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Research Article

Phenolic acids in black raspberry and in the gastrointestinal tract of pigs following ingestion of black raspberry

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Black raspberries (BRB) contain high levels of polyphenols and have been demonstrated to be chemopreventive. In order to investigate the underlying mechanism and study the metabolism of anthocyanins, pigs were fed freeze-dried BRB powder or purified diet (control) and three segments of the gastrointestinal (GI) tract (small intestine, cecum, and colon; 4 h after feeding) were collected for analysis of phenolic acids. Protocatechuic acid was the major phenolic acid (8.35 mg/100 g, dry weight (DW)) in BRB, followed by *p*-coumaric acid (1.63 mg/100 g, DW), caffeic acid (1.34 mg/100 g, DW), ferulic acid (0.24 mg/100 g, DW), and 3-hydroxybenzoic acid (0.20 mg/100 g, DW). Recoveries of these five phenolic acids in the whole GI tract were 199.9 ± 54.0%, 7.0 ± 3.0%, 37.0 ± 9.7%, 56.6 ± 31.3%, and 916.8 ± 642.3% (mean ± SEM, $n = 5$), respectively, and quantities in contents of the GI tract ranged from 0.13 ± 0.05 μmol (*p*-coumaric acid) to 23.47 ± 6.09 μmol (protocatechuic acid) (mean ± SEM, $n = 5$). Six other phenolic acids were detected primarily in the cecum and/or colon which were not in BRB, with total contents in the GI tract ranging from 0.18 ± 0.18 μmol (homovanillic acid) to 8.49 ± 4.31 μmol (homoprotocatechuic acid). Total phenolic acids in the GI tract were 49.32 ± 16.37 μmol (mean ± SEM, $n = 5$). Phenolic acids measured in the GI tract accounted for only 6.31% of the degraded anthocyanins.

Keywords: Anthocyanin / Black raspberry / Phenolic acids / Protocatechuic acid

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1 Introduction

Recent studies have indicated that black raspberries (*Rubus occidentalis* L.) (BRB) may inhibit the development of oral, esophageal, and colon cancer in rodents [1 – 3]. Molecular mechanisms involved in chemoprevention of BRB have been investigated extensively in the last few years [4 – 10]. However, the actual bioactive components have not been well characterized. BRB is rich in polyphenols, which primarily include anthocyanins (ACNs) and ellagitannins [11 – 13]. Total ACNs and ellagitannins were calculated to be ~4800 mg/100 g [14] and ~400 mg/100 g (Hager *et al*. unpublished data) lyophilized dry weight (DW), respectively. ACNs are thought to be at least partly responsible for the observed anticolon cancer activities [15 – 19]. In addition, phenolic acids, phytosterols, and other components in BRB have also been proposed as chemopreventive agents [20].

It is well known that flavonoids or other polyphenols undergo transformation by intestinal microorganisms and various products of degradation may be formed during this process [21 – 24]. Gut microflora plays an important role in

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Abbreviations: ACN, anthocyanin; **BRB,** black raspberry; **-OH BA,** -hydroxybenzoic acid, **3,4-DiOH BA,** 3,4-dihydroxybenzoic acid; **BA,** benzoic acid; **CA,** cinnamic acid; **DW,** dry weight; **GI,** gastrointestinal; **3-OMe-4-OH PAA,** 3-methoxy-4-hydroxyphenylacetic acid; **PAA,** phenylacetic acid; **PPA,** 3-phenylpropionic acid; **3,4-DiOH PAA,** 3,4-dihydroxyphenylacetic acid; **-OH PAA,** -hydroxyphenylacetic acid; **4-OH PPA,** 3-(4-hydroxyphenyl)-propionic acid; **3,4-DiOH PPA,** 3-(3,4-dihydroxyphenyl)-propionic acid

the metabolism, absorption, and biological activity of dietary flavonoids [23]. Simple phenolic acids are probably among the major end products of flavonoids by gut microflora. Jenner *et al*. [25] found that monophenolic acids were present in large amounts within the colon in healthy human subjects. Phenolic acids present in either the BRBs or generated by gut microflora from BRBs in gastrointestinal (GI) tract, may contribute to the protective effects of BRB against colon cancer. However, the role of phenolic acids in preventing colon cancer is not consistent. In one study, phenolic compounds did not suppress aberrant crypt foci induction by azoxymethane [26], whereas other studies showed positive chemopreventive effects of phenolic acids [27–29]. Recent studies suggested that whether or not phenolic acids show chemopreventive effects may depend upon their structure [30]. From data in the literature 3,4-hydroxybenzoic acid (3,4-OH BA) (protocatechuic acid) seems to be the most promising chemopreventive phenolic acid so far [28, 31–37]. Identifying individual phenolic acids and the concentrations of each present in the GI tract, rather than total amounts of phenolic acids, will be more important in understanding the underlying mechanism of cancer preventive effects of BRB.

In a previous study involving weanling pigs [38], we found that the recovery of total ACNs in the whole GI tract following consumption of BRB was only 41.7%. Combined with the fact that extremely low recoveries (<0.1%) from blood and urine were observed, the fate of a large portion of ACNs remains unaccounted for. These "missing" ACNs may have been broken down into simple phenolic compounds by gut microflora. Thus, evaluating phenolic acids in the GI tract will result in a greater understanding of the fate of ACNs within the body.

To our knowledge, there are no studies that have investigated the phenolic acids in GI tract after BRB consumption. The objective of this study was to characterize the major phenolic acids in BRBs followed by determination of the major phenolic acids within the GI tract following BRB consumption.

2 Materials and methods

2.1 Chemicals and materials

Standards for phenolic acids (Table 1) were obtained from Sigma Chemical (St. Louis, MO). Methanol was obtained from Fisher Scientific (Fair Lawn, NJ), formic acid from Aldrich Chemical Company (Milwaukee, WI), and TFA from Sigma Chemical (St. Louis, MO).

