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## Anthocyanin Absorption, Metabolism, and Distribution from a Wild Blueberry-Enriched Diet (*Vaccinium angustifolium*) Is Affected by Diet Duration in the Sprague–Dawley Rat

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The effect of wild blueberry consumption on anthocyanin (ACNs) distribution and metabolism in Sprague–Dawley (SD) rats was investigated. Thirty-two rats were fed for 4 or 8 weeks with a control (C) or a wild blueberry-enriched diet (8%) (WB). Anthocyanin profile in plasma, urine, feces, brain, and liver was evaluated by LC-MS/MS, and significantly increased in urine and not in feces after 8 weeks on the WB diet compared to that in 4 weeks, but no anthocyanins were detected in plasma, liver, and brain samples either in the C or WB groups. Metabolites of ACNs were detected in the plasma, urine, feces, and tissues of both the C and WB groups, but the urinary excretion of hippuric acid increased significantly after 4 and 8 weeks of WB consumption. Thus, it seems that ACNs are metabolized by the intestinal microflora to respective phenyl-alkyl acids, which can be further metabolized to benzoic acid. In conclusion, ACNs are bioavailable in rats, and the extent of their metabolism and excretion is based on diet duration. Additionally, urinary hippuric acid content could represent a potential biomarker of ACNs absorption and metabolism in the SD rat under the present experimental conditions.

**KEYWORDS:** Wild blueberry; *Vaccinium angustifolium*; rat; anthocyanins; metabolites; absorption; excretion

### INTRODUCTION

Anthocyanins (ACNs) are a wide group of natural compounds that provide colors from dark blue to purple to vegetables and fruits such as blueberries. Wild blueberry (*Vaccinium angustifolium*) is composed of different anthocyanidins such as delphinidin, malvidin, petunidin, cyanidin and peonidin (1). Studies have investigated the health effects of anthocyanin bioactive compounds suggesting anti-inflammatory and anticarcinogenic properties (2), improvement of lipid profiles and vasomotor tone, modulation of detoxifying enzymes (such as glutathione-S-transferase), reduction of blood pressure, and platelet aggregation as well as induction of apoptosis (3–8). Some of these biological activities and protective effects can be attributed to their antioxidant activity (9) against free radicals (10).

Anthocyanins are rapidly absorbed through the stomach and small intestine by different mechanisms that may involve specific enzymes such as bilitranslocases (11). Several pharmacokinetic animal studies have documented that ACNs from *Vaccinium angustifolium*, red fruits, and blackcurrants are absorbed mainly in their intact form and moved into the blood within 15 min–2 h after ingestion (12–14). Ingested ACNs are exposed to microbial populations, mainly in the colon, and may be degraded to phenolic acids and reabsorbed to exert their protective effect (15).

Anthocyanins enter the circulatory system after passing through the liver and are consequently distributed to different tissues. Talavera et al. (16) were the first to report intact as well as methylated and glucuronidated metabolites of ACNs in the jejunum, liver, and kidneys of rats.

Measurement of urinary excretion has often been used to assess bioavailability. Felgines et al. (17) documented that after 8 days of lyophilized blackberry powder consumption ACNs were excreted and detected in the urine as intact and methylated forms after blackberry consumption. Low ACN concentrations in plasma and tissues as well as aglycones detected in cecal contents have been linked to microbial degradation. In addition, ACNs and their metabolites (such as benzoic and hippuric acids) have been reported in bile after 20 min of ingestion, which suggests rapid absorption and metabolism (16).

Some human and animal studies have been performed in order to investigate the absorption and metabolism of ACN-rich foods in pharmacokinetic and short-term (10–12 days) diet studies (17–21). Even though there is a plethora of studies that focuses on ACNs bioavailability and their pharmacokinetic action in humans and animal models, very few studies have taken into account the effect of diet supplementation of normally consumed amounts of ACNs over time.

Thus, the aim of the present study was to investigate ACNs absorption, metabolism, and distribution in the plasma, liver, brain, and their excretion in urine and feces in the

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Sprague–Dawley (SD) rats fed a blueberry-enriched diet (*V. angustifolium*) (8%) over time (4 and 8 weeks).

## MATERIALS AND METHODS

**Chemicals and Dietary Ingredients.** Standards of cyanidin (Cy)-, delphinidin (Dp)-, peonidin (Pn)-, petunidin (Pt)- and malvidin (Mv)-3-*O*-glucoside (glc), -3-*O*-galactoside (gal), and Cy- and Mv-3-*O*-arabinoside (Ara) were purchased from Polyphenols Laboratory (Sandnes, Norway). Gallic acid (GA), 3,4-dihydroxybenzoic acid (DHBA), benzoic acid (BA), 4-hydroxybenzoic acid (4HBA), hippuric acid (HA), siringic acid (SA), vanillic acid (VA), 3-*O*-methyl-gallic acid, and veratric acid (internal standard) were purchased from ChromaDex (Irvine, CA). Cyanidin-3,5-diglucoside (CydG), as ACN's internal standard (IS), was purchased from Sigma (St. Louis, MO). Potassium chloride, hydrochloric acid, methanol, acetonitrile, formic acid, and trifluoroacetic acid (TFA) were from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q apparatus (Millipore, Milford, MA). The dietary ingredients were purchased from Laboratory Piccioni (Italy) where diets were pelleted. Freeze-dried wild blueberry powder (containing at least 1.5% total ACNs) was purchased from FutureCeuticals Company (Mokena, IL, USA) and utilized within 6 months.

