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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · MARCH 2008

Impact Factor: 2.91 · DOI: 10.1021/jf071998l · Source: PubMed

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Identification of Anthocyanins in the Liver, Eye, and Brain of Blueberry-Fed Pigs

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Dietary intervention with anthocyanins may confer benefits in brain function, including vision. Research to date indicates that animals have only a limited capacity to absorb anthocyanins, compared to other types of flavonoids. Pigs, which are a suitable model for human digestive absorption, were used to examine the deposition of anthocyanins in tissues including the liver, eye, and brain tissue. Pigs were fed diets supplemented with 0, 1, 2, or 4% w/w blueberries (*Vaccinium corymbosum* L. 'Jersey') for 4 weeks. Prior to euthanasia, pigs were fasted for 18–21 h. Although no anthocyanins were detected in the plasma or urine of the fasted animals, intact anthocyanins were detected in all tissues where they were sought. LC-MS/MS results are presented for the relative concentration of 11 intact anthocyanins in the liver, eye, cortex, and cerebellum. The results suggest that anthocyanins can accumulate in tissues, including tissues beyond the blood−brain barrier.

KEYWORDS: LC-MS; flavonoid; bioavailability; tissue; health

INTRODUCTION

Interest in the in vivo bioavailability of anthocyanins (ACN) arises from evidence supporting a role for these flavonoids in human health. To elucidate their effects in biological systems, ACN have been administered in a variety of ways in vitro and in vivo (for a review see ref *1*). Evidence suggests that ACN consumption can mitigate adverse physiological processes related to oxidative stress, obesity, and the degeneration of brain function (see references in ref *1*) and may be beneficial in vision and eye health (for a review, see ref *2*). Although research continues to characterize in vivo effects of ACN feeding, to fully understand the contribution that ACN could make to physiological processes, it is necessary to understand the nature of their bioavailability.

Although an understanding of ACN bioavailability has developed through recent research (*1*), there is a paucity of studies that examine bioavailability when ACN are administered at feasible dietary intake levels, over a long period, and as part of a complex basal diet. Also lacking is information on the deposition of ACN in the specific tissues and organs where ACN effects have been observed. Results from these types of studies will contribute to our understanding of the potential significance of ACN in human health.

Mainly on the basis of studies of ACN measurements in the urine, plasma, and gastrointestinal (GI) contents, after single-dose feeding, Prior and Wu (*1*) noted five major aspects that characterize ACN bioavailability from that of other flavonoids. These aspects are that (1) ACN can be absorbed as intact molecules, (2) ACN are rapidly absorbed and depleted from plasma and urine, (3) the chemical structure of ACN influences their absorption, (4) ACN can be degraded in the lower GI tract, and (5) ACN absorption can be affected by the food matrix.

In their review of 97 bioavailability studies, Manach et al. (*3*) reported a mean maximum plasma ACN concentration of 0.03 ± 0.02 μmol/L (range = 0.0001–0.2 μmol/L), whereas the plasma concentration for other common phenolics is higher, including 0.26 μmol/L for chlorogenic acid, 1.46 μmol/L for quercitin glycosides (gly), and 0.4 μmol/L for (epi)catechin. Total urinary excretion of ACN is typically <0.1% of the administered dose. Compared to their extremely low levels in plasma and urine (0.004−0.1% of the ingested dose) (*3*), the concentration of ACN remains high during transit through the GI tract. ACN and other dietary flavonoids may protect the GI tract against reactive species originating from foods and other sources (*4*). Wu et al. (*5*) accounted for 42% of the ACN and 47% of the antioxidant capacity in the GI tract 4 h after feeding weanling pigs freeze-dried black raspberries, with the ACN concentration in the ileum > cecum > colon. By feeding a relatively simple mixture of ACN from black raspberry fruit (six cyanidin glycosides and pelargonidin-3-rutinoside), Wu et al. (*5*) concluded that the degree of intestinal absorption of ACN was influenced by the specific anthocyanidin, the glycosydic group, the ACN dose, and the relative stability of the specific

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10.1021/jf071998l CCC: $40.75
Published on Web 01/23/2008

ACN. When ileostomy patients were fed blueberry (BB) ACN (7.8 mg of ACN/kg of food), malvidin-3-arabinoside (Mal ara) was recovered in the greatest proportion (85%), whereas cyanidin-3-glucoside (Cyn glu) was recovered in the least (28%) (*6*). The occurrence of ACN in ileostomy fluids indicates that ACN can reach the colon (*6*). Once in the colon, ACN can be degraded by the gut microflora, with phenolic acids as major products of catabolism (*7, 8*).

The high relative abundance of ACN in fruit crops, and especially berry crops (*9*), makes the study of berry ACN and, indeed, the study of the health functionality of berries appealing. In blueberries [*Vacccinium* species, including *V. corymbosum*, *V. angustifolium*, *V. asheii*, their various hybrids, and the bilberry (*V. myrtillus*)] ACN can be considered the "signature" health component, because of their abundance compared to other flavonoids. Blueberries contain a large variety of ACN compared to other berry crops (*9*). Notably, blueberries do not contain pelargonidin glycosides. Blueberries continue to be investigated for their beneficial effects in the brain (*10–14*), in cardiovascular disease (*15, 16*), and in aging (*17*).

In light of increasing evidence of the neuroprotective effects of BB (*10*), there is particular interest in ACN deposition in brain regions after BB feeding. Andres-Lacueva et al. (*18*) identified ACN in brain regions of BB-fed rats, providing evidence that these compounds cross the blood−brain barrier and can be localized in areas where BB feeding has been reported to confer protection. In relation to their possible effects in vision, Matsumoto et al. (*19*) recently reported the rapid appearance of black currant ACN in ocular tissues in both rats and rabbits after ACN were administered using different methods.

