INCREASING DOSES OF BLUEBERRY POLYPHENOLS ALTERS COLONIC METABOLISM AND CALCIUM ABSORPTION IN OVARIECTOMIZED RATS

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Abstract

Scope: Blueberries are rich sources of bioactive polyphenols that may provide health benefits when consumed regularly, leading to their increased marketing as dietary supplements. However, the metabolic changes associated with consuming concentrated doses of purified polyphenols, as may be present in dietary supplements, are unknown, especially when considering the colonic metabolites formed. This study aimed to evaluate the pharmacokinetics of high doses of purified blueberry polyphenols.

Methods and results: Five-month old, ovariectomized Sprague-Dawley rats were acutely dosed with purified blueberry polyphenols (0, 75, 350, and 1000 mg total polyphenols/kg bw) and ^{45}Ca to measure calcium absorption. Blood and urine were collected for 48h after dosing and phenolic metabolites measured via UPLC-MS/MS. The most prominent metabolites were colonically generated cinnamic and hippuric acids. Smaller amounts of other phenolic acids, flavonols, and anthocyanins were also detected. Most metabolites followed a dose-response relationship, though several showed saturated absorption. Maximal metabolite concentrations were reached within 12h for a majority of compounds measured, while some (e.g., hippuric acid) peaked up to 24h post-dosing. Calcium absorption was significantly increased in the highest dose group (p=0.03).

Conclusion: These results indicate that increased doses of blueberry polyphenols induce changes in intestinal phenolic metabolism and increase calcium absorption.

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Keywords

blueberry; polyphenol; colonic metabolism; pharmacokinetics; calcium absorption

1. INTRODUCTION

Fruit and vegetable derived polyphenols may have beneficial health effects by reducing the risk of developing many chronic diseases while also improving cardiovascular and neurocognitive health.[1–4] Blueberries are a particularly rich source of polyphenols, containing high amounts of anthocyanins, flavonols, and chlorogenic acid,[5] and demonstrating many of these same health benefits.[6]

To elucidate the connection between blueberry polyphenols and their health benefits, an understanding of their bioavailability and which forms are most biologically active is necessary. To that end, much recent work has been performed on the absorption, metabolism, and excretion of polyphenols. The metabolism of these compounds, in particular, has been challenging, as polyphenols undergo phase II metabolism (methylation, glucuronidation, and/or sulfation) within small intestinal epithelial cells before being absorbed or effluxed back into the intestinal lumen.[7–9] Small intestine-derived phase II metabolites are poorly absorbed (<2% for many classes of polyphenols),[10, 11] as evidenced by low plasma and urine concentrations (i.e., low C_{max} values) and rapid excretion (T_{max} 0.5–2h).[12] For years, researchers surmised that because the bioavailability of polyphenols and their *in vivo* residence time was low, they had a limited ability to exert beneficial health effects.[13]

However, in recent years, the emergence of the gut microbiome as an important and active part of the metabolic transformation of dietary phenolics has offered a new perspective. A large majority of orally ingested polyphenols reach the lower intestine intact, allowing them to interact extensively with the gut microbiota, in a bidirectional manner.[9, 14] Diets high in polyphenols have been reported to shift the composition of the gut microbial communities, often to a putatively healthier state, while also being efficiently metabolized and absorbed. [15] During this process, polyphenols are catabolized to smaller molecular weight phenolic acids that are more extensively absorbed (up to 10-fold higher) than in the small intestine (i.e., higher C_{max} and greater AUC) and persist longer in systemic circulation (i.e., later T_{max}).[8, 16] This not only increases the overall bioavailability of polyphenols, but raises the possibility that the colonic catabolites may be driving the observed health benefits.[10] Thus, much recent work has focused on understanding these catabolic pathways and fully characterizing the colonic metabolites produced from these interactions.

As knowledge of microbial metabolism of polyphenols continues to expand, there are several areas that remain understudied, including the full pharmacokinetics of colonic catabolites and the dose-response effects of elevated doses as may be found in dietary supplements that may contain up to 100x the amounts typically consumed in the Western diet.[17, 18] This is critical information for designing clinical trials. Most pharmacokinetic studies on polyphenols are completed within 8h of dosing, which may not be long enough to detect a number of colonic catabolites that may be produced as the ingested dose takes

~20h to traverse the full length of the GI tract.[19] Additionally, given the rapid rise – from <2% to 30% of U.S. adults – in popularity of polyphenol-rich herbal and botanical dietary supplements over the past 25 years,[20–23] expanding the range of doses studied is critical to understand the full scope of the metabolism of these compounds.

We evaluated the metabolism and dose-response of blueberry polyphenols. We accomplished this by quantitating urinary and plasma phenolic metabolites for 48h after an acute dose of blueberry polyphenols over a large range of doses. Given that women aged 51–70y are the most frequent consumers of botanical dietary supplements and are also susceptible to menopause-related bone loss,[20] we chose to use the ovariectomized (OVX) rat model for this study because it mimics hormonal changes and bone loss occurring in postmenopausal women.[24] With recent evidence suggesting that blueberry polyphenols may mitigate menopause-associated bone loss and reduce the incidence of osteoporosis in aging females by increasing calcium absorption,[25–27] we also evaluated the influence of blueberry polyphenols on calcium absorption. We hypothesized that the colonic catabolites of blueberry polyphenols would be absorbed much later than and to a much greater extent than small intestinal metabolites and would exhibit a dose-dependent increase in calcium absorption.

2.   EXPERIMENTAL SECTION

2.1   Chemicals/Materials and vendors

Commercial standards of cyanidin-3-O-glucoside chloride, delphindin-3-O-glucoside chloride, malvidin-3-O-glucoside chloride, gallic acid, caffeic acid, ferulic acid, ethyl gallate, taxifolin, chlorogenic acid, hippuric acid, 3-hydroxyhippuric acid, 4-hydroxybenzaldehyde, isovanillin, p-anisic acid, 4-hydroxyphenylacetic acid, 3-hydroxyphenylpropionic acid, 3-methoxyphenylacetic acid, isovanillic acid, homovanillic acid, 3-hydroxy-4-methoxyphenylpropionic acid, syringic acid, quercetin, myricetin, chlorogenic acid, quercetin-3-O-glucuronide, protocatechuic acid, p-coumaric acid, catechin, epicatechin, 4-methoxyquercetin, and quercetin-3-O-glucoside as well as sodium carbonate and Folin and Ciocalteu's reagent (2N) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Caffeic acid glucuronide was supplied by Synthose (Concord, Ontario, Canada). LC-MS grade solvents, including methanol, water, ACN, and formic acid as well as trace metal grade concentrated nitric acid were purchased from Thermo Fisher Scientific (Waltham, MA, USA). ^{45}Ca was purchased from PerkinElmer (Waltham, MA, USA). EcoLite (+) scintillation cocktail was purchased from MP Biomedicals (Santa Ana, CA, USA).

2.2   Animal protocols

2.2.1   Study overview.—The study design is illustrated in Figure 1. Upon arrival, animals were maintained on a polyphenol-free diet during a 1-week stabilization. Animals were then randomized to treatment groups (n=8/gp), with all groups (except control) switched to a 5% blueberry diet for the remainder of the study. After 1 week on the blueberry diet, animals were dosed with blueberry phenolics and ^{45}Ca, with blood and

urine collected over 48h for pharmacokinetics. Animals were then sacrificed via CO_2 asphyxiation, both femurs harvested, and ovariectomy verified by visual inspection.

2.2.2 Animal care.—Animal experiments were conducted in adherence to Purdue University Animal Care and Use Committee (PACUC) guidelines, following an approved protocol (1612001508). Thirty-two 5-month old, virgin, ovariectomized, female Sprague Dawley rats were purchased from Envigo (Indianapolis, IN, USA) and individually housed in stainless steel, wire-bottom cages in a temperature and humidity-controlled room with a 12h light/dark cycle and ad libitum access to food and water.

2.2.3 Diets.—Polyphenol-free diets were based on the AIN-93M diet, using corn oil in place of soybean oil to prevent confounding from soy isoflavones. The 5% blueberry chow diet (5% BB) incorporated lyophilized blueberry powder (Wild Blueberry Association of North America, Old Town, ME, USA) into the polyphenol-free base diet. To account for the fiber and sugar content of berries, these components were adjusted in the base diet to maintain isocaloricity with control group. Diets were prepared by Research Diets (New Brunswick, NJ, USA). To minimize phenolic degradation, all diets were stored at −20°C and changed daily for each animal.