**Experimental Design.** Thirty-two male SD rats (28 days old, about 90 g) were purchased from Charles River Laboratories (Calco, Italy) and were housed in metal cages in a temperature (21–23 °C), humidity (40–60%), and light (from 7 a.m. to 7 p.m.) controlled room.

Animals were divided into four groups of 8 rats each and were fed a control (AIN93) (C) or wild lowbush blueberry diet (WB) (C + 8% w/w blueberry powder substituting for dextrose) for a period of four or eight weeks (5).

Tap water and diet were provided ad libitum. Diet consumption was measured daily ( $20 \pm 4$  g (mean  $\pm$  standard deviation)), a dose equivalent to  $24.0 \pm 5.2$  mg/day of ACNs ( $\sim 48$  mg/kg body weight). The protocol was approved by the University of Milan Animal Care and Use Committee.

**Harvesting and Tissue Preparation.** Animals were anesthetized in a chamber with diethyl ether. Blood samples were collected from the abdominal aorta into a vacutainer containing heparin (0.2 mg/mL) as anticoagulant, and the plasma was obtained by centrifugation at 250g for 15 min at 4 °C. Samples were acidified with a hydrochloric acid solution (1% final concentration) to preserve anthocyanin stability, centrifuged at 4500g for 10 min, and the supernatants stored at  $-80$  °C in aliquots (1 mL) for no longer than 2 months. Animals were perfused via the portal vein with physiologic salt solution (PSS) (1.15% KCl), and organs such as the brain and liver were removed; feces and urine were collected and stored at  $-80$  °C until analysis. Animals were placed and fed in metabolic cages (Tecniplast, Varese, Italy) two days before euthanization (one day for acclimatization and another one for the collection of 24-h urine and feces). Urine was collected in tubes containing hydrochloric acid solution (10% v/v). Distilled water was added to give a final concentration of 1% HCl.

**Characterization of Wild Blueberry Powder by LC-DAD-MS-MS.** Wild blueberry powder (250 mg) was suspended in 40 mL of 1% TFA aqueous solution, sonicated for 10 min, and the volume adjusted to 50 mL. The solution was centrifuged at 1600g for 5 min, and 50  $\mu$ L of the supernatant was injected in the LC system.

The LC system consisted of an Alliance model 2695 (Waters, Milford, MA) equipped with a model 2996 photodiode array detector (Waters) and a triple quadrupole mass spectrometer model Quattro micro (Micromass, Beverly, MA). A 5  $\mu$ m C<sub>18</sub> Symmetry column (250  $\times$  4.6 mm, Waters) was used for the separation at a flow rate of 1.4 mL/min. The column was maintained at 30 °C and the flow-rate split 5:1 before electrospray (ESI). The separation was performed by means of a linear gradient elution, and the eluents were (A) 1% TFA and (B) 1% CH<sub>3</sub>OH/CH<sub>3</sub>CN/TFA (22:22:56, v/v/v). The gradient was as follows: 0–30 min from 20 to 35% B; 30–40 min from 35 to 45% B; 40–50 min from 45 to 55% B; 50–60 min from 55 to 65% B; 60–70 min from 65 to 80% B; 70–72 min from 80 to 100% B; 100% B for 8 min. Chromatographic data were acquired in the range of 200–700 nm and were integrated at 520 nm. The mass spectrometer operated in positive full-scan mode in the range 200–800 Da. The capillary voltage was set to 3.5 kV, the cone voltage to 20 V, the source temperature to 130 °C, and the desolvating temperature to 350 °C. Data were acquired by Masslynx 4.0 software (Micromass,

Beverly, MA) with Quan-Optimize option for the fragmentation study. Anthocyanin mother solutions were obtained by dissolving 10 mg of each standard in 10 mL of a solution of TFA/methanol (5:95, v/v). Working solutions were prepared by diluting the mother solution in methanol and 0.1% TFA, and the calibration curves were in the range of 2–50  $\mu$ g/mL.

**Anthocyanin Extraction from Biological Samples.** One milliliter of urine or plasma was diluted with water (2–4 mL respectively) containing 2.5 ng of Internal Standard (CydG); the resulting solution was loaded on a 3 mL HLB Oasis 100 mg solid phase extraction (SPE) cartridge (Waters) preactivated with methanol (3 mL) and then washed with water (5 mL). Furthermore, the SPE cartridge was washed with 3 mL of 1% TFA in water; the ACNs were eluted from the cartridge using 3 mL of methanol containing 0.1% TFA. The eluate was dried under vacuum, the residue dissolved in 50  $\mu$ L of methanol containing 0.1% TFA, and the solution was centrifuged at 2000g for 1 min, and 20  $\mu$ L were injected into UHPLC-MS/MS system for analysis.

Fecal samples were defrosted, and an aliquot of 0.5 g was homogenized in the Potter homogenizer (IKA-Werke, Staufen, Germany) using 4 mL of a solution of 1% CH<sub>3</sub>OH/TFA (20:80) containing 500 ng of the IS (CydG). Samples were centrifuged at 3645g for 10 min and the residue extracted twice. Supernatants were collected and brought to a final volume of 10 mL for the analysis.

Brain or liver samples (1 g) were homogenized in the Potter homogenizer (IKA-Werke, Staufen, Germany) with 3 mL of a solution of 1% CH<sub>3</sub>OH/TFA (20:80) containing 8 ng of the IS (CydG); the mixtures were then centrifuged at 3645g for 10 min and the residue extracted twice. Supernatants were collected and brought to a final volume of 10 mL. Samples (3 mL) were purified and concentrated by SPE as described above.