Pigs are a widely accepted model for human nutrition (*20*) because they are omnivores, with nutrient absorption processes and body weights similar to those of humans. The usefulness of the pig as a model for cardiovascular studies is also well-documented (*21*). Another more practical consideration for their use in ACN bioavailability studies is that large amounts tissue are available, as compared to a much smaller organism, such as a rat. The weanling pig has already been used in ACN bioavailability studies (*5, 22*).

Reported here are results obtained from an ACN bioavailability study conducted with pigs. One of the major goals of the study was to examine the distribution of ACN after feeding ACN doses (as powdered whole BB fruit) that would be realistically achievable in the human diet. This study provides evidence to suggest that the consumption of moderate doses of ACN can lead to their accumulation in tissues, even when they are not detected in the circulation.

MATERIALS AND METHODS

Animals. Studies were conducted with the approval of the University of Prince Edward Island Animal Care Committee, under the *Guidelines for the Care and Use of Experimental Animals* (Canadian Council on Animal Care). Healthy neutered male pigs (Yorkshire × Landrace) were obtained from a local producer at approximately 32–41 days old, 2 weeks after weaning. Pigs, which weighed 15 ± 2 kg at the beginning of the study, were housed in groups of five in pens that were 1.52 m wide, 3.04 m deep, and 1.07 m high. After an acclimation period of 2 days, during which they were fed 0% BB control diets, pigs were switched to experimental diets that had BB added (**Table 1**). Pigs (n = 5 per BB diet group) consumed food ad libitum, except that feed was removed from pens 18−21 h before euthanasia at 4 weeks. Weight gain was measured weekly on individual pigs, and feed intake was measured weekly as a mean value for each pen.

Table 1. Composition of Experimental Diets

feed ingredient	amount, g/kg of diet			
blueberry powder	0	10	20	40
fructose	13	10	7	0
glucose	12	9	6	0
salt	5.2	5.2	5.2	5.2
soy meal 48%	391	391	391	391
barley	200	200	200	200
oats	100	100	100	100
canola meal	75	75	75	75
Fat Pak 100	75	75	75	75
soy oil	63	63	63	63
corn no. 2 8%	24	24	24	24
Cal Carb Gran (limestone)	12	12	12	12
Dical Phos (Biofos)	11	11	11	11
bran, 22.7 kg bags	15	11	7	0
Gold Swine Ultra (2 kg/T)	2	2	2	2
methionine	1	1	1	1
lysine	0.5	0.5	0.5	0.5
choline chloride 70%	0.4	0.4	0.4	0.4
total	1000	1000	1000	1000

Table 2. Concentration of Anthocyanins in 'Jersey' Blueberry (BB) Powder

anthocyanin	μmol of ACN/g of BB powder	μg of ACN/g of BB powder
delphinidin 3-galactoside	5.43	2524
delphinidin 3-glucoside	5.66	2631
cyanidin 3-galactoside	0.069	31.0
cyanidin 3-glucoside	0.114	51.2
cyanidin 3-arabinoside	0.143	59.9
peonidin 3-galactoside	0.089	41.2
malvidin 3-galactoside	0.123	60.6
malvidin 3-glucoside	0.007	3.45
malvidin 3-arabinoside	2.26	1046
	13.9	6448[a]

[a] Compared to 7.97 mg/g of BB powder, based on colorimetric measurement.

Diets. Basal diets were supplemented with whole freeze-dried, powdered BB (*V. corymbosum* L. cv. 'Jersey') where indicated (**Table 1**). To measure ACN concentration, the BB powder was extracted in both water and in a solvent mixture of 40:40:20:0.1 acetone/methanol (MeOH)/water/formic acid. Colorimetric assays and HPLC with diode array detection were used to measure the phenolic (*23*) and ACN (*24, 25*) in the BB powder extracts (**Table 2**). BB powder was added to basal diets at concentrations of 1, 2, and 4% (w/w). Sufficient glucose and fructose were added to 0, 1, and 2% diets so that these sugars were present at the same concentration as found in the 4% BB powder. Soy, oats, and barley made up 70% w/w of the diet, which also contained 15% fat (**Table 1**).

Tissue Sample Preparation. Blood was collected from ketamine-sedated pigs 1 day before euthanasia using pentobarbitol. Tissues and organs were collected from the body within 0.5 h after euthanasia. Urine was collected from the bladder using a syringe. Where appropriate, tissues were rinsed with water to remove excess blood and were then frozen in liquid nitrogen. The time after tissue and urine were collected from the body, until freezing in liquid nitrogen, was between 5 and 10 min. All tissues and fluids were stored at −80 °C, and after freeze-drying, tissues were returned to −80 °C storage. Numerous tissues were collected; however, this paper will focus only on the analysis of intact ACN in the liver, eye, cortex, cerebellum, urine, and plasma.

Methods for the extraction of ACN from tissue were modified from those published by Tsuda et al. (*26*). To extract ACN, a representative portion of freeze-dried tissue was powdered, and a weighed amount [0.375 g, equivalent to approximately 1.5 g of fresh weight (FW)] was rehydrated in 1–2 mL of water (based on percent moisture of the fresh tissue), vortexed for 10 s, and then sonicated for 5 min. Samples were further dissolved with 2 mL of 0.4 M phosphate buffer (pH 3.9) containing 0.1% EDTA. This slurry was vortexed and then gently mixed for 10 min. Protein and phosphate were precipitated from the aqueous

Table 3. Total Concentration of 11 Anthocyanins Measured in Pig Tissues after Feeding Different Blueberry Doses to Pigs for 4 Weeks[a]

	tissue			
	liver	eyes	cortex	cerebellum
$^{10}\sqrt{}$AC/g of FW	2.89	2.98	2.72	2.65
SE	0.06	0.09	0.05	0.11
F prob dose	NS	NS	0.006	NS
ACN, pg/g of FW	584	709	394	298
SE	0.087	0.177	0.114	0.122
ACN, pmol/g of FW	1.30	1.58	0.878	0.664
F prob dose	NS	NS	0.017	NS

