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Absorption of Anthocyanins from Blueberry Extracts by Caco-2 Human Intestinal Cell Monolayers

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Recent studies have shown that dietary polyphenols may contribute to the prevention of cardiovascular disease and cancer. Anthocyanins from different plant sources including blueberries have been shown to possess potential anticancer activities. One of the key factors needed to correctly relate the in vitro study results to human disease outcomes is information about bioavailability. The objectives of the current study were to evaluate the absorption of blueberry anthocyanin extracts using Caco-2 human intestinal cell monolayers and investigate the effects of different aglycones, sugar moieties, and chemical structure on bioavailability of different types of anthocyanins. The results of this study showed that anthocyanins from blueberries could be transported through the Caco-2 cell monolayers although the transport/absorption efficiency was relatively low compared to other aglycone polyphenols. The transport efficiency of anthocyanins averaged ∼3−4% [less than 1% in delphinidin glucoside (Dp-glc)]. No significant difference in transport/absorption efficiency was observed among three blueberry cultivars. The observed trends among different anthocyanins generally agreed well with some published in vivo results. Dp-glc showed the lowest transport/absorption efficiency, and malvidin glucoside (Mv-glc) showed the highest transport/absorption efficiency. Our result indicates that more free hydroxyl groups and less OCH_3 groups can decrease the bioavailability of anthocyanins. In addition, cyanindin glucoside (Cy-glc) showed significantly higher transport efficiency than cyanidin galactoside (Cy-gal), and peonidin glucoside (Pn-glc) showed significantly higher transport efficiency than peonidin galactoside (Pn-gal), indicating that glucose-based anthocyanins have higher bioavailability than galactose-based anthocyanins.

KEYWORDS: Absorption; aglycone; anthocyanins; bioavailability; blueberry; Caco-2 monolayer; sugar moieties

INTRODUCTION

Flavonoids and anthocyanins are naturally occurring phenolic compounds that are present in fruits and vegetables. Recent studies have shown that dietary polyphenols may contribute to the prevention of cardiovascular diseases and cancer (*1*, *2*). Dietary consumption of anthocyanins has been reported to be higher than other flavonoids such as quercetin, probably because of their widespread distribution and occurrence in fruits and vegetables (*3*−*5*).

Anthocyanins are potent antioxidants. Fruits and vegetables with high anthocyanin contents generally have higher antioxidant capacity than other fruits and vegetables (*6*−*8*). Anthocyanins from different plant sources may also have potential anticancer activities (*9*−*12*). A few studies have reported the anticancer activities of blueberries, which are a rich source of anthocyanins (*13*−*15*). Our previous studies (*16*) evaluated the effects of polyphenol extracts on colon cancer cell growth and apoptosis and found that anthocyanin fractions showed the greatest bioactivity among all four fractions evaluated. It was, thus, suggested that anthocyanins were one of the major components that inhibited colon cancer cell proliferation and induced apoptosis.

Despite the statement that anthocyanins have potential in cancer prevention and other health benefits, a significant gap exists between what has been shown in many in vitro studies and what can be achieved under in vivo conditions. One of the key factors needed to correctly relate in vitro study results to human disease outcomes is information about bioavailability and metabolism (*17*). Although a few studies have been conducted to evaluate the bioavailability of anthocyanins (*18*−*21*), the information on absorption and metabolism is still very limited.

Compared to human and animal models that are highly complex and may be confounded by factors such as chemical

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instability and inadequate analytical methodology, it is easy to control these parameters in an in vitro study. An in vitro assay is simple, more convenient, and less expensive. Caco-2 cells have been the most extensively characterized and useful in vitro model in the field of drug permeability and absorption (*22*−*24*). The differentiated cell monolayers have been used for the study of unidirectional transport of phytochemicals such as quercetin, epicatechin, proanthocyanidins, and carotenoid (*25*−*29*), and much valuable information has been obtained using this model system.

Information on the bioavailability of pure compounds is a good start to clarify the bioavailability of complex mixtures of phytochemicals (*30*). The combination of phytochemicals in fruits and vegetables is thought to be critical to their powerful antioxidant and anticancer activity (*31*, *32*). Similarly, information on the bioavailability of complex mixtures of phytochemicals is essential. Thus, studies on the bioavailability of phenolic compounds such as anthocyanins from specific crops are a good way to provide direct and valuable information about their absorption. The objectives of the current study were to evaluate the absorption of blueberry anthocyanin extracts using Caco-2 human intestinal cell monolayers and investigate the effects of different aglycones, sugar moieties, and chemical structure on the bioavailability of different types of anthocyanins.

MATERIALS AND METHODS

Chemicals and Reagents. Acetonitrile, methanol, *O*-phosphoric acid (85% purity, HPLC grade), hydrochloric acid (analytical grade), formic acid, water (HPLC grade), and Hank's Balanced Salts Solution (HBSS) were purchased from Fisher Scientific (Norcross, GA). Anthocyanin standards were purchased from Polyphenols Laboratories (AS) (Sandnes, Norway). These standards were delphinidin 3-*O*-β-glucopyranoside (Dp-glc), cyanidin 3-*O*-β-galactopyranoside (Cy-gal), cyanidin 3-*O*-β-glucopyranoside (Cy-glc), petunidin 3-*O*-β-glucopyranoside (Pt-glc), peonidin 3-*O*-β-galactopyranoside (Pn-gal), peonidin 3-*O*-β-glucopyranoside (Pn-glc), malvidin 3-*O*-β-glucopyranoside (Mv-glc), and peonidin 3-*O*-α-arabinopyranoside (Pn-ara). Caco-2 human colorectal adenocarcinoma cells were purchased from ATCC (Manassas, VA). Blueberries were collected from the field in 2004. The blueberry cultivars collected were Briteblue (Alapaha, GA), Tifblue (Alma, GA), and Powderblue (Chula, GA). All cultivars were grown with irrigation or under conditions of adequate rainfall. Samples were frozen and stored at −40 °C until use.

