

**CONSUMING HIGH DOSES OF BLUEBERRY POLYPHENOLS IS SAFE
BUT INDUCES DOSE-DEPENDENT SHIFTS IN METABOLISM**

by
Dennis P Cladis

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THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Kee Hong Kim, Chair

Department of Food Science

Dr. Mario Ferruzzi

Department of Food, Bioprocessing and Nutrition Science, North Carolina State University

Dr. Jason Cannon

Department of Health Sciences

Dr. Lavanya Reddivari

Department of Food Science

Approved by:

Dr. Arun Bhunia

*Dedicated to those who believed in me,
even when I couldn't believe in myself,
especially my wife, Mary Ann,
my parents, Peter and Jane,
and my mentors, Connie and Mario.*

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette transporter
ADME	Absorption, distribution, metabolism, and excretion
A/G	Albumin:Globulin ratio
ALKP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of variance
AUC	Area under the curve
BALD	Benzaldehyde
BB	Lyophilized blueberry dose group
BMD	Bone mineral density
BSC	Botanical Safety Consortium
BUN	Blood urea nitrogen
BzA	Benzoic acid
C _{max}	Maximum concentration achieved in plasma or urine
CE	Concentrated blueberry polyphenol extract
COMT	Catechol- <i>O</i> -methyltransferase
CVD	Cardiovascular disease
DRI	Dietary Reference Intake
DSHEA	Dietary Supplements Health and Education Act
ECG	Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Administration
EGCG	Epigallocatechin gallate
EOS	Eosinophils
EPIC	European Prospective Investigation into Cancer and Nutrition
FD	Freeze-dried blueberry powder
FDA	Food and Drug Administration
FER	Food efficiency ratio
FITC	Fluorescein isothiocyanate
GI	Gastrointestinal
GTE	Green tea extract
HCT	Hematocrit
HESI	Health and Environmental Science Institute
HGB	Hemoglobin
ICPH	International Conference on Polyphenols and Health
i.p.	intraperitoneal
ISANH	International Society of Antioxidants in Nutrition and Health
LOD	Limit of detection
LOQ	Limit of quantitation
LYMPH	Lymphocytes
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MONO	Monocytes

MRM	Multiple reaction monitoring
MS	Mass spectrometry
nd	not detected
NG	Non-gavaged control group
NIH	National Institutes of Health
NIEHS	National Institute of Environmental Health Science
NOAEL	No observed adverse effect level
<i>O</i> -DMA	<i>O</i> -demthylangolensin
OECD	Organisation for Economic Co-operation and Development
-OH	Hydroxyl group
-OMe	Methoxyl group
OVX-SD	Ovariectomized Sprague Dawley
PAA	Phenyl acetic acid
PACUC	Purdue Animal Care and Use Committee
PPA	Phenyl propionic acid
PPF	Polyphenol free chow diet
RBC	Red blood cell
RDA	Recommended Dietary Allowance
RDW	Red blood cell distribution width
RETIC #	Reticulocyte number
SD	Standard deviation
SEG	Segmented neutrophils
SEM	Standard error of the mean
SERM	Selective estrogen receptor modulator
SGLT	Sodium-glucose cotransporter
SPE	Solid phase extraction
SULT	Sulfotransferase
T _{1/2}	Half-life
T _{max}	Time at which C _{max} is observed
trace	compound detected, but below LOQ
UL	Upper Limit
UPLC	Ultraperformance high pressure liquid chromatography
USDA	United States Department of Agriculture
WBANA	Wild Blueberry Association of North America
WBC	White blood cells
5% BB	5% blueberry chow diet

