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Identification of Cabernet Sauvignon Anthocyanin Gut Microflora Metabolites

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Anthocyanins are polyphenol antioxidants that have been shown to prevent many chronic diseases, including colon cancer. The compounds are largely metabolized by various enzymes and bacteria in the large intestine, and the health benefits of consuming foods rich in anthocyanins could be due mostly to the effects of these metabolites. In this study, the contents of the large intestine of pigs were used to model anthocyanin metabolism because pig and human intestinal microflora are similar. An anthocyanin extract from Cabernet Sauvignon grapes that contained delphinidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside was employed. The extract was incubated anaerobically in the contents of the large intestine of freshly slaughtered pigs for 0, 0.5, and 6 h (final concentrations of 20.9, 28.2, 61.4, and 298.0 μM of the above anthocyanin compounds, respectively, at $t = 0$ h). Anthocyanins and their metabolites were measured by LC-ESI-MS. After 6 h, anthocyanins were no longer detected, and three metabolites were identified as 3-*O*-methylgallic acid, syringic acid, and 2,4,6-trihydroxybenzaldehyde. Results from this study suggest that consumption of Cabernet Sauvignon grape anthocyanins could lead to the formation of specific metabolites in the human gut, and it is possible that these metabolites offer the protective effect against colon cancer attributed to anthocyanin consumption.

KEYWORDS: Anthocyanin; metabolism; phenolics; gut; microflora; flavonoids

INTRODUCTION

Polyphenols in wine have received attention due to powerful correlations in epidemiological studies relating wine intake to colon health. One type of polyphenol is a class belonging to the flavonoids, the anthocyanins. Anthocyanins are found in many foods and plants, but are found in abundance in the skins of red *Vitis vinifera* grapes. The class members commonly found in Cabernet Sauvignon grapes are delphinidin-3-glucoside, petunidin-3-glucoside, and malvidin-3-glucoside (*1*). On the basis of previous animal studies, anthocyanins are promising agents in the prevention of colon cancer (*2, 3*). Low amounts of anthocyanins, however, are absorbed into the bloodstream, where they are found unaltered or methylated, but mostly as deglycosylated/ring fission metabolites (*4, 5*). Because low levels of anthocyanins are actually absorbed into the bloodstream and are passed through the gastrointestinal tract, any beneficial effect anthocyanins have in the colon may be due to direct contact with epithelial colon cells and tumors (*6, 7*) and action in the intestinal lumen.

It may be that this protective effect is not only due to intact anthocyanins and their conjugated metabolites but also or exclusively due to anthocyanin metabolites formed from bacterial metabolism in the large intestine. The metabolites have been found in large concentrations within the gut and bloodstream (*8*), making them possible compounds responsible for the health benefits seen from drinking moderate quantities of wine in epidemiological studies (*9, 10*). In a study by Keppler and Humpf, various anthocyanin standards were metabolized to aldehydes and phenolic acids when incubated with pig cecum contents (*11*). This metabolism was owed to deglycosylation, followed by C-ring fission. Another group found that anthocyanins from red grapes were rapidly metabolized, and cyanidin glycosides were metabolized into protocatechuic acid (*12*). Cyanidin-3-glucoside is metabolized to protocatechuic acid in rats, which is better absorbed into the bloodstream than the anthocyanin (*5*). A study was recently published in which fecal products from human anthocyanin metabolism were measured. Protocatechuic acid was a microbial metabolite of cyanidin-3-glucoside and accounted for 28.1 and 44% of consumed cyanidin-3-glucoside in the feces and plasma, respectively (*8*). This indicates that certain anthocyanin metabolites are bioavailable in the colon and that they could be well absorbed into the bloodstream.

Due to the potentially high concentrations and bioavailability of anthocyanin microfloral metabolites, these phenolic acids and aldehydes could at least exert a local effect in the colon and perhaps prevent inflammatory bowel disease (IBD) and colon cancer. It is necessary to fully identify anthocyanin metabolites before their health effects are assessed. In this study, a whole

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extract of Cabernet Sauvignon grape anthocyanins was prepared and incubated in the contents of the large intestine of freshly slaughtered pigs to monitor the microbial metabolism of the anthocyanins over a period of 6 h, and the formation of anthocyanin metabolites was quantified.