2.2.4 Dosing Regimen.—VitaBlue Pure American Blueberry Extract, containing 28.8% total phenolics (w/w), was donated by FutureCeuticals (Momence, IL, USA) for use in this study. Oral gavage slurries were prepared with water at the following doses: 0, 75, 350, and 1000 mg phenolics/kg bw. The 75 mg/kg bw dose corresponds to an adult human consuming approximately 1–2 cups of fresh blueberries per day (i.e., a "dietary dose", calculated using the FDA's rat to human conversion factor).[28] The 350 and 1000 mg/ kg bw doses group were several-fold higher to mimic higher concentrations as may be present in dietary supplements. Immediately prior to receiving the oral gavage, each animal underwent a one-time, 8h fast.

2.2.5 Jugular catheter surgery, dosing, and blood/urine collection.—Two days prior to beginning pharmacokinetics, jugular catheters were placed in the right jugular vein of all animals as previously described.[29] Animals were anesthetized with isoflurane and given bruprinex (i.p., 0.1 mg/kg bw) to minimize pain. Catheters were flushed with heparinized saline (20 units/mL) every 12h to keep them patent.

Blood was collected via the implanted catheter immediately prior to (baseline) and every 6h after oral gavage until sacrifice. In total, 9 blood draws were collected (0, 6, 12, 18, 24, 30, 36, 42, and 48h). Plasma was separated from whole blood via immediate centrifugation in heparinized microfuge tubes at 4°C and 3500 *g* for 10 min. Plasma was acidified to a final concentration of 0.1% formic acid, flushed with nitrogen, and frozen at −80°C until analysis.

Urine was collected in 12h increments throughout the study. To capture baseline phenolic metabolites, 2×12h urine collections (0–12 and 12–24h) were obtained from all animals in the 24h leading up to the jugular catheter surgery. Then, after gavage, 4×12h urine collections (0–12, 12–24, 24–36, and 36–48h) were obtained. Collected urine was

centrifuged to remove particulates, acidified to a final concentration of 0.1% formic acid, blanketed with nitrogen, and frozen at −80°C until analysis.

2.2.6   [45]Ca absorption.—Immediately after gavaging animals with blueberry phenolics, six animals per group were given a second oral gavage containing 20 μCi [45]Ca and 100 mg calcium acetate (equivalent to ~25% daily calcium intake to replace calcium missed during fasting) in water. The remaining two animals in each group were also given a 500 μL oral gavage containing 100 mg calcium acetate and water, but were dosed with 10 μCi [45]Ca via jugular catheter. At sacrifice, both femurs were harvested, manually cleaned to remove soft tissue, wrapped in saline soaked gauze, and stored at −80°C until analysis.

2.3   Polyphenol analyses

2.3.1   Extraction and purification of phenolics in starting materials.—

Lyophilized whole blueberries, VitaBlue Pure blueberry extract, animal diets, and individual gavage doses were extracted in triplicate, as described elsewhere.[30] Extracts were resolubilized with 2% formic acid in water and purified via solid phase extraction (SPE) using Oasis HLB 1cc extraction cartridges (Waters, Milford, MA, USA), as described elsewhere. [30]

2.3.2   Extraction and purification of phenolic metabolites.—Phenolics in plasma and urine samples were extracted via SPE using the strataX, polymeric reversed phase microelution 96 well plate with a capacity of 2 mg/well (Phenomenex, Torrence, CA, USA). Wells were preconditioned with 200 μL 1% formic acid in methanol followed by 200 μL 1% formic acid in water. Samples were then loaded as a mixture of the biological sample (100 μL plasma or 50 μL urine), 200 μL 1% formic acid in water, and 20 μL of 50 μM taxifolin as an extraction efficiency control. Samples were washed with 2×200 μL 0.1% formic acid in water, and then dried under nitrogen for 30 minutes. Samples were eluted with 100 μL 0.1% formic acid in methanol into a 96-well plate (350 uL Acquity 96-well plate, Waters, Milford, MA, USA). All steps were aided by gentle, positive pressure nitrogen gas delivered via Waters Positive Pressure-96 Processor. To the eluate was added 20 μL 50 μM ethyl gallate as a volume control. Eluted samples were immediately capped with a pre-slit silicon mat (Cap-mat 96 well 7 mm round plug pre-slit silicone/PTFE, Waters) and frozen at −80°C until analysis.

2.3.3   Quantification of total phenolics in starting materials.—Total phenolics were quantified in crude extracts via the Folin method and corrected for water-soluble interferences (e.g., sugars and ascorbic acid) as described elsewhere.[31, 32]

2.3.4   Quantitation of phenolics via UPLC-MS/MS.—After purification via SPE, individual phenolics were quantified via UPLC-MS/MS using a Waters UPLC Acquity I Class system equipped with a TQD detector. Samples were injected and phenolics separated using an Acquity BEH C18 column (2.1 um, 1.7 mm id x 50 mm) with a flow rate of 0.5 mL/min. Samples were eluted using a biphasic gradient of solvent A (0.1% formic acid in acetonitrile) and solvent B (2.0% formic acid in water (for ESI+ mode) or 0.1% formic acid in water (for ESI-)) as follows: 0 min, 100% B; 0.5 min, 94% B; 2 min, 91% B; 3 min,

87% B; 4.5 min, 65% B; 5.2 min, 100% B; 6 min, 100% B. MS conditions were as follows: capillary voltage, 0.5 kV; probe temp, 150°C; source temp, 600°C; desolvation gas flow, 1000 L/hr; cone gas flow, 50 L/hr.

Identification and quantification of each compound was based on authentic standards, using calibration curves ranging from 0.001–100μM. When standards were not available, compounds (especially phase II metabolites) were confirmed based on retention times and the presence of multiple ion transitions consistent with each compound.[33] A complete list of phenolic compounds and metabolites measured, including corresponding MRMs and standards used for quantitation, is shown in Table SI-1.

2.4   Fractional calcium absorption

Total femoral calcium deposition was used to determine fractional calcium absorption, as described elsewhere.[34, 35] Briefly, each femur was ashed in a muffle furnace for 5d at 600°C, dissolved overnight in concentrated nitric acid, and diluted to 25 mL with ultrapure water. 1 mL of the resulting solution was mixed with 15 mL Ecolite in a scintillation vial and ^{45}Ca quantified by liquid scintillation counting (Tri-Carb 2910 TR Liquid Scintillation Analyzer, PerkinElmer, Waltham, MA, USA). Fractional absorption was calculated as a ratio of oral:i.v. ^{45}Ca in femurs as previously described.[35, 36]

2.5   Statistics

Statistics were completed using SAS (SAS Institute, Raleigh, NC). When data were not normal, appropriate transformations were performed before analysis to ensure normality. Outliers were detected and removed using Tukey's method. Plasma AUC was calculated using the trapezoidal method and qualitatively observed levels for C_{max} and T_{max} reported. Comparisons for total excretion and AUC were made via one-way ANOVA, while individual points on pharmacokinetic curves were analyzed via two-way ANOVA (factors: time and dose). Post hoc analyses were carried out with Tukey's HSD test and significance defined as $p < 0.05$ unless otherwise noted. Guidance in SAS coding was provided by the Statistical Consulting Service at Purdue University.

3.   RESULTS AND DISCUSSION

3.1   Phenolic profiles of raw materials, rat diets, and gavage doses.

3.1.1   Phenolic profiles of raw materials.—Two commercially available raw materials were used in this study: lyophilized blueberry powder (FD) and concentrated blueberry polyphenol extract (CE). Both materials were derived from commercially available wild blueberries and contained 3.75% and 28.8% (w/w) total polyphenols, respectively (Table 1). These raw materials were used to create the rat chow diets and gavage doses.

To further characterize these materials, a total of 30 individual phenolics were quantified. As shown in Table 1, anthocyanins were the most prevalent class of phenolics present, comprising nearly half of the total phenolics. In both FD and CE, malvidin glycosides were the most prevalent anthocyanins, followed by petunidin glycosides. Other classes of

phenolics, including phenolic acids, flavan-3-ols, and flavonols, were quantified. The most prevalent of these were chlorogenic acid and quercetin species, which collectively accounted for ~20% of total phenolics assayed by LC-MS. Comparatively lower levels of benzoic acids and flavan-3-ols were observed.

3.1.2   Phenolic profiles of rat diets.—The rat chow diets were created by incorporating either 0% or 5% of the FD berries into the AIN-93M diet (denoted PPF and 5% BB diet, respectively). During the manufacturing of these diets, thermal and oxidative degradation occur, resulting in phenolic losses. In preliminary experiments with several vendors, we have observed significant differences in phenolic content and potential losses (data not shown). For this study, we chose the manufacturer that demonstrated minimal losses, though we note ~25% of total phenolics were lost in the creation of our 5% BB diet (Table 1). When comparing the relative amounts of individual phenolics in the 5% BB diet with those in the FD berries, the losses appear to occur evenly across all compounds measured, indicating that the phenolic composition of the 5% BB diet is similar to the FD berries.