ABSTRACT

Fruit and vegetable derived polyphenols have been linked with many health benefits. In light of this, many consumers are seeking to increase their intake of polyphenols, with many turning to dietary supplements that contain concentrated doses of purified polyphenols. However, the safety of this consumption modality is not known, nor are the dose-dependent metabolic changes that may be present, especially when considering colonically generated phenolic metabolites. Using blueberry polyphenols as a model, we explored these phenomena in a rat model. Animals were dosed with blueberry polyphenols at levels up to 20 times what would be consumed in 1-2 servings of whole blueberries in an adult human. In the first study, animals were acutely dosed with blueberry polyphenols and urine and plasma pharmacokinetics measured. In the second study, animals were repeatedly dosed for 90d, with urinary metabolites monitored throughout the study and a complete necropsy performed following standard guidelines. In both studies, metabolite excretion patterns were similar: cinnamic acids accounted for a majority of the observed metabolites, followed by hippuric acids and then phenylpropionic acids (PPA). A dose-dependent shift in metabolite production was observed; as the dose increased, the relative amounts of PPA increased while hippuric acids decreased. No adverse or toxic effects were found, and, though there were several statistically significant differences in toxicological endpoints, all measured parameters remained in the normal range for these animals and thus were not deemed biologically significant. These results indicate that high doses of blueberry polyphenols, as may be present in dietary supplements, are safe for consumption. These results also demonstrate dose-dependent shifts in metabolism that may impact gut function and affect the health benefits derived from blueberry polyphenols.

CHAPTER 1. REVIEW OF LITERATURE

Fruits and vegetables have long been lauded as part of a healthy diet. Countless epidemiological and interventional studies have demonstrated that consistently consuming fruits and vegetables throughout the lifespan is instrumental in maintaining good health and delaying or preventing the development of many chronic diseases.¹ And, although fruit and vegetable consumption has been connected to many health benefits, the most promising and consistent benefits have been observed in connection with cardiovascular, neurocognitive, and gastrointestinal health.²

These health benefits may be due to a number of factors, but increasing evidence attributes these benefits to the bioactive polyphenols present in fruits and vegetables.³⁻⁵ *In planta*, polyphenols are secondary metabolites that serve multiple functions, including defense against predators (both herbivores and microbial pathogens) and attracting pollinators and seed-dispersal animals.⁶

Polyphenols have experienced a curious history since their initial discovery in the 1930s.⁷ They were first connected to a potential nutritional effect in the 1960s, when it was discovered that they could bind to proteins in foods. This led to the hypothesis that polyphenols may be “anti-nutrients”, as they may have an adverse effect on protein bioavailability.^{8, 9} By the 1990s, polyphenols were found to be excellent antioxidants *in vitro*, and the narrative changed: now polyphenols were viewed as a new health panacea that might relieve excessive oxidative stress and the negative health consequences arising from that stress.^{7, 8} However, by the early 2000s, researchers began to realize that polyphenols are rapidly metabolized by intestinal epithelial cells and are rarely observed intact in systemic circulation, making the antioxidant hypothesis dubious *in vivo*.⁷ This began a new wave of research into small intestinal metabolism and what forms of polyphenols became bioavailable. During this time, the presence of many phase-II metabolites of polyphenols were observed and reported in the blood and these were believed to be responsible for the health benefits observed in epidemiological trials.⁷ Because the small intestinal bioavailability of most polyphenols was extremely low and circulating metabolites were not observed at high enough levels *in vivo* to exert the health benefits observed *in vitro*, researchers began to question whether the phase-II metabolites were responsible for the health benefits. In the past 10 years, as the gut microbiome has become better appreciated as a major part of metabolism

and health, many researchers have begun looking at the colonic metabolism of polyphenols. This has again shifted the field, as many now report that the colonic metabolism of polyphenols produces smaller molecular weight phenolic acids that are far more bioavailable than their small intestinal counterparts.⁷ These compounds are able to reach systemic circulation at levels that may be capable of producing the health benefits associated with polyphenols.