MATERIALS AND METHODS

Chemicals. Methyl gallate for 3-*O*-methylgallic acid synthesis was purchased from K&K Laboratories, Inc. (Carlsbad, CA). Sodium tetraborate, sulfuric acid, sodium hydroxide, hydrochloric acid, and pyridine were purchased from Fisher (Pittsburgh, PA). Dimethyl sulfate and charcoal were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Ethyl acetate was purchased from Acros Organics (Geel, Belgium). *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce (Rockford, IL). Syringic acid was purchased from Eastman Organic Chemicals (Rochester, NY), and 2,4,6-trihydroxybenzaldehyde was purchased from Alfa Aesar (Ward Hill, MA). Carbonate/phosphate buffer was prepared according to a previous method (*13*) without addition of resazurin, nickel chloride, or ammonium molybdate tetrahydrate. Malvidin-3-glucoside was kindly prepared by Professor Peter Winterhalter (Braunschweig, Germany).

Anthocyanin Extract Preparation. The anthocyanin extraction from grapes was based on two previous methods (*14, 15*). Cabernet Sauvignon grapes, 400 g, were peeled, and the skins were shaken in 95% (v/v) ethyl alcohol (400 mL) for 24 h. The ethanolic grape skin solution was then centrifuged at 4000 rpm in Falcon tubes for 10 min at room temperature. The supernatants were pooled together, and ethanol was removed by rotary evaporation at 35 °C. The aqueous grape skin extract was then filtered through glass filters and applied to an Amberlite XAD-7 HP (Rohm and Haas, Philadelphia, PA) column with dimensions of 500 mm × 38 mm i.d. The column was rinsed with distilled water (250 mL) to remove the sugars and organic acids, and phenolic material was eluted with methanol/acetic acid (19:1, 250 mL). Distilled water (100 mL) was added to the eluted material, and methanol was removed by rotary evaporation. The resulting solution was brought to 200 mL with distilled water and the pH adjusted to 7.0 with 1 N sodium hydroxide. The extract was fractionated by applying 20 mL of grape extract per 60 mL solid phase extraction (SPE) cartridge (C18/10 g/60 mL), in batches of six cartridges, until all of the extract was fractionated. At a flow rate of 1 mL/min, the phenolic acids were eluted with 60 mL of 10% methanol (v/v) per cartridge. Each cartridge was then rinsed with 20 mL of 0.01 N hydrochloric acid. The anthocyanins and catechins were then eluted with 60 mL of 16% acetonitrile (pH 2.0). The anthocyanin/catechin fraction was further separated by liquid/liquid extraction by adding the fraction to 50 mL of ethyl acetate in a separatory funnel. The anthocyanins were retained in the aqueous fraction, and any organic solvent that remained was removed by rotary evaporation. The anthocyanin mixture was then further concentrated for the intestinal incubation phase. The concentrations and purity of the extract were monitored by high-performance liquid chromatography with electrospray mass spectrometric detection (LC-ESI-MS) analysis.

Anthocyanin Intestinal Incubation. The anthocyanin extract was incubated in the large intestinal contents (LIC) of three freshly slaughtered PIC (PIC Genetics, Berkeley, CA) white line adult female pigs to look for metabolites (Meat Laboratory, Department of Animal Science, University of California, Davis). The pigs were fed a defined mixture of wheat, corn, soybean, minerals, and vitamins with no polyphenol content. We used the large intestinal contents of pigs because they have gut microflora similar to that of humans (*16, 17*). The incubation method was modified from that of Keppler and Humpf (*11*). Briefly, the gastrointestinal tracts of freshly slaughtered pigs were removed, and samples of LIC were sampled immediately and placed into a jar containing Anaerocult A (EM Science, Gibbstown, NJ) to maintain an anaerobic environment and an equal amount of carbonate/phosphate buffer. The solution was homogenized with a magnetic stir bar for 10 min. The mixture was then filtered through a 1 mm sieve, divided into three containers, flash frozen, and stored at −80 °C (pH 7.57). The three replicate incubation experiments were then conducted on three separate days. For each replicate the thawed intestinal content/buffer mixture was divided into two anaerobic containers. One container was autoclaved for 20 min to inactivate bacteria. The incubations were performed under anaerobic conditions in 100 mL round-bottom flasks at 37 °C, to which 9 mL of either active or inactive intestinal matrix was added and stirred with magnetic stir bars. The flasks were gassed with a defined mixture of N_2/CO_2 (4:1 v/v, 4 L/min), to maintain an anaerobic environment. One experimental group had 1 mL of the anthocyanin extract added to three active intestinal matrix flasks. The anthocyanin extract (1 mL) was also added to three inactive matrix flasks. The initial concentrations of the anthocyanins in the flasks were 20.9, 28.2, 61.4, and 298.0 μM delphinidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside, respectively (all measured as malvidin-3-glucoside). Control flasks (three) had 1 mL of water added instead of the extract. Each treatment and control incubation was sampled at 0, 0.5, and 6 h. The contents of each flask were transferred to glass vials containing 10 mL of 10% formic acid (Acros Organics) in methanol to inactivate bacterial activity. The vials were sonicated for 10 min, the contents were transferred to Falcon tubes and centrifuged for 20 min at 4000 rpm and 4 °C. The samples were then filtered through 0.45 μm PTFE membrane filters and analyzed by LC-ESI-MS. Spike recoveries for anthocyanin metabolites were also conducted, whereby 1 mL of 20 mM 3-*O*-methylgallic acid, syringic acid, and 2,4,6-trihydroxybenzaldehyde was added to the active and inactive matrices in triplicate.

