

## Anthocyanins Are Efficiently Absorbed from the Stomach in Anesthetized Rats

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**ABSTRACT** After consumption, anthocyanins are rapidly absorbed as glycosides. Their rapid appearance in plasma could result from absorption through the gastric wall. The aim of this study was to evaluate the fate of anthocyanins in the stomach. Absorption of purified anthocyanins (14  $\mu\text{mol/L}$ ) as well as blackberry (14 and 750  $\mu\text{mol/L}$ ) and bilberry (88  $\mu\text{mol/L}$ ) anthocyanins was compared after *in situ* gastric administration for 30 min. A high proportion ( $\sim 25\%$ ) of anthocyanin monoglycosides (glucoside or galactoside) was absorbed from the stomach, whereas absorption of cyanidin 3-rutinoside was lower. Bilberry anthocyanins were also efficiently absorbed, but absorption varied greatly (19–37%) according to the anthocyanin structure; delphinidin glycosides were the most absorbed. When a high concentration of blackberry anthocyanins (750  $\mu\text{mol/L}$ ) was injected into the gastric lumen, the percentage of cyanidin 3-glucoside (Cy 3-glc) absorption was lower than after administration of a low concentration (14  $\mu\text{mol/L}$ ). After administration of this high concentration, blackberry anthocyanins were observed in plasma from gastric vein and aorta, whereas neither aglycones nor metabolites were detected. Analysis of bile samples revealed that Cy 3-glc appeared in bile after as little as 20 min. Peonidin 3-glucoside (the methylated form of Cy 3-glc) as well as unknown anthocyanin metabolites were also observed in bile. Thus, this study demonstrated that anthocyanin glycosides were quickly and efficiently absorbed from the stomach and rapidly excreted into bile as intact and metabolized forms. *J. Nutr.* 133: 4178–4182, 2003.

**KEY WORDS:** • rats • anthocyanins • bilberry • blackberry • stomach

Anthocyanins are a group of naturally occurring phenolic compounds responsible for the color of many flowers, fruits (particularly berries) and vegetables. Their daily intake in humans has been estimated to be  $\sim 200$  mg/d in the United States (1) due to their widespread distribution and occurrence in fruits and vegetables. Consumption of anthocyanins has been shown to reduce the risk of coronary heart disease and to prevent some chronic diseases (2,3). Moreover, it has been reported that anthocyanins inhibit platelet aggregation (4), improve visual function (2,5), possess vasoprotective properties (6–8) and could exert neurological beneficial effects (9). *In vitro* experiments have also shown that anthocyanins inhibited cellular growth and induced apoptosis in cancer cells (10,11). The positive effects of these pigments could be related to their potent antioxidant activity demonstrated in various *in vitro* and *in vivo* studies (12–16).

Anthocyanins are rapidly absorbed as glycosides in rats and humans (17–21). Indeed, the intact glycosidic forms were recovered in plasma only a few minutes after oral administration of anthocyanins (17,19,20). This rapid appearance in plasma could result from absorption through the gastric wall. Thus, the aim of this study was to evaluate the fate of antho-

cyanins in the stomach. For this purpose, we compared the absorption of various anthocyanins in the stomach using *in situ* gastric administration. Four purified anthocyanins with various aglycones and glycosidic moieties were studied. Moreover, because berries are rich dietary sources of anthocyanins (22), gastric absorption of blackberry anthocyanins and of a commercially available bilberry extract was also evaluated.

### MATERIALS AND METHODS

**Chemicals.** Cyanidin 3-glucoside (Cy 3-glc),<sup>2</sup> cyanidin 3-galactoside (Cy 3-gal), cyanidin 3-rutinoside (Cy 3-rut), malvidin 3-glucoside (Mv 3-glc) and cyanidin 3,5-diglucoside (Cy 3,5-diglc) were purchased from Extrasynthèse (Genay, France). Deep-frozen blackberries were from a deep-frozen food product supplier (Szymczak-Nadreau, Romagnat, France). Bilberry (*Vaccinium myrtillus* L.) anthocyanin extract (Antho 50) was from Ferlux Mediolanum (Cournon d'Auvergne, France).