1.1 Polyphenol structure and food sources

1.1.1 Major structural families of polyphenols

Plant-derived polyphenols are characterized by the presence of at least one phenolic ring in their chemical structure. To date, over 8,000 polyphenols have been identified,¹⁰ and can be broadly grouped into two major categories: flavonoids and non-flavonoids. Flavonoids are characterized by a C6-C3-C6 backbone with two hydroxylated aromatic rings linked by a heterocyclic, oxygen-containing ring. Because flavonoids are the most heavily studied class of polyphenols and exhibit the most consistent health benefits,^{11, 12} a separate section of this review is dedicated to describing their structural differences and common food sources (see section 1.1.2). Non-flavonoids, on the other hand, are more structurally diverse and are made up of phenolic acids, stilbenes, lignans, and several other minor categories (Figure 1).¹¹

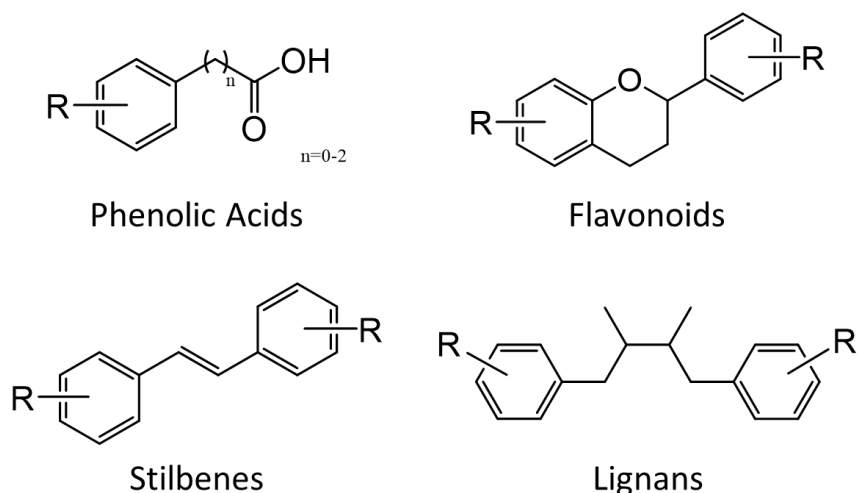


Figure 1.1 – Structure of major classes of polyphenols. In each molecule, at least one hydroxyl group is attached to the aromatic ring(s) present. Additional substitutions, including methoxyl groups, glycosides, and acylated moieties are commonly found in nature, as are polymeric forms of these molecules. All substitutions are represented by R-groups.

Phenolic acids are characterized by a C6-C1 to C6-C3 structure, with a single, hydroxylated aromatic ring and a carboxylic acid group attached to a short carbon chain (1-3 carbons in length). They are commonly found in their free form in fruits and vegetables, while in many grains, they are bound to other molecules.¹³ Phenolic acids may also be conjugated to other molecules, most commonly via an ester bond (e.g., chlorogenic acid is a linkage of caffeic and quinic acids). Because phenolic acids are small compounds with low molecular weight, they are widely distributed in plants that often contain other polyphenols in addition to their phenolic acids. Several commonly studied phenolic acid-rich foods include coffee, cocoa, curcumin, and many fruits, vegetables, and grains.¹³

Stilbenes are characterized by a C6-C2-C6 structure, with two aromatic rings on either side of a carbon-carbon double bond.¹³ Either or both aromatic rings in stilbenes may be decorated with one or more hydroxyl groups. The most heavily studied stilbene is resveratrol, commonly found in grapes and red wines. As a regular part of the diet, moderate consumption of resveratrol in red wine is thought to be responsible for the so-called “French paradox”, in which improved cardiovascular outcomes were observed despite a high fat diet in French people.¹⁴