LC-MS Analysis. Each sample was injected (25 μL) into a Hewlett-Packard (Agilent, Santa Clara, CA) 1100 series LC instrument. Separation was achieved with a 150 mm × 4.6 mm i.d., 5 μm, Hypersil C18 column (Phenomenex, Torrance, CA). The column temperature was 35 °C. Solvents used for the separation were 0.1% TFA (solvent A) and acetonitrile (solvent B). The gradient began at 0−5 min (10% B), then 20 min (15% B), 25 min (15% B), 30 min (18% B), 50 min (35% B), 55 min (10% B), and finally 65 min (10% B) at a constant flow rate of 0.5 mL/min. UV−vis DAD signals were monitored at 280, 315, 365, and 520 nm, and scanning was from 190 to 600 nm. A quadrupole mass analyzer was used for mass spectrometric detection using ESI as the ion source in positive polarity. Compounds of interest were monitored in selective ion monitoring (SIM) mode. The drying gas flow/temperature was 12 L min^{-1}/350 °C, nebulizer pressure was 35 psig, and capillary voltage was 3000 V.

3-*O*-Methylgallic Acid Synthesis and Analysis. 3-*O*-Methylgallic acid was synthesized according to the method of Scheline (*18*). The method was followed very closely with a few minor changes: the synthesis scale was reduced 10-fold, the final continuous extraction was performed with ethyl acetate instead of ether, and the synthesis product remained in ethyl acetate for purification by preparative thin layer chromatography (TLC) (*19*). The identification of the prepared standard was confirmed by GC-MS. Briefly, 5 mg of freeze-dried 3-*O*-methylgallic acid was incubated in a glass vial with 200 μL each of BSTFA and pyridine. The vial was incubated in an oven at 60 °C for 1 h. The derivatized solution was then cooled, and 1 μL was injected for GC-MS analysis (*19*). The column was a 30 m × 0.25 mm i.d., 0.25 μm, HP-5MS (Agilent). The synthesized product was used for 3-*O*-methylgallic acid standard curves, spike recoveries, and identification.

Statistics. Two-tailed Student *t* tests were performed in Microsoft (Bellevue, WA) Excel 2003 software. Differences were considered significant at $P < 0.05$, $P < 0.01$, and $P < 0.005$.

RESULTS AND DISCUSSION

Anthocyanin Preparation. The anthocyanin class members in the extract from Cabernet Sauvignon grapes included delphinidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside (**Figure 1**). The purity of the extract was 98.3% by monitoring UV absorbance at 280 nm on our LC instrument. Scarce information is available on preparative methods for anthocyanin extraction. Counter-current chromatography (*20, 21*) offers an effective way of isolating anthocyanin extracts, yet less expensive methods exist for the preparation of smaller quantities. We have built on two previous