Extraction and Fractionation. Anthocyanin fractions were obtained using a modified procedure of Youdim et al. (*33*) and Oszmianski et al. (*34*), which we previously reported (*16*). Briefly, 100 g of blueberries was homogenized in 300 mL of acetone:methanol:water:formic acid (40:40:20:0.1, v/v/v/v). The crude extract was freeze-dried using a UNITOP 600L freeze-dryer (Virtis, Gardiner, NY). The dried extract was resolubilized in water, applied to an activated Oasis HLB cartridge (Waters Corp., Milford, MA), and washed with water, 15% methanol in water, and finally methanol acidified with 5% formic acid. The acidified methanol eluted the anthocyanins and other flavonoids. The fraction containing the anthocyanins was dried again and applied to a Sephadex LH20 column (Amersham Biosciences AB, Uppsala, Sweden). The column was then washed with 70% methanol acidified with 10% formic acid to elute anthocyanins and flavonols. After freeze-drying the anthocyanin and flavonol fraction, the fraction was applied to the second Oasis HLB cartridge. The cartridge was washed with 5% formic acid, followed by ethyl acetate and then 10% formic acid in methanol. The acidified methanol eluted the anthocyanins, which we used for further analysis.

HPLC Analysis. Anthocyanin measurements were conducted following a procedure previously reported in our laboratory (*16*). HPLC was performed with a Hewlett-Packard (Avondale, PA) model 1100 liquid chromatograph with quaternary pumps and a diode array UV−visible detector. The mobile phases were Solvent A, *O*-phosphoric acid/methanol/water (5:10:85, v/v/v), and Solvent B, acetonitrile. The flow rate was 0.5 mL/min. The gradient system was 100% solvent A at 0 min, 90% solvent A and 10% solvent B at 5 min, and 50% solvent A and 50% solvent B at 25 min with 5 min postrun with HPLC-grade water. Anthocyanins were detected at 520 nm.

Cell Cultures. Caco-2 human colon cancer cells were cultured in ATCC Medium: Minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate, 80%, fetal bovine serum, 20%. Cancer cells were grown in an incubator with 5% CO_2 and 95% humidity at 37 °C. Medium was changed 2−3 times per week.

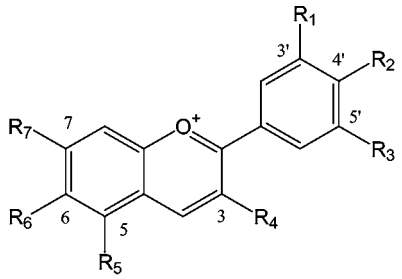
Transport/Absorption. Transport experiments were carried out using Transwell inserts (polycarbonate membrane, 0.4 μm pore size, 24 mm diameter, Corning Inc., Corning, NY). Inserts were placed in 6-well plates. Caco-2 cells (passage 30−50) were seeded at 1.5×10^5/cm^2 on the membrane inserts with 1.5 mL of medium in the apical/luminal side and 2.5 mL of medium in the basolateral side. Cells were allowed to grow and differentiate to confluent monolayers for 20−26 days post seeding by changing the medium three times a week. Transepithelial electrical resistance (TEER) of cells grown in the Transwell was measured using the Millicell-ERS voltmeter (Millipore Co., Billerica, MA). Only monolayers with a TEER value higher than 400 Ω cm^2 were used for experiments. TEER values were also obtained after completion of transport experiments.

Medium was removed, and the cells were washed with HBSS (pH = 7.4, 37 °C). Anthocyanin extract solutions in HBSS (approximately 50 μg/mL) were added to the apical side of the cells. Transepithelial transport was followed as a function of time. At 0, 1, and 2 h the anthocyanin concentrations at apical and basolateral sides were sampled, adjusted with formic acid to make pH $\approx$ 2, cooled in an ice bath, and measured using HPLC. The cellular absorption of anthocyanins was also measured following the procedure below. The cell membranes were washed three times with HBSS (pH = 7.4, 37 °C) and then removed/tore off from the insert. Anthocyanins were extracted using methanol with 5% formic acid. Cells were sonicated for 15 min and centrifuged at 2000*g*. The supernatant was collected, and cells were rinsed two more times with acidified methanol and centrifuged again at 2000*g*. The supernatant solutions were evaporated under nitrogen and reconstituted in 600 μL of methanol with 5% formic acid following the methodology reported by Boyer et al. (*30*). The extract solutions were then injected into HPLC.

Statistical Analysis. The transport and absorption efficiencies of different anthocyanins and different anthocyanin extracts from blueberry cultivars were statistically compared. Statistical analysis was conducted using the general linear model (GLM) followed by Duncan's multiple range test at $\alpha = 0.05$.