Lignans (not to be confused with lignins) are formed by linking two phenylpropanoid (i.e., C6-C3) units to form the characteristic C6-C4-C6 skeleton of lignans. As with other polyphenols, lignans have at least one hydroxyl group attached to the aromatic ring(s) and may have additional substitutions, cyclizations, and/or glycosidic linkages *in planta*.¹⁵ They are found in high levels in flax, sesame, and chickpea, with lower concentrations found in many grains and legumes as well as several fruits and vegetables.¹⁵ In connection with health effects, flax and sesame are the most frequently studied lignan-rich foods. Although many promising health benefits have been noted with lignans, no consensus has yet been reached on their definitive role in health.¹⁶

1.1.2 Subclasses of flavonoids and common food sources

Flavonoids are the most heavily studied polyphenols, owing to their ubiquitous presence in vegetal foods as well as their consistently demonstrated health benefits. The full range of health benefits and studies associated with each subclass of flavonoids will not be detailed here, as the goal of this section is to introduce and differentiate between key structural features of each subclass of flavonoids and common food sources of each.

As shown in Figure 2, flavonoids contain two aromatic rings (the A- and B-rings) linked by a three-carbon bridge that, when bound to oxygen, forms a heterocyclic ring (the C-ring). Although there are differences in where the hydroxyl groups and other moieties attach to the A- and B-rings, the key structural differences between flavonoid subclasses are found in the C-ring. These structural differences can modulate the chemical properties and potential health benefits of these molecules.¹⁷