No significant difference in food consumption or food efficiency ratio was found between groups (data not shown). Based on total diet consumption while on the 5% BB diet, rats consumed 50–60 mg total polyphenols/kg bw/d from their diets (data not shown). This is nearly as much as the low dose received via oral gavage.

3.1.3   Phenolic profiles of gavage doses.—Gavage doses were created using the CE, with target doses of 75, 350, and 1000 mg total polyphenol/kg bw. As shown in Table 1, our actual doses were quite close to these targets. And, as expected, the relative amounts of individual phenolics in the gavage doses mirrored the amounts in the CE.

3.2   Urinary excretion of phenolic metabolites.

3.2.1   Summary of urinary phenolic excretion.—A total of 43 phenolic metabolites were detected in the urine, including 17 anthocyanins, 19 phenolic acids, 2 hippuric acids, and 5 flavonols. A majority of the metabolites demonstrated dose-dependent excretion and were maximally detected within 12h of dosing (Table 2). Blueberry polyphenols were extensively metabolized, with <5% of total urinary metabolites being detected in their unmetabolized forms. The most prominent metabolites were *trans*-cinnamic acids, followed by hippuric acids, with smaller amounts of other phenolic acids, flavonols, and anthocyanins also detected (Figure 2). These metabolites exhibited a dose-dependent shift in excretion, with higher doses showing increased proportions of cinnamic and phenylpropionoic acids and a concomitant decrease in the proportion of hippuric acids.

3.2.2   Urinary excretion of phenolic acids.—Of the phenolic acids quantitated in the urine, 7 were maximally excreted within 12h after dosing, with pharmacokinetic curves similar to the one shown in Figure 3a. For the other phenolic acids, 4 exhibited similar excretion in both the 0–12h and 12–24h urine collections (exemplified in Figure 3c), while the 3 remaining metabolites did not have a clear peak excretion time. (C_{max} and T_{max} values

can be found in Table 2; Pharmacokinetic curves for all phenolic acids can be found in the Supporting Information, Figures SI-1–14.)

Cinnamic acid derivatives accounted for more than half of all urinary metabolites observed. These metabolites can come from a variety of parent compounds, though their main precursors are thought to be anthocyanins [9] and chlorogenic acids.[37, 38] Additionally, cinnamic acid derivatives have been observed in other studies using polyphenol-rich berries. In a recent clinical study of cranberries, for example, a total of 24 cinnamic acid derivatives were observed, including ferulic and caffeic acid glucuronides and sulfates. The authors note that the cinnamic acids are most likely derived from both chlorogenic acid and anthocyanins present in the cranberries.[39] Similar results were observed after blueberry intake in healthy adults, as cinnamic acid derivatives (especially glucuronidated and sulfated forms) were prominent metabolites.[40–42] However, in contrast to our results, in which cinnamic acid derivatives accounted for up to 75% of total urinary metabolites, cinnamic acid derivatives in these studies accounted for <20% of total urinary metabolites. This may be due to a number of factors, though the most likely are the large amounts of hippuric acids observed in these studies (see below) or the lack of authentic standards for all cinnamic acid derivatives, which can significantly alter the quantitation of these metabolites.[8]

Other phenolic acids, including benzoic acids, phenylacetic acids (PAA), and phenylpropionic acids (PPA), are commonly reported as colonic metabolites of polyphenols, though their total contribution to phenolic metabolism varies widely. In previous studies of blueberries, phenolic acid metabolites varied considerably, with different studies reporting benzoic acids,[40, 43] PAA,[41, 42] or benzaldehydes and PAA [44] as the most prominent phenolic acid metabolites. In contrast to these studies, we found higher amounts of PPA in urine than PAA or benzoic acids.

3.2.3 Urinary excretion of flavonoids.—Flavonol metabolites observed in urine followed similar excretion patterns observed for phenolic acids, with 3 maximally excreted within 12h of dosing and 1 having similar excretion in the 0–12h and 12–24h urine collections (Figure SI-15–18).

Anthocyanins and anthocyanin metabolites (i.e., glucuronidated and sulfated anthocyanins) were also detected in the urine. Of those quantitated, 12 were maximally excreted within 12h of dosing (exemplified in Figure 3b), 3 had similar excretions during the 0–12h and 12–24h time points (exemplified in Figure 3d), and 2 did not exhibit a discernable peak excretion. (See Table 2 for C_{max} and T_{max} as well as Figures SI-19–35 for pharmacokinetic urinary excretion.) The relative excretion of unmetabolized 3-O-glucoside and 3-O-galactoside anthocyanins vs. the phase II glucuronidated and sulfated forms varied based on the aglycone (Figure SI-36). Cyanidins and malvidins were observed almost exclusively as unmetabolized 3-O-glucoside and 3-O-galactoside anthocyanins, while delphinidins and peonidins were extensively metabolized to the glucuronidated and sulfated forms; petunidins were a mixture of both unmetabolized and metabolized forms.

Although observed in small amounts, anthocyanin and flavonol derivatives were detected in the urine. This is noted in other studies,[40, 44] though it is rare for others to report the

presence of unmetabolized anthocyanin glucosides and galactosides in the urine, especially in the dose-dependent manner observed here. However, despite the lack of reporting on these metabolites in the literature, it is known that small amounts of unmetabolized anthocyanins are present in systemic circulation, though the mechanism for their absorption is currently unknown.[45]

3.2.4 Urinary excretion of hippuric acids.—The two hippuric acid metabolites did not follow the excretion patterns exhibited by other metabolites. These metabolites were maximally excreted during the 12–24h urine collection (Figures 3e and SI-37–38). There was no difference in the amount of hippuric acid excreted after dosing with either the medium or high dose.

Hippuric acids were the second most prominent urinary metabolite observed in the current study. Hippuric acid and its derivatives are frequently noted as a major metabolite in studies of polyphenol metabolism, and they can be derived in large quantities from flavanols,[46] anthocyanins,[9, 47] and chlorogenic acids.[37, 38] In studies on polyphenol-rich berries, hippuric acids are often observed as the most prominent metabolite, accounting for up to half of total metabolites quantitated.[41, 48–51] We found hippuric acid to be a major metabolite, but as shown in Figures 3e and SI-37–38, there appears to be a saturation effect, whereby urinary excretion of hippuric acid is no different between the medium and high dose groups. As a result, hippuric acid accounts for a smaller portion of total polyphenol metabolites as the dose increases (Figure 2). This may indicate that hippuric acid production is reaching a point of saturation by the medium dose. It could also be the result of hippuric acid being one of the end stage metabolites formed in the microbial metabolism of polyphenols, meaning that, at higher doses, the total polyphenol content may be too high for the colonic microbes to fully metabolize prior to fecal excretion (see section 3.2.5 for details).

3.2.5 Metabolic sinks in colonic metabolism of blueberry polyphenols.—A summary of colonic metabolism of polyphenols is shown in Figure 4. Although not commonly discussed in relation to polyphenol metabolism, the ideas of rate limiting steps in metabolism, competition for metabolites, and "metabolic sinks" where various metabolites may accumulate are common to many metabolic pathways. Given the large quantities of cinnamic acid sulfates and glucuronides, it appears that the phase II metabolism of cinnamic acids occurs more readily than the conversion to PPA. However, once converted to PPA, the metabolites appear to be rapidly metabolized through the rest of the chain, being converted to different phenolic acid metabolites and accumulating as hippuric acids. As the dose increases, the decreasing proportion of hippuric acids (accompanied by the relative increases in PPA and cinnamic acids) suggests that this conversion is the rate limiting step. This may also slow the production at earlier steps of the process, which would cause an accumulation of other forms (in this case, PPA). Additionally, this notion of competition and diversion of metabolites at the cinnamic acid step is supported by the timing of peak production of metabolites. As shown in Table 2, cinnamic acids and the phase II metabolites are observed most prominently within the first 12h after dosing, while other phenolic acids (PPA, PAA, and benzoic acids) were generally observed somewhat later, with hippuric acids not peaking

until 12–24h after dosing. This indicates that cinnamic acids were formed in large amounts and were largely diverted to phase II metabolism rather than being further metabolized into phenolic and hippuric acids. However, of those that were shuttled through the phenolic acid pathway, it appears that most went all the way to the terminal hippuric acid step until the saturation point was reached.