[a] At the top of the table is the mean anthocyanin concentration expressed as the root$_{10}$ ($^{10}\sqrt{}$) of MS area counts (AC) per gram of fresh weight (FW) of pig tissue. At the bottom of the table is the estimated concentration, based on calibration standards of pure anthocyanins.

sample using 12.5 mL of acetone containing 0.1% trifluoroacetic acid (TFA) with gentle mixing for 10 min. Precipitated material was removed from the acetone solution by centrifugation (5 min at 14000 rpm, 4 °C), and the supernatant was acidified further using 100 μL of neat TFA. Solvent partitioning was used to selectively remove nonpolar and polar lipids and to remove acetone from the aqueous portion of the supernatant. To the acidified supernatant was added 18 mL of solvent and 6 mL each of hexane, dichloromethane (DCM), and ethyl acetate, in that order. The solution was vortexed and centrifuged, and the upper layer of organic solvent was discarded. The aqueous portion was finally extracted with 6 mL of DCM, followed by vortexing and centrifugation, and the top aqueous portion was removed and freeze-dried.

Liquid samples of plasma and urine were handled differently from tissue samples. Plasma or urine (1 mL) was acidified with 0.2 mL of 0.44 M TFA in water, vortexed, incubated for 15 min in the dark, and then centrifuged at 14000 rpm for 10 min, all at room temperature. The supernatant (1 mL) was loaded on Nexus 200 mg solid-phase extraction (SPE) cartridges (Varian, Mississauga, ON, Canada) that had been preconditioned with 2 mL of MeOH and 4 mL of 0.1% TFA in water. Cartridges were washed with 2 mL of 0.1% aqueous TFA, 2 mL of DCM, and 2 mL of hexane, in that order. The SPE bed was dried before and after DCM washing. ACN was eluted in 2 mL of 0.1% TFA in MeOH, and solvent was removed under vacuum at room temperature. Once the solvent was removed, samples were freeze-dried and stored at −80 °C until they were analyzed.

LC-MS Analysis. Freeze-dried extracts were dissolved in 130 μL of a solution containing 5% acetonitrile (Fisher Co., Fair Lawn, NJ) in 1% aqueous formic acid (HFO) (Sigma Chemical Co., St. Louis, MO). Once dissolved, the extract was drawn into a syringe to determine its volume prior to microcentrifuging at 10000 rpm for 10 min.

Chromatographic separation of ACN extracted from tissue, plasma, and urine was conducted using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) fitted with dual C_{18} analytical columns, in series. These columns were protected with an Eclipse XDB-C_{18} (3 μm) analytical guard column, 4.6 × 12.5 mm (Agilent P/N: 820950-925). The first analytical column was a Perkin-Elmer reversed phase 3, C_{18} (3 μm) 4.6 × 83 mm monofunctional Pecosphere cartridge column (Perkin-Elmer P/N: 0258-0166). The second analytical column was a ZORBAX SB-C_{18} (3.5 μm) 4.6 × 150 mm (Agilent P/N: 863953-902). ACN separation was achieved using a gradient between 4.5% HFO in water (mobile phase A) to 4.5% HFO in 100% acetonitrile (mobile phase B) over an 80 min analytical run at a flow rate of 0.3 mL/min. The gradient profile was as follows: mobile phase B, 5% at 0 min, 12% at 12 min, 24% at 40 min, 40% between 45 and 50 min, 100% from 55 to 70 min, and then 5% from 75 to 80 min.

Using a six-port valve, the eluant flowing from the first analytical column was directed to waste until just before ACN began to elute at 14 min. The flow was then redirected to the second analytical column between 14 and 56 min and into an Agilent UV G1315A diode array detector (DAD), where absorbance was monitored between 250 and 700 nm. Once through the DAD, the eluant was directed to a Bruker Esquire ion trap MS/MS (Bruker Daltonics Inc., Billerica, MA). This MS unit was fitted with an electrospray interface and was operated in the positive ion mode with alternating MS and MS/MS scans from m/z 150 to 1000.

MS/MS scans of ACN were compared with authentic ACN standards that were obtained from either Polyphenols AS (Sandnes, Norway) or Extrasynthase (Genay, France). Eight pure ACN standards were used including cyanidin 3-glucoside (Cyn glu), cyanidin 3-galactoside (Cyn gal), cyanidin 3-arabinoside (Cyn ara), delphinidin 3-glucoside (Del glu), peonidin 3-glucoside (Peo glu), peonidin 3-galactoside (Peo gal), peonidin 3-arabinoside (Peo ara), and malvidin 3-glucoside (Mal glu). MS data were handled using Bruker Daltronics Esquire LC 4.5 (Build 21) software for collection and data analysis using software version 3.0 (Build 49). Compounds were identified on the basis of a match of their HPLC retention time, UV absorption profile, m/z of their molecular ions, and their MS/MS fragmentation pattern. ACN concentration in tissues and standards was calculated from area counts (AC) for the ACN aglycone fragment obtained by MS/MS. The response for delphinidin 3-galactoside (Del gal) was calculated at each level of anthocyanin standard by multiplying (AC for Cyn gal/AC for Cyn glu) by the AC for Del glu. Using the same approach, malvidin galactoside (Mal gal) and malvidin arabinsode (Mal ara) were calculated using the response for Mal glu, multiplied by the appropriate ratios of AC obtained from calibration curves for Cyn glu, Cyn gal, and Cyn ara.