2.2 Experimental materials

Freeze-dried BRB powder was provided by Oregon Raspberry and Blackberry Commission as reported previously [13].

2.3 Animals and study design

All animal protocols were approved by the UAMS Animal Care and Use Committee. Healthy pigs (Hampshire/Duroc Cross) (n = 8–21 days of age) were purchased from a local swine producer and brought to the Arkansas Children's Nutrition Center animal facility and allowed to adapt for a period of 7 days before surgery. At surgery, an in-dwelling cannulae was placed in the femoral artery for blood collection. Four days before administration of berry powder, the pigs were fed a purified diet [39], which was free of any polyphenolic- or flavonoid-like compounds. At the time of the experiment, the pigs weighed 13.5 ± 3.5 kg (mean ± SD, n = 8).

Pigs were placed in a metabolic cage and fasted overnight with water freely available before the experiment. Freeze-dried BRB powder was mixed with water (1:3, w/w) and given *via* gastric incubation. This dose provided 50.5 ± 3.7 mg *per* kilogram of body weight (BW) of total ACNs (mean ± SD, n = 5). Three pigs were used as control animals. They had free access to food and water. After 4 h, five BRB-fed pigs along with control pigs were sacrificed by euthanasia with Nembutal given IV. Three sections of the GI tract, namely small intestine, cecum, and colon were collected. The contents of each section were flushed out with saline. The contents with saline were weighed and mixed. A portion of each sample of gut contents was taken and 0.44 M TFA was added to produce a final concentration of 20% of the sample to stabilize the ACNs. Gut content samples were stored at −70°C until analysis.

2.4 Sample preparation

Five grams of contents from each GI tract segment was weighed, and then extracted twice (10 mL × 2) using methanol/water/acetic acid (MWA) (70:29.5:0.5 v/v). The mixture of sample and solvent was centrifuged at 4550 × g for 10 min and the supernatants from the two extractions were combined. The combined supernatant was transferred to a 25 mL volumetric flask, and MWA was added to make up the final volume to 25 mL.

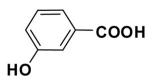
2.5 LC/ESI-MS analysis

The LC-MS/MS analyses were carried out using an Agilent 1100 HPLC system including an autosampler, a binary pump, and a diode array detector (Agilent Technologies, Palo Alto, CA), coupled with the 4000 QTRAP™ mass spectrometer (Applied Biosystems, Forest City, CA). Separation was performed on a Phenomenex Synergi Max-RP column (150 × 3.00 mm^2, 4 μm) using a flow rate of 0.4 mL/min. The solvent consisted of: (i) 0.1% v/v of formic acid in water and (ii) methanol. The 33 min linear gradient was as follows: 0–7–9–14–16–26–30–33 min, 25–25–35–40–45–75–75–25% of methanol, followed by 6 min of re-equilibration of the column before the next

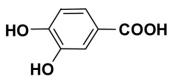
Table 1. Phenolic acid standards, their abbreviations (Abbr.), MW, transition pairs for MRM mode (Trans. pair), and retention time (t_R) from HPLC-MS/MS

Peak no.	Phenolic acids	Abbr.	MW	Trans. pair	t_R (min)
Benzoic acid derivatives					
9	4-Hydroxybenzoic acid	4-OH BA	138	136.9/93.1	15.1
1	3,4-Dihydroxybenzoic acid	3,4-DiOH BA	154	152.7/109.0	7.3
6	3-Methoxy-4-hydroxybenzoic acid	3-OMe-4-OH BA	168	166.8/123.0	13.6
Phenylacetic acid derivatives					
8	3-Hydroxyphenylacetic acid	3-OH PAA	152	150.7/106.7	13.8
4	4-Hydroxyphenylacetic acid	4-OH PAA	152	150.7/106.7	11.9
2	3,4-Dihydroxyphenylacetic acid	3,4-DiOH PAA	168	166.8/123.0	7.7
5	3-Methoxy-4-hydroxyphenylacetic acid	3-OMe-4-OH PAA	182	180.9/136.8	13.5
3-Phenylpropionic acid derivatives					
14	3-Phenylpropionic acid	PPA	150	148.8/105.0	27.1
13	3-(3-Hydroxyphenyl)-propionic acid	3-OH PPA	166	164.9/121.2	22.2
10	3-(4-Hydroxyphenyl)-propionic acid	4-OH PPA	166	164.9/121.2	19.5
3	3-(3,4-Dihydroxyphenyl)-propionic acid	3,4-DiOH PPA	182	180.9/136.8	11.3
Cinnamic acid derivatives					
11	*p*-Coumaric acid	4-OH CA	164	162.9/118.8	19.8
7	Caffeic acid	3,4-DiOH CA	180	178.5/134.7	13.8
12	Ferulic acid	3-OMe-4-OH CA	194	192.8/133.8	20.7

Benzoic acid derivatives (BA)

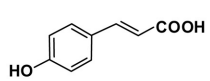


3-OH BA

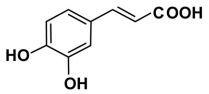


3,4-DiOH BA

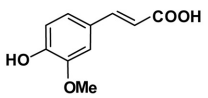
Cinnamic acid derivatives (CA)



4-OH CA

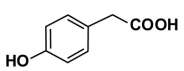


3,4-DiOH CA

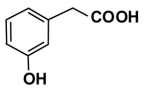


3-OMe-4-OH CA

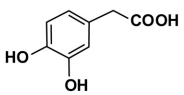
Phenylacetic acid derivatives (PAA)



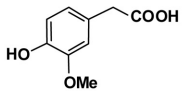
4-OH PAA



3-OH PAA

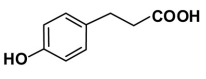


3,4-DiOH PAA

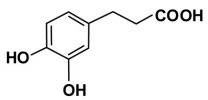


3-OMe-4-OH PAA

3-Phenylpropionic acid derivatives (PPA)



4-OH PPA



3,4-DiOH PPA

Figure 1. Groups and chemical structures of 14 phenolic acid standards.

run. The mass spectrometer used an electrospray interface in negative ionization mode. ESI-MS/MS was conducted using a QTRAP quadrupole-linear IT (QLIT) instrument equipped with a Turbo Ion Spray (TISP) interface (Applied Biosystems/MDS Sciex, Concord, ON). The whole LC/MS system was controlled by Analyst software (v. 1.4.1, Applied Biosystems/MDS Sciex).