3.3 Plasma pharmacokinetics.

A total of 16 metabolites (6 anthocyanins, 6 phenolic acids, 3 flavonols, and hippuric acid) were detected in the plasma (Table 3). Anthocyanin responses were low and inconsistent, which is not surprising given their rapid metabolism and short half-lives *in vivo*. Of the remaining 10 metabolites, 7 were quantitated, with most demonstrating a dose-dependent response and exhibiting peak plasma concentrations 6h after dosing (Table 3 and Figures SI-39–45). Most plasma metabolites had T_{max} values consistent with urinary excretion. Metabolites that had early T_{max} values in the urine were detected early in the plasma, and later appearing metabolites tended to appear later in both urine and plasma.

3.4 Calcium absorption.

To link phenolic absorption to physiological effects, fractional calcium absorption was measured using ^{45}Ca, an isotopic tracer. When comparing all groups to control (using Dunnett's test), fractional calcium absorption was significantly higher in the highest dose group ($p = 0.034$, Figure 5). This relationship was demonstrated for both the right and left femurs when analyzed separately and together. Additionally, when comparing all groups to each other (using Tukey's test), fractional calcium absorption was nearly significantly higher for the high dose compared to control group ($p = 0.058$) and high dose compared to medium dose ($p = 0.056$).

Maintaining bone health throughout the lifespan is key to preventing osteoporosis, and this can be accomplished through either increasing calcium absorption, increasing bone formation, or inhibiting bone resorption.[52] Our results indicate that calcium absorption is only increased with the highest dose of blueberry polyphenols; the control, low, and medium dose groups exhibit ^{45}Ca absorption levels that are typically observed for this animal model (~35%).[35, 53, 54] Our results agree with a previous study of grape polyphenols in rats, where, at low doses of polyphenols, calcium absorption was not acutely altered by administration of a grape-enriched diet.[55] These studies indicate that polyphenols consumed at dietary doses do not acutely alter calcium absorption.

Although grape and blueberry polyphenols did not to alter calcium absorption at low doses, a recent study found that repeated administration of low doses of blueberry polyphenols increased bone calcium retention.[56] Low doses of polyphenols may improve bone quality through pathways other than increased calcium absorption. This should direct future mechanistic studies aimed at understanding the effects of polyphenols on bone health.

4. CONCLUDING REMARKS

The strength and innovation of this study is the unique perspective it provides on the dose-dependent shift in the production of colonic metabolites after an acute dose of

purified blueberry polyphenols as may be present in dietary supplements. Given the growing popularity and use of botanical dietary supplements, our approach of using a concentrated phenolic extract at elevated doses is directly relevant to dietary supplement consumption. By focusing on the pharmacokinetics and colonic metabolism of elevated doses, this study provides a novel glimpse into metabolic shifts that may occur when consuming botanical dietary supplements. Additionally, by not only measuring dose-dependent phenolic metabolism but also measuring the influence on calcium absorption, we have demonstrated that shifts in phenolic metabolism can have physiological effects.

However, there were also several limitations to the current study. First, the lack of authentic standards for some of the metabolites may have caused inaccurate estimates of the actual amounts present, as certain metabolites (e.g., sulfates) ionize more and may easily be overestimated.[8] This is a challenge that many studies of phenolic metabolism face, as commercially available standards are rarely found for specific metabolites and are challenging to synthesize. Second, the time points chosen to sample and measure plasma metabolites likely missed peak plasma concentrations for many metabolites, as many phenolics are absorbed and excreted quickly (T_{max} <2h).[12] Our original hypothesis was that many of the colonic metabolites would be observed at later times in the plasma, leading us to select later times for blood collection. Our hypothesis appears to hold true for the urinary appearance of metabolites, but we were unable to detect many of these metabolites in appreciable amounts in the plasma. Finally, using metabolic cages to collect urine samples from animals presents two important challenges in measuring phenolic metabolites: oxidation and contamination. Because urine was collected continuously in 12h increments, samples were exposed to oxygen for several hours and small food particles and debris were observed. We judged this to be the best method for collecting urine from the animals and, because none of the major phenolic metabolites observed are present in the chow diet, this had a minimal impact on the results and their interpretation.

In conclusion, we have shown that increasing doses of blueberry polyphenols result in dose-dependent shifts in phenolic metabolite profiles and increased calcium absorption. Taken together, these results indicate that high doses of blueberry polyphenols may alter gut function. Future studies examining the consequences of these metabolic changes with repeated dosing will help determine if these changes have systemic effects. Repeated dosing studies with high doses of purified polyphenols mimics typical dietary supplement consumption and will help elucidate the safety and potential health consequences – both positive and negative – of this consumption modality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations:

5% BB	5% blueberry chow diet
CE	concentrated blueberry polyphenol extract
FD	lyophilized blueberry powder
MRM	multiple reaction monitoring
OVX	ovariectomized
PAA	phenylacetic acid
PPA	phenylpropionic acid
PPF	polyphenol-free chow diet
SPE	solid phase extraction

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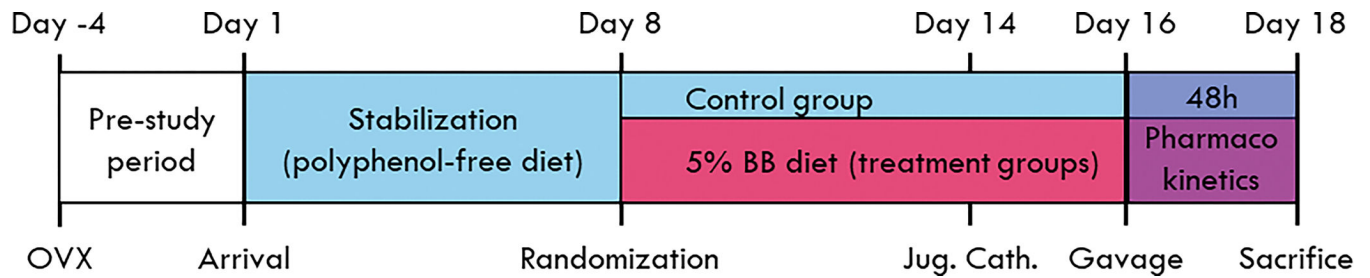
Figure 1 –.
Study design and timeline. All animals were ovariectomized (OVX) by the vendor prior to shipping (day -4). Upon arrival (day 1), animals were stabilized for one week on a polyphenol-free diet. Randomization took place on day 8, with control animals remaining on the polyphenol-free diet and all others placed on a 5% blueberry diet for the remainder of the study. Jugular catheters were placed on day 14, with animals receiving blueberry phenolics and [45]Ca via oral gavage two days later. Blood and urine were collected for 48h until sacrifice.
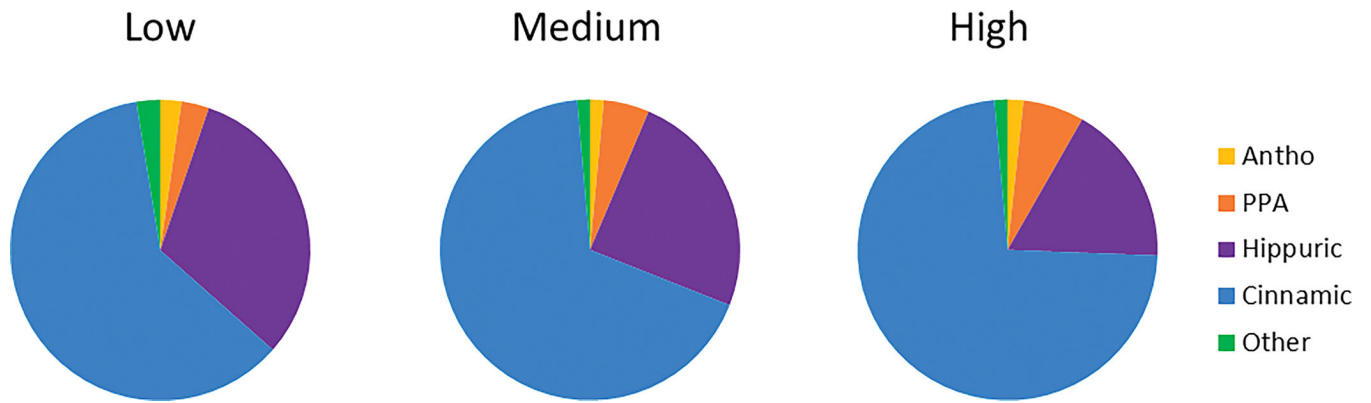
Figure 2 –.