**Anthocyanin Determination in Biological Samples.** The chromatographic system was an Acquity UHPLC (Waters) coupled to a model Quattro micro triple quadrupole mass spectrometer. The analyses were carried out in gradient mode by a 1.8  $\mu$ m C<sub>18</sub> HSS column (150  $\times$  2.1 mm, Waters) maintained at 80 °C, and the flow-rate was 0.6 mL/min. The eluents were (A) TFA 0.1% and (B) CH<sub>3</sub>OH/CH<sub>3</sub>CN/TFA 0.1% (22:22:56), and the gradient was as follows: 25 to 40% B in 3 min, from 40 to 90% B in 1 min, and 90% B for 3 min.

Routine analyses were carried out in multiple reaction monitoring (MRM) mode, monitoring the following transitions: ( $m/z$ )<sup>+</sup> 449  $\rightarrow$  287 Cy-gal and Cy-glc; 465  $\rightarrow$  303 Dp-gal and Dp-glc; 463  $\rightarrow$  301 Pn-gal and Pn-glc; 479  $\rightarrow$  317 Pt-glc and Pt-gal; 493  $\rightarrow$  331 Mv-gal and Mv-glc; 419  $\rightarrow$  287 Cy-ara; 433  $\rightarrow$  301 Pn-ara; 435  $\rightarrow$  303 Dp-ara; 449  $\rightarrow$  317 Pt-ara; 463  $\rightarrow$  331 Mv-ara; and the acetylated (-ac) forms of Pn, Pt, Mv (505  $\rightarrow$  301 Pn-glc-ac; 521  $\rightarrow$  317 Pt-glc-ac; 535  $\rightarrow$  331 Mv-glc-ac).

The capillary voltage was set to 3.5 kV, and the cone voltage and collision energy were specific for each compound; source and desolvation temperatures were 130 and 350 °C, respectively. The argon for the fragmentation in the collision cells was  $3.2 \times 10^{-3}$  mbar. The calibration curves were prepared in 0.1% TFA in methanol in the range of 2–40 ng/mL.

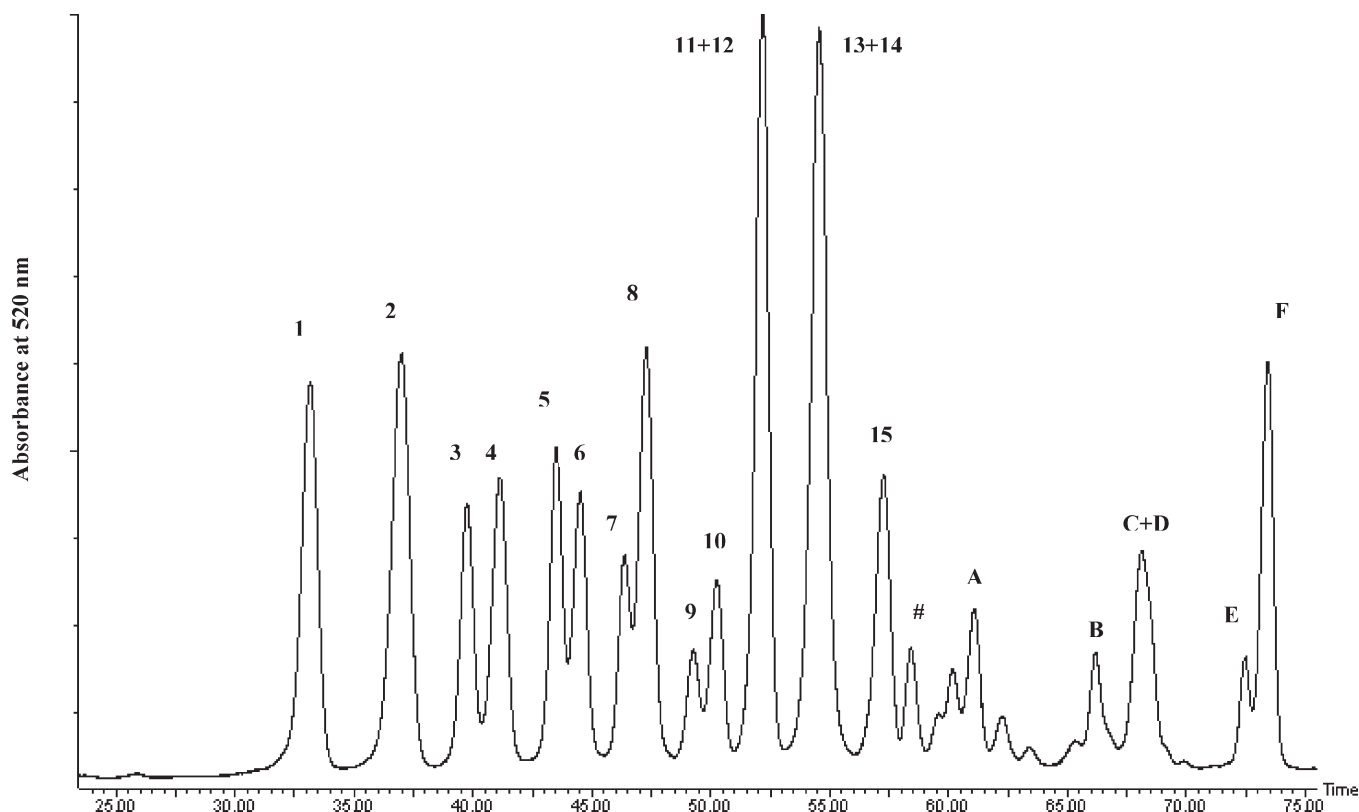
Acetylated forms were assayed using the calibration curves of the unacetylated standard compounds, and their amounts were then normalized by molecular mass ratios.