Multireaction monitoring (MRM) mode scan was performed. MRM uses the combination of a specific parent mass and a unique fragment ion to selectively monitor the

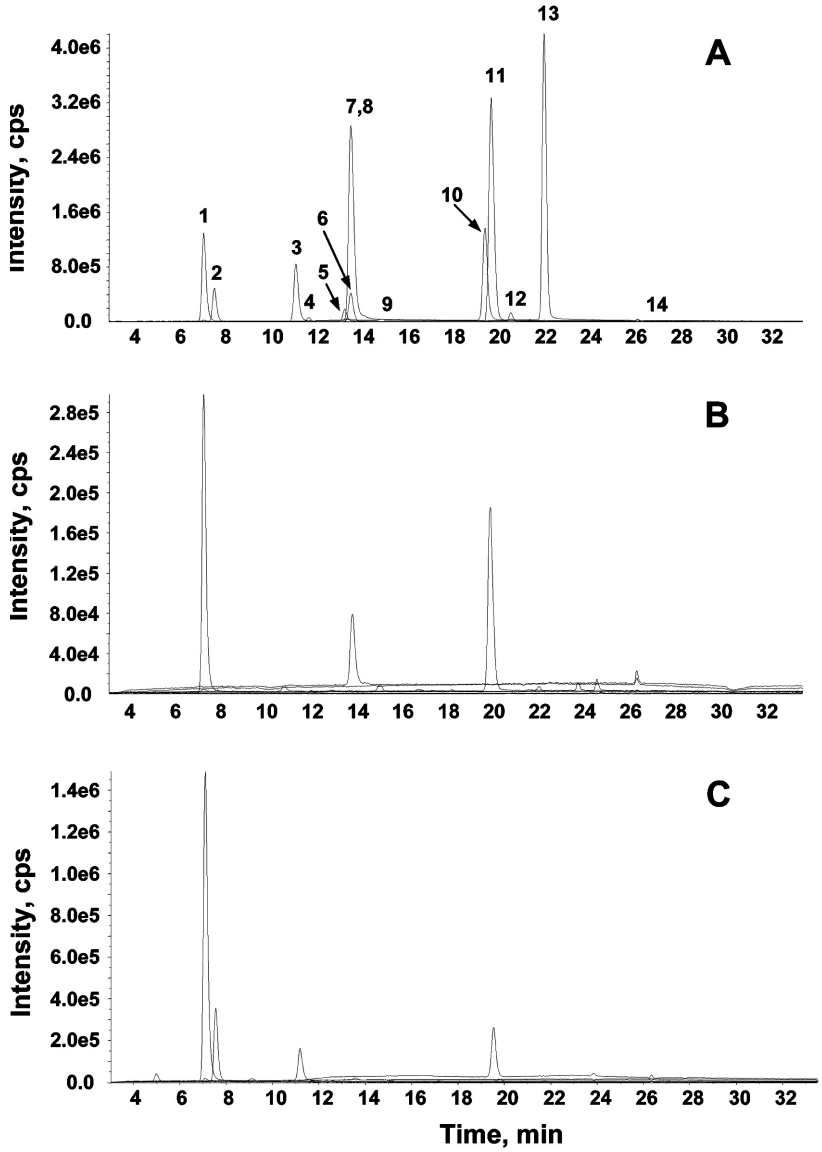


Figure 2. MRM-MS spectra of phenolic acid standards (A), phenolic acids in BRB (B), and representative phenolic profile in one section of GI tract (cecum) (C). Peaks numbered 1 – 14 correspond to (1) 3,4-OH BA; (2) 3,4-diOH PAA; (3) 3,4-diOH PPA; (4) 4-OH PAA; (5) 3-OMe-4-OH PAA; (6) 3-OMe-4-OH BA; (7) 3,4-diOH CA; (8) 3-OH PAA; (9) 3-OH BA; (10) 4-OH PPA; (11) 3-OH CA; (12) 3-OMe-4-OH CA; (13) 3-OH PPA; and (14) PPA. See Table 1 for full names of phenolic acids.

compound to be quantified. The MRM transition pairs used for all 14 phenolic acids are listed in Table 1. The standards for the calibration curve were prepared at concentrations of 40, 100, 200, 400, 1000, and 2000 ng/mL. Major parameters were 20 for curtain gas (CUR), −4500 V for potential (IS) of electrospray capillary, 450°C for source temperature, 30 and 50 for nebulizing (GS1), and turbo spray gas (GS2). The entrance potential (EP) was set at −10 V. The declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) were optimized individually with each standard. Peak areas from the chromatogram for each of the MRM pairs were determined and used for quantitation.

2.6 Statistics

All data with a sample number equal or larger than three was expressed as mean ± SEM if not mentioned specifically. Charts and graphs were made using Sigma Plot 2001 (SPSS, Chicago, IL).

