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ARTICLE

Bioavailability of Cyanidin Glycosides from Natural Chokeberry (*Aronia melanocarpa*) Juice with Dietary-Relevant Dose of Anthocyanins in Humans

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The aim of this study was to investigate the bioavailability of anthocyanins from chokeberry juice with a dietary-relevant dose of anthocyanins. Thirteen healthy volunteers consumed chokeberry juice providing 0.8 mg of anthocyanins/kg of body weight. Before and after juice consumption, blood and urine were collected. Concentration of anthocyanins was measured with HPLC-PDA−MS-ESI. Cyanidin-3-galactoside comprised 66% of total chokeberry anthocyanins. Eight cyanidin derivatives were found in blood and urine after juice consumption. The maximum plasma anthocyanin concentration of 32.7 ± 2.9 nmol/L was reached at 1.3 ± 0.1 h after juice consumption. The anthocyanins' urine excretion rate (62.9 ± 5.0 nmol/h) was the highest within the first 2 h. In total, $0.25 \pm 0.02\%$ of the ingested anthocyanins was excreted by the renal route during 24 h, mainly as metabolites of cyanidin. According to these observations, after consumption of a dietary-relevant dose of anthocyanins as natural chokeberry juice, anthocyanins and their metabolites were present in plasma and urine of volunteers.

KEYWORDS: Anthocyanins; chokeberry; bioavailability; metabolites; pharmacokinetics

INTRODUCTION

Anthocyanins are red, orange, blue and purple water-soluble pigments, and they are abundant in fruits and colorized vegetables. As inherent components of plant-derived food, anthocyanins are consumed in amounts which may be significant from the consumer physiology point of view. Daily anthocyanin consumption has been estimated at approximately 200 mg and in comparison with other flavonoids is substantially higher (*1*, *2*).

Within the past two decades, many studies have focused on the occurrence of anthocyanins, their potential biological activities and health effects, and their bioavailability (*3−8*). Numerous studies have suggested that anthocyanins present in food products have protective potential against chronic degenerative diseases, but mechanisms of this action are not completely explained. The anthocyanins have been demonstrated to have anticancer, cardioprotective and anti-neurodegenerative activities (*9−12*). They may be responsible for improvement of vision (*13*) and for the prevention of diabetes (*14*). Taking the above into account, measurement of anthocyanins' bioavailability and determination of their metabolites appearing in human tissues are essential requirements for investigations explaining the physiological function of anthocyanins. Hitherto, it has been shown that anthocyanins are absorbed and occur in animal and human plasma and urine in unchanged form of glycosides and conjugated derivatives, but their concentration in blood plasma after the intake was very low (*15−18*). The percentage of the ingested anthocyanin

dose recovered from urine was also low (*19−21*). Unfortunately, unequivocal reasons for anthocyanins' low bioavailability have not yet been found. Some studies suggest that, in describing the fate of anthocyanins in human and animal organisms, low-molecular-weight derivatives should be taken into account since anthocyanins can be degraded to phenolic acids (*22−24*). At present, the aspects of anthocyanins' bioavailability including absorption, metabolism and excretion are still not fully recognized.

It is noteworthy that in most of the studies extracts or concentrates of anthocyanins were used. The doses of anthocyanins were unnaturally high, even several dozen mg/kg body weight (bw). In bioavailability studies anthocyanins with a natural food matrix were rarely served (*5*, *6*). In earlier studies it was indicated that flavonols' bioavailability was strongly affected by the native food matrix (*15*, *16*, *25*, *26*). Based on these studies, the bioavailability of anthocyanins consumed as immanent components of food may be different from the bioavailability of anthocyanins consumed in the form of concentrates. Moreover, the bioavailability and metabolism of anthocyanins administered in low doses may be different from bioavailability of anthocyanins provided in high concentrated doses. Also, from the nutritional point of view, the application of a dietary source with natural concentration of anthocyanins for bioavailability research seems to be more appropriate.

Taking the above into consideration, the determination of the bioavailability of cyanidin glycosides from natural chokeberry juice with a dietary-relevant dose of anthocyanins was the aim of the present study. The chokeberry (*Aronia melanocarpa*) was

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chosen as it is an excellent source of anthocyanins such as cyanidin, a strong *in vitro* antioxidant, and a component of the human diet, mainly as an additive for fruit drinks, wine and jam, as well as gaining in popularity as a source of intensive color and phytochemicals beneficial to health (*3, 27, 28*). Because cyanidin compounds possess strong biological activities (*9, 29–31*), therefore, to estimate the potential beneficial effect of this substance on the body of the consumer after absorption, our investigation focused only on the study of compounds whose main core is flavylium cation structure.

MATERIALS AND METHODS

Chemicals. All reagents including acetonitrile, methanol and formic acid were purchased from Merck KGaA (Darmstadt, Germany). Cyanidin-3-galactoside (Cy-3-gal), cyanidin-3-glucoside (Cy-3-glu), cyanidin-3-arabinoside (Cy-3-ara), cyanidin-3-xyloside (Cy-3-xyl) (Extrasynthese, Genay, France), peonidin-3-galactoside (Pe-3-gal) and peonidin-3-arabinoside (Pe-3-ara) (Polyphenols, Sandnes, Norway) were used for identification. Natural 100% chokeberry juice (without additives) was obtained from a Polish organic farm (Eko-Ar, Lewin Klodzki, Poland) cultivating chokeberry.

Subjects and Study Design. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and study protocol approved by the Bioethical Committee of the Regional Warmia and Mazury Medical Chamber in Olsztyn. All subjects were fully informed about the study and signed an informed consent form.

Thirteen healthy volunteers (seven women and six men) aged 25 ± 1 years and with a mean body mass index of 23 ± 1 kg/m^2 participated in this study, which was carried out at the premises of the Institute of Animal Reproduction and Food Research in Olsztyn under the medical supervision of Pantamed Clinic, Olsztyn. The volunteers that were enrolled to participate in the study had to meet the following criteria: they had to be certified healthy at medical interview; they could not: participate in other clinical trials within 90 days prior to the survey, have intolerance and hypersensitivity to berries, take drugs, abuse alcohol, smoke more than 15 cigarettes a day, drink more than 5 cups of coffee a day, be pregnant and breast-feeding, or take any medications or vitamin supplements.