**Animals and diets.** Male Wistar rats ( $n = 42$ ; Iffa-Credo, L'Arbresle, France) weighing  $\sim 200$  g were housed two per cage in temperature-controlled rooms (22°C), with a dark period from 2000 to 800 h and access to food from 1600 to 800 h. They were fed a

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<sup>2</sup> Abbreviations used: ara, arabinase; Cy, cyanidin; diglc, diglucoside; Dp, delphinidin; gal, galactoside; glc, glucoside; Mv, malvidin; PEG, polyethylene glycol; Pn, peonidin; Pt, petunidin; rut, rutinoside.

semipurified control diet (755 g/kg wheat starch, 150 g/kg casein, 50 g/kg peanut oil, 35 g/kg AIN-93M mineral mixture, 10 g/kg AIN-76A vitamin mixture) for 2 wk (21). The rats were maintained and handled according to the recommendations of the Institutional Ethic Committee (INRA), in accordance to the decree No 87-848.

**Anthocyanin administration.** Rats were deprived of food for 24 h and anesthetized with sodium pentobarbital (40 mg/kg body weight); they were kept alive and under anesthesia throughout the experiments. Pentobarbital could have affected the rate of anthocyanin absorption, but determination of this effect was beyond the scope of this work. After cannulation of the biliary duct, the pylorus was ligated and a physiologic buffer was injected into the stomach across the cardia. To prevent any gastroesophageal reflux, this sphincter was ligated. The stomach was filled in situ with 5 mL of a buffer devised to mimic the osmotic and pH conditions found in the stomach during a meal. This buffer (pH 3) contained  $\text{KH}_2\text{PO}_4$  (7.5 mmol/L), NaCl (50 mmol/L), KCl (50 mmol/L),  $\text{CaCl}_2$  (2 mmol/L), acetic acid (25 mmol/L), lactic acid (25 mmol/L),  $\text{MgSO}_4$  (1 mmol/L) and polyethylene glycol (PEG) 6000 (5 g/L) and was maintained at 37°C. It was supplemented with  $\sim 14 \mu\text{mol/L}$  purified molecules (Cy 3-glc, Cy 3-gal, Cy 3-rut or Mv 3-glc), blackberry extract (14 and 750  $\mu\text{mol/L}$ ) or bilberry extract (88  $\mu\text{mol/L}$ ). The amounts of anthocyanins infused into the stomach corresponded to those used previously in similar models (23,24). Berry anthocyanin extracts were obtained as described below. At 30 min after the administration, stomach contents were collected and blood samples were withdrawn from the gastric vein and abdominal aorta into heparinized tubes. Bile was collected in two fractions at 0–20 min and 20–30 min. Plasma and bile samples were acidified with 0.02 volume of 12 mol/L HCl, whereas stomach contents were acidified with 0.003 volume of 12 mol/L HCl. All samples were stored at  $-20^\circ\text{C}$  before analysis.

**Anthocyanin extracts.** Two blackberry anthocyanin extracts (low concentration, 14  $\mu\text{mol/L}$ ; high concentration, 750  $\mu\text{mol/L}$ ) were prepared from a powder obtained from frozen blackberries that were lyophilized, pulverized and then sieved to eliminate seeds (21). They were obtained from 4 g of powder treated for 30 min under agitation with 100 mL of 0.12 mol/L HCl in 10% ethanol, then centrifuged for 5 min at  $12,000 \times g$ . The supernatant was diluted 80-fold in the gastric buffer to obtain the 14- $\mu\text{mol/L}$  extract. To obtain the 750- $\mu\text{mol/L}$  extract, the supernatant was dried using a rotary evaporator at 35°C, and finally dissolved in 12 mL of the gastric buffer.

Bilberry anthocyanin extract (Antho 50, Ferlux Mediolanum) was dissolved in 0.12 mol/L HCl in 10% ethanol and then diluted 80-fold in the gastric buffer to obtain a final anthocyanin concentration of 88  $\mu\text{mol/L}$ . This concentration, higher than that of purified anthocyanins (14  $\mu\text{mol/L}$ ), was chosen to allow quantification of each anthocyanin present in the bilberry extract.