**Phenolic Acid Extraction from Biological Samples.** Plasma (1 mL) was diluted with water (4 mL) containing veratric acid (10 ng) as IS and treated as the plasma ACN extraction with the following modifications. The SPE cartridge was washed with 3 mL of 0.1% acetic acid instead of 0.1% TFA.

Urine samples (0.2 mL) were treated with 1.8 mL of 0.1% formic acid solution, centrifuged at 3600g for 10 min, and the supernatant used for analysis.

Brain, liver (1 g), and fecal samples (0.5 g) were homogenized in the Potter homogenizer (IKA-Werke, Staufen, Germany) with 3 and 5 mL, respectively, of H<sub>2</sub>O/CH<sub>3</sub>OH/acetic acid (80:20:0.1) solution containing veratric acid as the IS (1  $\mu$ g), the mixtures were centrifuged at 3600g for 10 min, and the residue was extracted twice. Supernatants were collected and brought to a final volume of 10 mL.

**Validation Method.** Cyanidin-3,5-diglucoside (Cy-dG) or veratric acid was used as the internal standard to correct the loss of anthocyanins or phenolic acids during sample preparation, respectively. Calibration



**Figure 1.** HPLC profile of the anthocyanins in wild blueberries (*Vaccinium Angustifolium*) detected at 520 nm. See **Table 1** for peak identification.

curves were constructed for each standard at five concentration levels, and two independent determinations were performed at each concentration.

The LC-MS/MS methods were validated for linearity, limit of quantization and detection, peak purity, precision, and repeatability. Limit of quantization ( $S/N$  ratio of 6) and limit of detection ( $S/N$  ratio of 3) were determined by serial dilution of standards in the different biological matrixes. The accuracy (matrix effect) was evaluated according to Matuszewski et al. (22). Specifically, three sets with different concentrations of anthocyanins (2, 10, and 40 ng/mL) or phenolic acids (5, 25, 100, 200, and 400 ng/mL) and IS (50 ng/mL) were prepared. The first set consisted of standards plus IS; the second set was represented by a biological matrix (plasma, urine, liver, or brain) containing standards and IS subjected to extraction treatment and SPE purification, and the third was obtained adding the standards and IS after extraction and purification of the different biological matrix. All of the tests and LC-MS/MS analyses were carried out in triplicate. Peak purity and identity were confirmed by LC-MS/MS experiments. Intra- and interday precision of the assay was verified by analyzing sample sets 3 times on five consecutive days. Repeatability was confirmed by evaluating standard deviations of the retention times.

Calibration curves were constructed for each standard at five concentration levels, and three independent determinations were performed at each concentration. Regression analysis was employed to determine the linearity of the calibration graphs.

**Phenolic Acid Analysis in Biological Samples.** The analyses were carried out with the UHPLC-MS/MS system in gradient mode by a 1.8  $\mu$ m C<sub>18</sub> HSS column (100  $\times$  2.1 mm, Waters) maintained at 70  $^{\circ}$ C, with a flow rate of 0.6 mL/min, and an injection volume of 2  $\mu$ L. The eluents were (A) 0.1% CH<sub>3</sub>COOH and (B) CH<sub>3</sub>CN. The gradient was as follows: 5 to 15% B in 1.5 min, from 15 to 25% B in 1 min, from 25 to 30% B in 0.5 min, and then from 30 to 50% B in 0.5 min.

Analyses were carried out in an ESI negative mode monitoring the following transitions: BA 121  $\rightarrow$  77; 4HBA 137  $\rightarrow$  93; DHBA 153  $\rightarrow$  109; GA 169  $\rightarrow$  125; HA 178  $\rightarrow$  134; VA 167  $\rightarrow$  152; SA 197  $\rightarrow$  182; IS 181  $\rightarrow$  137.

Capillary voltage was fixed at 3.00 kV, source and desolvation temperature at 130 and 350  $^{\circ}$ C, respectively, and argon pressure

at  $1.2 \times 10^{-3}$  mbar, while cone voltage and collision energy were specific for each compound.

The calibration curves were obtained from standard solutions prepared by dissolving 10 mg of each standard in 10 mL of methanol, and the calibration curves were in the range of 5–400 ng/mL.

**Statistical Analysis.** The STATISTICA software (Statsoft Inc., Tulsa, OK) was used for statistical analysis of data. A two-way ANOVA analysis was used to compare the effect of diet (WB vs C) and diet duration (4 vs 8 weeks) on anthocyanins and phenolic acid concentration in the plasma and different rat tissues (liver and brain). The values are presented as the mean  $\pm$  standard deviation. A value of  $p < 0.05$  was considered statistically significant.

## RESULTS

**Anthocyanin Profile in the Blueberry Powder.** Liquid Chromatography combined with UV–vis detection and tandem mass spectrometry is a reliable approach to identify anthocyanin fractions since blueberry is a complex food matrix. **Figure 1** presents the HPLC profile of ACNs in the WB powder sample, integrated at 520 nm, while in **Table 1**, the peak identity and the mean ACN content in freeze-dried WB is reported. The peak identity was established by cochromatography (peaks 1–3, 5–9, and 11–15), on-line UV–vis spectra comparison, and molecular ion and product ion evaluation.

Triplicate analyses were performed on the freeze-dried WB powder. Twenty-one different ACNs were detected, and their total amount was  $1.6 \pm 0.2$  mg/100 mg. The main ones were Mv-3-gal and Pn-3-glc, while Dp-gal and Dp-glc were in lower concentrations.