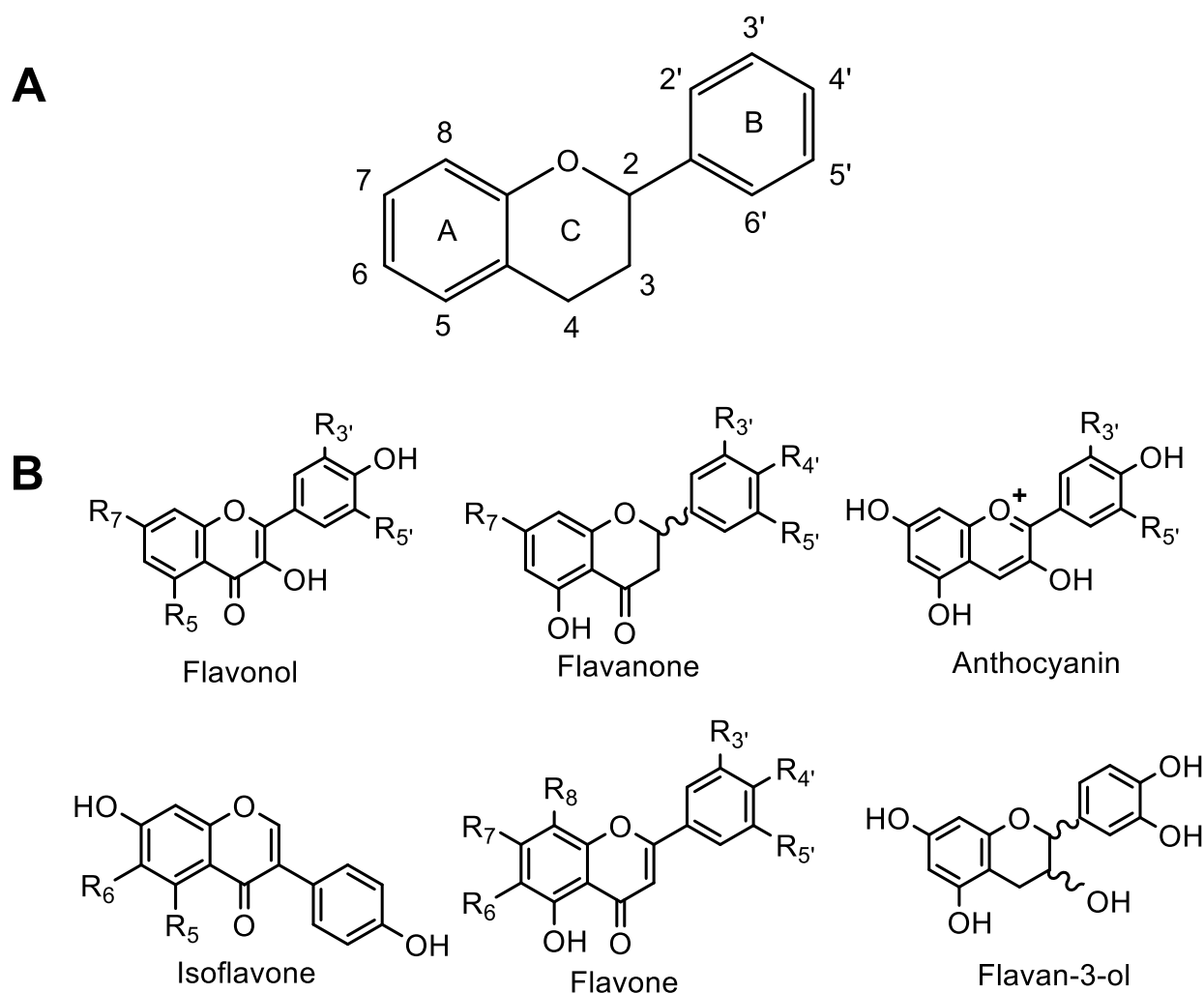


Figure 1.2 – Flavonoid structures. A) Generic structure and numbering of carbons in the flavonoid backbone. The A- and B-rings are aromatic and contain at least one hydroxyl group, while different bonding patterns within the heterocyclic C-ring determine which subclass the flavonoid belongs to. B) The six major subclasses of flavonoids and their characteristic substitution pattern in the heterocyclic C-ring.

Anthocyanins

Anthocyanins are vibrant colorants that give red, blue, and purple hues to many different fruits and vegetables.¹⁸ They are present in high amounts in berry fruits, grapes, red cabbage, and purple carrots.¹⁸ The unique structural marker of anthocyanins is the presence of the flavylum cation, which is only stable at low pH. Although numerous anthocyanins have been identified and structurally characterized nearly all anthocyanins are derived from six different aglycones (defined by different substitutions of hydrogen, hydroxyl, or methoxyl groups at the 3', 4' - and 5' -positions of the B-ring), including pelargonidin, cyanidin, delphinidin, malvidin, petunidin, and peonidin.¹⁷ Anthocyanins are rarely found as aglycones in nature, rather, they are routinely glycosylated at the 3-position of the C-ring to one or more mono- or di-saccharides and may be further acylated, with these attached groups imparting color and stability to the molecules.¹⁸

Flavonols

Flavonols are present in apples, onions, peppers, and berries and have been noted for their bioactivity as anti-cancer and anti-inflammatory agents.^{13, 19} Structurally, flavonols are similar to anthocyanins, as they are differentiated primarily by different substitutions at the 3' - and 5' -positions of the B-ring and are commonly glycosylated at the 3-position of the C-ring, though some forms have glycosidic linkages at the 7-position of the A-ring.²⁰ They are differentiated from anthocyanins by the lack of a flavylum cation and the presence of a ketone in the C-ring. The most common flavonol aglycones are quercetin, kaempferol, and myricetin.²⁰

Flavan-3-ols

Flavan-3-ols are a unique class of polyphenols that have been extensively studied in connection with numerous health benefits. The most common flavan-3-ols are catechins (catechin, epicatechin, and their gallated forms) and proanthocyanidins (polymeric forms of the catechins) that are thought to be responsible for the health benefits in green tea and cocoa.^{21, 22} Epigallocatechin gallate (EGCG), in particular, accounts for a majority of the phenolic content of green tea and has been connected to myriad health benefits.¹³ Flavan-3-ols are distinguished from other polyphenols by their non-planar structure leading to the formation of several stereoisomers based on substitutions at the 2- and 3-positions of the C-ring. Unlike anthocyanins and flavonols,

flavan-3-ols are typically observed as aglycones without being conjugated to glycosidic or acylated moieties.²⁰

Flavanones

Flavanones are commonly found in citrus fruits and tomatoes, with naringenin and hesperidin being the most commonly observed forms in nature.²³ They are typically observed with hydroxyl groups at the 5- and 7-positions of the A-ring, with glycosylation frequently occurring at the 7-position.²³ Although structurally similar to other flavonoids, the distinguishing C-ring features are a ketone moiety at the 4-position of the C-ring and a C2-C3 single bond.