Dose dependent excretion of phenolics over 48h. For all doses, cinnamic acids were the predominant metabolite found in urine, followed by hippuric acids. As dose increased, hippuric acids decreased as a percentage of total metabolites excreted, while cinnamic acids and phenylpropionic acids increased. (Antho = sum of all anthocyanins excreted; PPA = sum of phenylpropionic acids and phase-II metabolites excreted; Hippuric = sum of hippuric acids excreted; Cinnamic = sum of cinnamic acids and phase-II metabolites excreted; Other = sum of benzoic acids, phenylacetic acids, and flavonols excreted.)
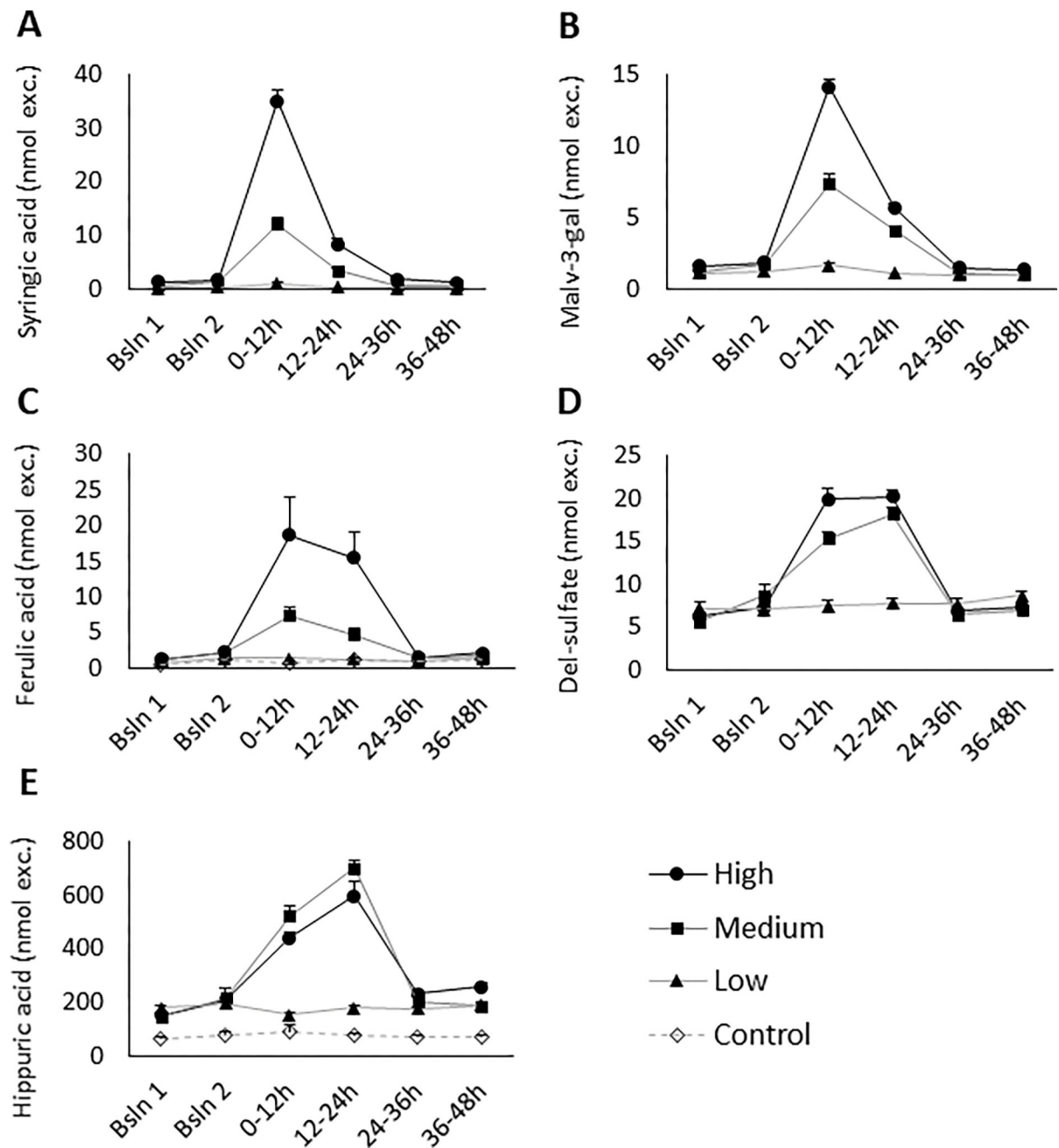
Figure 3 –.

Urinary excretion pharmacokinetics of selected metabolites. Most phenolic acid, flavonol, and anthocyanin metabolites demonstrated a dose-response relationship and were maximally excreted within 12h of dosing (exemplified by syringic acid (A) and malvidin-3-O-galactoside (B)), while others had similar levels of excretion at both the 0–12h and 12–24h urine collections (e.g., ferulic acid (C) and delphinidin sulfate (D)). Hippuric acid (E), one of the last metabolites formed in colonic metabolism, exhibited maximal excretion during the 12–24h urine collection and appeared to have saturated absorption at the medium dose. Data shown as mean ± SEM. Bsln 1 = Baseline 0–12h; Bsln 2 = Baseline 12–24h.
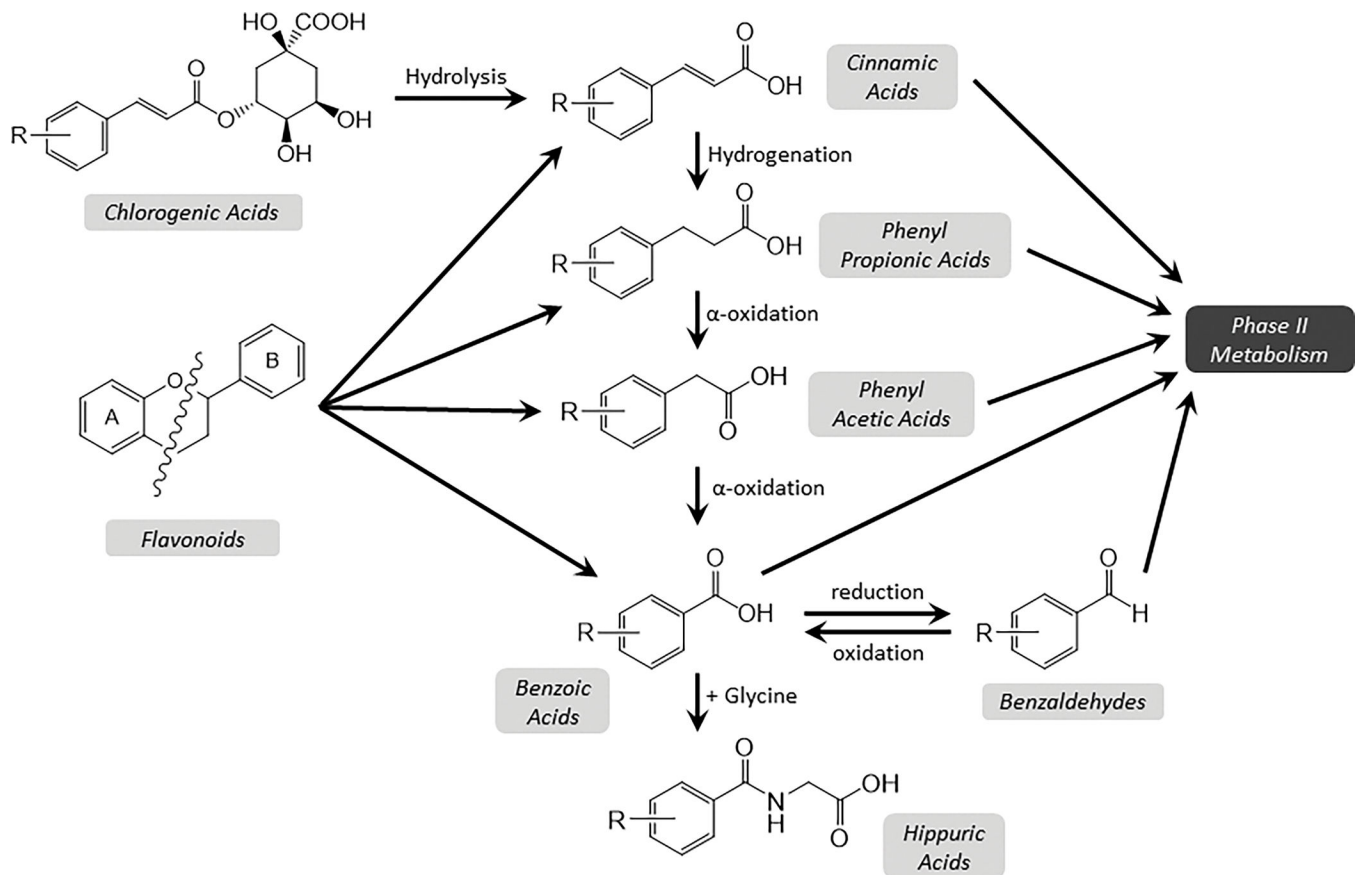
Figure 4 –.