The MS and MS/MS data (at lower collision energy values, 15–25 eV), combined with the UV–vis and chromatographic behavior suggest that peaks A–F were the acetylated forms (Dp-glc-ac, Cy-glc-ac, Pt-glc-ac, Mv-gal-ac, Pn-glc-ac, and Mv-glc-ac). The unknown peak (#) had an UV–vis spectra comparable to that of anthocyanins, but in MS/MS, it does not



**Table 1.** Qualitative and Quantitative Composition of Wild Blueberry-Powder Anthocyanins

peak	[M] <sup>+</sup>	product ion	compound	%
1	465	303	delphinidin-galactose (Dp-gal)	7.8
2	465	303	delphinidin-glucose (Dp-glc)	9.0
3	449	287	cyanidin-galactose (Cy-gal)	3.6
4	435	303	delphinidin-arabinose (Dp-ara)	5.4
5	449	287	cyanidin-glucose (Cy-glc)	3.6
6	479	317	petunidin-galactose (Pt-gal)	4.8
7	419	287	cyanidin-arabinose (Cy-ara)	1.8
8	479	317	petunidin-glucose (Pt-glc)	6.6
9	463	301	peonidin-galactose (Pn-gal)	1.8
10	449	317	petunidin-arabinose (Pt-ara)	3.0
11	463	301	peonidin-glucose (Pn-glc)	13.2
12	493	331	malvidin-galactose (Mv-gal)	13.2
13	433	301	peonidin-arabinose (Pn-ara)	6.0
14	493	331	malvidin-glucose (Mv-glc)	2.4
15	463	331	malvidin-arabinose (Mv-ara)	0.6
#	487	275, 185	unidentified	
A	507	303	delphinidin-glucose-acetyl (Dp-glc-ac)	1.2
B	491	287	cyanidin-glucose-acetyl (Cy-glc-ac)	2.4
C	521	317	petunidin-glucose-acetyl (Pt-glc-ac)	2.4
D	535	331	malvidin-galactose-acetyl (Mv-gal-ac)	1.8
E	505	301	peonidin-glucose-acetyl (Pn-glc-ac)	2.4
F	535	331	malvidin-glucose-acetyl (Mv-glc-ac)	7.2
			total ACNs	100

give product ions related to anthocyanin aglycones; thus, it cannot be considered an anthocyanin.

**Growth Rate and Body Weight.** Control and WB rat groups exhibited the same growth rate at 4 weeks and 8 weeks. The final mean body weights were  $351 \pm 21$  g for the WB group and  $354 \pm 32$  g for the C group at 4 weeks, and  $459 \pm 21$  g for the WB group and  $444 \pm 20$  g for the C group at 8 weeks respectively. No statistically significant difference in body weight or food intake was detected between the diet groups either at 4 or at 8 weeks of diet duration.

**Validation Method.** The lower limit of detection (LLOD) was 1 ng/mL for Mv-glc, 4 ng/mL for D-glc, and about 2 ng/mL for the other anthocyanins. Regarding phenolic acids, the LLOD was 2 ng/mL for BA and IS and about 4 ng/mL for the other acids. The accuracy of the extraction for anthocyanins and phenolic acids was  $89.2 \pm 4.1$  and  $91.1 \pm 3.6$ , respectively. The precision of the method was tested by both intraday ( $n = 3$ ) and interday (5 days,  $n = 3$ ) reproducibility, and the coefficient of variation was below 12.5%. Regarding repeatability, a maximum relative standard deviation of 5.2% was observed for triplicate injections.

**Evaluation of ACNs in the Plasma, Liver, Brain, Urine, and Feces.** Anthocyanins were not detected in plasma, tissues, urine, and feces in group C and, at time zero, in the WB group. ACNs were found in the urine of the WB group and not of the C group after 4 or 8 weeks of dietary treatment, and their concentrations are reported in **Table 2**. Total ACNs in urine significantly increased after 8 weeks of WB consumption ( $1989.6 \pm 929.2$  ng/24 h) with respect to 4 weeks ( $886.8 \pm 298.8$  ng/24 h). In particular, we obtained significantly higher differences after 8 weeks of WB consumption ( $p < 0.05$ ) for the following ACNs: Cy-gal, Cy-glc, Pt-gal, Pn-gal, Pt-ara, Mv-gal, and Pt-glc-ac. Total ACN content in the feces after 4 and 8 weeks of WB treatment was  $76.7 \pm 43.5$   $\mu$ g/24 h and  $55.4 \pm 39.9$   $\mu$ g/24 h, respectively, with main ACNs, Pn-gal, Pt-ara, and Mv-glc-ac. Individual ACN concentrations decreased at 8 weeks with respect to 4 weeks, but the differences were not statistically different. Three different acetylated forms were also identified in the feces: Mv-glc-ac, Pt-glc-ac, and Pn-glc-ac.

