. This was suggested to be due to competitive binding of Cy-3-glc and sugar to the intestinal sugar carriers. Even though some of the 79% carbohydrates in the rice cake are broken down to glucose in the intestine, this has apparently no effect on the absorption of the anthocyanin glucosides in comparison with the rutinosides ($p = 0.6$).

Human Absorption and Excretion of Anthocyanins. The amount of dose found in plasma and excreted through urine was found to be similar for all treatment groups. The human study was found to include two subjects in the high-dose group with extraordinarily high excretions of anthocyanins as reported previously (*27*, *28*). When these subjects were excluded as outliers from the data analyses, the mean excretion in the high-dose group was more similar to that of the other groups (0.043 ± 0.022%). In comparison to two previously reported studies (*13*, *16*) we find a similar dose-adjusted plasma level of Cy-3-glc at 1 h after dosage. Thus, on the basis of the present and previous studies it may be assumed that the increase in plasma anthocyanin concentrations is proportional with dose at least within the range of 0.165 (*13*) to 2.7 mg/kg of bw (*16*). During the first 4 h after dosage the urinary excretions of anthocyanins in the present study were around 0.044% for the two low-dose groups and 0.072% for the high-dose group, inclusive of the high excreters. Previous papers on urinary excretion of anthocyanins report an excretion between 0.016 and 0.11% of dosage within the first 2−8 h (*16*, *19*, *20*). Lapidot et al. (*28*) found urinary excretions as high as 1.5 and 5.1% of dose, but the methodology might, however, according to the authors, have resulted in the detection of falsely high levels of anthocyanins in the urine samples (*28*).

Rabbits and Humans. The absorption and excretion of anthocyanins in rabbits and humans were compared using all subjects in the human study and the rabbit juice group. Panels A and B of **Figure 4** show similar absorptions of anthocyanins in the two species with plasma t_{max} at 30−45 min. After t_{max}, the elimination of anthocyanins from plasma seems to be a little faster in rabbits than in humans. **Tables 3** and **4** indicate that the AUC_{dose} may be higher in humans than in rabbits, suggesting

that the latter species would need about twice the human dose to obtain a similar amount of anthocyanin in plasma but still, the AUC_{dose} values for humans must be regarded indicative and not absolute. No differences were detected between the two species in the percentage of the ingested dose excreted in urine at 4 h after ingestion of black currant juice. In the literature, the urinary excretion in humans has been followed up until 24 h at the most (*21*), showing a total excretion of 0.023−0.11% (*16*, *19*), whereas the rabbits in the present study excreted a total of 0.36% of the dose during the first 48 h after dosage. The previously published studies did not use acidification of the urine during their collection intervals of up to 12 h and did not use an internal standard to correct for degradation of the anthocyanins. It is therefore difficult to compare their human excretion data with our rabbit data, and a longer and better-controlled human intervention study with optimal sample treatment would thus be needed to compare the total urinary excretion of anthocyanins in the two species.

Effect of Aglycon and Sugar Moiety. No aglycom-dependent difference was detected between the absorption and excretion of the different types of anthocyanins in either rabbits or humans. This is supported by Netzel et al. (*20*), whereas Matsumoto et al. (*16*) might have found a larger proportion of the delphinidin glycosides than of cyanidin glycosides in plasma, but these differences were not supported by the urinary excretion data in that study. Neither of those two studies was looking specifically into this matter though.

In both species the AUC_{dose} values for the anthocyanin rutinosides were significantly higher than for the anthocyanin glucosides. This difference was probably also observed by Matsumoto et al. (*16*) and Netzel et al. (*20*), although the papers do not address this specific question in detail either. This difference between the anthocyanin glucosides and rutinosides was confirmed in the rabbit urine (**Figure 5**). In the human study (**Table 5**) the same tendency was observed in the 4-h urine sample ($p = 0.06$). According to the literature (*12*, *19*) the concentration of anthocyanin glucosides in plasma increases and decreases more quickly than the anthocyanin rutinosides. This was reflected in the 2-h urine sample in the rabbits (**Figure 5**, anthocyanin glucosides = 0.020%, anthocyanin rutinosides = 0.0094%, $p = 0.10$), whereafter this shifted toward an increased excretion of the rutinosides. This initially higher plasma level of the anthocyanin glucosides probably also influences the composition of the human 4-h urine, diminishing the differences between the anthocyanin glucosides and rutinosides in these samples. Flavonoids have been found to be transported into the epithelial cell layer of the intestine (*31*), where they are cleaved by the β-glucosidases (*29*) before transport into the blood lumen. An explanation for the different proportions of the two anthocyanin glycosides found in plasma and urine might be that the compounds are transported or diffuse into the epithelial cell layer of the intestine and that a part of the anthocyanin glucosides but not the rutinosides are cleaved there by the β-glucosidases at the formation of their corresponding aglycon, as for the other flavonoids. The aglycons of anthocyanins are, if at all, absorbed only in small quantities (*17*, *21*). The cleavage of a part of the anthocyanin glucosides, but not of the anthocyanin rutinosides by the β-glucosidases, would thus result in a larger proportion of intact anthocyanin rutinosides that would be accessible for absorption and distribution in plasma and urine. We can thus conclude that the glycoside moiety, but not the aglycon itself, has an influence on the absorption of the individual anthocyanins and that this effect is evident in both humans and WHHL rabbits, suggesting similar mechanisms of absorption and excretion in the two species.