The eligible subjects were provided with instructions for a strict anthocyanin-free diet for 72 h prior to the morning of the starting day and throughout the experiment. For this purpose, a list of permitted foods and beverages was given to the participants. Moreover, the subjects were not allowed to drink alcohol for 2 weeks before and during the study and to take any medicine during the study, except for oral contraceptives. Next, during the first four hours after the challenge, subjects were allowed to drink water and then lunch (sandwich with cheese and ham) was provided. After that period, the volunteers were allowed to resume their daily diet with restriction to non-anthocyanin foods.

After an overnight fast, chokeberry juice providing 0.8 mg of anthocyanins/kg of body weight (250 mL/60 kg of subject, 0.67 mmol cyanidin/L of juice) with 100 of mL water was served to volunteers. Before (control) and after chokeberry juice consumption (0.5, 1.0, 2.0, 4.0, 6.0, 12.0, 24.0 h), elbow or forearm vein blood samples were taken to heparinized vacutainers. After each sampling, blood was centrifuged at 4 °C in two stages (500*g* for 10 min and 1000*g* for 5 min) and the obtained plasma was frozen and stored at −80 °C until analysis. Moreover, before chokeberry juice consumption and between 0 and 2, 2 and 4, 4 and 8, 8 and 12, and 12 and 24 h, urine samples were collected from subjects. The samples were immediately acidified with 0.44 mol of trifluoroacetic acid/L (1:0.2; v:v), frozen and stored at −80 °C until analysis.

Sample Preparation. To test the recovery of anthocyanins from the samples, blanks of urine and blood plasma samples were spiked with cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside, peonidin-3-galactoside or peonidin-3-arabinoside and were prepared using the procedure as below.

Extraction of anthocyanin was carried out using Sep-Pak C$_{18}$ cartridges (J. T. Baker, USA). The chokeberry juice, plasma and urine samples were applied to Sep-Pak C$_{18}$ cartridges conditioned with methanol and 5% formic acid aqueous solution. After the sample application, the cartridges were washed with 10 mL of 5% formic acid aqueous solution, followed by elution of anthocyanin derivatives with 5 mL of 5% formic acid

Table 1. Content of Cyanidin Derivatives in Chokeberry Juice

compound	content (mg/100 mL)
cyanidin-3-galactoside	12.60
cyanidin-3-glucoside	0.73
cyanidin-3-arabinoside	5.18
cyanidin-3-xyloside	0.59
total	19.10

methanolic solution. The eluents were evaporated to dryness with a stream of nitrogen at 37 °C and dissolved in 100 μL of 15% acetonitrile containing 4% formic acid. Before HPLC injection the solution was centrifuged (20 min, 13000*g*).

HPLC Analysis. Aliquots (20 μL) of sample solutions were injected into a HPLC system (Shimadzu, Kyoto, Japan) equipped with a 250 × 4.6 mm i.d. Synergi 4 μ Polar-RP 80A column (Phenomenex, USA). The HPLC system consisted of two pumps (LC-10 AD$_{VP}$), DAD detector (SPD-M10 A$_{VP}$) set at 520 nm, MS detector with single quadruple (QP8000α), autosampler (SIL-10 AD$_{VP}$), column oven (CTO-10 AS$_{VP}$) and system controller (SCL-10 A$_{VP}$). All chromatographic determinations were performed at 37 °C with a flow rate of 1.0 mL/min. The elution was done using a solvent gradient system consisting of solvent A (4% formic acid aqueous solution) and solvent B (4% formic acid acetonitrile solution). Gradients were as follows: 8−16−23−40−80−8−8% B at gradient time, t_G = 0−18−26−40−45−50−60 min. Anthocyanins were identified based on the comparison of their retention time, UV−visible spectrum and presence of the respective parent and daughter ion pairs (*m/z* values) with authentic compounds (if available) and previously published data (*16, 17*). The mass spectrometer with electrospray ionization (ESI) worked at positive mode with the following parameters: CDL temperature of 150 °C, CDL voltage of 10 V, probe voltage of 5.0 kV, nebulizer gas (N$_2$) flow of 4.0 L/min, and defragmentation voltage of 45 V. Quantification of all anthocyanins was expressed as cyanidin-3-galactose equivalents.

Pharmacokinetic Methods and Statistical Analysis. The data are presented as mean ± SEM. The pharmacokinetic parameters of anthocyanins in plasma (maximum anthocyanin concentration, time to reach maximum concentration, elimination half-life, elimination rate constant, lag time, volume of distribution, clearance, the area under the first moment of curve and the area under the concentration−time curve) and pharmacokinetic parameters of anthocyanin excretion with urine (elimination rate constant in urine, clearance, total amount of substance excreted in urine, volume of distribution, fraction of dose eliminated in urine and the area under the urinary excretion rate curve) were determined using Biokinetica (Poland) software. To measure the differences between means of measurement points, a repeated ANOVA measure with Tukey's post hoc test was applied. $P < 0.05$ was considered significant. The statistical analysis was performed using Statistica v. Six (Stat Soft, USA).

RESULTS

Recovery Test. Results of recovery tests on SPE of cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside, peonidin-3-galactoside and peonidin-3-arabinoside from urine were $88.2 \pm 1.6\%$, $86.2 \pm 1.4\%$, $86.6 \pm 1.7\%$, $89.0 \pm 1.4\%$ and $87.7 \pm 1.5\%$, respectively; and from plasma were $84.2 \pm 1.5\%$, $83.3 \pm 1.6\%$, $81.5 \pm 1.7\%$, $84.9 \pm 1.5\%$ and $83.3 \pm 1.8\%$ (for all tests, $n = 3$), respectively. The results were not corrected by the recovery factors.