Statistical Analysis. Tissue ACN results were analyzed using procedures in GenStat (*27*). The relative tissue ACN concentration was calculated from AC for the ACN aglycone fragment obtained by MS/MS, which was converted to molar quantities by comparison with MS results for pure ACN standards, using coefficients determined from their calibration curves. Numerous tissue extracts had MS/MS signals that were below that of the lowest concentration of ACN standard employed (0.5 ng per ACN, on-column); therefore, unless otherwise indicated, data are reported only on the basis of AC. Root$_{10}$ ($^{10}\sqrt{}$) transformation was applied to AC to normalize variation arising from the wide range of AC values, including numerous zeroes. The $^{10}\sqrt{}$ transformation is useful because the $^{10}\sqrt{}$ back transformation of zero is zero, whereas the back transformation of $\log_{10}$ zero is undefined. Tissue ACN was analyzed as $^{10}\sqrt{}$ AC per gram of FW tissue and also as a percentage of total ACN per gram of FW tissue.

ANOVA was conducted using a randomized block design for each dose (0, 1, 2, and 4% blueberry) for each of four tissues (liver, eyes, cortex, and cerebellum). The four anthocyanidins and the three glycosidic units were also analyzed by ANOVA. Mean ACN values ($^{10}\sqrt{}$ AC per gram of FW tissue) for the tissue × dose combinations and correlations among the 11 ACN were used to carry out principal component analysis (PCA) to describe the relationships among the tissue × BB dose combinations and to determine how particular ACN contributed to these relationships.

RESULTS AND DISCUSSION

ACN Consumed by Pigs. Feed intake was not different among the BB diet groups. However, there was a significantly lower weight gain in pigs receiving 4% BB in their diet. A more detailed description of these results is reported elsewhere (*16*). Colorimetric analysis of the ACN concentration of the BB powder after extraction into water or solvent indicated 1.57 and 7.97 mg of Cyn glu equivalents (equiv)/g of BB powder, respectively. It is unknown how effectively ACN are extracted from the food matrix during transit through the GI tract; however, the concentration of ACN in the water extract will most likely reflect the extent of ACN extraction during mastication. HPLC-MS of the BB powder indicated nine major identified ACN peaks (**Table 2**) that made up about 93% of the peaks that were monitored. Although Peo glu and Mal gal were not chromatographically separated, the 'Jersey' variety has been reported earlier (*28*) to not contain Peo glu. Therefore, this unresolved peak has been designated Mal gal in this study. Petunidin glycosides, which were reported (*28*) to make up 20.2% of 'Jersey', were not monitored.

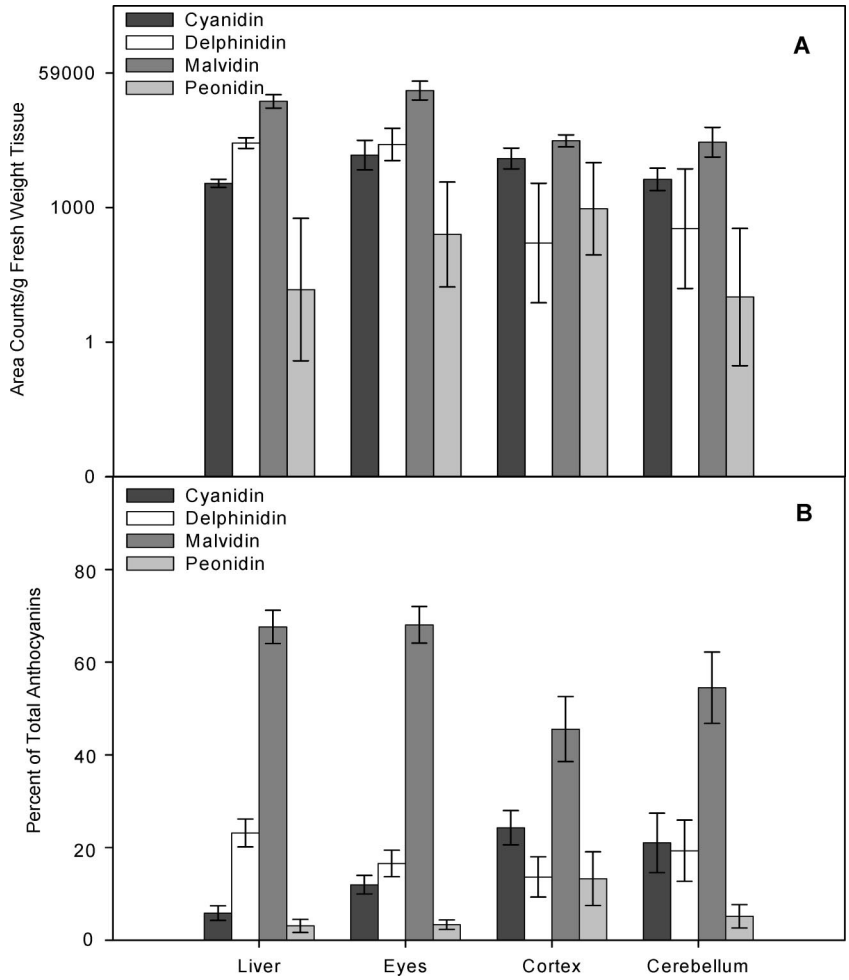

Figure 1. Mean total anthocyanidin concentration measured in pig tissues after 4 weeks of blueberry feeding. Results are the mean values for five pigs in each of three blueberry dosage groups for the three glycosides of cyanidin, malvidin, or peonidin or two glycosides of delphinidin. Panel **A** shows total anthocyanidin concentration back transformed from $^{10}\sqrt{}$area counts per gram of fresh weight. Panel **B** is the same analysis, based on percent of total anthocyanins.

On the basis of pig weights, average feed consumption, and the 1, 2, and 4% BB doses, pigs consumed daily ACN ranging from approximately 1.3−8.5 to 6.6−42.3 μmol/kg of BW, based on the ACN concentration of water-extracted or solvent-extracted BB powder, respectively. The 0% BB basal diets and the diets fed to pigs at weaning were found to contain small quantities of ACN; the basal diet contained 165 ng of Cyn glu equiv/g of feed, and the weaning feed contained 130 ng of Cyn glu equiv/g of feed. Therefore, during their weaning and 4 week treatment period, all pigs consumed ACN equivalent to a 0.0002% BB diet in addition to their specific BB dose. These ACN were presumably present in the soy, oats, and barley, which made up 70% of the basal diet.