**Table 2.** Anthocyanin Content in Urine and Feces of Sprague–Dawley Rats after 4 and 8 Weeks on the Wild Blueberry Diet<sup>a</sup>

	urine (ng/24 h)		feces ( $\mu$ g/24 h)	
	4 weeks	8 weeks	4 weeks	8 weeks
Dp-gal	$42.7 \pm 16.4$	$84.5 \pm 61.7$	$0.8 \pm 0.6$	$0.8 \pm 0.8$
Dp-glc	$45.4 \pm 20.4$	$81.4 \pm 67.8$	$0.9 \pm 0.8$	$0.9 \pm 1.0$
Cy-gal	$74.3 \pm 27.6$ a	$219.2 \pm 149.8$ b	$0.9 \pm 0.8$	$1.1 \pm 1.1$
Dp-ara	$60.9 \pm 24.6$	$123.9 \pm 93.8$	$1.1 \pm 0.8$	$1.4 \pm 1.6$
Cy-glc	$45.5 \pm 17.9$ a	$108.5 \pm 80.0$ b	$0.6 \pm 0.4$	$1.2 \pm 1.1$
Pt-gal	$61.3 \pm 18.7$ a	$153.5 \pm 33.8$ b	$2.4 \pm 0.8$	$2.6 \pm 2.3$
Cy-ara	$29.2 \pm 13.6$	$46.9 \pm 25.5$	$1.0 \pm 0.8$	$1.3 \pm 1.1$
Pt-glc	$38.4 \pm 13.6$	$60.1 \pm 31.1$	$1.9 \pm 0.9$	$2.3 \pm 2.2$
Pn-gal	$192.1 \pm 57.5$ a	$553.7 \pm 157.0$ b	$6.6 \pm 2.1$	$9.6 \pm 6.0$
Pt-ara	$157.6 \pm 42.2$ a	$323.9 \pm 146.3$ b	$9.1 \pm 2.6$	$11.5 \pm 8.1$
Mv-gal	$55.4 \pm 12.1$ a	$98.0 \pm 14.3$ b	nd	nd
Pn-glc	$16.4 \pm 9.0$	$25.3 \pm 14.2$	nd	nd
Mv-glc	$4.3 \pm 3.0$	$13.3 \pm 19.3$	$2.5 \pm 2.3$	$4.7 \pm 5.3$
Pn-ara	$7.7 \pm 4.0$	$15.5 \pm 8.5$	$2.4 \pm 2.0$	$0.8 \pm 0.4$
Mv-ara	$5.6 \pm 5.1$	$9.8 \pm 6.2$	$3.6 \pm 2.5$	$1.2 \pm 0.8$
Pt-glc-ac	$50.1 \pm 13.1$ a	$72.3 \pm 19.7$ b	$6.0 \pm 4.1$	$2.2 \pm 1.2$
Pn-glc-ac	nd	nd	$5.1 \pm 3.8$	$1.9 \pm 0.9$
Mv-glc-ac	nd	nd	$31.7 \pm 18.1$ b	$11.9 \pm 5.9$ a
total	$886.8 \pm 298.8$ a	$1989.6 \pm 929.2$ b	$76.7 \pm 43.5$	$55.4 \pm 39.9$

<sup>a</sup>Data are expressed as the means  $\pm$  standard deviation. For urine, means between groups not having the same letters are statistically different at  $p < 0.05$ . nd: not detectable.

Anthocyanins were not detected in the plasma, liver, and brain samples after 4 or 8 weeks of dietary treatment either in the C or WB diet groups.

**Identification of Phenolic Acids in Urine, Plasma, Feces, Brain, and Liver.** **Table 3** presents the phenolic acid concentration in plasma (ng/mL) and the content in urine and feces ( $\mu$ g/24 h) of rats after the consumption of a C or a WB diet for either 4 or 8 weeks.

No significant differences were detected in total plasma metabolite concentrations between the C and the WB groups after 4 and 8 weeks of diet treatment. Hippuric acid was the main metabolite present in the urine of the C and WB groups, and its concentration significantly increased ( $p < 0.05$ ) after 8 weeks of WB consumption compared to that after 4 weeks. Hippuric acid concentration in the C group was significantly lower compared to that of the WB group either after 4 or 8 weeks of dietary treatment (**Table 3**). Lower amounts of GA and SA were detected in the urine of the WB group which slightly increased from 4 to 8 weeks, whereas DHBA significantly increased after 8 weeks of WB diet. 4HBA was also present in the C group but was higher ( $p < 0.05$ ) in the WB group. Benzoic acid was not detected in the urine possibly due to its poor solubility or/and rapid conjugation with glycine to produce HA.

The principal phenolic acid present in feces after 4 weeks of WB consumption was DHBA ( $6.7 \pm 2.4$   $\mu$ g/24 h), while after 8 weeks, a significant reduction ( $p < 0.05$ ) was observed, indicating that this compound may be metabolized to BA by intestinal microflora. Indeed, after 8 weeks of WB intake, BA remained unmodified. Some metabolites such as 4HBA and HA were also present in the C groups, but they were lower and significantly different ( $p < 0.05$ ) from those found in the WB group at four weeks.

Benzoic acid was the main metabolite present in the liver and brain with other phenolic acids present in trace amounts (data not shown). Total concentration of BA in the liver was  $40.6 \pm 6.8$   $\mu$ g/g in the C groups and  $42.8 \pm 5.3$   $\mu$ g/g and  $47.6 \pm 6.9$   $\mu$ g/g after 4 and 8 weeks of WB supplementation, respectively. Regarding the brain, the total amount of phenolic acids was  $69.0 \pm 0.6$   $\mu$ g/g in the C groups and  $64.9 \pm 3.9$   $\mu$ g/g and  $63.0 \pm 3.1$   $\mu$ g/g after 4 and 8 weeks of WB intake, respectively.