RESULTS

Highly purified anthocyanin extracts (with ~90% purity of identified anthocyanins) were obtained from three cultivars of blueberries. Seven monoglycoside anthocyanins were identified: Dp-glc, Cy-gal, Cy-glc, Pt-glc, Pn-gal, Pn-glc, and Mv-glc. Their structures are shown in **Figure 1** in the flavylium cation form. Our previous reports (*16*) have shown additional detail on the anthocyanin extracts. The anthocyanin extracts were dissolved in HBSS and added to the apical side of the cell monolayers. Transport of anthocyanin fractions across the Caco-2 monolayers was studied in the apical to basolateral direction. **Figure 2** shows the chromatogram of analytical HPLC of Tifblue anthocyanins in the basolateral solutions after a 2 h incubation. Four peaks (a−d) could not be identified because of a lack of standards. Anthocyanins in the pure extract are also shown in **Figure 2**. **Figure 3** shows the anthocyanin concentrations in the apical solutions after incubation with Caco-2 monolayers over time. A drastic decrease was observed in the seven anthocyanins from all three cultivars. Total anthocyanins refers to the total amount of all seven anthocyanins identified. In Briteblue, the total anthocyanins decreased from 28.7 to 19.0



Anthocyanin	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Dp-glc	-OH	-OH	-OH	-O-glc	-OH	-H	-OH
Cy-gal	-OH	-OH	-H	-O-gal	-OH	-H	-OH
Cy-glc	-OH	-OH	-H	-O-glc	-OH	-H	-OH
Pt-glc	$-OCH_3$	-OH	-OH	-O-glc	-OH	-H	-OH
Pn-gal	$-OCH_3$	-OH	-H	-O-gal	-OH	-H	-OH
Pn-glc	$-OCH_3$	-OH	-H	-O-glc	-OH	-H	-OH
Mv-glc	$-OCH_3$	-OH	$-OCH_3$	-O-glc	-OH	-H	-OH

Figure 1. Anthocyanin structure. The anthocyanin shown here is in the flavylium cation form.

μg/mL (66% retained) in 1 h and 13.3 μg/mL (46% retained) in 2 h. In the case of the seven individual Briteblue anthocyanins, the concentration in the apical solution decreased over time. The concentration after 1 h incubation ranged from 49% to 66% of those at 0 h, and the concentration after 2 h incubation ranged from 29% to 50%. Similarly, in Tifblue, the total anthocyanins decreased from 48.2 to 33.0 μg/mL (68% retained) in 1 h and 24.5 μg/mL (51% retained) in 2 h. The concentrations of seven individual Tifblue anthocyanins after 1 h incubation ranged from 50% to 75% of those at 0 h, and the concentration after 2 h incubation ranged from 35% to 59%. In Powderblue, the total anthocyanins decreased from 41.1 to 24.6 μg/mL (60% retained) in 1 h and 19.8 μg/mL (48% retained) in 2 h. The concentrations of seven individual Powderblue anthocyanins after 1 h incubation ranged from 44% to 71% of those at 0 h, and the concentration after 2 h incubation ranged from 29% to 60%.

All of the seven major anthocyanins in the extracts were detected in the basolateral solutions on the basis of retention time and characteristic spectra. The anthocyanin concentration in the basolateral solutions after incubation with Caco-2 monolayers over time is shown in **Figure 4**. In general, the concentration of anthocyanins increased with incubation time. In Briteblue, the total amount of identified anthocyanins reached 1.22 μg/mL after 1 h incubation and 1.66 μg/mL after 2 h. Among the seven individual anthocyanins, Pt-glc and Pn-glc showed the highest concentration in the basolateral solutions with 0.50 and 0.41 μg/mL after 2 h incubation, respectively. The lowest concentration (0.06 μg/mL) was observed in Dp-glc. In Tifblue, the total anthocyanins in the basolateral solution reached 1.79 and 2.57 μg/mL after 1 and 2 h incubation periods, respectively. As in Briteblue, Pt-glc and Pn-glc showed the highest concentration among the seven anthocyanins with 0.62 and 0.71 μg/mL after 2 h incubation, respectively. Dp-glc was the lowest with concentration at 0.06 μg/mL. For Powderblue, the total anthocyanins in the basolateral solution reached 0.95 and 2.12 μg/mL after 1 and 2 h incubation period, respectively. Similarly, the highest concentration among the seven anthocyanins was observed in Pt-glc and Pn-glc and the lowest in Dp-glc.