**Sample preparation.** Anthocyanins present in plasma samples were extracted with a solid phase extraction cartridge (Sep-Pak  $\text{C}_{18}$  Plus, Waters, Milford, MA) as follows. The cartridge was washed with 10 mL of methanol and equilibrated with 10 mL of 12 mmol/L aqueous HCl before use. Plasma samples (1 mL) spiked with 0.41 nmol cyanidin 3,5-diglucoside as an internal standard were loaded onto the cartridge. The cartridge was then washed with 10 mL of 12 mmol/L aqueous HCl, and anthocyanins were eluted with 3 mL of 12 mmol/L HCl in methanol. The methanolic extract was dried under reduced pressure using a rotary evaporator at 35°C. The dried extract was dissolved with 100  $\mu\text{L}$  of 0.12 mol/L aqueous HCl. After centrifugation for 5 min at  $12,000 \times g$ , the supernatant (60  $\mu\text{L}$ ) was analyzed by HPLC as described below. Proteins from bile samples were eliminated as previously described (23). Supernatants were evaporated under a nitrogen stream to the initial volume of bile. A 60- $\mu\text{L}$  aliquot was immediately analyzed by HPLC as described below. Stomach contents were centrifuged for 5 min at room temperature, then filtered on fritted glass and analyzed (20  $\mu\text{L}$ ) by HPLC.

**HPLC analysis.** Analysis of anthocyanins was conducted by HPLC using a photodiode array detector (991, Waters) and an UV-visible detector (785A, Perkin Elmer, Courtabœuf, France) at 524 nm. Elution was performed using water/ $\text{H}_3\text{PO}_4$  (99:1) as solvent A and acetonitrile as solvent B. All samples except those from

bilberry extract were analyzed as previously described (25). Samples from bilberry extract were loaded onto an Uptisphere 30DB C18–3 $\mu\text{m}$  column (150  $\times$  4.6 mm) protected by a guard column (Uptisphere 30DB C18–3 $\mu\text{m}$ , 10  $\times$  4 mm; Interchim, Montluçon, France). The chromatographic conditions were as follows (flow rate 1 mL/min): 0–10 min, 9% B; 10–25 min, linear gradient from 9% B to 12% B; 25–40 min, linear gradient from 12% B to 16% B; 40–45 min, 16% B; 45–50 min, linear gradient from 16% B to 40% B. The identification of the compounds present in samples was made by comparison with the authentic compounds based on the retention time in the HPLC analysis and UV-visible spectrum, and by spiking with individual compounds. Absorption through the gastric wall was estimated by the difference between the amount of anthocyanins administered into stomach and the amount recovered at the end of the incubation.

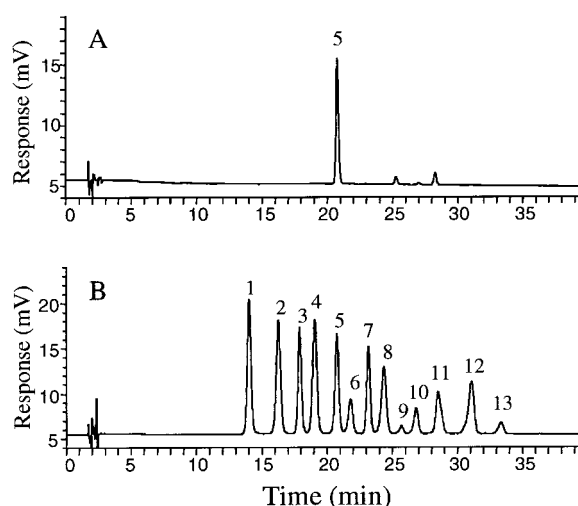
**PEG measurements.** PEG, a compound that is not absorbed by the stomach, was added to the gastric buffer. Its concentration in the gastric buffer was determined by the method of Powell and Malawer (26). The ratio between the initial concentration and that measured at the end of the experiment reflected the intensity of the gastric secretion. This parameter must be taken into account to obtain the correct concentration of anthocyanins at the end of the experiment.