ACN in Plasma and Urine. Wu et al. (*22*) administered a single feeding of ACN (140–228 μmol/kg of BW) from different berries to weanling pigs. They concluded that the specific anthocyanidin, the glycosylating moiety, and the quantity consumed can all influence the nature of the ACN occurrence in urine. When He et al. (*29*) conducted a 13 week study, feeding ACN from grape, chokeberry, and bilberry to rats, they found both intact and ACN metabolites in urine and reported that the proportion of methylated ACN in the urine was greater than in studies of shorter duration (8 days). He et al. (*29*) suggested that this result may reflect changes over time in ACN metabolism and distribution, including their possible accumulation in tissues.

In the present study ACN was not detected in either plasma or urine (data not shown). The absence of ACN from plasma and urine may be expected because animals were fasted for 18−21 h prior to euthanasia. The $t_{1/2}$ for ACN in plasma is typically <2 h (*1*). Kay et al. (*30*) reported that the urine ACN concentration was about 1000 times lower 24 h after ACN ingestion, compared to its maximum concentration, observed within 5 h after consumption. Another factor in the present study contributing to the absence of ACN in plasma and urine was the low dose of ACN employed. The ACN consumed by pigs, based on water-extracted BB powder, ranged between 1.3 and 8.5 μmol/kg of BW/day, whereas 140–228 μmol/kg of BW was administered by Wu et al. (*22*).

ACN in Liver, Eye, Cortex, and Cerebellum Tissues. Intact ACN were detected in tissues after consumption of BB-enriched diets for 4 weeks, whereas ACN were absent from plasma or urine, suggesting that ACN had accumulated in tissues, rather than being in a rapid equilibrium with ACN in blood circulation.

The total concentration of 11 intact anthocyanins for the four tissues was not different (**Table 3**) based on $^{10}\sqrt{}$AC per gram of FW tissue. The estimated molar quantities of ACN in the tissue have also been included in **Table 3** to compare with other studies. On the basis of these calibrated values, the ACN concentration ranged between about 300 pg of ACN/g of FW of cerebellum tissue to 700 pg of ACN/g of FW of eye tissue. The ACN concentration was greater in the cortex of pigs fed

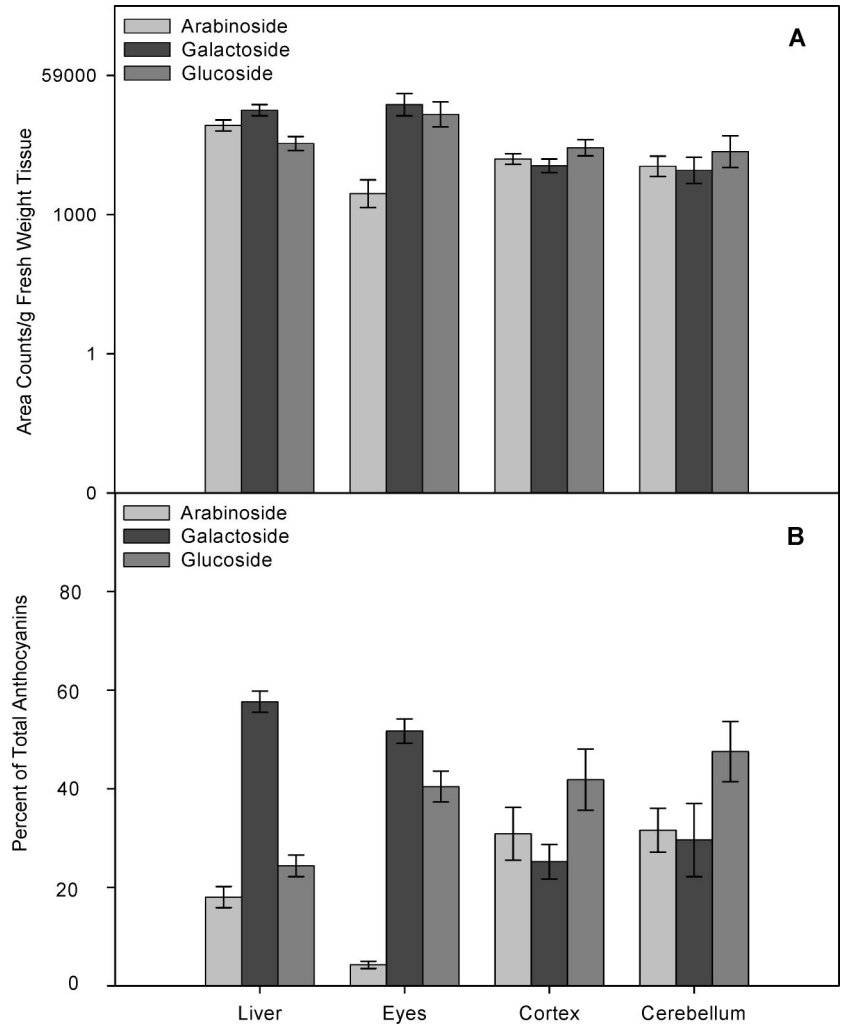

Figure 2. Mean total glycoside concentration measured in pig tissues after 4 weeks of blueberry feeding. Results are the mean values for five pigs in each of three blueberry dosage groups for each of the total arabinosides, galactosides, and glucosides. Panel **A** shows total glycoside concentration back transformed from $^{10}\sqrt{}$area counts per gram of fresh weight. Panel **B** is the same analysis, based on percent of total anthocyanins.