3 Results

3.1 Major phenolic acids and their concentration in BRB

The chemical structures of the 14 phenolic acids studied are presented in Fig. 1. By comparing to phenolic acid stand-

Table 2. Concentrations of major phenolic acids in BRB and their amounts and recoveries in the entire GI tract

Phenolic acids	Conc. in BRB (mg/100 g DW)[a)]	Dose[b)] (μmol)	Amounts in GI tract[b)] (μmol)	Recovery[b)] (%)
3,4-DiOH BA (protocatechuic acid)	8.35	11.60 ± 1.22	23.47 ± 6.09	199.9 ± 53.98
4-HydroxyCA (*p*-Coumaric acid)	1.63	2.13 ± 0.22	0.13 ± 0.05	7.0 ± 3.0
3,4-DihydroxyCA (3,4-DiOH CA)	1.34	1.59 ± 0.17	0.52 ± 0.12	37.0 ± 9.7
3-Methoxy-4-hydroxyCA (Ferulic acid)	0.24	0.27 ± 0.03	0.17 ± 0.09	56.6 ± 31.3
3-Hydroxybenzoic acid	0.20	0.31 ± 0.03	3.68 ± 2.58	916.8 ± 642.3
3,4-DiOH PAA (homoprotocatechuic acid)	0	0	8.49 ± 4.31	NA
4-OH PPA (phloretic acid)	0	0	4.28 ± 2.11	NA
3,4-DiOH PPA	0	0	3.74 ± 1.58	NA
4-OH PAA	0	0	3.50 ± 1.28	NA
3-OH PAA	0	0	1.16 ± 0.48	NA
3-OMe-4-OH PAA (homovanillic acid)	0	0	0.18 ± 0.18	NA

a) DW: dry weight.
b) Data expressed as mean ± SEM, $n = 5$.

ards (Fig. 2A), five major phenolic acids were identified in BRB (Fig. 2B), namely 3,4-OH BA (protocatechuic acid), *p*-coumaric acid, caffeic acid, ferulic acid, and 3-OH BA. However, compared to ACNs or ellagitannins, their concentrations were low. Of these five phenolic acids, 3,4-OH BA (protocatechuic acid) was the most abundant one and its concentration was 8.35 mg/100 g DW (Table 2). Concentrations of the other four phenolic acids were lower than 2 mg/100 g DW (Table 2).

3.2 Identification of phenolic acids in gut contents of control animals

A representative profile of 14 phenolic acid standards is shown in Fig. 2A. In control animals, four phenolic acids, 4-(4-hydroxyphenyl)propionic acid, 4-hydroxyphenylacetic acid (4-OH PAA), 3-hydroxyphenylacetic acid (3-OH PAA), and *p*-coumaric acid were detected in the cecum. Their amounts in the GI tract were 1.46 ± 1.32, 0.14 ± 0.14, 0.14 ± 0.14, and 0.11 ± 0.11 μmol, respectively.

3.3 Identification and recoveries of phenolic acids in gut contents of BRB fed animals

Identification of phenolic acids in gut contents was accomplished by comparing their retention time and MS data with that of standards. A representative profile of the phenolic acids in the contents of one section of the GI tract (cecum) of a pig following BRB feeding is shown in Fig. 2C. There were 11 phenolic acids detected and quantified in the gut contents of pigs fed BRB. The total amount of all phenolic acids combined in the whole GI tract was calculated to be 49.32 ± 16.37 μmol.

Among all five major phenolic acids in BRB, 3,4-OH BA (protocatechuic acid), 4-hydroxycinnamic acid (*p*-coumaric acid), and 3-OH BA were detected in all three sections of GI tract, whereas 3,4-dihydroxycinnamic acid (caffeic acid) and 3-methoxy-4-hydroxycinnamic acid (ferulic

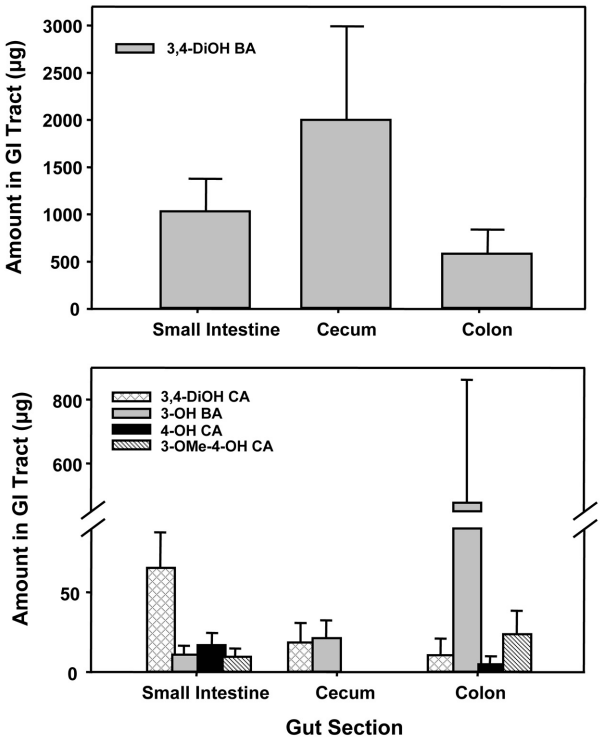


Figure 3. Amounts of original phenolic acids of BRB in three sections of GI tract (μg) of weanling pigs after BRB feeding.

acid) were only detected in small intestine and colon. The total recoveries of all the phenolic acids in the GI tract were calculated based on intake and are presented in Table 2. Remarkably, recoveries of 3,4-dihydroxybenzoic acid (3,4-DiOH BA) and 3-OH BA were 199.9 and 916.8%, respectively, indicating that they were generated from multiple sources within the GI tract. The five BRB phenolic acids were present in all three sections of the GI tract (Fig. 3).