**Data analysis.** Values are means  $\pm$  SEM; when appropriate, significance of differences between means was determined by one-way ANOVA followed by Student-Newman-Keuls test (GraphPad, Instat, San Diego, CA). Values of  $P < 0.05$  were considered significant.

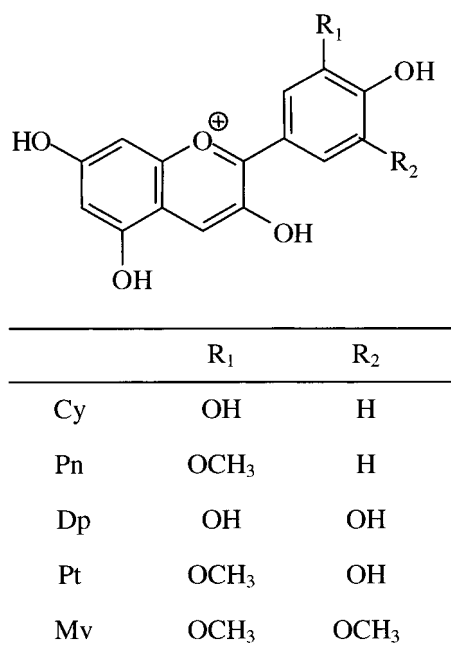
## RESULTS

We first determined that the anthocyanins had not degraded in the gastric buffer. All of the tested compounds were stable under the experimental conditions, i.e., incubated for 30 min at 37°C in the defined buffer.

As previously reported (21), Cy 3-glc is the major anthocyanin in blackberries (> 98% of total anthocyanins) (Fig. 1A). Thus, only Cy 3-glc was quantified in experiments conducted with blackberry extracts. The bilberry extract, Antho 50, is a commercially available extract that contains  $\sim 61\%$  anthocyanins, making it very rich in these compounds. Fifteen anthocyanins derived from five aglycones (Fig. 2) were identified in this extract. They eluted as 13 peaks (Fig. 1B) due to



**FIGURE 1** HPLC chromatograms of blackberry (A) and bilberry (B) extracts. Detection was performed at 524 nm. Peaks are as follows: 1, Dp 3-gal; 2, Dp 3-glc; 3, Cy 3-gal; 4, Dp 3-ara; 5, Cy 3-glc; 6, Pt 3-gal; 7, Cy 3-ara; 8, Pt 3-glc; 9, Pn 3-gal; 10, Pt 3-ara; 11, Pn 3-glc + Mv 3-gal; 12, Pn 3-ara + Mv 3-glc; 13, Mv 3-ara. Abbreviations: Dp, delphinidin; gal, galactoside; glc, glucoside; Cy, cyanidin; ara, arabinoside; Pt, petunidin; Pn, peonidin; Mv, malvidin.



**FIGURE 2** Structure of the aglycones of anthocyanins present in the bilberry extract. Cy, cyanidin; Pn, peonidin; Dp, delphinidin; Pt, petunidin; Mv, malvidin.

technical difficulties in resolving all components. Their quantification was expressed as Cy 3-glc equivalents.

In experiments conducted with 14  $\mu\text{mol/L}$  purified molecules and blackberry extract, Cy 3-rut was significantly less absorbed through the gastric wall than other anthocyanins (Table 1). Absorption of Cy 3-glc present in blackberries tended to be higher ( $P = 0.07$ ) than that of purified anthocyanin. No metabolites of anthocyanins were observed in the stomach content after 30 min of incubation. Moreover, no anthocyanins were detected in plasma or bile samples.