Because Pt-glc and Pn-glc possessed two of the highest contents of anthocyanins in the apical solutions, the transport of anthocyanins across the cell monolayers could be related to the original concentrations of individual anthocyanins in the apical solution. Therefore, transport efficiency percentages were calculated. Calculation was based on (anthocyanin concentrations at the basolateral side over time)/(anthocyanin concentrations at the apical side at 0 h) $\times$ 100%. **Table 1** shows the

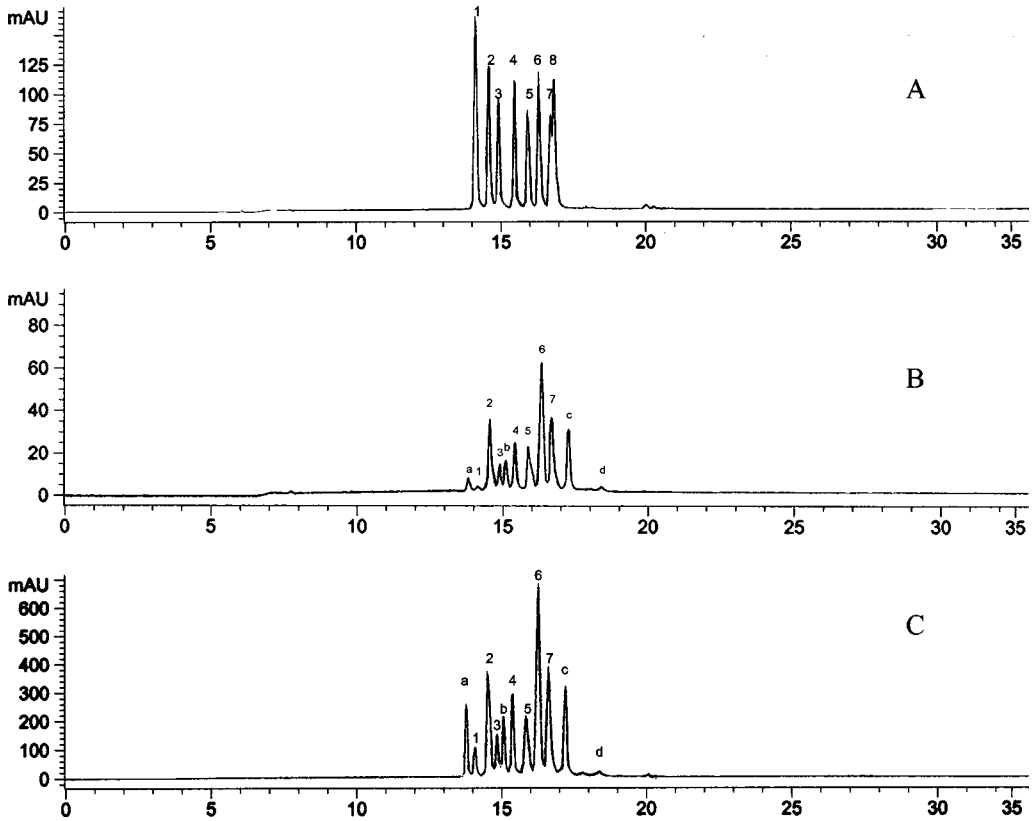


Figure 2. Chromatogram of analytical HPLC of Tifblue berry anthocyanins in the basolateral solutions after 2 h incubation: (A) Anthocyanin standards; (B) Tifblue berry anthocyanins in the basolateral solutions; (C) Tifblue berry anthocyanins in the pure extract. (1) Dp-glc, (2) Cy-gal, (3) Cy-glc, (4) Pt-glc, (5) Pn-gal, (6) Pn-glc, (7) Mv-glc, (8) Pn-ara. (a– d) unidentified peaks.

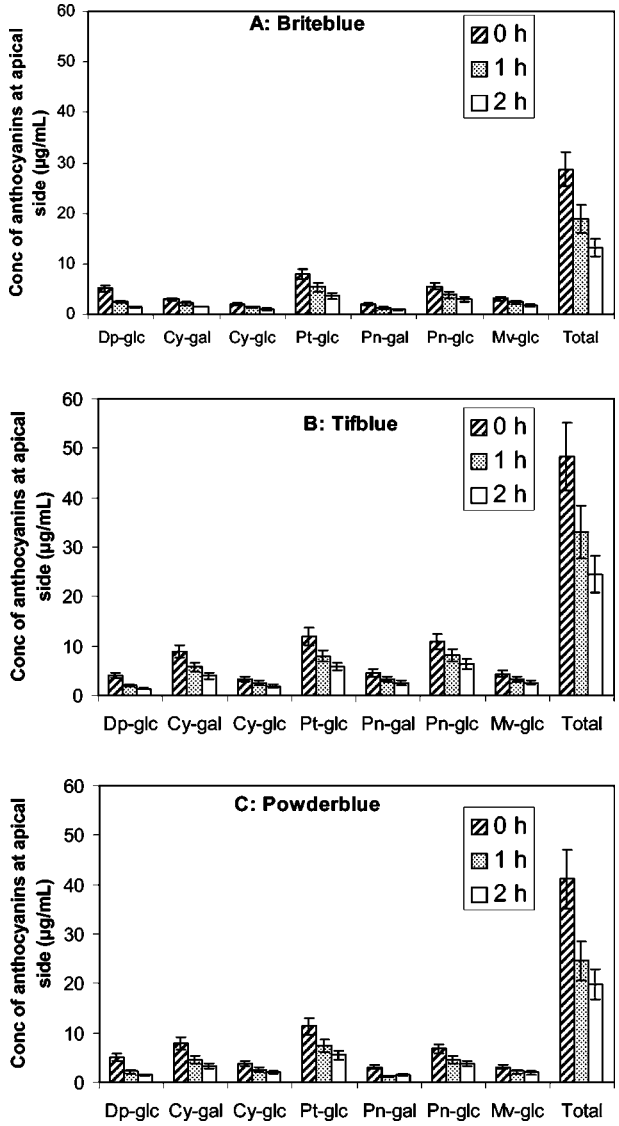


Figure 3. Anthocyanin concentrations at the apical side of cell monolayer over time (mean ± SD, $n = 3$). Total means the total amount of all seven anthocyanins identified: (A) Briteblue; (B) Tifblue; (C) Powderblue.