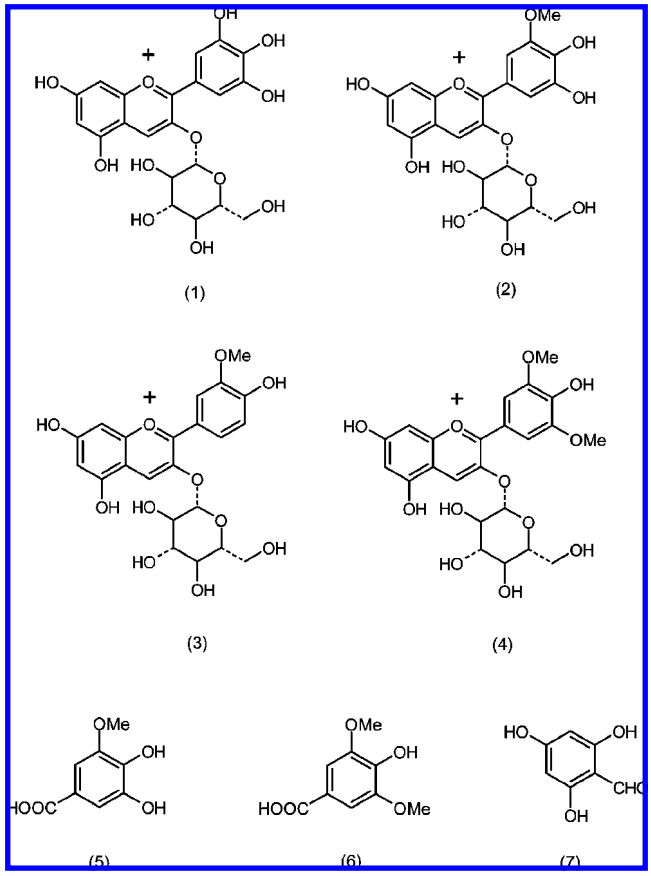

Figure 1. Structures of anthocyanins and metabolites: (**1**) delphinidin-3-glucoside; (**2**) petunidin-3-glucoside; (**3**) peonidin-3-glucoside; (**4**) malvidin-3-glucoside; (**5**) 3-*O*-methylgallic acid; (**6**) syringic acid; (**7**) 2,4,6-trihydroxybenzaldehyde.

methods for anthocyanin extraction by increasing the size of SPE cartridges and incorporating a liquid/liquid extraction as a final separation to isolate the anthocyanins from the catechins. This appeared to effectively separate the two classes of polyphenols from each other and could be scaled-up further with flash chromatography using C-18 packing.

Anthocyanin Degradation in LIC. The anthocyanin extract was incubated in the active and inactive large intestinal contents of freshly slaughtered pigs for 0, 0.5, and 6 h. The added concentrations of the anthocyanin compounds were 20.9 μM delphinidin-3-glucoside, 28.2 μM petunidin-3-glucoside, 61.4 μM peonidin-3-glucoside, and 298.0 μM malvidin-3-glucoside, which are reasonable gastrointestinal values for humans that consume moderate amounts of red wine (*22*). After 0.5 h, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside were greatly metabolized by the microflora in the active LIC, and residual amounts were 10.9 $\pm$ 1.18, 12.1 $\pm$ 3.02, and 104 $\pm$ 12.7 μM, respectively. Initial data included a time point after 2 h (data not shown). Because all anthocyanin concentrations were below the limit of detection at this time point, it was changed to 0.5 h. After 6 h of active incubation, all four anthocyanins were metabolized below the limit of detection.

No significant difference was found between concentrations of delphinidin-3-glucoside in the active and inactive flasks at 0.5 h (9.82 $\pm$ 0.744 and 10.2 $\pm$ 0.413 μM, respectively), indicating that the loss of delphinidin-3-glucoside after 0.5 h of incubation was due only to either chemical degradation or protein binding and that no metabolism of delphinidin-3-glucoside had occurred. At 6 h, however, delphinidin-3-glucoside was below the limit of detection in the active matrix, but some remained in the inactive matrix, indicating a metabolism effect. Petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside displayed significant losses ($P < 0.005$) due to metabolism within the first 0.5 h and were all below the limit of detection by 6 h, presenting a clear metabolism effect. In the inactive LIC (**Figure 2A**,**2B**) there was significant loss ($P < 0.05$) of each anthocyanin over time. This could be due to chemical degradation or protein binding (*11, 23*). The loss of anthocyanins in the inactive LIC, however, was significantly less than in the active LIC.