After infusion of 484 nmol bilberry anthocyanins, 134  $\pm$  13 nmol ( $n = 6$ ) were absorbed from the gastric lumen, corresponding to a global percentage of absorption of 27.4  $\pm$  1.9% ( $n = 6$ ). The amount of anthocyanins infused varied from 3.20

**TABLE 1**

*Anthocyanin absorption after administration of various anthocyanins into the stomach of rats for 30 min<sup>1,2</sup>*

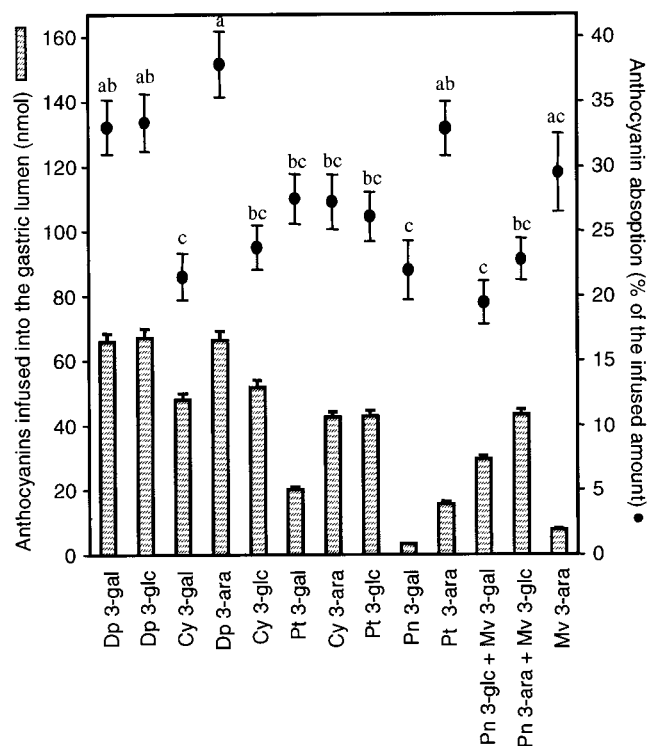
Compound	Anthocyanins injected into the gastric lumen	Anthocyanins absorbed from the gastric lumen	Anthocyanin absorption from the gastric lumen
	nmol	% of the injected dose	
Cy 3-glc	71.7 $\pm$ 0.8	16.4 $\pm$ 2.0 <sup>a</sup>	23.0 $\pm$ 3.0 <sup>a</sup>
Cy 3-gal	67.3 $\pm$ 0.9	17.6 $\pm$ 2.0 <sup>a</sup>	26.2 $\pm$ 2.9 <sup>a</sup>
Cy 3-rut	68.1 $\pm$ 1.0	5.7 $\pm$ 0.9 <sup>b</sup>	8.4 $\pm$ 1.5 <sup>b</sup>
Mv 3-glc	71.4 $\pm$ 1.7	16.8 $\pm$ 1.4 <sup>a</sup>	23.6 $\pm$ 2.2 <sup>a</sup>
Blackberry extract (Cy 3-glc)	70.9 $\pm$ 3.1	23.0 $\pm$ 3.8 <sup>a</sup>	32.4 $\pm$ 4.9 <sup>a</sup>

<sup>1</sup> Values are means  $\pm$  SEM,  $n = 5-6$ . Means in a column without a common letter differ,  $P < 0.01$ .

<sup>2</sup> Abbreviations: Cy, cyanidin; glc, glucoside; gal, galactoside; rut, rutinose; Mv, malvidin.

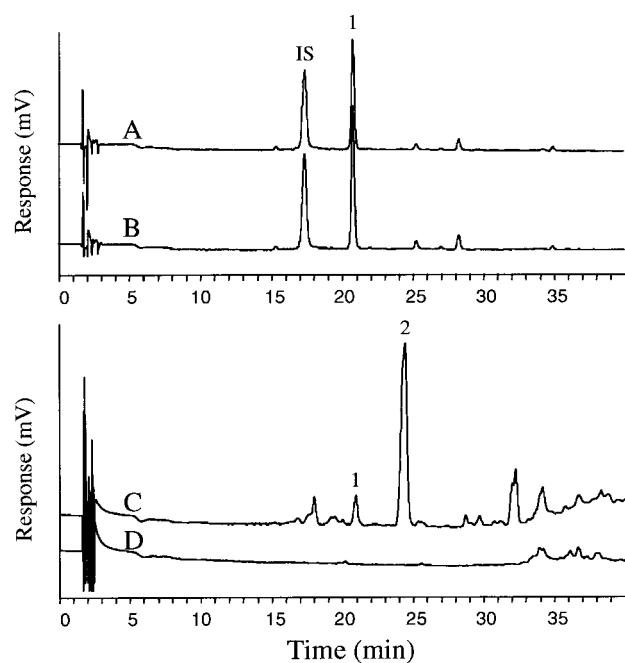
nmol (Pn 3-gal) to 64.5 nmol (Dp 3-glc) according to the anthocyanin considered, and the percentage of absorption was between 18.7 and 36.6% for Pn 3-glc + Mv 3-gal, and Dp 3-ara, respectively (Fig. 3). Because of the low amounts of each molecule infused, no anthocyanin was detected in plasma or bile samples.