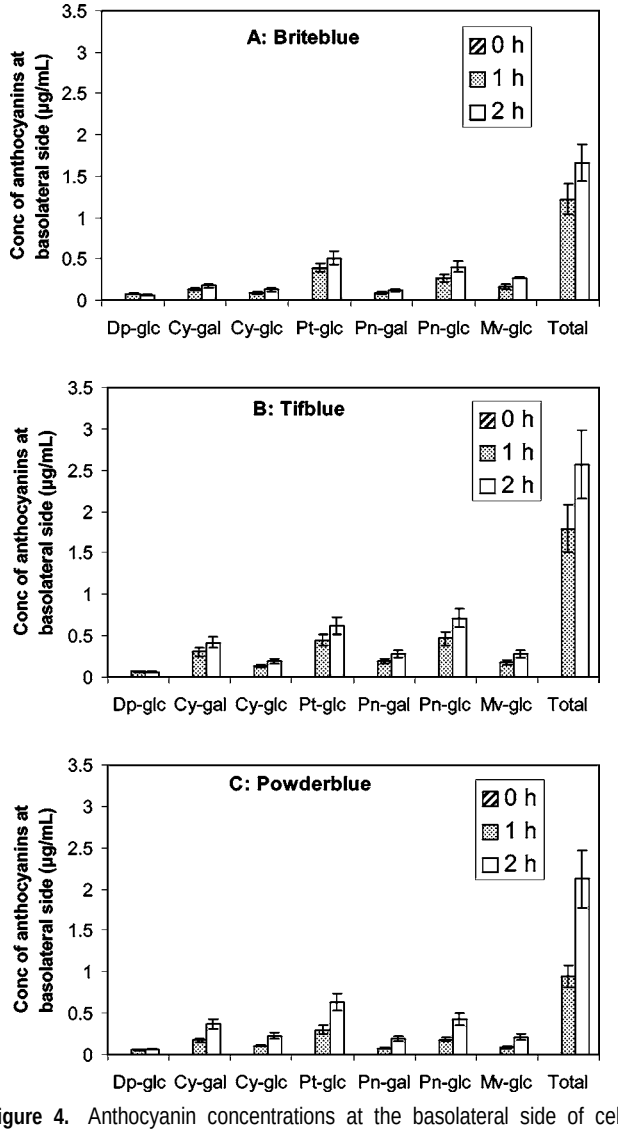


Figure 4. Anthocyanin concentrations at the basolateral side of cell monolayer over time (mean ± SD, $n = 3$). Total means the total amount of all seven anthocyanins identified: (A) Briteblue; (B) Tifblue; (C) Powderblue.

transport efficiency percentages of anthocyanins from the apical to the basolateral side of cell monolayers. The transport efficiency of the total anthocyanins from different cultivars of blueberry extracts was also calculated. Statistical analysis showed no significant difference among the three cultivars in the transport efficiency of total anthocyanins except that Powderblue extract showed significantly lower (1.61%) transport efficiency than the other two cultivars after 1 h incubation. In the case of individual anthocyanins, Dp-glc showed the lowest transport efficiency (ranging from 0.72% to 1.18%) in all three cultivars. In Tifblue and Powderblue, Pn-gal, Pn-glc, and Mv-glc showed the highest transport efficiency after 2 h incubation with transport efficiency ranging from 4.15% to 4.72%. In Briteblue, Pn-glc and Mv-glc showed the highest transport efficiency with 5.17% and 6.03% after 2 h incubation, respectively. Unlike in Tifblue and Powderblue, the transport efficiency of Pn-gal (4.18%) was not one of the highest.

In Briteblue extract, Cy-glc showed significantly higher transport efficiency (4.58%) than Cy-gal (3.94%); similarly, Pn-glc (5.17%) showed significantly higher transport efficiency than Pn-gal (4.18%). The difference is less significant at 1 h than at 2 h incubation (**Table 1**). Similar trends were observed in Tifblue and Powderblue extract. Cy-glc showed significantly higher transport efficiency (4.01 and 4.18%) than Cy-gal (3.29 and 3.28% in Tifblue and Powderblue, respectively). Meanwhile, Pn-glc showed higher transport efficiency than Pn-gal in both Tifblue and Powderblue, yet statistical analysis did not identify the significant difference, probably because of the relatively high standard deviations.