Summary of colonic catabolism of major blueberry polyphenols. Chlorogenic acids are hydrolyzed to cinnamic acids, while the heterocyclic ring of flavonoids (anthocyanins and flavonols in blueberries) is cleaved, producing two, smaller molecular weight phenolic acids from the A and B rings. These smaller phenolic acids can then be further metabolized via glucuronidation or sulfation (phase II metabolism) or to other phenolic acids. Hippuric acids are formed by conjugation with glycine, and are the terminal step in the metabolic chain. Various metabolites within each family are formed by different substitutions on the benzene ring (represented by –R).
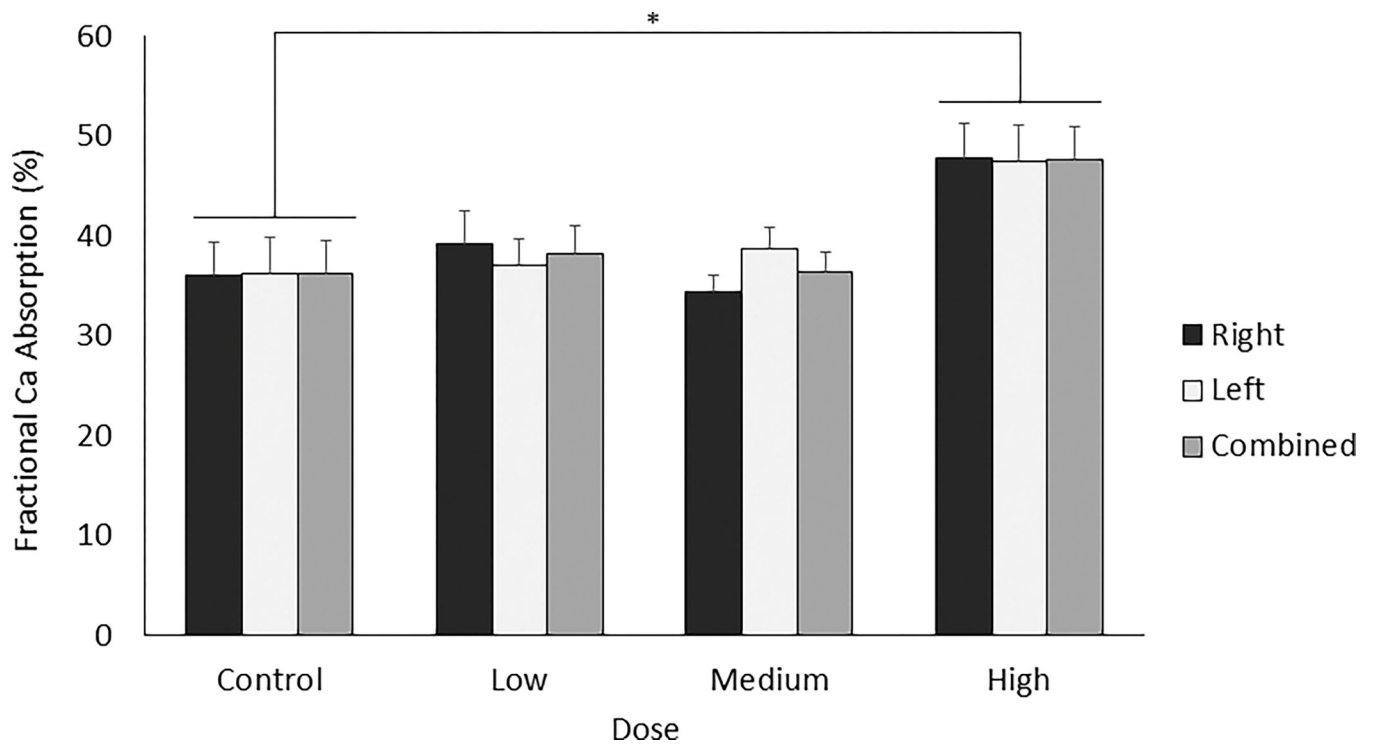
Figure 5 –.
Fractional ^{45}Ca absorption. Fractional calcium absorption was significantly higher in the high dose group for both the right and left femurs separately and when analyzed together. *p<0.05 significantly different from respective control, using Dunnett's test. Data shown as mean ± SEM.

Table 1 –

Phenolic content of raw materials, rat diets, and gavage doses. [a]

Polyphenol	Raw Materials		Rat Diets		Gavage Doses			
	FD	CE	PPF	5% BB	Control	Low	Medium	High
Anthocyanins								
Cyanidins								
Arabinoside	48.5 ± 7.19	227 ± 14.2	nd	1.85 ± 0.47	nd	0.63 ± 0.14	3.59 ± 1.03	6.98 ± 1.18
Galactoside	102 ± 16.5	581 ± 52.8	nd	3.92 ± 1.11	nd	1.54 ± 0.35	8.60 ± 2.70	16.0 ± 3.14
Glucoside	102 ± 16.8	271 ± 24.4	nd	3.97 ± 0.98	nd	0.74 ± 0.16	4.20 ± 1.18	8.15 ± 1.32
Delphinidins								
Arabinoside	31.1 ± 4.51	484 ± 28.1	nd	1.07 ± 0.24	nd	1.26 ± 0.28	8.03 ± 2.01	15.6 ± 2.62
Galactoside + Glucoside	58.6 ± 7.72	662 ± 35.3	nd	2.43 ± 0.45	nd	1.78 ± 0.26	11.8 ± 2.72	23.4 ± 3.98
Malvidins								
Arabinoside	144 ± 22.5	2313 ± 95.3	nd	5.44 ± 1.19	nd	6.33 ± 1.12	38.4 ± 8.72	79.2 ± 11.7
Galactoside	237 ± 24.3	3976 ± 153	nd	11.6 ± 1.22	nd	10.7 ± 0.78	76.2 ± 9.22	169 ± 13.2
Glucoside	319 ± 40.7	3248 ± 136	nd	15.6 ± 1.52	nd	8.75 ± 0.54	63.7 ± 6.73	142 ± 12.1
Peonidins								
Arabinoside	42.0 ± 6.64	202 ± 13.7	nd	1.61 ± 0.42	nd	0.58 ± 0.13	3.19 ± 0.91	6.24 ± 1.06
Galactoside	24.5 ± 3.9	180 ± 18.3	nd	0.95 ± 0.27	nd	0.49 ± 0.11	2.58 ± 0.81	4.84 ± 0.94
Glucoside	42.2 ± 6.29	164 ± 10.8	nd	1.72 ± 0.36	nd	0.45 ± 0.08	2.65 ± 0.62	5.27 ± 0.82
Petunidins								
Arabinoside	48.0 ± 7.47	847 ± 65.6	nd	1.84 ± 0.48	nd	2.34 ± 0.57	13.6 ± 3.75	26.4 ± 4.38
Galactoside	78.9 ± 12.1	1814 ± 170	nd	3.07 ± 0.88	nd	4.75 ± 1.13	27.4 ± 7.70	51.8 ± 9.02
Glucoside	132 ± 20.0	946 ± 105	nd	5.12 ± 1.23	nd	2.59 ± 0.61	14.6 ± 4.25	27.8 ± 4.99
Phenolic Acids								
Benzoic Acids								
Gallic acid	0.04 ± 0.07	9.8 ± 2.43	nd	trace	nd	0.083 ± 0.018	0.44 ± 0.05	0.86 ± 0.25
Protocatechuic acid	0.85 ± 0.17	13 ± 3.05	nd	0.07 ± 0.01	nd	0.047 ± 0.007	0.26 ± 0.06	0.51 ± 0.19
Cinnamic Acids								
Caffeic acid	0.51 ± 0.2	32.5 ± 7.4	nd	0.27 ± 0.07	nd	0.085 ± 0.022	0.44 ± 0.13	0.73 ± 0.23
Chlorogenic acid	593 ± 65.9	1916 ± 165	nd	21.9 ± 3.61	nd	5.21 ± 0.72	26.8 ± 5.07	52.1 ± 9.08