Determination of Anthocyanins in Chokeberry Juice. Four anthocyanins were detected in chokeberry juice: Cy-3-gal, Cy-3-glu, Cy-3-ara and Cy-3-xyl. As shown in **Table 1**, Cy-3-gal comprised 66% of total chokeberry anthocyanins and was the main anthocyanin in chokeberry juice consumed in this study.

Determination of Chokeberry Anthocyanins in Plasma and Urine Samples. As a result of HPLC-PDA−MS-ESI analysis of human blood plasma and urine collected after chokeberry juice consumption, eight anthocyanin derivatives were found (**Tables 2−4**). Apart from the native anthocyanins (Cy-3-gal, Cy-3-glu, Cy-3-ara), their methylated and glucuronided metabolites were found to be

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Table 2. Anthocyanins Identified in Chokeberry Juice, Plasma and Urine

compound	retention time (min)	*m/z*	sample
cyanidin-3-galactoside	11.9	449, 287	juice, plasma, urine
cyanidin-3-glucoside	12.9	449, 287	juice, plasma, urine
cyanidin monoglucuronide	13.5	463, 287	plasma, urine
cyanidin-3-arabinoside	14.7	419, 287	juice, plasma, urine
peonidin-3-galactoside	16.6	463, 301	plasma, urine
peonidin monoglucuronide	18.6	477, 301	plasma, urine
peonidin monoglucuronide	19.1	477, 301	plasma, urine
peonidin-3-arabinoside	21.1	433, 301	plasma, urine
cyaniding-3-xyloside	22.3	419, 287	juice

**Table 3.** The Proportion of Different Cyanidin Derivatives Detected in Blood Plasma over Time[a]

anthocyanin	proportion (% area at 520 nm)								
	0	0.5	1.0	2.0	4.0	6.0	12.0	24.0	total
Cy3Gal	0.0	7.2	9.1	4.1	1.7	0.7	0.0	0.0	22.8
Cy3Glu	0.0	0.8	1.0	0.4	0.1	0.0	0.0	0.0	2.3
Cy3Ara	0.0	1.9	2.2	0.9	0.3	0.0	0.0	0.0	5.3
native	0.0	9.9	12.3	5.4	2.1	0.7	0.0	0.0	30.4
CyGluc	0.0	2.0	4.0	4.4	3.1	1.6	0.2	0.0	15.3
Pe3Gal	0.0	0.8	1.1	0.7	0.1	0.0	0.0	0.0	2.7
PeGluc	0.0	7.3	14.2	16.0	8.0	3.6	0.5	0.0	49.6
Pe3Ara	0.0	1.0	0.6	0.3	0.1	0.0	0.0	0.0	2.0
metabolites	0.0	11.1	19.9	21.3	11.3	5.2	0.7	0.0	69.6

[a] 0−24 h.

Table 4. The Proportion of Different Cyanidin Derivatives Detected in Urine over Time[a]

anthocyanin	proportion (% area at 520 nm)					
	0	0−2	2−4	4−8	8−12	12−24	total
Cy3Gal	0.0	13.1	5.8	3.3	0.6	0.0	22.8
Cy3Glu	0.0	1.5	0.7	0.4	0.1	0.0	2.7
Cy3Ara	0.0	2.6	1.2	0.7	0.2	0.0	4.7
native	0.0	17.2	7.7	4.4	0.9	0.0	30.2
CyGluc	0.0	4.7	5.2	2.3	0.7	0.0	12.9
Pe3Gal	0.0	2.4	1.6	1.0	0.3	0.0	5.3
PeGluc	0.0	13.5	18.2	10.6	2.5	0.9	45.7
Pe3Ara	0.0	2.7	2.2	0.9	0.1	0.0	5.9
metabolites	0.0	23.3	27.2	14.8	3.6	0.9	69.8

[a] 0−24 h.

present: Pe-3-gal, Pe-3-ara, cyanidin monoglucuronide (Cy-gluc) and peonidin monoglucuronides (Pe-gluc). The Cy-3-gal, Cy-3-glu, Cy-3-ara, Pe-3-gal, and Pe-3-ara were identified on the basis of authentic compounds. The peak with retention time of 13.5 min and the parent and daughter ion of *m/z* 463 and *m/z* 287, respectively, was identified as Cy-gluc (**Table 2**). Similar UV−visible spectrum and presence of the respective parent and daughter ion pairs (*m/z* 477 and *m/z* 301) were noted for two peaks (18.6 and 19.1 min) (**Table 2**). These peaks were designated as peonidin monoglucuronides. Previous studies (*32*) showed that different position of methylation in cyanidin molecule is possible, resulting in 3′-methylated (peonidin) or 4′-methylated (isopeonidin). On the other hand, flavonoid glucuronidation can occur at different hydroxyl groups within their structure. Therefore, the several monoglucuronides of peonidin or isopeonidin are possible (*19*). Given these elucidations several possibilities could appear. Since the analytical equipment used in our investigation has not permitted determination of the methylation and glucuronidation position, both compounds were designated as peonidin monoglucuronides and calculated together. In plasma and urine samples Cy-3-xyl, which appeared
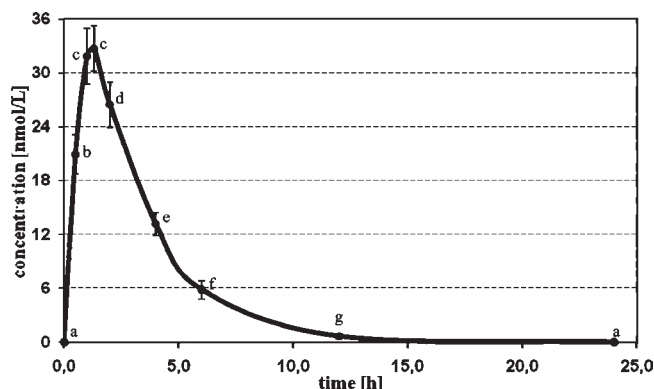
Figure 1. Plasma anthocyanin concentration in subjects who consumed chokeberry juice providing 0.8 mg of anthocyanins per kg body weight. Values are means ± SEM, *n* = 13. Values with different letters are significantly different at *P* < 0.05.