The direct absorption of anthocyanins by Caco-2 cells was also evaluated. The anthocyanins absorbed by cells were extracted and reconstituted in 600 μL of methanol with 5% formic acid. **Figure 5** shows the anthocyanin concentrations absorbed by cells over time. The concentration of anthocyanins in Caco-2 cells increased with incubation time. In Briteblue, the total amount of identified anthocyanins was 0.06 μg/mL after 1 h incubation and 0.16 μg/mL after 2 h. In Tifblue, the total anthocyanins in the cells were 0.13 and 0.24 μg/mL after 1 and 2 h incubation, respectively. In Powderblue, the total anthocyanins were 0.12 and 0.22 μg/mL after 1 and 2 h incubation periods, respectively. Dp-glc was not detected in any of the three treatments. Compared with other anthocyanins, Pt-glc and Pn-glc exhibited the highest contents. As in transport efficiency, absorption efficiency was calculated. The calculation

Table 1. Transport Efficiency of Anthocyanins from Apical to Basolateral Side of Caco-2 Cell Monolayer[a]

	Briteblue		Tifblue		Powderblue	
anthocyanins	1 h	2 h	1 h	2 h	1 h	2 h
Dp-glc	1.07 a[c]	0.89 a	1.18 a	1.07 a	0.72 a	0.92 a
Cy-gal	3.10 b	3.94 b	2.39 b	3.29 b	1.54 b	3.28 b
Cy-glc	3.28 bc	4.58 c	2.77 bc	4.01 c	1.93 c	4.18 c
Pt-glc	3.44 bc	4.44 bc	2.63 bc	3.67 bc	1.86 bc	3.93 c
Pn-gal	3.01 b	4.18 bc	2.82 bc	4.15 cd	1.74 bc	4.21 c
Pn-glc	3.47 bc	5.17 d	2.96 c	4.54 d	1.89 c	4.41 cd
Mv-glc	3.74 c	6.03 e	2.94 c	4.56 d	2.01 c	4.72 d
total anthocyanins[b]	3.10 B[d]	4.20 C	2.60 B	3.73 C	1.61 A	3.61 C

[a] Transport efficiency percentages werecalculated based on (anthocyanin concentrations at the basolateral side over time)/(anthocyanin concentrations at the apical side at 0 h) × 100%. Values are averages of triplicates. [b] Total anthocyanins refers to the total amount of all seven anthocyanins identified. [c] Means within the same column followed by the same lower case letters are not significantly different at $\alpha = 0.05$ using Duncan's Multiple Range Test. [d] Means within the same row followed by the same upper case letters are not significantly different at $\alpha = 0.05$ using Duncan's Multiple Range Test.

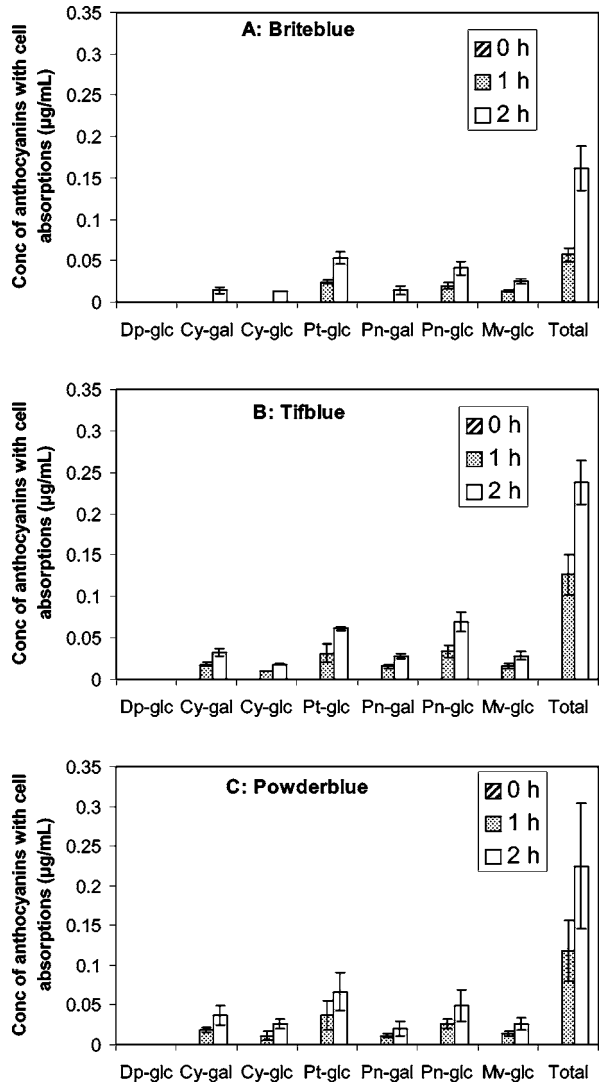


Figure 5. Anthocyanin concentrations absorbed by Caco-2 cells over time (mean ± SD, $n = 3$). Total means the total amount of all seven anthocyanins identified: (A) Briteblue; (B) Tifblue; (C) Powderblue.

was based on (anthocyanin concentrations extracted from Caco-2 cells over time)/(anthocyanin concentrations at the apical side at 0 h) × 100%. **Table 2** shows the absorption efficiency of anthocyanins by Caco-2 cells. As in transport efficiency, Pn-gal, Pn-glc, and Mv-glc showed the highest absorption efficiency among all of the seven anthocyanins. The absorption efficiency after 2 h incubation ranged from 0.26% to 0.28% in Briteblue, 0.21% to 0.23% in Tifblue, and 0.23% to 0.29% in Powderblue. Cy-glc showed significantly higher absorption efficiency than Cy-gal. The absorption efficiency of Cy-glc and Cy-gal after a 2 h incubation was 0.24% versus 0.17% in Briteblue, 0.19% versus 0.13% in Tifblue, and 0.23% versus 0.16% in Powderblue. Although similar trends were observed in Pn-glc and Pn-gal, statistical analysis did not show significant differences.