In addition to the five phenolic acids discussed above, six other phenolic acids were also detected in the GI tract. They

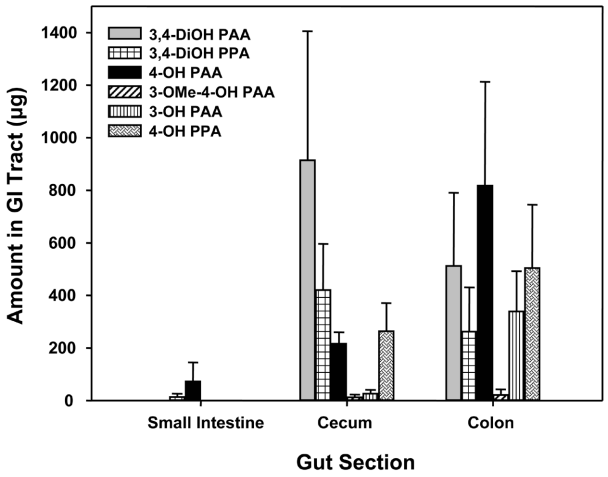


Figure 4. Amounts of newly formed phenolic acids in three sections of the GI tract (µg) of weanling pigs after BRB feeding.

were 3,4-dihydroxyphenylacetic acid (3,4-DiOH PAA) (homoprotocatechuic acid), 3-(4-hydroxyphenyl) propionic acid (4-OH PPA) (phloretic acid), 3-(3,4-dihydroxyphenyl) propionic acid (3,4-DiOH PPA), 4-OH PAA, 3-OH PAA, and 3-methoxy-4-OH PAA (3-OMe-4-OH PAA) (homovanillic acid) (Table 2). Almost all of them were detected in either the cecum or colon (Fig. 4).

4 Discussion

Early studies indicated that red raspberry contained phenolic acids, which were mainly derivatives of cinnamic acid (CA) including caffeic, *p*-coumaric, and ferulic acids [40, 41]. However, the phenolic acids in BRBs have not been characterized. Our previous study showed that the anthocyanin profile of BRB is quite different from that of red raspberries [13]. This suggested that the phenolic acid profile of BRB may also vary from that of red raspberry. In this study, the phenolic acids in BRB were characterized for the first time. In addition to the three CA derivatives mentioned above, two benzoic acid derivatives, 3,4-OH BA and 3-OH BA were also found as major phenolic acids. 3,4-DiOH BA (protocatechuic acid) was the most abundant phenolic acid in BRB. Notably, protocatechuic acid is a demonstrated chemopreventive agent against various cancers including colon cancer [28, 31 – 37].

These five phenolic acids behaved differently in the GI tract. 3,4-DiOH BA, *p*-coumaric acid, and 3-OH BA were detected in all three sections of GI tract, but their relative amounts in the different sections were different (Fig. 3). In particular, 3-OH BA displayed a roughly 20-fold increase in the colon compared to that in the small intestine or cecum, which indicated that the bulk of 3-OH BA was generated by microflora exclusively in the colon. Caffeic acid and ferulic acid were detected in small intestine and colon, but not in the cecum. Recoveries of 3,4-OH BA and 3-OH BA in the whole GI tract were calculated as 199.9 and 916.8%, respectively, whereas recoveries of the three CA derivatives were 7.0% for *p*-coumaric acid, 37.0% for caffeic acid, and 56.6% for ferulic acid. Benzoic acid derivatives were found to be the major products of intestinal microflora transformation of BRB phenolics. For three CA derivatives, stability may explain their different recoveries in GI tract. Phenolic acid stability seems to be greater for those with more hydroxyl or methoxyl groups.

In addition to these five phenolic acids, six other phenolic acids, which were not present in BRB, were also identified by comparing their retention time and MS data to standards in the GI contents (Table 2). They were considered degradation products or microfloral transformation products of polyphenols found in BRB; four of them were phenylacetic acid (PAA) derivatives, namely, 3,4-DiOH PAA, 4-OH PAA, 3-OH PAA, and 3-OMe-4-OH PAA (homovanillic acid). Two others were 3-phenylpropionic acid (PPA) derivatives: 4-OH PPA (phloretic acid) and 3,4-DiOH PPA. Among the newly formed phenolic acids, 3,4-DiOH PAA was the most abundant one. The amount in the whole GI tract was lower than protocatechuic acid but higher than all other phenolic acids. Homoprotocatechuic acid is another promising chemoprevention agent. It was demonstrated as the only one of the major phenolic acids formed during human microbial fermentation of tea, citrus, and soy flavonoid supplements to have antiproliferative activity [30]. Protocatechuic acid and homoprotocatechuic acid, the two most promising chemoprevention agents accounted for 65% (31.96 in 49.32 µmol) of all phenolic acids in whole GI tract.

In control animals, four phenolic acids were detected in relatively low amounts. Huge variations were observed among these four compounds. Only 4-(4-hydroxyphenyl) propionic acid was found in all three animals. The other three were found in each of the three different animals. However, except for *p*-coumaric acid, the amounts of these in the GI tract were much lower than that which was found in the GI tract of BRB fed animals. It is reasonable to conclude that the sources of phenolic acids detected in GI tract were mostly from BRB.