Table 5. Plasma Pharmacokinetic Parameters of Anthocyanins in Subjects Who Consumed Chokeberry Juice Providing 0.8 mg of Anthocyanins per kg Body Weight[a]

parameter	$x \pm$ sem
β	0.3 ± 0.0
$t_{1/2}$	2.4 ± 0.2
t_{max}	1.3 ± 0.1
C_{max}	32.7 ± 2.9
$AUC_{(0-t)}$	109.4 ± 10.9
$AUMC_{(0-t)}$	309.7 ± 42.5
$MRT_{(0-t)}$	2.7 ± 0.2
$Vd_{(s-s,0-t)}$	185.2 ± 11.5
$Cl_{(b)(0-t)}$	73.7 ± 7.6

[a] Values are means ± SEM, *n* = 13; β (h^{-1}), elimination rate constant; $t_{1/2}$ (h), half life; t_{max} (h), time describing C_{max}; C_{max} (nmol/L), maximum concentration; $AUC_{(0-t)}$ (nmol/L × h), the area under the curve; $AUMC_{(0-t)}$ (nmol/L × h), the area under the first moment of curve; $MRT_{(0-t)}$ (h), mean residence time; $Vd_{(s-s,0-t)}$ (L), volume of distribution; $Cl_{(b)(0-t)}$ (L/h), clearance.

in chokeberry juice, was not found. This could be due to very low concentration of this compound in the drink served and/or the kind of sugar attached to the main structure (*33*, *34*).

The chokeberry anthocyanins' plasma concentration is shown in **Figure 1** and **Table 3**. None of the anthocyanins were detected in plasma prior to intake of chokeberry juice. Anthocyanins appeared in plasma within 30 min after dosing (21.0 ± 2.2 nmol/L). At this time the concentration of native anthocyanins (9.8 ± 2.1 nmol/L) and their metabolites (11.1 ± 1.1 nmol/L) was similar. The highest total anthocyanin plasma concentration was observed 1 h after the test when anthocyanins' metabolites began to prevail. Intensive metabolism resulted in domination of anthocyanins' metabolites in all plasma samples collected 1 h after consumption of juice. The peonidin monoglucuronide was the predominant metabolite, and 2 h after chokeberry juice intake its highest concentration (15.8 ± 1.8 nmol/L) was found (**Table 3**). Finally, twelve hours after juice intake, only anthocyanin metabolites (Cy-gluc and Pe-gluc) were presented in plasma. Their concentrations were 0.2 ± 0.1 nmol/L and 0.5 ± 0.2 nmol/L, respectively. A significant difference (*P* < 0.05) between control and other time points has been observed. The pharmacokinetic parameters of plasma anthocyanins in humans after consumption of chokeberry juice are presented in **Table 5**. The area under the concentration−time curve, a bioavailability marker of anthocyanins from chokeberry juice, was 109.4 ± 10.9 nmol/L × h. The time to reach maximum concentration occurred more than 1 h after intake while the elimination rate constant of anthocyanins
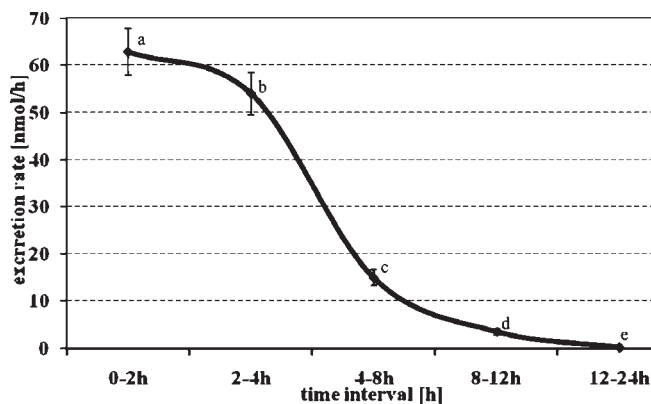
Figure 2. Urine excretion rate of anthocyanins in subjects who consumed chokeberry juice providing 0.8 mg of anthocyanins per kg body weight. Values are means ± SEM, $n = 13$. Values with different letters are significantly different at $P < 0.05$.
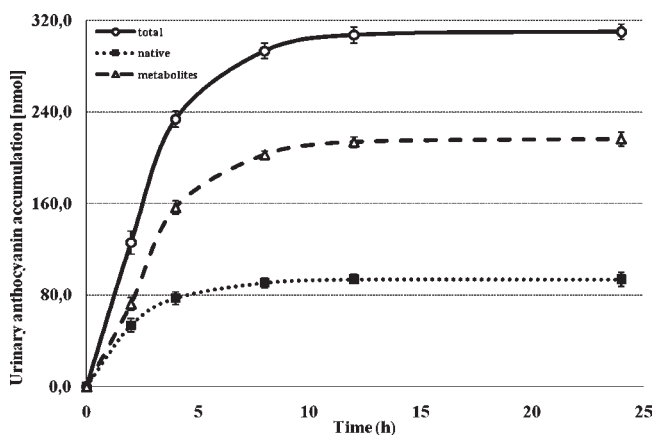

Figure 3. Accumulation of native and metabolites of anthocyanins in urine of subjects who consumed chokeberry juice providing 0.8 mg of anthocyanins per kg body weight. Values are means ± SEM, $n = 13$.

Figure 4. Accumulation of individual anthocyanins in urine of subjects who consumed chokeberry juice providing 0.8 mg of anthocyanins per kg body weight. Values are means ± SEM, $n = 13$.