Using the pig as a model for human anthocyanin metabolism was validated by Keppler and Humpf, when selected anthocyanin standards were incubated with pig cecum microflora (*11*). In this study, we were able to model the metabolism of a whole anthocyanin extract. From our results it appears that microflora found in the large intestine metabolize each anthocyanin class member at different rates. All anthocyanins were greatly metabolized after 6 h, as is seen by the degradation trends, yet no phenolic acid metabolites were detected for either delphinidin-3-glucoside (gallic acid) or peonidin-3-glucoside (vanillic acid). This indicates that these two phenolic acid metabolites were themselves metabolized, not formed, or were below the limit of detection.

It has been shown that anthocyanins are not chemically deglycosylated at the low pH of the stomach or small intestine in vivo (*7*). Anthocyanins are largely found in their glycosidic forms in the large intestine, where they can be acted on by gut microbes. It is difficult to distinguish, however, whether the anthocyanins are being chemically degraded versus microbially degraded in the large intestine. At the pH of the large intestine it appears that anthocyanins can degrade to similar products through a carbinol pseudobase form. This has been observed before where substantial loss of anthocyanins and formation of phenolic acids occurred in inactive samples (*11*). We found the same result, yet there was clearly more degradation of the anthocyanins and formation of metabolites in flasks with active bacteria than in flasks with inactive bacteria.

The concentration of anthocyanins incubated in this experiment would be roughly equivalent to consuming 1 L of a young Cabernet Sauvignon wine (*22*), assuming the anthocyanins are largely able to reach the large intestine. Anthocyanin intake, however, can be highly variable depending on individual daily intake of fruits, vegetables, and beverages. The average person living in the United States consumes far less than the anthocyanin levels used in this study (*24*). Human anthocyanin metabolism studies need to be conducted, where anthocyanin consumption history and amounts are considered.

Metabolite Formation and Spike Recoveries. After 0.5 h of anthocyanin metabolism in active LIC, three metabolites were formed and identified (**Figure 2C**). These were 3-*O*-methylgallic acid, syringic acid, and 2,4,6-trihydroxybenzaldehyde (52.5 $\pm$ 10.5, 40.3 $\pm$ 11.1, and 38.1 $\pm$ 10.6 μM, respectively) (**Figure 1**). The compounds were still present after 6 h in the active flasks, yet at significantly lower ($P < 0.01$) concentrations (16.1 $\pm$ 4.68, 11.3 $\pm$ 5.00, and 7.24 $\pm$ 3.73 μM, respectively). This indicates further metabolism of these metabolites, yet the breakdown products were not detected in this study. Spike recoveries for all four anthocyanins and three metabolites are given in **Table 1**.

3-*O*-Methylgallic acid and 2,4,6-trihydroxybenzaldehyde were not found in any of the inactive flasks. Low concentrations of syringic acid were found in the inactive LIC flasks, presumably due to chemical degradation of malvidin-3-glucoside. The levels