Table 2. Absorption Efficiency of Anthocyanins by Caco-2 Cells[a]

	Briteblue		Tifblue		Powderblue	
anthocyanins	1 h	2 h	1 h	2 h	1 h	2 h
Dp-glc	0 a[c]	0 a	0 a	0 a	0 a	0 a
Cy-gal	0 a	0.17 b	0.07 b	0.13 b	0.08 b	0.16 b
Cy-glc	0 a	0.24 c	0.11 cd	0.19 cd	0.10 c	0.23 cd
Pt-glc	0.11 b	0.23 c	0.09 c	0.18 c	0.11 c	0.20 c
Pn-gal	0 a	0.26 cd	0.12 d	0.21 d	0.13 d	0.23 cd
Pn-glc	0.13 c	0.27 cd	0.11 cd	0.22 d	0.14 d	0.25 d
Mv-glc	0.14 d	0.28 d	0.13 e	0.23 d	0.16 e	0.29 e
total anthocyanins[b]	0.07 A[d]	0.20 B	0.09 A	0.17 B	0.10 A	0.19 B

[a] Absorption efficiency was calculated based on (anthocyanin concentrations extracted from Caco-2 cells over time)/(anthocyanin concentrations at the apical side at 0 h) × 100%. Values are averages of triplicates. [b] Total anthocyanins means the total amount of all seven anthocyanins identified. [c] Means within the same column followed by the same lower case letters are not significantly different at $\alpha = 0.05$ using Duncan's Multiple Range Test. [d] Means within the same row followed by the same upper case letters are not significantly different at $\alpha = 0.05$ using Duncan's Multiple Range Test.

DISCUSSIONS

It has been reported that Caco-2 cells can undergo spontaneous differentiation in culture conditions and exhibit the characteristics of mature enterocytes (*35*). The cell surface facing the top medium develops a brush border that resembles the luminal membrane of the intestinal epithelium. The cell surface attaching to the permeable membrane and facing the bottom medium develops into the basolateral membrane. In the current study, we evaluated the absorption of purified blueberry anthocyanin extracts using a Caco-2 cell model. We found that blueberry anthocyanins could be transported through the Caco-2 cell monolayers in intact glycone forms.

It was originally believed that anthocyanins needed to be hydrolyzed to an aglycone form before they can be absorbed. Only recently, several studies have reported the absorption of anthocyanins as intact forms (*19*, *20*). Our results showed that anthocyanins from blueberries could be transported through the Caco-2 cell monolayers, although the transport/absorption efficiency was relatively low compared with some aglycone polyphenols. Using a Caco-2 cell model, Steensma et al. (*36*) observed 10−15 μM (~3−4 μg/mL) of genistein and daidzein in the basolateral side and 10 times lower absorption of the glycosides genistin and daidzin. In the current study, the anthocyanin concentration observed in the basolateral solution ranged from less than 0.1 to 0.7 μg/mL depending on different individual anthocyanins and the original concentration. In the case of transport rates, 30−40% of genistein and daidzein at the apical side was transported to the basolateral side in 6 h (*36*). In our study, the transport efficiency of anthocyanins averaged ~3−4% (less than 1% in Dp-glc). This low absorption rate probably resulted from the poor lipophilic properties of anthocyanins.

The nature of aglycone of the anthocyanin could influence their bioavailability (*18*, *21*). Using an animal model, Wu et al. (*18*) reported that Pg-glc had a much higher total urinary excretion than cyanidin-based anthocyanins. McGhie et al. (*21*) also reported the relative concentrations of Dp-based anthocyanins were lower than those of Mv-based anthocyanins in the urine of rats and humans. The authors suggested that this may be a result of the greater number of hydroxyl groups in Dp or the greater hydrophobic nature of Mv that facilitated increased portioning into cells and tissues. This result is in good agreement with our results. In our current study, Dp-glc showed the lowest transport/absorption efficiency and Mv-glc the highest transport/absorption efficiency.

Free hydroxyl groups in flavonoids can hinder transport in Caco-2 cell monolayers (*37*). Ollila et al. (*38*) stated that polyhydroxylated flavonoids show longer retention delays in membranes, and this is most likely due to hydrogen-bond formation between the flavonoids hydroxyl groups and polar groups of the lipid molecules at the lipid/water interface. In the current study, Dp-glc (which has six hydroxyl groups in Dp) showed the lowest transport efficiency in all three cultivars. In Tifblue and Powderblue, Pn-gal, Pn-glc, and Mv-glc showed the highest transport efficiency. Both Pn and Mv have four hydroxyl groups, the lowest among all the anthocyanins evaluated in the current study. In addition, Dp has no OCH_3 group, while Pn has one and Mv has two OCH_3 groups. Our results provided further evidence that aglycone structure, especially hydrophilic and hydrophobic groups, is important for the bioavailability of anthocyanins.