Table 6. Urine Pharmacokinetic Parameters of Anthocyanins in Subjects Who Consumed Chokeberry Juice Providing 0.8 mg of Anthocyanins per kg Body Weight[a]

parameter	$x \pm$ SEM
K	0.2 ± 0.0
Cl	0.3 ± 0.0
Ae	310.1 ± 23.3
Vu	1.5 ± 0.1
Fe	0.25 ± 0.02
D	122.4 ± 4.8
AURC$_{(0-24)}$	66.9 ± 12.2

[a] Values are means ± SEM, $n = 13$; K (h^{-1}), elimination rate constant in urine; Cl (L/h), clearance; Ae (nmol), total amount of substance excreted in urine; Vu (L), volume of distribution; Fe (%), fraction of dose eliminated in urine; D (μmol), dose; AURC$_{(0-24)}$ (L × nmol), the area under the urinary excretion rate curve.

was 0.3 ± 0.0 h^{-1}. Moreover, in addition to the elimination rate constant of anthocyanins, other plasma pharmacokinetic parameters of anthocyanins, such as the area under the first moment of curve (309.7 ± 42.5 nmol/L × h), mean residence time (2.7 ± 0.2 h), volume of distribution (185.2 ± 11.5 L) and clearance (73.7 ± 7.6 L/h), were determined for the first time. Despite the high interindividual variation in plasma anthocyanin concentration among the subjects, maximum concentration after the consumption of chokeberry juice was measured in most subjects in blood sampled 1 h after the test, and it ranged between 20.4 nmol/L and 51.8 nmol/L.

Figure 2 and **Table 4** shows urinary excretion rate of anthocyanins after consumption of chokeberry juice. Similarly to blood plasma analysis, at baseline all urine samples did not contain any anthocyanins. Their urine excretion rate (62.9 ± 5.0 nmol/h) was the highest within the first 2 h after the juice intake and decreased to 0.2 ± 0.1 nmol/h within the 12−24 h time interval. Also, the urinary excretion rate of native anthocyanins (26.4 ± 2.9 nmol/h) reached maximum within the first 2 h after juice intake, whereas the maximum urinary excretion rate of anthocyanins metabolites (42.1 ± 4.6 nmol/h) was between 2 and 4 h after juice consumption. Urinary accumulation of anthocyanins with time is presented in **Figures 3** and **4**. The pattern of accumulation was similar for each anthocyanin. The rate of anthocyanin accumulation was highest in the period of 0−4 h. The amount of anthocyanins
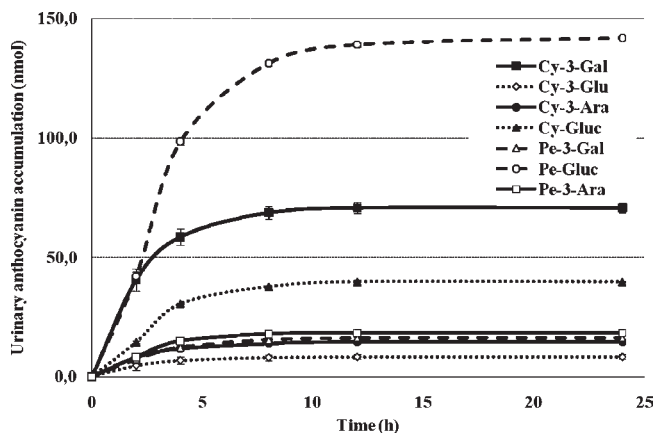
accumulated at 8 h was as much as 96% of that at 24 h. In total, during 24 h after the challenge 0.25 ± 0.02% of the ingested dose was excreted with urine: 70% as anthocyanins' metabolites and 30% as native compounds (**Table 4**, **Figure 3**) with the peonidin monoglucuronide as the dominant metabolite detected in urine (**Table 4**, **Figure 4**). The excretion parameters of anthocyanins determined after consumption of chokeberry juice are presented in **Table 6**. Similarly as in the case of the plasma pharmacokinetic parameters of anthocyanins, some of the excretion parameters of anthocyanins, such as elimination rate constant in urine (0.2 ± 0.0 h^{-1}), clearance (0.3 ± 0.0 L/h) and the area under the urinary excretion rate curve (66.9 ± 12.2 L × nmol), were determined for the first time.

DISCUSSION

In order to understand the biological activity of food compounds, it is important to find out their fate in the human body. Since the bioavailability study allows determination of real exposure of the human organism to the tested compounds, their results are an important and essential topic: there are "golden standards" for other experiments aiming at explanation of anthocyanins' physiological functions.

In our study we have examined the bioavailability of anthocyanins after consumption of the chokeberry juice with dietary-relevant doses of anthocyanins. Chokeberry is rich in astringent proanthocyanidins restricting the volume of disposable consumption, therefore subjects drank a glass of chokeberry juice providing 0.8 mg of anthocyanins/kg bw.

The chokeberry juice contained one major (Cy-3-gal) and three minor (Cy-3-ara, Cy-3-glu, Cy-3-xyl) anthocyanins. The main

compound covered almost 70% of the total anthocyanins. A similar profile of anthocyanins in chokeberry was presented by Oszmiański and Wojdylo (*3*).

In the present investigation, neither native anthocyanins nor metabolites of anthocyanin were identified in the plasma or urine from fasting volunteers, indicating that the three-day washout phase with a strict anthocyanin-free diet that preceded the study was sufficient. After consumption of the experimental beverage, analysis of both plasma and urine of all volunteers showed that the chokeberry anthocyanins were absorbed intact as cyanidin glycosides as well as being metabolized to methylated and/or glucuronidated derivatives, which is consistent with the previous studies (*32*, *35*).

Early studies show that anthocyanin glycosides are absorbed from both stomach and small intestine without modification, but the absorption is limited by the kind of glycosidic moiety attached (*33*, *34*). Moreover, Passamonti et al. (*36*) suggested that an organic anion carrier, bilitranslocase (TC 2.A.65.1.1), present in the gastric epithelium, could be involved in the absorption of anthocyanins.

In the case of intestinal absorption, anthocyanin glycosides could be transferred through the intestinal brush border membrane and enter enterocytes as a result of an interaction with the sodium-dependent glucose transporter SGLT1 as reported by Gee et al. (*37*). Other results which also show that a sugar carrier might play a role in anthocyanin absorption have been presented by Mülleder et al. (*38*). After consumption of elderberry anthocyanins in two models, with and without sugar addition, the total anthocyanin excretion in the model with sugar was lower than in that without sugar. The authors concluded that it was possible because the ingestion of sugar may lead to saturation of the glucose transporter and change anthocyanin intake.