From this data, it seems the rate and extent of degradation of polyphenols depends on their structures as well as the composition of gut microflora [42]. Cyanidin-based ACNs are the major polyphenols in BRB, followed by ellagitannins and pelargonidin-based ACNs [14] (Hager *et al.*, unpublished data). Considering the extremely high concentration of cyanidin-based ACNs in BRB, they are likely to be the major sources of phenolic acids formed in the gut. Protocatechuic acid has been detected in rat plasma and tissues after administration of cyanidin-3-glucoside [43]. Most recently, protocatechuic acid was found to be the major human metabolite of cyanidin glucosides. Analyses

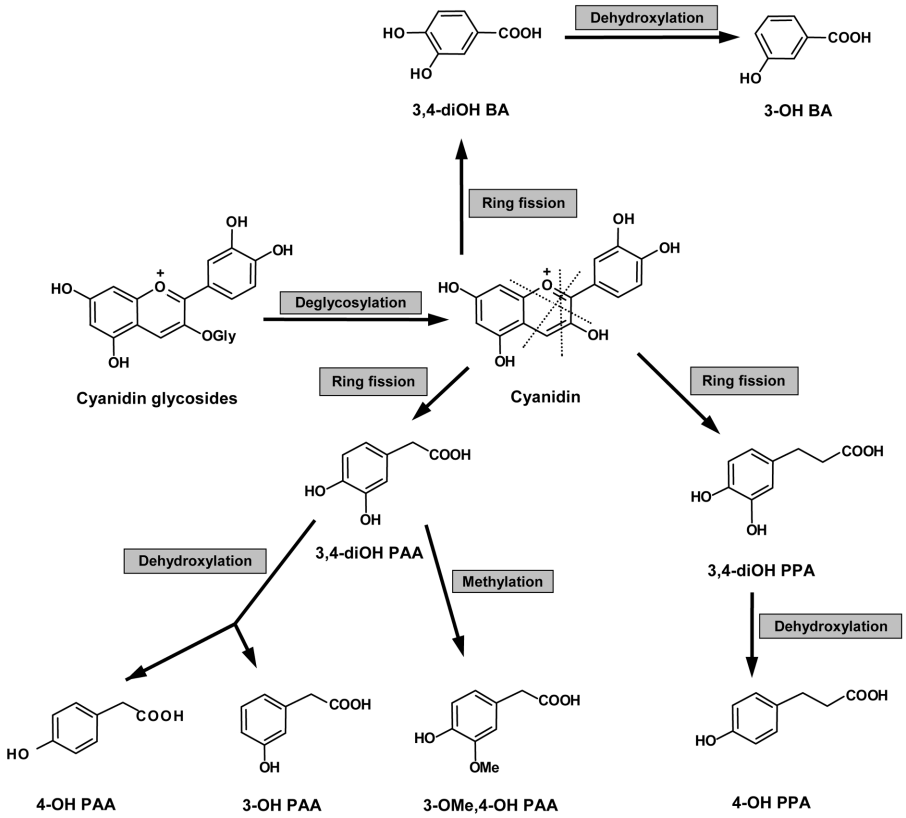


Figure 5. Proposed pathways of phenolic acids generated from cyanidin glycosides by gut microflora.

of fecal samples collected over 24 h from human subjects showed that after ingesting 140 μmol of cyanidin-3-glucoside, recovered amounts of cyanidin glucoside and protocatechuic acids were 0.28 and 41.6 μmol, respectively [44]. One possible explanation is that in the above study, only cyanidin monoglycosides were present. In our previous study [38], we demonstrated that cyanidin monoglycoside (cyanidin-3-glucoside) behaved very differently from cyanidin di- or triglycosides, with cyanidin monoglucosides being very unstable. Similar to findings of Vitaglione *et al*. [44], we found that about 98% of cyanidin-3-glucosides disappeared from the gut in 4 h after feeding BRB. The major degradation products from these unstable monoglycosides were probably protocatechuic acid and its further degradation products. However, except for protocatechuic acid and its further degradation products, we found that several other phenolic acids were also formed. They were apparently formed through different ring fission pathways (Fig. 5). Cyanidin di- and triglycosides are present in BRB and they may be responsible for the formation of the other types of phenolic acids to some extent. Possible degradation pathways are proposed according to published data (Fig. 5) [42]. Furthermore, distributions of these phenolic acids in different sections of GI tract were evaluated for the first time. In order to provide protection against colon cancer, one would hope the compounds would be present in high amounts in the colon. Among the major phenolic acids in colon, we found that 4-OH PAA was the most abundant one, followed by 3,4-diOH PAA, 3-(4-hydroxyphenyl)-propionic acid (4-OH PPA), protocatechuic acid, and 3-OH BA (Figs. 3 and 4). 3,4-diOH PAA and protocatechuic acid are two promising anti-colon cancer agents [28, 30, 35, 37]; whether the others show anticancer properties needs further investigation.

In an attempt to determine the fate of the "missing" ACNs, we studied the phenolic acids and ACNs in the GI tract. The recovery of total ACNs in the whole GI tract was only 41.7% [38]. In this study, the dose of total ACNs was calculated as 1331.0 ± 175.5 μmol. The total phenolic acid in whole GI tract was 49.3 ± 16.4 μmol. Even if we postulate that all the phenolic acids in the GI tract were generated from nonrecovered (considered as being transformed to other forms) ACNs in BRB, the recovery of ACNs as phenolic acids would be only 6.31%. Considering the fact that BRB does contain some phenolic acids and some phenolic acids may be generated in the GI tract from sources other than ACNs, such as ellegitannins, the actual recovery of phenolic acids from ACNs would be even lower. Our results do not support the concept that phenolic acids are the major degradative products of ACNs in GI tract. Nonetheless, some phenolic acids, such as protocatechuic acid [44], still could be major degradation products and exert health effects in the GI tract.

In conclusion, phenolic acids, including those present in BRB or formed by gut microflora, were present in considerable amounts within the GI tract after BRB feeding. Protocatechuic acid and homoprotocatechuic acid were the most abundant compounds. These two phenolic acids, along with others may contribute in part to the chemopreventative effects of BRB against colon cancer. The recovery of all phenolic acids in the GI tract calculated as a percentage of the total dose of ACNs was only 6.3%. This indicated that phenolic acids that we were able to measure were probably not the major degradative products of ACNs in the GI tract. Other possibilities need to be explored to account for the "missing" ACNs.