**Table 3.** Phenolic Acid Concentration in Plasma (ng/mL) and Phenolic Acid Content ( $\mu\text{g}/24\text{ h}$ ), in Urine and Feces of Sprague–Dawley Rats after 4 and 8 Weeks on Control and Wild Blueberry Diets<sup>a</sup>

phenolic acids	4 weeks		8 weeks	
	control	WB diet	control	WB diet
Plasma (ng/mL)				
gallic acid (GA)	nd	nd	nd	nd
siringic acid (SA)	nd	nd	nd	nd
3-4-dihydroxybenzoic acid (DHBA)	nd	nd	nd	nd
4-hydroxybenzoic acid (4HBA)	4.0 $\pm$ 0.4	5.0 $\pm$ 1.2	3.8 $\pm$ 0.3	3.3 $\pm$ 0.7
benzoic acid (BA)	430.6 $\pm$ 64.1	489.8 $\pm$ 54.4 a	408.1 $\pm$ 90.5	319.4 $\pm$ 39.3 b
hippuric acid (HA)	14.0 $\pm$ 13.6	29.6 $\pm$ 12.4	35.3 $\pm$ 13.6	31.2 $\pm$ 4.6
total	448.6 $\pm$ 78.1	524.4 $\pm$ 68.0 a	447.2 $\pm$ 104.4	353.9 $\pm$ 44.6 b
Urine ( $\mu\text{g}/24\text{ h}$ )				
gallic acid (GA)*	nd	136.4 $\pm$ 22.2	nd	159.8 $\pm$ 39.7
siringic acid (SA)*	nd	nd	nd	721.4 $\pm$ 801.1
3-4-dihydroxybenzoic acid (DHBA)*	nd	122.3 $\pm$ 12.9 a	nd	182 $\pm$ 67.8 b
4-hydroxybenzoic acid (4HBA)*	564.1 $\pm$ 294.9 a	2749.4 $\pm$ 1353.1 b	617.5 $\pm$ 63.1 a	3760.9 $\pm$ 1956.1 b
benzoic acid (BA)*	nd	nd	nd	nd
hippuric acid (HA)	301.8 $\pm$ 124.9 a	13020.1 $\pm$ 7190.1 b	653.9 $\pm$ 239.1 c	25230.2 $\pm$ 4730.1 d
total	302.4 $\pm$ 125.2 a	13023.1 $\pm$ 7191.4 b	654.5 $\pm$ 239.2 c	25234.8 $\pm$ 4733.5 d
Feces ( $\mu\text{g}/24\text{ h}$ )				
gallic acid (GA)	nd	1.6 $\pm$ 0.7 a	nd	0.7 $\pm$ 0.5 b
siringic acid (SA)	nd	nd	nd	nd
3-4-dihydroxybenzoic acid (DHBA)	nd	6.7 $\pm$ 2.4 a	nd	1.6 $\pm$ 1.1 b
4-hydroxybenzoic acid (4HBA)	0.7 $\pm$ 0.3 b	2.1 $\pm$ 1.5 a	1.0 $\pm$ 0.5 b	0.7 $\pm$ 1.1 b
benzoic acid (BA)	nd	1.9 $\pm$ 2.1	nd	2.0 $\pm$ 4.3
hippuric acid (HA)	0.6 $\pm$ 0.2 b	23.9 $\pm$ 42.1 a	1.3 $\pm$ 0.9 c	10.0 $\pm$ 19.3 ac
total	1.3 $\pm$ 0.5	36.2 $\pm$ 48.8	2.3 $\pm$ 1.4	14.9 $\pm$ 26.3

<sup>a</sup> Data are expressed as means  $\pm$  standard deviation. Means between groups not having the same letters are statistically different at  $p < 0.05$ . (\*) Data are as  $\mu\text{g}10^{-3}/24\text{ h}$ . WB: wild blueberry. nd: not detectable.

## DISCUSSION

Only few studies have investigated the metabolic fate of ACNs introduced through food sources by analyzing the distribution of ACNs and their metabolites in plasma and tissues and their excretion in urine and feces following long periods of supplementation.

Since ACNs are rapidly metabolized, the majority of studies have taken the pharmacokinetic approach (i.e., one dose, acute study) that has the advantage of allowing one to track the compounds under study in the short term, but the disadvantage is that one is not able to examine their absorption, metabolism, and excretion after long-term exposure.

To our knowledge, this is the first study that has investigated the time effect of exposure to ACNs (i.e., 4 weeks vs 8 weeks) on the absorption, metabolism, and distribution of ACNs and their metabolites following the consumption of wild blueberries. Data obtained in the present study confirm previous observations on ACN absorption and include some new information on ACN metabolism as well as their metabolites.

The bioavailability of ACNs has been investigated in different human and animal pharmacokinetic studies demonstrating that the maximum level of ACNs in serum is found between 2 and 4 h after consumption and very low concentrations are reached (4, 14). The rapid absorption of ACNs could be dependent and mediated by specific enzymes such as bilitranslocase present in the stomach (11). From these studies, the time/rate of absorption of ACNs does not seem to be different between rats and humans.

In a recent study, ACNs were not detected in the plasma of rats at 3, 6, and 24 h after a meal containing blackberry powder (17). This is also in accordance with data by Kalt et al., (23) where no anthocyanins were detected in the plasma of pigs supplemented

with blueberry powder after 18–21 h of fasting. Accordingly, in the present study, we also could not detect anthocyanins in the plasma of rats since blood samples were obtained from animals that consumed the last blueberry-enriched meal 3–4 h before euthanization. Thus, the above data confirm the rapid absorption and metabolism of ACNs.

Urinary excretion is often used to assess ACN absorption and metabolism. Matsumoto et al. (14) indicated that ACN 3-glycosides can be excreted in urine as intact forms in rats within 4 h from ingestion (14, 24). The excretion of ACNs after ingestion of cranberry juice was also investigated in humans; ACN urinary levels reached a maximum, between 3 and 6 h after consumption (25). Similarly in another human study, urinary levels of ACNs reached a maximum concentration after 4–8 h of black raspberry consumption and decreased during the following 8–12 h (26). These data suggest that ACNs are excreted rapidly with kinetics that appears to be comparable in rats and humans.