Although native anthocyanins were found in blood plasma and urine, the metabolites of anthocyanins as main compounds were identified. Both methylated and glucuronidated conjugates of chokeberry anthocyanins were observed. Two methylated derivatives of cyanidin (3′-*O*-methyl and 4′-*O*-methyl cyanidin) have been already reported (*32*). We also found methylated cyanidin which additionally was monoglucuronidated. Moreover, cyanidin monoglucuronoide, peonidin-3-galactoside and peonidin-3-arabinoside were found in blood plasma and urine, as in the previous study (*32*). Finally, a total of 8 different cyanidin derivatives, including 3 native forms and 5 metabolites, were identified. In comparison, Wu et al. (*32*) identified 14 metabolites of cyanidin after a single administration of chokeberry freeze-dried powder to pigs providing over 100 mg of anthocyanins/kg bw, while Kay et al. (*21*) found 4 metabolites of cyanidin after single consumption of encapsulated chokeberry extract providing 18 mg of anthocyanin/kg bw to humans. The lower number of identified anthocyanin metabolites in our study may result from lower dosage of anthocyanins served and possible differences in metabolism between humans and pigs. There are some reports indicating that *O*-methylation plays an important role in the metabolism of flavonoids in animals (*26*, *39*) but not in humans (*40*, *41*). The dose of anthocyanins/kg bw used in our experiment was respectively 125 and 22 times lower than in the above studies. It is possible that too high a dose of these compounds activates different metabolic routes as indicated in other investigations (*1*, *41*). Other studies suggest that the metabolism of flavonoids may be modulated by both dose and bioaccessibility. In the first case, the urinary recovery of anthocyanins varied in inverse proportion to the dose size of anthocyanins (*16*, *42*). In the case of bioaccessibility, the efficiency of intestinal absorption of phytochemicals is strongly affected by its solubility in the vehicles and the composition of the diet. For example, when quercetin (one of the most

common phytochemicals) in water and/or water−propylene glycol suspension was administered to rats, low concentrations of quercetin metabolites were found in plasma after administration with water because of quercetin's low solubility (*43*). Moreover, in other animal studies (*44*, *45*) it was demonstrated that the absorption of quercetin was enhanced when the diets were enriched with lipids. It was concluded that the presence of lipids could promote quercetin solubility, transport through the unstirred water layer and uptake into the enterocytes. Possibly, in the case of consumption of quercetin with lipids, quercetin may partially permeate into formed micelles in the lumen, and this could have promoted its transport.

Anthocyanins appeared in the blood within 30 min after the consumption of chokeberry juice. This indicates that absorption occurred in the upper part of the digestive tract (*33*, *34*, *46*). This phenomenon may at least in part result from absorption of anthocyanin from the stomach (*33*, *46*). The total plasma concentration of anthocyanins reached the maximum concentration of 32.7 ± 2.9 nmol/L at 1.3 ± 0.1 h after chokeberry juice consumption and then decreased with time. Uptake and excretion profiles of each native anthocyanin in the plasma were similar. Summing up, the glucuronidation was the major metabolic route of chokeberry anthocyanins while methylation played a minor role in the metabolism of those compounds (**Tables 3** and **4**).

The elimination half-life of plasma total anthocyanins was calculated as 2.4 ± 0.2 h and was slightly higher than the 2.1 and 2.2 h reported by Mullen et al. (*47*) and Cao et al. (*48*), respectively, and similar to catechins and flavanones (*5*, *25*, *49*). Contrarily, isoflavones (3.4−10.8 h) and flavonols (10.9−45.7 h) are characterized by longer elimination half-life (*5*, *21*), which suggests that these components can be accumulated in tissues.

The total amount of anthocyanins excreted with urine during 24 h was 310.0 ± 23.0 nmol, accounting for 0.25 ± 0.02% of the ingested dose. This value is around 2 times higher than that observed following the consumption of chokeberry freeze-dried powder and encapsulated chokeberry extract (*21*, *32*). A number of other studies reported that the urinary excretion of anthocyanins amounted from 0.004% to 0.1% of the intake (as reviewed in ref *5*). During the first 2 h after intake the cumulative urinary excretion of anthocyanins in the present study was around 0.1%, while the values in the periods 0−4 h, 0−8 h, 0−12 h and 0−24 h were 0.19%, 0.24%, 0.25% and 0.25%, respectively (**Figures 3** and **4**). Early reports on human urinary excretion of chokeberry anthocyanins show a lower value of this parameter (*35*). Generally, despite the higher values of fraction of dose eliminated in urine (0.25%), the bioavailability of anthocyanins from chokeberry juice described by parameters which were determined for the first time for characterization of anthocyanins' bioavailability, such as elimination rate, clearance, the area under the first moment of curve, volume of distribution, elimination rate constant in urine, the area under the urinary excretion rate curve, half-life and mean residence time (**Tables 5** and **6**), was also low. This study focused only on anthocyanin metabolites having an anthocyanin core/structure and being thus recorded at 520 nm with MS-ESI identification. One has to keep in mind that anthocyanins can be converted into degradation products at physiological pH in the small and large intestine which are still uncharacterized as well, as they could be metabolized by colon microbiota to low molecular derivatives which were not the objective of this study (*22*−*24*).

To our knowledge this is the first time when fresh chokeberry juice with dietary-relevant dose of anthocyanins has been used as source of anthocyanins in bioavailability study. We have shown that, after consumption of this natural chokeberry juice, the native compounds and their glucuronidated and methylated metabolites are present in human plasma and urine during 24 h. Moreover,

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our study showed that 70% of anthocyanins were excreted with urine as metabolites with the dominance of peonidin mono-glucuronide. The presence of these compounds in the blood and urine may be beneficial for the health of consumers. Further studies, considering the metabolism of chokeberry polyphenols, are necessary to demonstrate the health benefits of chokeberry.

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LITERATURE CITED

(1) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073−2085.

(2) Clifford, M. N. Anthocyanins − nature, occurrence and dietary burden. *J. Sci. Food Agric.* **2000**, *80*, 1063−1072.