In the current study, Cy-glc showed significantly higher transport efficiency than Cy-gal and Pn-glc showed significantly higher transport efficiency than Pn-gal. In terms of absorption efficiency, Cy-glc showed significantly higher efficiency than Cy-gal, yet, no significant differences were found between Pn-glc and Pn-gal. Sugar moieties may influence the absorption of anthocyanins, although the mechanisms are far from clear. A higher absorption percentage of anthocyanin glucosides in rabbit urine was observed than anthocyanin rutinosides after 2 h, and this trend turned into an opposite effect after 10−24 h (*39*). The authors suggested the possible reason could be that the anthocyanin glucosides in plasma increase and decrease more quickly than anthocyanin rutinosides. Some studies reported that monoglucosides of flavonoids/quercetin can be transported across the apical membrane of enterocytes by the sodium dependent glucose transporter SGLT1 (*40*, *41*). Mailleau et al. (*42*) reported that SGLT1 activities rapidly increased from day 12 up to day 20 post seeding of Caco-2 cells. In addition, quercetin-glc uptake in Caco-2 cells was inhibited by adding glucose (*40*). Milbury et al. (*43*) suggested that the absorption of anthocyanins in their unchanged glycosylated forms may indicate the involvement of the glucose transport receptors, since quercetin and anthocyanidins (aglycones of anthocyanins) share a similar basic flavonoid structure. The reason that no significant differences were found between Pn-glc and Pn-gal in the absorption efficiency could be that they already reached maximum absorption capacity.

Anthocyanins can undergo reversible structural transformations and dramatic changes in color with changes in pH. More importantly, anthocyanins are unstable at pH 7.0. Kang et al. (*10*) reported that anthocyanins and cyanidin could spontaneously degrade to chalcone and benzoic acid derivatives at pH 7.0 and 37 °C. The fast decrease of anthocyanin in the apical solution and relatively low concentrations in the basolateral side could also be due to the degradation that may occur under the culture conditions. We evaluated the change of anthocyanins under neutral pH and 37 °C in the absence of cells. Our results showed that more than a 30% loss could occur. In the case of Dp-glc, a nearly 60% loss was observed in 2 h. This was significantly higher than the remaining six anthocyanins. Except for Dp-glc, no significant difference was observed in the percentage loss among Cy-gal, Cy-glc, Pt-glc, Pn-gal, Pn-glc, and Mv-glc. The significantly higher loss of Dp-glc could explain to some degree its remarkable lower transport efficiency and direct cell absorption efficiency. Yet, the transport efficiency and absorption efficiency of Dp-glc were still significantly lower than the remaining six anthocyanins even after conversion of loss percentage. In addition, demethylation can occur under culture conditions, as it does in the presence of intestinal microflora (*44*). Demethylation of methoxyls could result in underestimation of the transport/absorption efficiency of related anthocyanins. However, it will not impact the trend of structure and bioavailability relationship suggested in the current study. Anthocyanins with more methoxyl groups showed higher bioavailability withstanding the loss due to demethylation. The degradation and low stability under neutral pH could be another reason low absorption and excretion of anthocyanins has been observed compared with other flavonoids (*19*). The anthocyanin absorption efficiency could be underestimated, not only under in vitro, but also under in vivo conditions. It is worthy to note that not only anthocyanins in their original form, but anthocyanin degradation products/metabolites which have higher chemical and microbial stability may also be the active forms responsible for their bioactivities (*9*, *44*). Scalbert et al. (*45*) suggested that the active compounds may not be the native polyphenols found in food which are most often tested in in vitro studies; they are more likely to be metabolites. Although there have been some recent reports about metabolites of anthocyanins, the metabolism process is very complicated and far from clear (*3*, *18*, *19*). Further studies are essential in this area.

In the current study, we used HBSS at pH 7.4, since this is the widely accepted buffered pH in Caco-2 monolayer (*46*). Although the pH in the cellular interstice and blood is ∼7.4, the pH in the upper GI tract under fasting conditions ranges from 5.0 to 6.5, and the acidic microclimate operating just above the epithelial cell layer has been reported to be 5.8-6.3 (*47*). Modification of this typical method such as using pH-gradient condition will be helpful. This is especially important for anthocyanin studies since their stability is low under neutral and high pH.

A Caco-2 cell model can only serve as a one-way screen such that compounds with high permeability in this model are typically well absorbed in vivo, yet it cannot be assumed that compounds with low permeability are poorly absorbed in vivo (*22*). Using a Caco-2 cell model, we have shown that anthocyanins can be transported across cell monolayers. However, whether there are other transport mechanisms involved will require further studies.

In conclusion, the results of this study demonstrated that anthocyanins from blueberries can be transported through Caco-2 cell monolayers, although the transport/absorption efficiency was relatively low compared with some aglycone polyphenols. No significant difference in transport/absorption efficiency was observed among the three blueberry cultivars. The transport efficiency of anthocyanins averaged ∼3−4% (less than 1% in Dp-glc). This low absorption rate probably resulted from the poor lipophilic properties of anthocyanins. The observed trend among different anthocyanins is in general agreement with some existing in vivo reports. Dp-glc showed

the lowest transport/absorption efficiency, and Mv-glc showed the highest transport/absorption efficiency. We proposed that more free hydroxyl groups and less OCH_3 groups in anthocyanins may decrease their bioavailability in vivo. In addition, Cy-glc showed significantly higher transport efficiency than Cy-gal, and Pn-glc showed significantly higher transport efficiency than Pn-gal, indicating that glucose-based anthocyanins have higher bioavailability than galactose-based anthocyanins. The Caco-2 cell monolayer is a good model system to investigate the impact of molecular structure on bioavailability of anthocyanins, especially when some parameters are difficult to control under in vivo conditions.

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