Antioxidant Capacity of Rabbit Plasma. No difference was detected in TEAC, whereas FRAP was initially increased in the juice group in comparison to the other groups. This increase indicates that even though the juice group reached a higher anthocyanin plasma concentration than the other anthocyanin groups (**Figure 4A**), the observed difference in FRAP is probably caused by components in the juice other than the anthocyanins. According to the literature, anthocyanins decrease TBARS in rat plasma (*30*) and act as an antioxidant in vitro on human LDL (*7*). The present study does, however, not suggest any direct antioxidant effect of the anthocyanins in vivo that are measurable by the TEAC and FRAP assays. It cannot be excluded, though, that the anthocyanins can have more indirect antioxidant properties on, for example, the plasma lipids, which could be responsible for a possible protective effect of anthocyanins on the development of atherosclerosis.

In conclusion we have seen that the developed methodologies for the collection, workup, and analyses of anthocyanins in plasma and urine are well controlled by employing internal standards and repeatedly using spiked samples. The methodology had high recoveries and low limits of quantification in comparison to previous methodologies. In the rabbit study a food matrix effect was observed resulting in a higher absorption of anthocyanins from a black currant juice than from purified anthocyanin fractions dissolved in aqueous citric acid. In the human study the plasma concentration and urinary excretion of anthocyanins were found to be proportional to dose and not influenced by the additional ingestion of a rice cake. Furthermore, we found a higher plasma concentration and urinary excretion of anthocyanin rutinosides than of anthocyanin glucosides in relation to dose. This is probably due to the cleavage of the anthocyanin glucosides but not of the rutinosides in the small intestine by the β-glucosidases. We found that the same parameters influenced the plasma and urinary levels of anthocyanins in WHHL rabbits and humans, indicating a similar mechanism of uptake in the two species, although there might be a difference in the achievable plasma levels within the first 4 h after dosage. The anthocyanins did not affect the antioxidant capacity of plasma measured as TEAC or FRAP in comparison to placebo in rabbits. This indicates that a possible protective effect of anthocyanins on the development of atherosclerosis is unlikely to be due to an increased redox capacity of plasma. This study shows that because the kinetics and excretion of anthocyanins in WHHL rabbits are comparable to those in the humans, the WHHL rabbit will be a useful model to study the effects of anthocyanins on the development of atherosclerotic cardiovascular disease.

SAFETY

Only healthy volunteers were qualified for participating in the study. Disposable glass- and plasticware were used wherever possible, and all biological samples were treated as potentially infectious.

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ABBREVIATIONS USED

ACN, acetonitrile; AUC_{dose}, area under the plasma/dose curve (each point at the plasma dose curve was calculated as ng/mL(t)/

(mg/kg of bw); LDL, low-density lipoproteins; SPE, solid phase extraction; WHHL rabbits, Watanabe heritable hyperlipidemic rabbits; LOD, limit of detection; LOQ, limit of quantification; TEAC, Trolox equivalent antioxidant capacity; FRAP, ferric reducing ability of plasma; Cy-3,5-diglu, cyanidin 3,5-di-*O*-glucoside; Dp-3-glc, delphinidin 3-*O*-glucoside; Dp-3-rut, delphidin 3-*O*-rutinoside; Cy-3-glc, cyanidin 3-*O*-glucoside; Cy-3-rut, cyanidin-3-*O*-rutinoside; Pg-3-glc, pelargonidin 3-*O*-glucoside; Dp-3-gly, delphinidin 3-*O*-glucoside and delphidin 3-*O*-rutinoside.

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