(3) Oszmiański, J.; Wojdylo, A. Aronia melanocarpa phenolics and their antioxidant activity. *Eur. Food Res. Technol.* **2005**, *10*, 1−5.

(4) Wu, X.; Prior, R. Identification and characterization of anthocyanins by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry in common foods in the United States: vegetables, nuts, and grains. *J. Agric. Food Chem.* **2005**, *53*, 3101−3113.

(5) Manach, C.; Williamson, G.; Morand, Ch.; Scalbert, A.; Remesy, Ch. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 243S−255S.

(6) McGhie, T. K.; Walton, M. C. The bioavailability and absorption of anthocyanins: Towards a better understanding. *Mol. Nutr. Food Res.* **2007**, *51*, 702−713.

(7) Prior, R. L.; Wu, X. Anthocyanins: structural characteristic that result in unique metabolic patterns and biological activities. *Free Radical Res.* **2006**, *40*, 1014−1028.

(8) Forester, S. C.; Waterhouse, A. L. Gut metabolites of anthocyanins, gallic acid, 3-O-methylgallic acid, and 2,4,6-trihydroxybenzaldehyde, inhibit cell proliferation of Caco-2 cells. *J. Agric. Food Chem.* **2010**, *58*, 5320−5327.

(9) Galvano, F.; La Fauci, L.; Lazzarino, G.; Fogliano, V.; Ritieni, A.; Cappellano, S.; Battistini, N. C.; Gavazzi, B.; Galvano, G. Cyanidins: metabolism and biological properties. *J. Nutr. Biochem.* **2004**, *15*, 2−11.

(10) Wang, L. S.; Stoner, G. D. Anthocyanins and their role in cancer prevention. *Cancer Lett.* **2008**, *269*, 281−290.

(11) Nielsen, I. L. F.; Rasmussen, S. E.; Mortensen, A.; Ravn-Haren, G.; Ma, H. P.; Knuthsen, P.; Hansen, B. F.; McPhail, D.; Freese, R.; Breinholt, V.; Frandsen, H.; Dragsted, L. O. Anthocyanins increase low-density lipoprotein and plasma cholesterol and do not reduce atherosclerosis in Watanabe Heritable Hyperlipidemic rabbits. *Mol. Nutr. Food Res.* **2005**, *49*, 301−308.

(12) Rahman, M. M.; Ichiyanagi, T.; Komiyama, T.; Sato, S.; Konishi, T. Effects of anthocyanins on psychological stress-induced oxidative stress and neurotransmitter status. *J. Agric. Food Chem.* **2008**, *56*, 7545−7550.

(13) Yanamala, N.; Tirupula, K. C.; Balem, F.; Klein-Seetharaman, J. pH-dependent interaction of *Rhodopsin* with Cyanidin-3-glucoside. 1. Structural Aspects. *Photochem. Photobiol.* **2009**, *85*, 454−462.

(14) Nizamutdinova, I. T.; Jin, Y. C.; Chung, J. I.; Shin, S. C.; Lee, S. J.; Seo, H. G.; Lee, J. H.; Chang, K. C.; Kim, H. J. The anti-diabetic effect of anthocyanins in streptozotocin-induced diabetic rats through glucose transporter 4 regulation and prevention of insulin resistance and pancreatic apoptosis. *Mol. Nutr. Food Res.* **2009**, *53*, 1419−1429.

(15) Charron, C. S.; Kurilich, A. C.; Clevidence, B. A.; Simon, P. W.; Harrison, D. J.; Britz, S. J.; Baer, D. J.; Novotny, J. A. Bioavailability of anthocyanins from purple carrot juice: effects of acylation and plant matrix. *J. Agric. Food Chem.* **2009**, *57*, 1226−1230.

(16) Kurilich, A. C.; Clevidence, B. A.; Britz, S. J.; Simon, P. W.; Novotny, J. A. Plasma and urine responses are lower for acylated *vs* nonacylated anthocyanins from raw and cooked purple of carrots. *J. Agric. Food Chem.* **2005**, *53*, 6537−6542.

(17) Millbury, P. E.; Vita, J. A.; Blumbergs, J. B. Anthocyanins are bioavailable in humans following an acute dose of cranberry juice. *J. Nutr.* **2010**, *140*, 1099−1104.

(18) Gonzalez-Barrio, R.; Borges, G.; Mullen, W.; Crozier, A. Bioavailability of anthocyanins and ellagitannins following consumption of raspberries by healthy humans and subjects with an ileostomy. *J. Agric. Food Chem.* **2010**, *58*, 5933−5939.

(19) Felgines, C.; Talavera, S.; Texier, O.; Gil-Izquierdo, A.; Lamaison, J. L.; Remesy, Ch. Blackberry anthocyanins are mainly recovered from urine as methylated and glucuronidated conjugates in humans. *J. Agric. Food Chem.* **2005**, *53*, 7721−7727.

(20) Tian, Q.; Giusti, M. M.; Stoner, G. D.; Schwartz, S. J. Urinary excretion of black raspberry (*Rubus occidentalis*) anthocyanins and their metabolites. *J. Agric. Food Chem.* **2006**, *54*, 1467−1472.

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

(22) Keppler, K.; Humpf, H. U. Metabolism of anthocyanins and their phenolic degradation products by the intestinal microflora. *Biol. Med. Chem.* **2005**, *13*, 5195−5205.

(23) Vitaglione, P.; Donnarumma, G.; Napolitano, A.; Galvano, F.; Gallo, A.; Scalfi, L.; Fogliano, V. Protocatechuic acid is the major human metabolite of cyaniding-glucosides. *J. Nutr.* **2007**, *137*, 2043−2048.

(24) Prior, R. L.; Rogers, T. R.; Khanal, R. C.; Wilkes, S. E.; Wu, X. L.; Howard, L. R. Urinary excretion of phenolic acids in rats fed cranberry. *J. Agric. Food Chem.* **2010**, *58*, 3940−3949.