Polyphenol	Raw Materials		Rat Diets			Gavage Doses		
	FD	CE	PPF	5% BB	Control	Low	Medium	High
Ferulic acid	4.48 ± 1	97.2 ± 8.18	nd	0.58 ± 0.12	nd	0.27 ± 0.05	1.39 ± 0.26	2.67 ± 0.61
Feruloylquinic acid	16.2 ± 2.3	60.3 ± 5.75	nd	0.6 ± 0.14	nd	0.18 ± 0.03	0.86 ± 0.15	1.64 ± 0.27
Flavan-3-ols								
Catechin	8.91 ± 1.44	43.8 ± 5.68	nd	0.39 ± 0.06	nd	0.083 ± 0.023	0.45 ± 0.05	0.79 ± 0.19
Epicatechin	5.91 ± 0.74	13.6 ± 3.1	nd	0.22 ± 0.04	nd	0.025 ± 0.009	0.12 ± 0.02	0.20 ± 0.08
Gallocatechin	2.93 ± 0.51	3.2 ± 0.58	nd	0.1 ± 0.02	nd	0.0065 ± 0.002	0.034 ± 0.004	0.059 ± 0.024
Epigallocatechin	nd	30.1 ± 7.58	nd	nd	nd	0.091 ± 0.031	0.36 ± 0.09	0.63 ± 0.18
Flavonols								
Myricetin	2.68 ± 0.45	38.8 ± 5.19	nd	0.1 ± 0.03	nd	0.11 ± 0.04	0.49 ± 0.16	0.86 ± 0.21
Kaemperol	trace	trace	nd	trace	nd	trace	trace	trace
Galactoside + Glucoside	53.9 ± 8.2	194 ± 34	nd	1.78 ± 0.58	nd	0.46 ± 0.13	2.24 ± 0.89	3.48 ± 1.01
Quercetin	11.7 ± 2.23	951 ± 93.1	nd	1.12 ± 0.29	nd	2.24 ± 0.71	11.7 ± 3.47	21.4 ± 4.62
Galactoside + Glucoside	181 ± 24.9	1095 ± 108	nd	8.35 ± 1.58	nd	3.01 ± 0.56	14.9 ± 3.94	28.2 ± 5.53
Rutin	82.7 ± 13.5	245 ± 29	nd	3.63 ± 0.81	nd	0.71 ± 0.11	3.67 ± 0.77	7.64 ± 1.10
Total Phenolics (via Folin assay)	*3747 ± 68.1*	*28824 ± 1608*	*nd*	*141 ± 11.6*	*nd*	*75.3 ± 3.30*	*342 ± 16.1*	*1011 ± 11.7*

FD = freeze dried whole blueberries; CE = concentrated blueberry phenolic extract; PPF = polyphenol-free diet; nd = not detected; trace = compound detected, but below LOQ.

[a] Raw materials and rat diets shown in mg phenolic/100 g dw; gavage doses shown in mg phenolic/kg bw. Data are comprised of three analytical replicates and presented as mean ± SD.

Table 2 –

Total urinary excretion of phenolic metabolites for 48h after gavage.[a]

	Total Urinary Excretion (nmol)				Qualitative T_{max} [b]				C_{max} (nmol excreted)		
	Control	Low	Medium	High	Control	Low	Medium	High	Low	Medium	High
Anthocyanins											
Cyanidin											
Galactoside	nd	0.19 ± 0.04 [w]	0.41 ± 0.08 [x]	0.68 ± 0.08 [y]	-	np	0–12h	0–12h	np	0.19 ± 0.04	0.46 ± 0.09
Glucoside	nd	0.17 ± 0.03 [w]	0.31 ± 0.08 [x]	0.41 ± 0.04 [y]	-	np	0–24h	0–12h	np	0.11 ± 0.05	0.22 ± 0.04
Glucuronide	nd	nd	0.05 ± 0.02 [w]	0.13 ± 0.05 [x]	-	-	0–12h	0–12h	-	0.02 ± 0.01	0.09 ± 0.04
Total Cyanidins	-	*0.36*	*0.77*	*1.22*							
Delphinidin											
Galactoside	nd	0.76 ± 0.06 [w]	2.08 ± 0.21 [x]	2.81 ± 0.23 [y]	-	np	0–12h	0–12h	np	0.98 ± 0.20	1.74 ± 0.22
Glucoside	nd	0.79 ± 0.07 [w]	1.94 ± 0.19 [x]	2.00 ± 0.29 [x]	-	np	0–24h	0–24h	np	0.81 ± 0.14	0.94 ± 0.22
Glucuronide	nd	4.21 ± 0.92 [w]	12.7 ± 2.18 [x]	31.4 ± 1.33 [y]	-	np	0–12h	0–12h	np	6.35 ± 1.60	20.8 ± 5.1
Sulfate	nd	31.8 ± 5.83 [w]	46.9 ± 6.01 [x]	54.4 ± 4.23 [x]	-	np	0–24h	0–24h	np	18.2 ± 2.4	20.2 ± 2.4
Total Delphinidins	-	*37.5*	*63.6*	*90.6*							
Malvidin											
Galactoside	nd	4.75 ± 0.44 [w]	13.5 ± 2.51 [x]	22.7 ± 1.30 [y]	-	np	0–12h	0–12h	np	7.36 ± 2.00	14.2 ± 1.3
Glucoside	nd	6.06 ± 0.66 [w]	14.3 ± 0.88 [x]	19.7 ± 1.02 [y]	-	np	0–12h	0–12h	np	6.57 ± 0.57	10.7 ± 0.9
Total Malvidins	-	*10.8*	*27.8*	*42.4*							
Peonidin											
Galactoside	nd	0.03 ± 0.01 [w]	0.08 ± 0.01 [x]	0.14 ± 0.02 [y]	-	np	0–12h	0–12h	np	0.04 ± 0.01	0.09 ± 0.02
Glucoside	nd	0.05 ± 0.01 [w]	0.08 ± 0.02 [x]	0.14 ± 0.02 [y]	-	np	0–12h	0–12h	np	0.03 ± 0.01	0.09 ± 0.02
Glucuronide	nd	2.12 ± 0.83 [w]	3.01 ± 1.01 [w,x]	3.38 ± 1.27 [x]	-	np	np	np	np	np	np
Sulfate	nd	0.59 ± 0.17	0.64 ± 0.13	0.58 ± 0.08	-	np	np	np	np	np	np
Total Peonidins	-	*2.79*	*3.83*	*4.24*							
Petunidin											
Galactoside	nd	0.44 ± 0.09 [w]	1.05 ± 0.30 [x]	2.49 ± 0.32 [y]	-	np	0–12h	0–12h	np	0.49 ± 0.23	1.73 ± 0.38
Glucoside	nd	0.44 ± 0.08 [w]	0.90 ± 0.25 [x]	1.47 ± 0.17 [y]	-	np	0–12h	0–12h	np	0.37 ± 0.14	0.94 ± 0.19

	Total Urinary Excretion (nmol)				Qualitative T_{max} [b]				C_{max} (nmol excreted)		
	Control	Low	Medium	High	Control	Low	Medium	High	Low	Medium	High
Glucuronide	nd	0.69 ± 0.25 w	1.81 ± 0.47 x	5.63 ± 0.56 y	-	np	0–12h	0–12h	np	0.92 ± 0.24	3.49 ± 0.46
Sulfate	nd	0.32 ± 0.09 w	0.43 ± 0.08 x	0.44 ± 0.09 w,x	-	np	12–24h	12–24h	np	0.16 ± 0.03	0.15 ± 0.02
Total Petunidins	-	*1.89*	*4.19*	*10.0*							
Total Anthocyanins	**-**	**53.3**	**100.2**	**148.5**							
Phenolic Acids											
Benzaldehydes (BALD)											
4-OH-BALD	nd	nd	trace	trace	-	-	-	-	-	-	-
3-OH-4-OMe-BALD	nd	nd	trace	trace	-	-	-	-	-	-	-
Total BALD	*-*	*-*	*-*	*-*							
Benzoic Acids (BzA)											
Gallic acid	nd	nd	trace	trace	-	-	-	-	-	-	-
3-OH-4-OMe-BzA	nd	nd	trace	trace	-	-	-	-	-	-	-
Syringic acid	nd	1.90 ± 0.32 w	17.0 ± 2.89 x	48.2 ± 4.49 y	-	0–12h	0–12h	0–12h	1.03 ± 0.30	12.2 ± 3.0	35.1 ± 6.0
BzA sulfate	11.2 ± 1.72 w,x	7.96 ± 1.20 y	12.6 ± 1.75 x	9.62 ± 1.40 w,y	np	np	np	np	np	np	np
BzA glucuronide	1.45 ± 0.30 w	1.14 ± 0.16 x	2.25 ± 0.21 y	1.95 ± 0.31 y	np	np	12–24h	12–24h	np	0.85 ± 0.17	0.76 ± 0.17
Total BzA	*12.7*	*11.0*	*31.9*	*59.8*							
Phenyl Acetic Acids (PAA)											
4-OH-PAA	3.08 ± 0.81 w	0.95 ± 0.20 x	1.87 ± 0.52 y	1.22 ± 0.23 x	np	0–24h	0–24h	0–24h	0.30 ± 0.12	0.84 ± 0.38	0.51 ± 0.16
3-OMe-PAA	nd	nd	nd	trace	-	-	-	-	-	-	-
3-OH-4-OMe-PAA	nd	33.3 ± 8.61 w	34.4 ± 6.10 w	28.6 ± 3.82 w	-	np	np	np	np	np	np
Total PAA	*3.08*	*34.3*	*36.3*	*29.8*							
Phenyl Propionic Acids (PPA)											
3-OH-PPA	nd	57.2 ± 11.4 x	328 ± 49.6 y	579 ± 67.1 z	-	0–12h	0–12h	12–24h	25.4 ± 6.9	177 ± 24	275 ± 34
3-OH-4-OMe-PPA	nd	2.00 ± 1.01 w	4.77 ± 1.99 x	6.76 ± 5.20 x	-	0–12h	12–24h	12–24h	0.82 ± 0.58	2.69 ± 1.83	2.48 ± 2.69
Total PPA	*nd*	*59.2*	*333*	*586*							
trans-Cinnamic acids											
Caffeic acid	nd	4.37 ± 0.99 w	8.78 ± 3.77 x	8.52 ± 1.73 x	np	np	0–12h	0–12h	np	3.18 ± 1.64	3.97 ± 1.38