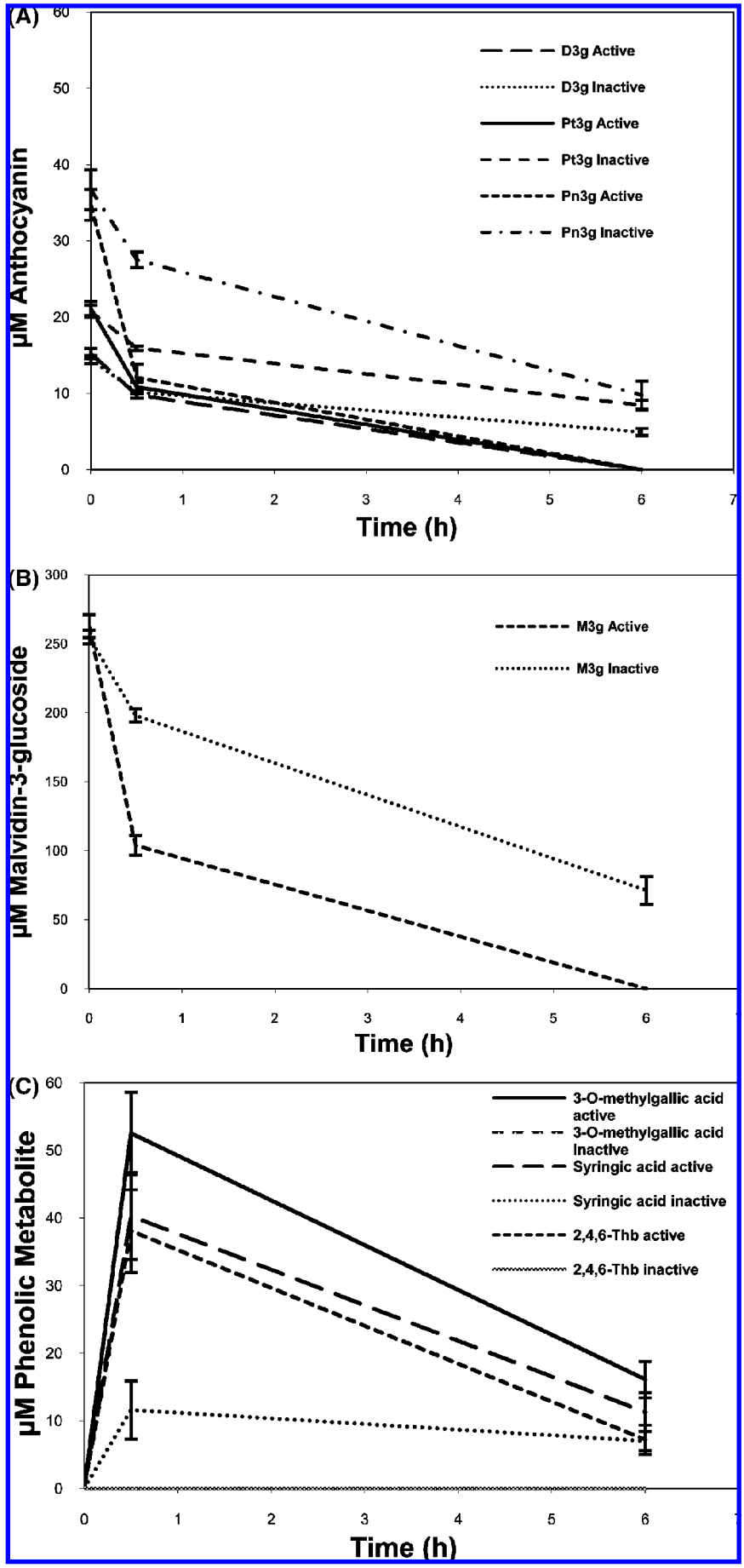

Figure 2. (**A**) Metabolism of delphinidin-3-glucoside (d3g), petunidin-3-glucoside (pt3g), and peonidin-3-glucoside (pn3g) over 6 h. (**B**) Metabolism of malvidin-3-glucoside (m3g) over 6 h. (**C**) Formation of 3-*O*-methylgallic acid, syringic acid, and 2,4,6-trihydroxybenzaldehyde (2,4,6-Thb) over 6 h.

Table 1. Analytical Information for Anthocyanins and Metabolites

compound	retention time (min)	*m/z* values	spike recoveries[a] (%) active LIC	spike recoveries[a] (%) inactive LIC
3-*O*-methylgallic acid	8.2	185	96.6 ± 0.147	100.0 ± 1.72
syringic acid	17.0	199	99.0 ± 1.11	99.2 ± 0.278
delphinidin-3-glucoside	18.5	465, 303	73.1 ± 5.36	69.1 ± 4.25
2,4,6-trihydroxybenzaldehyde	21.5	155	106 ± 1.21	105 ± 0.572
petunidin-3-glucoside	25.0	479, 317	75.1 ± 5.64	73.8 ± 5.13
peonidin-3-glucoside	31.0	463, 301	56.6 ± 5.70	59.9 ± 7.30
malvidin3-glucoside	32.5	493, 331	88.2 ± 4.83	85.6 ± 2.88

[a] Each spike recovery is given as an average ± standard deviation ($n = 3$).

of syringic acid in the inactive flasks did not change significantly when levels at 0.5 and 6 h were compared (11.6 ± 7.53 and 7.04 ± 2.45 μM, respectively). The levels of syringic acid, however, at 0.5 h were significantly different ($P < 0.05$) when active and inactive flasks were compared, with the active levels being much higher, presenting a clear metabolism effect. After 6 h, the levels of syringic acid in the active flasks were not significantly different from the levels in the inactive flasks.