(25) Wiczkowski, W.; Romaszko, J.; Buciński, A.; Szawara-Nowak, D.; Honke, J.; Zieliński, H.; Piskuła, M. K. Quercetin from shallot (*Allium cepa* var. *Aggregatum*) is more bioavailable than its glucosides. *J. Nutr.* **2008**, *138*, 885−889.

(26) Wiczkowski, W.; Nemeth, K.; Buciński, A.; Piskuła, M. K. Bioavailability quercetin from fresh scales and dry skin of onion in rats. *Pol. J. Food Nutr. Sci.* **2003**, *53*, 95−99.

(27) Gonzalez-Molina, E.; Moreno, D. A.; Garcia-Viguera, C. Aronia-enriched lemon juice: a new highly antioxidant beverage. *J. Agric. Food Chem.* **2006**, *56*, 11327−11333.

(28) Kokotkiewicz, A.; Jaremicz, Z.; Luczkiewicz, M. Aronia plants: a review of traditional use, biological activities, and perspectives for modern medicine. *J. Med. Food* **2010**, *13*, 255−269.

(29) Solomon, A.; Golubowicz, S.; Yablowicz, Z.; Bergman, M.; Grossman, S.; Altman, A.; Kerem, Z.; Flaishman, M. A. EPR studies of O-2(center dot-), OH, and O-1(2) scavenging and prevention of glutathione depletion in fibroblast cells by cyanidin-3-rhamnoglucoside isolated from fig (*Ficus carica* L.) fruits. *J. Agric. Food Chem.* **2010**, *58*, 7158−7165.

(30) Ho, M. L.; Chen, P. N.; Chu, S. C.; Kuo, D. Y.; Kuo, W. H.; Chen, J. Y.; Hsieh, Y. S. Peonidin 3-glucoside inhibits lung cancer metastasis by down regulation of proteinases activities and MAPK pathway. *Nutr. Cancer* **2010**, *62*, 505−5016.

(31) Tarozzi, A.; Morroni, F.; Merlicco, A.; Bolondi, C.; Teti, G.; Falconi, M.; Cantelli-Forti, G.; Hrelia, P. Neuroprotective effects of cyanidin 3-O-glucopyranoside on amyloid beta (25−35) oligomer-induced toxicity. *Neurosci. Lett.* **2010**, *473*, 72−76.

(32) Wu, X.; Pittman, H. E.; Mckay, S.; Prior, R. L. Aglycones and sugar moieties alter anthocyanin absorption and metabolism after berry consumption in weanling pigs. *J. Nutr.* **2005**, *135*, 2417−2424.

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

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

(35) Kay, C. D.; Mazza, G.; Holub, B. J. Anthocyanins exist in the circulation primarily as metabolites in adult men. *J. Nutr.* **2005**, *135*, 2582−2588.

(36) Passamonti, S.; Vrhovsek, U.; Mattivi, F. The interaction of anthocyanins with bilitranslocase. *Biochem. Biophys. Res. Commun.* **2002**, *296*, 631−636.

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

(38) Mülleder, U.; Murkovic, M.; Pfannhauser, W. Urinary excretion of cyaniding glycosides. *J. Biochem. Biophys. Methods* **2002**, *53*, 61−66.

(39) Day, A. J.; Rothwell, J. A.; Morgan, R. A. Characterization of polyphenol metabolites. In *Phytochemicals in Health and Disease*; Bao, Y., Fenwick, R., Eds.; Dekker: New York, 2004; pp 57−76.

(40) Day, A. J.; Mellon, F.; Barron, D.; Sarrazin, G.; Morgan, M. R. A.; Williamson, G. Human metabolism of dietary flavonoids: identification of plasma metabolites of quercetin. *Free Radical Res.* **2001**, *35*, 941−952.

(41) Manach, C.; Morand, Ch.; Crespy, V.; Demingné, Ch.; Texier, O.; Régérat, F.; Rémésy, Ch. Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. *FEBS Lett.* **1998**, *426*, 331−336.

(42) Charon, C. S.; Clevidence, B. A.; Britz, S. J.; Novotny, J. A. Effects of dose size on bioavailability of acylated and nonacylated anthocyanins from red cabbage (*Brassica oleracea L. var. capitata*). *J. Agric. Food Chem.* **2007**, *55*, 5354−5362.

(43) Piskula, M. K.; Terao, J. Quercetin's solubility affects its accumulation in rat plasma after oral administration. *J. Agric. Food. Chem.* **1998**, *46*, 4313−4317.

(44) Lesser, S.; Cermak, R.; Wolffram, S. Bioavailability of quercetin pigs is influenced by the dietary fat content. *J. Nutr.* **2004**, *134*, 1508−1511.

(45) Azuma, K.; Ippoushi, K.; Ito, H.; Horie, H.; Terao, J. Enhancing effect of lipids and emulsifiers on the accumulation of quercetin metabolites in blood plasma after short-term ingestion of onion by rats. *Biosci., Biotechnol., Biochem.* **2003**, *67*, 2548−2555.

(46) Passamonti, S.; Vrhovsek, U.; Vanzo, A.; Mattivi, F. The stomach as a site for anthocyanins absorption from food. *FEBS Lett.* **2003**, *544*, 210−213.

(47) Mullen, W.; Edwards, Ch. A.; Serafini, M.; Crozier, A. Bioavailability of pelargonidin-3-*O*-glucoside and its metabolites in humans following the ingestion of strawberries with and without cream. *J. Agric. Food Chem.* **2008**, *56*, 713−719.

(48) Cao, G.; Muccitelli, H. U.; Sanchez-Moreno, C.; Prior, R. L. Anthocyanins are absorbed in glycated forms in elderly womenn: a pharmacokinetic study. *Am. J. Clin. Nutr.* **2001**, *73*, 920−926.

(49) van Amelsvoort, J. M.; Hoft, K. H. V. H.; Mathot, J. N.; Muller, T. P.; Wiersza, A.; Tijburg, L. B. Plasma concentrations of individual tea catechins after a single oral dose in humans. *Xenobiotica* **2001**, *31*, 891−901.