Table 4. Concentration of 11 Anthocyanins, Normalized to 100%, Measured in Pig Tissues after 4 Weeks of Blueberry Feeding[a]

anthocyanin	fruit	liver	eyes	cortex	cerebellum
cyanidin 3-arabinoside	6.05	2.58 (±0.97)	3.65 (±086)	6.15 (±1.47)	3.80 (±1.99)
cyanidin 3-galactoside	9.41	2.21 (±0.76)	3.22 (±0.57)	9.58 (±2.09)	12.9 (±5.53)
cyanidin 3-glucoside	7.57	1.32 (±0.78)	5.12 (±1.21)	7.25 (±1.67)	4.26 (±3.07)
delphinidin 3-galactoside	11.9	11.9 (±1.38)	9.42 (±1.06)	8.24 (±2.71)	15.3 (±6.49)
delphinidin 3-glucoside	6.64	12.7 (±3.93)	8.26 (±1.13)	4.72 (±1.45)	3.96 (±2.38)
malvidin 3-arabinoside	14.9	15.3 (±1.91)	0.09 (±0.10)	22.1 (±4.26)	0.26 (±0.36)
malvidin 3-galactoside	23.6	41.5 (±1.47)	39.0 (±4.70)	0.92 (±0.75)	28.6 (±6.74)
malvidin 3-glucoside	15.0	10.8 (±1.94)	30.2 (±0.91)	22.5 (±5.63)	25.6 (±4.38)
peonidin 3-arabinoside	0.00	0.75 (±0.36)	0.36 (±0.25)	2.57 (±2.22)	0.68 (±0.50)
peonidin 3-galactoside	4.93	2.22 (±1.12)	2.85 (±1.18)	5.05 (±2.20)	20.5 (±0.91)
peonidin 3- glucoside	0.00	0.00 (±0.00)	0.00 (±0.00)	4.28 (±1.48)	2.16 (±1.23)
total	100	100	100	100	100

[a] Results are the mean values for five pigs in each of three dosage groups (1, 2, and 4% blueberries in diet).

higher doses of BB ($P = 0.006$); there were no BB dose-related effects among the other three tissues.

Interestingly, ACN were also found in the tissues of pigs fed the 0% BB diet. ACN content measured in the 0% BB-fed pigs was included in the statistical analysis and may have influenced possible dose–effects among the BB diets. The occurrence of ACN in the tissues of animals fed very small doses of ACN (the equivalent of 0.0002% BB diet) suggests a very high ACN bioavailability under these circumstances and warrants further investigation.

The total ACN measured (**Table 3**) probably underestimates their actual concentration in tissue, for the following reasons. First, ACN metabolites (i.e., methylated or glucuronidated forms) have not been included in these measurements. Kay et al. (*30*) reported that approximately two-thirds of ACN were present as methylated and glucuronidated metabolites in plasma

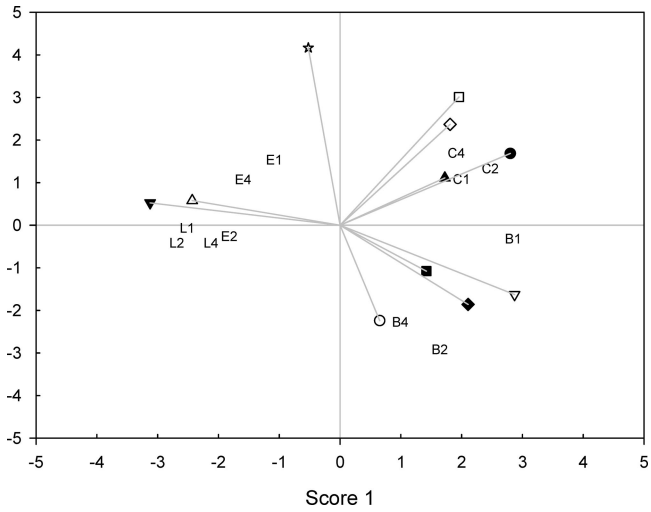

Figure 3. Principal component analysis illustrating distinctive tissue × dose combinations and anthocyanins: B, cerebellum; C, cortex; E, eye; L, liver; 1, 1% blueberry; 2, 2% blueberry; 4, 4% blueberry. Symbols: ◇, cyanidin arabinoside; ▽, cyanidin galactoside; □, cyanidin glucoside; ○, delphinidin galactoside; △, delphinidin glucoside; ◆, malvidin arabinoside; ▼, malvidin galactoside; ■, malvidin glucoside; ▲, peonidin arabinoside; ☆, peonidin galactoside; ●, peonidin glucoside.

and urine. Second, petunidin glycosides, which make up 20% of 'Jersey' ACN (*28*), were not monitored in this study. Third, during the course of this study, when ACN stability in fresh frozen and freeze-dried tissue was compared, ACN levels were found to be substantially lower in freeze-dried as compared to fresh frozen tissue, even though all tissues were stored at −80 °C. Freeze-dried tissue was used exclusively in this study.

Total ACN concentration was analyzed on the basis of the four ACN aglycones (i.e., anthocyanidins) to determine if anthocyanidin concentration differed among tissues and with the dose of BB fed (**Figure 1**). Cyn glycosides (gly) were highest in eyes and cortex, whereas Del gly were greatest in eyes and liver, as were Mal gly. Peo gly were present at the lowest levels and were not different among the tissues. Only in the liver was there a significant effect of BB dose on Del ($P = 0.008$) and Mal ($P = 0.001$) gly, increasing with higher doses of BB. In **Figure 1B**, which shows the anthocyanidin content as a percentage of the total ACN in the tissue, Mal gly were the most abundant, ranging between 45% (cortex) and 68% (eyes). The least abundant among the four anthocyanidins was Peo, which ranged between 3 and 13% of the total ACN in tissues.