Syringic acid and 2,4,6-trihydroxybenzaldehyde were previously identified from metabolized anthocyanin standards (*11*). Our results include a novel anthocyanin metabolite, 3-*O*-methylgallic acid, previously identified as a black tea metabolite (*19*). The rapid metabolism of petunidin-3-glucoside within 0.5 h appears to correlate with the formation of 3-*O*-methylgallic acid. Methylation of gallic acid was considered as an additional source of 3-*O*-methylgallic acid, yet was soon ruled out due to the fact that no metabolism of delphinidin-3-glucoside was observed within the first 0.5 h of incubation. Another possible pathway to forming 3-*O*-methylgallic acid is the demethylation of syringic acid. On the basis of the concentration of 3-*O*-methylgallic acid found after 30 min of active metabolism, it is likely that both pathways are occurring.

Bacteria that could be involved in human anthocyanin metabolism have been identified as *Bacteroides*, *Clostridium*, *Eubacterium*, *Ruminococcus*, and *Eggertheilla* genera (*25*). Some bacterial enzymes involved are α,L-rhamnosidase, β,D-glucosidase, catechol-*O*-methyltransferase, and aryl-sulfotransferase (*12*), and the human enzyme lactase phlorizin hydrolase could also participate (*26*). A more detailed understanding, however, of specific bacteria and enzymes responsible for anthocyanin metabolism remains to be achieved.

Phenolic compounds have also been shown to undergo secondary metabolism in the intestinal lumen and liver in vivo. In the lumen and liver, various flavonoids can be glucuronidated, sulfated, or methylated (*27*). Glucuronide and methyl anthocyanin metabolites, along with intact anthocyanins, are found in the urine and intestine (*2*), and methylated anthocyanins can also be found in the liver and kidney (*5*). Peak plasma concentrations of absorbed compounds roughly occur 30 min after ingestion, leading to a slight enhancement of antioxidant capacity in the plasma (*28*). The total absorbed compounds are usually in the nanomoles per liter range; after 4 h, a large part of these plasma anthocyanins are passed through the urine (*29*). Stability and absorption of anthocyanins appear to depend on the sugar type and number attached to the aglycone (*30*). The phenolic acids and aldehydes produced by anthocyanin metabolism could also be further metabolized and produced as glucuronidated, sulfated, or methylated conjugates in vivo and potentially better absorbed. Because protocatechuic acid (*8*) appears to be absorbed into the bloodstream without further conjugation, more work needs to be done in assessing the bioavailability of other anthocyanin metabolites. The modes of transport across the blood barrier and any enzymes responsible also need to be identified for these metabolites.

Recent work in our laboratory showed that gallic acid, an anthocyanin metabolite, can reduce inflammation and tumor load in vivo (unpublished data). Gallic acid also inhibits cell proliferation when incubated with colon cancer cells. Epigallocatechin gallate has a similar effect in vitro, emphasizing the importance of a galloyl group to inhibit cancer cell proliferation (*31*). It seems that the antiproliferative properties of gallic acid are due to its ability to be a prooxidant (*32*) and that the antioxidant power of gallic acid could be more important for preventing mutation of cancer-related genes (*33*). Therefore, it seems that these compounds are able to act as redox buffering molecules, scavenging radicals under oxidative stress (*34*), and acting as prooxidants under normal conditions. The antioxidant activity of phenolic acids can also be well correlated with the up-regulation of metabolic enzymes, leading to the detoxification of damage-causing compounds (*35*).

Other phenolic acids such as syringic acid, protocatechuic acid, caffeic acid, and 3,4-dihydroxyphenylacetic acid (PAA) can affect breast and colon cancer cell proliferation in vitro. The antioxidant effect of these compounds does not appear to correlate with their antiproliferative effects, but their mechanism of action leads to the induction of apoptosis and certain metabolic enzymes (*36, 37*). Similarly, gallic acid induces apoptotic related genes in a few cancer cell lines, but has no such effect in normal cells (*38*).

We have shown that anthocyanins from a *V. vinifera* grape whole extract are metabolized into two phenolic acids and one aldehyde. To our knowledge this is the first time 3-*O*-methylgallic acid has been identified as an anthocyanin metabolite. Due to the rate and extent of formation of these metabolites produced by gut microflora, their absorption and interaction with chronic disease processes should be pursued. Future work should focus on the health effects of these metabolites using cell and animal models as well as their relevance to humans.

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