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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5 References

[1] Casto, B. C., Kresty, L. A., Kraly, C. L., Pearl, D. K., *et al*., Chemoprevention of oral cancer by black raspberries, *Anticancer Res.* 2002, *22*, 4005 – 4015.

[2] Harris, G. K., Gupta, A., Nines, R. G., Kresty, L. A., *et al*., Effects of lyophilized black raspberries on azoxymethane-induced colon cancer and 8-hydroxy-2′-deoxyguanosine levels in the Fischer 344 rat, *Nutr. Cancer* 2001, *40*, 125 – 133.

[3] Kresty, L. A., Morse, M. A., Morgan, C., Carlton, P. S., *et al*., Chemoprevention of esophageal tumorigenesis by dietary administration of lyophilized black raspberries, *Cancer Res.* 2001, *61*, 6112 – 6119.

[4] Stoner, G. D., Wang, L. S., Zikri, N., Chen, T., *et al*., Cancer prevention with freeze-dried berries and berry components, *Semin. Cancer Biol.* 2007, *17*, 403 – 410.

[5] Chen, T., Hwang, H., Rose, M. E., Nines, R. G., *et al*., Chemopreventive properties of black raspberries in *N*-nitrosomethylbenzylamine-induced rat esophageal tumorigenesis: Down-regulation of cyclooxygenase-2, inducible nitric oxide synthase, and c-Jun, *Cancer Res.* 2006, *66*, 2853 – 2859.

[6] Chen, T., Rose, M. E., Hwang, H., Nines, R. G., *et al*., Black raspberries inhibit *N*-nitrosomethylbenzylamine (NMBA)-induced angiogenesis in rat esophagus parallel to the suppression of COX-2 and iNOS, *Carcinogenesis* 2006, *27*, 2301 – 2307.

[7] Huang, C., Li, J., Song, L., Zhang, D., *et al*., Black raspberry extracts inhibit benzo(a)pyrene diol-epoxide-induced activator protein 1 activation and VEGF transcription by targeting the phosphotidylinositol 3-kinase/Akt pathway, *Cancer Res.* 2006, *66*, 581 – 587.

[8] Kresty, L. A., Frankel, W. L., Hammond, C. D., Baird, M. E.,, *et al*., Transitioning from preclinical to clinical chemopreventive assessments of lyophilized black raspberries: Interim results show berries modulate markers of oxidative stress in Barrett's esophagus patients, *Nutr. Cancer* 2006, *54*, 148 – 156.

[9] Lu, H., Li, J., Zhang, D., Stoner, G. D., *et al*., Molecular mechanisms involved in chemoprevention of black raspberry extracts: From transcription factors to their target genes, *Nutr. Cancer* 2006, *54*, 69 – 78.

[10] Reen, R. K., Nines, R., Stoner, G. D., Modulation of *N*-nitrosomethylbenzylamine metabolism by black raspberries in the esophagus and liver of Fischer 344 rats, *Nutr. Cancer* 2006, *54*, 47 – 57.

[11] Clifford, M. N., Scalbert, A., Ellagitannins – nature, occurrence and dietary burden, *J. Sci. Food Agric.* 2000, *80*, 1118 – 1125.

[12] Seeram, N. P., Ellagitannins in human health and disease, *Abstracts of Papers, 232nd ACS National Meeting*, San Francisco, CA, United States, Sept. 10-14, 2006, CELL-090.

[13] Wu, X., Prior, R. L., Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: Fruits and berries, *J. Agric. Food Chem.* 2005, *53*, 2589 – 2599.

[14] Wu, X., Beecher, G. R., Holden, J. M., Haytowitz, D. B., *et al*., Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption, *J. Agric. Food Chem.* 2006, *54*, 4069 – 4075.

[15] Hecht, S. S., Huang, C., Stoner, G. D., Li, J., *et al*., Identification of cyanidin glycosides as constituents of freeze-dried black raspberries which inhibit anti-benzo[a]pyrene-7,8-diol-9,10-epoxide-induced NFkappaB and AP-1 activity, *Carcinogenesis* 2006, *27*, 1617 – 1626.

[16] Coates, E. M., Popa, G., Gill, C. I., McCann, M. J., *et al*., Colon-available raspberry polyphenols exhibit anti-cancer effects on in vitro models of colon cancer, *J. Carcinog.* 2007, *6*, 4.

[17] Kang, S. Y., Seeram, N. P., Nair, M. G., Bourquin, L. D., Tart cherry anthocyanins inhibit tumor development in Apc(Min) mice and reduce proliferation of human colon cancer cells, *Cancer Lett.* 2003, *194*, 13 – 19.

[18] Lala, G., Malik, M., Zhao, C., He, J., *et al*., Anthocyanin-rich extracts inhibit multiple biomarkers of colon cancer in rats, *Nutr. Cancer* 2006, *54*, 84 – 93.

[19] Zhao, C., Giusti, M. M., Malik, M., Moyer, M. P., *et al*., Effects of commercial anthocyanin-rich extracts on colonic cancer and nontumorigenic colonic cell growth, *J. Agric. Food Chem.* 2004, *52*, 6122 – 6128.

[20] Stoner, G. D., Chen, T., Kresty, L. A., Aziz, R. M., *et al*., Protection against esophageal cancer in rodents with lyophilized berries: Potential mechanisms, *Nutr. Cancer* 2006, *54*, 33 – 46.

[21] Scalbert, A., Morand, C., Manach, C., Rémésy, C., Absorption and metabolism of polyphenols in the gut and impact on health, *Biomed. Pharmacother.* 2002, *56*, 276 – 282.