The total ACN concentration based on the three glycosides measured (**Figure 2**) showed that eye tissue had the lowest ara and highest glu and gal concentrations, compared to the other three tissues. Like the eyes, the liver had a higher gal concentration than the cortex and cerebellum. The proportion of the three glycosides was just about equal in the cortex and cerebellum. In **Figure 2B**, which shows the specific glycoside content as a percentage of the total ACN, ara ranged between approximately 4% (eyes) and 30% (cortex and cerebellum). Gal were approximately 50% of the ACN in eyes and liver and about 25% in the cortex and cerebellum. Glu ranged between approximately 45% in cortex, cerebellum, and eyes and 24% in liver tissue.

Wu et al. (*22*) demonstrated that both the specific anthocyanidin and the glycosidic moiety can influence the absorption of ACN in the gastrointestinal tract. The current results generally support this finding, where differences in the anthocyanidin and glycoside profiles among the different tissues may reflect selectivity of ACN absorption, metabolism, and retention among tissues and sites such as the blood−brain barrier and the blood−retinal barrier.

The specific ACN measured in the four tissues are shown in **Table 4** as a mean percentage of the total ACN for all BB dosage levels. Some distinctive patterns were apparent in the distribution of Mal gly among tissues. For example, whereas Mal ara ranged between 15.3 and 25.6% of the total ACN in liver, cortex, and cerebellum, it was only 0.09% of the total ACN in eyes. In contrast, Mal gal was <1% of the total ACN in cortex and cerebellum, but it was approximately 40% of the total ACN in eyes and liver. The proportion of Mal glu was more consistent among the four tissues and ranged between 10.8 and 28.6% of the total ACN. The high relative abundance of Mal gly may be due to the methylation of other anthocyanidins, which can occur via catechol-*O*-methyltransferase. It is worth noting that Peo glu was present in the brain at between 4.28% (cortex) and 2.16% (cerebellum); however, this particular ACN is reported to not be present in the 'Jersey' BB (*28*). This result suggests that deglycosylation and reglycosylation with other sugars may have occurred. Although some dose-related differences in content of specific ACN were observed, there was, in most cases, no clear pattern of an increase in the ACN with increased doses of BB ACN. As mentioned earlier, the occurrence of ACN in tissues of 0% BB pigs affected our ability to detect significant effects.

PCA was used to identify relationships among tissue × BB dose combinations and to indicate which ACN contributed to these relationships. In the PCA biplot (**Figure 3**), points that are <90° to each other are positively correlated, whereas those with angles >90° are negatively correlated. Angles of 0° or 180° indicate a perfect positive or negative relationship. As a two-dimensional array, sample points that are nearest are most closely related to each other. The two scores obtained from the latent vectors (**Figure 3**) accounted for 61.6% of the variation among the tissue × BB dose combinations. The four tissues are separated in the biplots from left to right along score 1 (41.9% of variation), with the profile of ACN in eyes and liver contrasted with that in cortex and cerebellum. Cortex and cerebellum separated vertically from each other in score 2 (19.7% of variation). Liver and eye were similarly, but less, separated along score 2. The greatest distinguishing features of the ACN profiles among tissues were the preponderance of Mal gal in liver and eye and its very low level in two brain regions. This is illustrated in the biplot where Mal gal appears at almost 180° to, and furthest from, the cortex and cerebellum (**Figure 3**). Cortex was distinguished from other samples by having the highest concentration of Cyn ara and glu. Peo glu was grouped with the cortex and cerebellum samples, because this ACN occurred only in these two tissues. The eyes had only 0.09% of Mal ara, which was apparent by the large distance between Mal ara and the eye samples (**Figure 3**). PCA was an effective means to illustrate the complex relationships among 11 ACN and 16 tissue × BB dose combinations.

Table 5 is included to facilitate a comparison of the present study with other studies that examine distribution of ACN in tissues. Only two other studies (*18, 31*) examine tissue ACN concentrations after more than a single dose of ACN, and in both cases using a rat model; these longer term feeding studies may be most relevant to the present study. Andres-Lacueva et al. (*18*) fed BB ACN to rats for 70 days and, although ACN concentrations were too low to quantify, it appeared that the cortex was a region of higher ACN bioavailability than the

Table 5. Selected Studies Reporting Anthocyanin in Tissues

ref	model	ACN[a] (μmol[b] of dose/kg of BW[c])	days	administration	tissue where ACN identified
26	rat	±900 as cyanidin3-glu	1 dose	gavage	stomach, jejunum, kidney, liver
33	rat	890 as bilberry extract	1 dose	orally	kidney, liver
19	rat	220 as black currant powder	1 dose	oral	eye regions
19	rat	240 as black currant powder	1 dose	intraperitoneal	eye regions
19	rabbit	44 as black currant powder	1 dose	intravenous	eye regions
32	rat	17.8 as purified grape	1 dose	gastric absorption	whole brain
18	rat	27[d] as blueberry extract	70	ad libitum	brain regions
31	rat	1480 as blackberry extract	15	ad libitum	whole brain, stomach, jejunum, kidney, liver
present	pig	8.5 blueberry powder[e]	30	ad libitum	brain regions, whole eye

[a] ACN, anthocyanins. [b] Based on MW of 450 ACN. [c] BW, body weight. [d] Based on ACN concentration in 2% diet in current study. [e] Based on ACN concentration in 4% blueberry diet with water extraction.

cerebellum. In their study, of 10 ACN examined, 8 were identified in the rat cortex, compared to only 2 ACN in the cerebellum (*18*). In the present study, ACN appeared to be approximately equally present in these two brain regions (**Table 3**). Andres-Lacueva et al. (*18*) noted Mal gal to be the most prevalent ACN in the cortex, and the next most prevalent ACN were Cyn gal and Del gal. These results differ from the present study, in particular because Mal gal contributed <1% to the total cortex ACN, whereas Mal glu and Mal ara contributed together 41% of the total cortex ACN. These differences may be due to methodologies or may suggest that rats and pigs differ in their ACN metabolism.