	Total Urinary Excretion (nmol)				Qualitative T_{max} [b]				C_{max} (nmol excreted)		
	Control	Low	Medium	High	Control	Low	Medium	High	Low	Medium	High
Caffeic acid sulfate	1.30 ± 0.03 [x]	9.55 ± 4.01 [x]	15.4 ± 6.56 [x]	18.1 ± 2.69 [x]	np	np	0–12h	0–12h	np	6.43 ± 4.57	9.74 ± 0.78
Caffeic acid glucuronide	443 ± 28.4 [w]	402 ± 61.8 [w]	677 ± 97.7 [x]	897 ± 163 [y]	-	np	0–12h	0–12h	np	261 ± 50	379 ± 134
Ferulic acid	nd	5.76 ± 1.26 [w]	22.6 ± 4.34 [x]	38.8 ± 0.81 [y]	np	np	0–24h	0–24h	np	11.7 ± 3.0	29.5 ± 5.9
Ferulic acid sulfate	4.01 ± 1.03 [w]	5.17 ± 1.70 [w]	15.2 ± 3.47 [x]	37.8 ± 13.7 [y]	np	np	0–12h	0–12h	np	7.41 ± 3.52	18.7 ± 14.9
Ferulic acid glucuronide	469 ± 82.8 [w]	936 ± 147 [x]	3611 ± 450 [y]	4772 ± 674 [y]	-	np	0–12h	0–12h	np	2069 ± 354	2943 ± 302
Chlorogenic acid	nd	40.1 ± 10.4 [w]	256 ± 13.5 [x]	360 ± 61.1 [y]	-	np	0–24h	0–12h	np	66.1 ± 23.4	238 ± 67
Total Cinnamic Acids	*917*	*1403*	*4606*	*6122*							
Total Phenolic Acids	**935**	**1507**	**5007**	**6798**							
Hippuric Acids											
Hippuric acid	304 ± 70.2 [w]	706 ± 66.8 [x]	1611 ± 154 [y]	1528 ± 193 [y]	np	np	12–24h	12–24h	np	700 ± 78	598 ± 146
3-OH-hippuric acid	3.94 ± 1.01 [w]	18.4 ± 3.14 [x]	64.2 ± 13.4 [y]	42.6 ± 9.1 [z]	np	np	12–24h	12–24h	np	31.8 ± 8.1	26.3 ± 7.6
Total Hippuric Acids	**308**	**724**	**1675**	**1571**							
Flavonols											
Myricetin	nd	trace	trace	trace	-	-	-	-	-	-	-
Quercetin	nd	11.5 ± 4.47 [w]	19.7 ± 7.26 [x]	26.9 ± 2.24 [x]	-	0–12h	0–12h	0–12h	3.88 ± 1.46	10.1 ± 7.4	11.5 ± 4.0
Quercetin glucuronide	nd	0.49 ± 0.07 [w]	1.48 ± 0.18 [x]	2.70 ± 0.19 [y]	-	np	0–12h	0–12h	np	0.72 ± 0.14	1.83 ± 0.28
4-OMe-quercetin	nd	0.60 ± 0.25 [w]	1.39 ± 0.32 [x]	2.49 ± 0.61 [y]	-	0–12h	0–12h	12–24h	0.19 ± 0.08	0.75 ± 0.33	1.29 ± 1.02
Me-quercetin glucuronide	nd	0.82 ± 0.16 [w]	2.93 ± 0.58 [x]	7.17 ± 1.31 [y]	-	np	0–12h	0–12h	np	1.61 ± 0.43	4.16 ± 0.58
Total Flavonols	**nd**	**13.4**	**25.5**	**39.3**							
Total Phenolics Excreted	**1243**	**2298**	**6808**	**8557**							

nd = not detected; trace = compound detected, but below LOQ; np = no peak observed.

[a] Note: only phenolics detected via chromatography presented here. Data are comprised of biological replicates (n=6–8) and presented as mean ± SD. Superscript letters indicate significant differences between doses (p<0.05 with Tukey's HSD test).

[b] Qualitative T_{max} represents the urine collection during which excretion of the metabolite was greatest; np indicates no peak excretion (i.e., consistent excretion at all time points), 0–12h indicates peak excretion within 12h after dosing, 12–24h indicates peak excretion 12–24h after dosing, and 0–24h indicates peak excretion was similar at 0–12h and 12–24h.

Table 3 –

Phenolics detected in plasma.[a]

	Plasma AUC (uM*h)				Qualitative T_{max}[b]				Cmax (uM)			
	Control	Low	Medium	High	Control	Low	Medium	High	Control	Low	Medium	High
Phenolic Acids												
Syringic acid	nd	nd	nd	trace	-	-	-	-	-	-	-	-
3-OH-PPA	nd	nd	2.46 ± 0.95 w	8.00 ± 2.22 x	-	-	12h	12h	-	-	0.161 ± 0.092	0.507 ± 0.107
Caffeic gcnd	nd	0.36 ± 0.06 w	0.55 ± 0.11 x	0.92 ± 0.20 y	-	np	6h	6h	-	np	0.021 ± 0.008	0.084 ± 0.020
Ferulic acid	nd	nd	nd	trace	-	-	-	-	-	-	-	-
Ferulic sulf	1.88 ± 0.26 w	6.16 ± 1.50 x	6.33 ± 3.04 x	9.12 ± 2.89 x	np	np	6h	6h	np	np	0.262 ± 0.169	0.825 ± 0.317
Ferulic gcnd	nd	5.78 ± 0.75 w	7.17 ± 1.05 x	11.3 ± 0.72 y	-	np	6h	6h	-	np	0.217 ± 0.073	0.797 ± 0.141
Hippuric acid	3.19 ± 0.36 w	25.8 ± 3.08 x	47.7 ± 5.13 y	74.3 ± 7.54 z	np	np	12h	24–36h	np	np	1.535 ± 0.510	2.065 ± 0.806
Flavonols												
Quer gcnd	nd	0.32 ± 0.01 w	0.34 ± 0.05 w	0.57 ± 0.14 x	-	np	6h	6h	-	np	0.008 ± 0.002	0.023 ± 0.005
4-OMe-quer	nd	nd	nd	trace	-	-	-	-	-	-	-	-
Me-quer gcnd	nd	3.27 ± 0.29 w	3.56 ± 0.73 w	4.31 ± 0.20 x	-	np	np	6–18h	-	np	np	0.104 ± 0.007

nd = not detected; trace = compound detected, but below LOQ; np = no peak observed; gcnd = glucuronide; sulf = sulfate; quer = quercetin; OH = hydroxy; PPA = phenyl propionic acid; OMe = methoxy.

[a] Several anthocyanins (delphinidin glucuronide, delphinidin sulfate, peonidin glucuronide, petunidin-3-O-galactoside, petunidin-3-O-glucoside, and petunidin glucuronide) were detected at trace levels in medium and high doses, though responses were inconsistent and below LOQ. Data are comprised of biological replicates (n=6–8) and presented as mean ± SD. Superscript letters indicate significant differences between doses (p<0.05 with Tukey's HSD test).

[b] Qualitative Tmax represents the blood collection during which plasma levels of the metabolite was greatest; time ranges demonstrate similar amounts quantitated at multiple time points.