[22] Blaut, M., Schoefer, L., Braune, A., Transformation of flavonoids by intestinal microorganisms, *Int. J. Vitam. Nutr. Res.* 2003, *73*, 79 – 87.

[23] Tamura, M., Hirayama, K., Itoh, K., Role of intestinal flora on the metabolism, absorption, and biological activity of dietary flavonoids, *Biosci. Microflora* 2003, *22*, 125 – 131.

[24] Aura, A. M., Martin-Lopez, P., O'Leary K, A., Williamson, G. *et al*., In vitro metabolism of anthocyanins by human gut microflora, *Eur. J. Nutr.* 2005, *44*, 133 – 142.

[25] Jenner, A. M., Rafter, J., Halliwell, B., Human fecal water content of phenolics: The extent of colonic exposure to aromatic compounds, *Free Radic. Biol. Med.* 2005, *38*, 763 – 772.

[26] Femia, A. P., Caderni, G., Buzzigoli, C., Cocca, E., *et al*., Effect of simple phenolic compounds on azoxymethane-induced aberrant crypt foci in rat colon, *Nutr. Cancer* 2001, *41*, 107 – 110.

[27] Kawamori, T., Tanaka, T., Kojima, T., Suzui, M., *et al*., Suppression of azoxymethane-induced rat colon aberrant crypt foci by dietary protocatechuic acid, *Jpn. J. Cancer Res.* 1994, *85*, 686 – 691.

[28] Tanaka, T., Kojima, T., Suzui, M., Mori, H., Chemoprevention of colon carcinogenesis by the natural product of a simple phenolic compound protocatechuic acid: Suppressing effects on tumor development and biomarkers expression of colon tumorigenesis, *Cancer Res.* 1993, *53*, 3908 – 3913.

[29] Yi, W., Fischer, J., Krewer, G., Akoh, C. C., Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis, *J. Agric. Food Chem.* 2005, *53*, 7320 – 7329.

[30] Gao, K., Xu, A., Krul, C., Venema, K., *et al*., Of the major phenolic acids formed during human microbial fermentation of tea, citrus, and soy flavonoid supplements, only 3,4-dihydroxyphenylacetic acid has antiproliferative activity, *J. Nutr.* 2006, *136*, 52 – 57.

[31] Lin, H. H., Chen, J. H., Huang, C. C., Wang, C. J., Apoptotic effect of 3,4-dihydroxybenzoic acid on human gastric carcinoma cells involving JNK/p38 MAPK signaling activation, *Int. J. Cancer* 2007, *120*, 2306 – 2316.

[32] Zheng, Q., Hirose, Y., Yoshimi, N., Murakami, A., *et al*., Further investigation of the modifying effect of various chemopreventive agents on apoptosis and cell proliferation in human colon cancer cells, *J. Cancer Res. Clin. Oncol.* 2002, *128*, 539 – 546.

[33] Mori, H., Tanaka, T., Sugie, S., Yoshimi, N., *et al*., Chemoprevention by naturally occurring and synthetic agents in oral, liver, and large bowel carcinogenesis, *J. Cell. Biochem. Suppl.* 1997, *27*, 35 – 41.

[34] Hirose, Y., Tanaka, T., Kawamori, T., Ohnishi, M., *et al*., Chemoprevention of urinary bladder carcinogenesis by the natural phenolic compound protocatechuic acid in rats, *Carcinogenesis* 1995, *16*, 2337 – 2342.

[35] Tanaka, T., Kojima, T., Kawamori, T., Mori, H., Chemoprevention of digestive organs carcinogenesis by natural product protocatechuic acid, *Cancer* 1995, *75*, 1433 – 1439.

[36] Tanaka, T., Kawamori, T., Ohnishi, M., Okamoto, K., *et al*., Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis by dietary protocatechuic acid during initiation and postinitiation phases, *Cancer Res.* 1994, *54*, 2359 – 2365.

[37] Tanaka, T., Kojima, T., Kawamori, T., Yoshimi, N., *et al*., Chemoprevention of diethylnitrosamine-induced hepatocarcinogenesis by a simple phenolic acid protocatechuic acid in rats, *Cancer Res.* 1993, *53*, 2775 – 2779.

[38] Wu, X., Pittman, H. E., IIIrd, Prior, R. L., Fate of anthocyanins and antioxidant capacity in contents of the gastrointestinal tract of weanling pigs following black raspberry consumption, *J. Agric. Food Chem.* 2006, *54*, 583 – 589.

[39] Wu, X., Pittman, H. E., Prior, R. L., Pelargonidin is absorbed and metabolized differently than cyanidin after marionberry consumption in pigs, *J. Nutr.* 2004, *134*, 2603 – 2610.

[40] Torronen, R., Hakkinen, S., Karenlampi, S., Mykkanen, H., Flavonoids and phenolic acid in selected berries, *Cancer Lett.* 1997, *114*, 191 – 192.

[41] Mosel, H. D., Herrmann, K., Phenolics of fruits. IV. Phenolics of blackberries and raspberries and their changes during development and ripening of the fruits, *Z. Lebensm. Unters. Forsch.* 1974, *154*, 324 – 327.

[42] Rechner, A. R., Smith, M. A., Kuhnle, G., Gibson, G. R., *et al*., Colonic metabolism of dietary polyphenols: Influence of structure on microbial fermentation products, *Free Radic. Biol. Med.* 2004, *36*, 212 – 225.

[43] Tsuda, T., Horio, F., Osawa, T., Absorption and metabolism of cyanidin 3-O-beta-D-glucoside in rats, *FEBS Lett.* 1999, *449*, 179 – 182.

[44] Vitaglione, P., Donnarumma, G., Napolitano, A., Galvano, F. *et al*., Protocatechuic acid is the major human metabolite of cyanidin-glucosides, *J. Nutr.* 2007, *137*, 2043 – 2048.