When Talavéra et al. (*31*) fed to rats a dose of 1.48 mmol of ACN/kg of BW (consisting of >90% Cyn glu) for 15 days (**Table 5**), they measured 108 ng of Cyn glu equiv/g of FW in a whole brain extract, of which 85% was Cyn glu. This ACN concentration is about 120 times greater than the mean ACN concentration found in the two brain regions in the present study, approximately 350 pg/g of FW. However, the dose administered by Talavéra et al. (*31*) was about 175 times greater than the ACN in the water-extracted 4% BB powder (**Table 5**). As mentioned earlier, the ACN measurements in the present study probably significantly underestimate tissue levels. Together these results suggest that ACN absorption may become saturated at high dosage levels.

Passimonti et al. (*32*) surgically introduced a single dose of 8 mg of ACN/kg of BW of purified grape ACN into the stomachs of anesthetized rats ($n = 13$) and found ACN in the brain within 10 min, almost exclusively as Mal glu and Mal 3-(6-coumaroyl) glu. The maximum ACN concentration observed in the whole brain was 192.2 ± 57.7 ng/g of FW. Interestingly, although Mal glu was present in the whole grape at 47.2% and Mal 3-(6-coumaroyl) glu at 1.86%, in the brain Mal 3-(6-coumaroyl) glu was more prevalent than Mal glu. Ichiyanagi et al. (*33*) found that after oral administration of bilberry ACN, the plasma retention of ACN varied according to their gly moiety with gal > glu > ara. This is consistent with the present study, in which ara were the least abundant anthocyanosides found (**Figure 2**). They (*33*) also report that the profile of ACN, including ACN conjugates, differed between plasma and kidney tissue, similar to the present study, in which distinctive differences were found in the profile of intact ACN among the tissues.

In the eyes of BB-fed pigs, 10 of the 11 ACN were identified, with Mal gal and Mal ara making up 57% of the total ACN (**Table 4**). Deposition of ACN in eye tissues is of great interest because ACN have been associated with improvements in vision ((*2*); see references in ref *19*). Matsumoto et al. (*19*) found high levels of ACN differentially distributed among ocular tissues of rats 1 h after intraperitoneal administration of about 0.24 mmol of ACN/kg of BW. Among seven ocular regions examined, total ACN was highest in the sclera and choroid (245 μg/g of tissue) and exceeded the plasma ACN concentration by about 100 times. When ACN (20 mg/kg of BW) was administered intravenously to rabbits, lower levels of ACN were found in the ocular tissues (*19*). As with the rats, the highest ACN concentration was in the rabbit sclera (3.02 μg/g of tissue), but this concentration was lower than in plasma (12.36 μg/mL). The vitreous, which is a large proportion of the total eye tissue, had a substantially lower ACN concentration compared to other ocular tissues (*19*). In the current study the ACN concentration measured in the whole eye may have been higher if the vitreous had been excluded from the tissue sample. Our results support the results of Matsumoto et al. (*19*) in that, compared to other tissues, the eye appears to have a high relative capacity for ACN absorption.

Analytical Methods. When the recovery of ACN was examined in pig liver tissue that had been stored either fresh frozen or freeze-dried, it was found that ACN was substantially less abundant in tissue that had been freeze-dried. Unfortunately, this was unknown at the beginning of the study. All of the results reported here were obtained from ACN extracted from freeze-dried tissue and have not been corrected for ACN losses during freeze-drying and storage. Details of a study examining the stability of ACN in tissues during long-term storage will be described elsewhere, along with other methods for ACN tissue bioavailability research.

The methods described by Tsuda et al. (*26*) for the extraction of ACN from tissue were modified to use larger amounts of tissue and to take additional steps to remove lipids, which may have been particularly abundant in tissues such as the brain. ACN recovery from liver tissue was lowest and averaged 57.0 ± 19.1%, whereas ACN recovery was higher from kidney (89 ± 13%), muscle (86 ± 9.7%), and cortex (87 ± 10.3%). No correction was made in the results for losses in ACN during extraction.

In closing, this paper describes the presence of ACN in animal tissues, where ACN were absent from plasma and urine, suggesting that ACN may accumulate in tissues. The identification of ACN in brain regions and the eye suggests that these compounds may act directly at the sites where their benefits have been documented in neuroprotection (*10–14*) and in vision ((*2*); see references in ref *19*). Differential transport, metabolism, and retention may have contributed to differences in the profile of ACN among tissues. The tissue concentrations of ACN reported here are likely to be substantially underestimated. The detection of ACN in the pig tissues collected at the beginning of the study ($n = 5$ pigs, $t = 2$ days), as well in the tissues of pigs fed the 0% BB diet, was remarkable and suggests that low levels of ACN may be highly bioavailable and well-retained in tissues of newly weaned pigs. Although ACN bioavailability has been reported to be low (for a review see ref *1*), this study

suggests that reasonable dietary levels of ACN may be well absorbed; however, their uptake mechanisms may be readily saturated. Taken together, this study provides further evidence to support a role for ACN in human health and wellness.

ACKNOWLEDGMENT

For their excellent assistance, we thank Basil Dickson and Ken McRae, Ph.D., of AAFC, Ted Van Lunen, Ph.D., of the Canadian Forestry Service, Kim Foote, DVM, of the Charlottetown Veterinary Clinic, Alfred Mitchell of the Atlantic Veterinary College, Kristen Doncaster of AAFC, and Susan Monro of Acadia University.

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Received for review July 3, 2007. Revised manuscript received November 22, 2007. Accepted November 27, 2007. We gratefully acknowledge financial support from the U.S. Highbush Blueberry Council, the Agriculture and Agri-Food Canada (AAFC) Matching Investment Initiative, and the U.S. Department of Agriculture, under Agreement 58-1950-7-707. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the USDA.

JF071998L