In the present study, the content of ACNs in the 24 h urine of rats fed a WB diet for 4 and 8 weeks was reported. Studies in which a short-term (10–12 days) feeding with blackberry or red orange juice were performed, ACNs were excreted in the urine as intact forms, while no aglycones or conjugated forms were detected (17, 27).

Anthocyanin content in the urine significantly increased at 8 weeks compared to that at 4 weeks (i.e., Cy-gal, Cy-glc, Pt-gal, Pn-gal, Pt-ara, Mv-gal, and Pt-glc-ac). This observation may be explained by saturation of storage.

Anthocyanins that were not absorbed or excreted by bile were detected in the feces. Pn-gal, Pt-ara, and Mv-glc-ac were the dominant excretory products after WB consumption. An increase (but not significant) of ACNs was detected in the feces of rats fed

for 8 weeks compared to those for 4 weeks particularly for Dp-ara, Cy-glc, Pt-gal, Cy-ara, Pt-glc, Pn-gal, Pt-ara, and Mv-glc. Interestingly, Pn-gal and Pt-ara were among the major ACNs excreted not only in the urine but also in the feces. Since their content in the WB powder was very low, the presence in urine and feces may be due to the methylation of Cy and Dp as reported by different studies (13, 17, 28). Differences in the type of monosaccharide present in the molecule may affect absorption and ACN metabolism (29–31).

It is known that ACNs and polyphenols in general can be metabolized and transformed by intestinal microflora to phenolic acids (32, 33). These compounds can be further absorbed and detected in the blood following ACN intake. In fact, intestinal microflora exhibit a significant hydrolytic potential since they can cleave glycosidic bonds and generate degradation products such as aglycons and phenolic acids that are metabolized by the liver (34).

Vitaglione et al. (33) demonstrated that protocatechuic acid (i.e., DHBA) was the main metabolite present in the bloodstream and was excreted in the feces of human volunteers consuming one liter of blood orange juice providing mainly Cy-glc. In the present study, we detected different phenolic acids in the plasma and in particular, BA. Since BA was also present in the control rats, we may postulate that benzoic acid is not only related to ACN metabolism but its metabolism may depend on many other factors such as intake of fiber or amino acids and the type of intestinal microflora (35–38). Moreover, our results suggest that the concentration of BA (but also HA) in the plasma was not dependent on the duration of the dietary treatment and cannot be considered a good marker of ACN absorption under our present experimental conditions.

Recently, Nurmi et al. (39) studied the metabolism of berry ACNs to phenolic acids in subjects consuming bilberry–lingonberry purée. The excretion of phenolic acids was demonstrated at 4–6 h after the consumption of the purée. The principal anthocyanin metabolites detected in the urine were homovanillic and VA. In our study, the principal ACN metabolite in the urine was HA; this metabolite is produced in the liver through a conjugation of glycine with aromatic phenolic acids such as BA (37). Hippuric acid represents the final product of the metabolic pathway of ACNs but also of amino acids and fiber.

In the present study, HA concentration in the urine significantly increased after 4 and 8 weeks of blueberry consumption with respect to the control group. These results confirm that HA represents an important product of ACN metabolism. The BA, a partially insoluble compound, is absorbed and conjugated with glycine by liver microsomes to produce the more polar compound HA, which is easily excreted in the urine. In fact, unlike benzoic acid, HA did not reach a steady-state in the urine but significantly increased at 8 weeks with respect to 4 weeks. This increase in excretion could be attributed to an increase in the extent of absorption, saturation of storage, or to an activation of detoxification systems. Thus, the presence of HA in the urine could be related to ACN absorption. This hypothesis is well supported by the fact that the HA content of the control group at 4 and 8 weeks was much lower ( $p < 0.05$ ) than that of the WB group.

In the feces, the principal phenolic acids were DHBA at 4 weeks and HA at 8 weeks. This is in agreement with data obtained in humans by Vitaglione et al. (33).

Traces of BA were also present in the liver and brain of all diet groups, while no ACNs were detected. Talavera et al. (40) reported that the methylated and glucuronidated forms were the main metabolites present in the liver, while just traces of native ACNs were found. The native forms of ACNs were instead found in the brain of rats that were sacrificed 3 h after the beginning of

the last meal composed of blackberry extract in contrast with our study in which no ACNs were detected.

We may conclude that ACNs from a WB diet are bioavailable and are detected in the feces and urine of rats after 4 and 8 weeks; this process is enhanced on the basis of the duration of diet exposure. However, since they are rapidly metabolized and our animals were sacrificed 3–4 h from the last meal, we were not able to detect their native forms in the plasma, liver, or brain tissues. Phenolic acids and in particular HA were the principal metabolites detected in all analyzed tissues. The amount of metabolites increased with time of exposure to the ACNs, suggesting a modulatory effect on metabolic pathways or an increased efficiency in absorption/excretion in the older animals (8 weeks vs 4 weeks). Additionally, HA (the ultimate product of ACNs degradation) detected in urine could represent a potential marker of ACN absorption under our experimental conditions. However, more studies will be necessary to understand the metabolic fate of ACNs.

#### NOTE ADDED AFTER ASAP PUBLICATION

The order of author names in the original ASAP posting of December 23, 2009, has been changed, and the spelling of *Vaccinium angustifolium* has been corrected. These corrections are incorporated in the ASAP posting of January 14, 2010. Several corrections of anthocyanin notations throughout the text are incorporated in the ASAP posting January 21, 2010.

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