Because the low doses administered previously did not allow detection of metabolites in plasma or bile samples, we injected a high amount of blackberry anthocyanins (3750 nmol) into the gastric lumen. Under these conditions, 261  $\pm$  52 nmol (i.e., 6.94  $\pm$  1.33%,  $n = 5$ ) Cy 3-glc was absorbed after 30 min. Examination of plasma samples (from gastric vein and aorta) indicated the presence of blackberry anthocyanins (Fig. 4A, B). Small peaks corresponding to minor unidentified blackberry anthocyanins (cf. Fig. 1A) were also detected in plasma. Plasma samples from the gastric vein were pooled to have sufficient volume to be treated on the Sep-Pak C18. In this pooled sample, Cy 3-glc was present at a concentration of 0.13  $\mu\text{mol/L}$ . In plasma from the aorta, Cy 3-glc concentration was 0.17  $\pm$  0.07  $\mu\text{mol/L}$  ( $n = 6$ ). Analysis of bile samples revealed that Cy 3-glc appeared in bile in the first fraction (0–20 min) (Fig. 4C). Moreover, the presence of a greater amount of peonidin 3-glucoside (Pn 3-glc; methylated form of Cy 3-glc) compared with Cy 3-glc was detected in bile samples by comparison of the UV-visible spectrum and retention time and by spiking samples with a commercially available standard. No aglycone was detected but some small peaks that could correspond to unknown anthocyanin metabolites were observed in bile samples. However, small peaks with retention times  $> 33$  min were also detected in bile from control rats (Fig. 4D). With the exception of one rat, antho-



**FIGURE 3** Amounts of anthocyanins from the bilberry extract infused into the stomach and their respective percentages of absorption from the gastric lumen after in situ gastric administration in rats. Values are expressed as Cy 3-glc equivalents. Values are means  $\pm$  SEM,  $n = 6$ . Means without a common letter differ,  $P < 0.05$ . For abbreviations, see legend for Figure 1.





**FIGURE 4** Representative HPLC chromatograms of plasma from gastric vein (A) and aorta (B) and of bile collected in rats 30 min after in situ gastric administration of 3750 nmol blackberry anthocyanins (C) or collected from control rats (D). Detection was performed at 524 nm. IS, internal standard (cyanidin 3,5-diglucoside). Peaks are as follows: 1, cyanidin 3-glucoside; 2, peonidin 3-glucoside.

anthocyanins were present in bile in amounts that were too low to be quantified.

## DISCUSSION

The purpose of this work was to study the fate of various anthocyanins in the stomach. Indeed, anthocyanins appeared rapidly in plasma after feeding (17,19,20). On the other hand, because anthocyanin stability is highly influenced by pH (27), the acidity of the gastric content should constitute a favorable environment for these molecules. We hypothesized, therefore, that anthocyanins were efficiently absorbed from the stomach. To test this assumption, we evaluated the absorption of various anthocyanins after direct administration into the rat stomach using an in situ gastric administration model (23). Because cyanidin is the main anthocyanin aglycone encountered in plants, we compared the absorption of three glycosides of cyanidin (glucoside, galactoside and rutinoside) and of the 3-glucoside of a highly methylated aglycone, malvidin. Our results first indicated that a high proportion (~25%) of anthocyanin monoglycosides was rapidly absorbed from the stomach whatever the aglycone. This result could explain the rapid appearance of anthocyanins in plasma (17,18). Moreover, Tsuda et al. (17) reported the presence of Cy 3-glc in the gastric tissue at a concentration that reached a maximum only 15 min after its administration by gavage. Thus, this organ seems to play an essential role in anthocyanin absorption. A recent paper demonstrated that grape anthocyanins were able to permeate the gastric mucosa, but the rate of this absorption was not quantified (24). Gastric absorption was influenced by the glycosidic moiety because Cy 3-rut absorption was nearly three times lower than that of the glucoside or galactoside of cyanidin. This lower absorption of Cy 3-rut in the stomach could explain why the absorption and excretion of anthocya-

nin rutinosides are slightly delayed compared with glucosides (19).

Among berries, bilberry is one of the richest in anthocyanins, pigments that have been reported to exert numerous beneficial health effects (2,6,7). Therefore, we studied gastric absorption of anthocyanins from a bilberry anthocyanin extract (Antho 50). Although total absorption of bilberry anthocyanins was similar to that obtained with purified molecules, a high variability of absorption was observed when considering each component individually. Taken as a whole, delphinidin glycosides were better absorbed than others. Because delphinidin glycosides are potent antioxidants among anthocyanins (28) as well as main bilberry pigments, they could thus contribute efficiently to the health-promoting effects of bilberry anthocyanins. Moreover, anthocyanin arabinosides appeared to be better absorbed than the corresponding glucosides or galactosides. This result will require further investigation especially because Wu et al. (29) suggested that arabinosides may be absorbed or metabolized in a different manner than other glycosides.

Taken as a whole, our results have clearly shown that glycosides of anthocyanins were absorbed through the gastric wall. This result highlights once again the difference in the metabolism of anthocyanins compared with other flavonoids. Indeed, flavonoid glycosides are not absorbed from the stomach, whereas their aglycones (such as quercetin or daidzein) are (23,30).

Gastric administration of a high dose of blackberry anthocyanins lowered the percentage of Cy 3-glc absorption (~7%) compared with that observed after administration of a lower dose. Recently, Passamonti et al. (31) suggested that an organic anion carrier, bilitranslocase, expressed in the gastric epithelium, could be involved in the absorption of anthocyanins at the gastric level. Thus, we could hypothesize that administration of high amounts of anthocyanins induces saturation of this transport. However, further experiments with several doses and time points will be necessary to confirm this hypothesis. Examination of plasma from gastric veins did not reveal the presence of anthocyanin metabolites, thus suggesting that anthocyanins were absorbed through the gastric wall without modification.

This work demonstrated for the first time the presence of anthocyanins in bile. Absorption and further metabolism of Cy 3-glc occurred very quickly because only 20 min after the beginning of the experiment, methylated and likely conjugated metabolites of Cy 3-glc were found in bile. However, although Pn 3-glc was the major anthocyanin recovered in bile, it was not found in plasma from the aorta even after 30 min. That suggested that this methylated form of Cy 3-glc was formed in the liver and preferentially eliminated by bile rather than distributed into blood. This result agrees with previous studies (17,18) that demonstrated the presence of high amounts of methylated anthocyanins in rat liver but failed to detect them in plasma. Miyazawa et al. (18) hypothesized that these metabolites may be excreted from liver directly into bile. On the other hand, small peaks around Cy 3-glc and Pn 3-glc could be conjugated forms of anthocyanins. Indeed, we demonstrated recently that pelargonidin 3-glucoside was excreted in humans mainly as glucuro- or sulfoconjugated forms (25). We also noted that peak 2 (Pn 3-glc) of Figure 4C was broad, and when it was enlarged, a shoulder was observed. That could correspond to a glucuronide of Pn because glucuronides of pelargonidin eluted very near to or with their glucoside under identical gradient elution conditions (25).

In conclusion, this study demonstrated that anthocyanin glycosides were rapidly and efficiently absorbed from the stom-

ach. Because their bioavailability, evaluated by urinary excretion, is low (19,21,29), their distribution to various tissues will have to be investigated further.

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