Flavones

Flavones are commonly found in herbs, vegetables, juices, and oils, with apigenin and luteolin being the most commonly observed aglycones.²³ They are structurally similar to flavanones, with the main difference being the presence of a double bond between C2-C3 in the C-ring. They are commonly found in their glycosylated form in nature.¹³

Isoflavones

Isoflavones are commonly found in soy beans and other soy products. Isoflavones are able to bind to the estrogen receptors and are classified as selective estrogen receptor modulators (SERM).^{24, 25} The most prevalent isoflavone aglycones are genistein and daidzein, which are typically linked to glycosides *in planta*.²³ In contrast to other flavonoids, they are structurally unique, as the B-ring is attached at the 3-position (as opposed to the 2-position in other flavonoids) of the C-ring. This key structural difference is what allows it to align and bind to the estrogen receptor and alter estrogen signaling pathways.²⁵

1.1.3 Blueberry polyphenols

Health benefits have been associated with many different polyphenols, though the most promising effects have been linked to polyphenols present in tea, cocoa, and especially berry fruits.¹¹ As a rich source of polyphenols, blueberries (*Vaccinium spp.*) have been used in many recent clinical trials. Blueberries are able to improve markers of cardiovascular function (e.g.,

blood pressure, arterial blood flow, and cholesterol levels),²⁶ decrease the risk of developing cardiovascular disease (CVD),²⁷ while also aiding in neurocognition, memory, and slowing age-related cognitive decline.^{28, 29}

Blueberries contain a number of polyphenols, though the most studied in connection with the health benefits derived from blueberries are anthocyanins.³⁰ They account for approximately half of the total polyphenols present in blueberries and give them their distinctive blue-purple color.³¹ The remaining polyphenol content is comprised of a mixture of other flavonoids (including quercetin and myricetin glycosides) and phenolic acids (primarily chlorogenic acid).³² Due to their high phenolic content, their increased production, and growing popularity among consumers,³³ blueberries serve as a good model system for the examination of colonic metabolism and safety of high doses of polyphenols. The remainder of this literature review will summarize concepts related to all polyphenols, but will focus on phenolics prominent in blueberries.

1.2 Polyphenol metabolism

Many publications have summarized the metabolism of different polyphenols and families of polyphenols. The extensive and ever-expanding literature on this topic highlights just how complex this issue is and how much our understanding of the full picture of metabolism is still developing. In this section, a broad overview of the absorption, distribution, metabolism, and excretion (ADME) of ingested polyphenols will be summarized, with a particular emphasis placed on the colonic metabolism of flavonoids and phenolic acids present in high amounts in blueberries. It is important to note that although orally ingested polyphenols may affect digestion as early as the cephalic phase, with early absorption occurring in the stomach,²³ these areas of phenolic research are still in their infancy, and will not be covered here.

1.2.1 Models used to measure polyphenol ADME

Many different models and techniques have been used to measure the ADME of polyphenols. Typically, when designing experiments to determine the metabolic fate of polyphenols, researchers must make two important choices: what *form* of the molecule(s) is going to be employed and what *model system* is going to be used.³⁴

The *form* of the molecule refers to the choice of a single, specific polyphenolic molecule, a radio- or isotopically-labeled polyphenol, or a mixture of polyphenols with or without a food matrix present. Because all polyphenol-containing vegetal food sources contain a complex mixture of polyphenols that interact with the food matrix they are presented in, this choice is often determined by the researchers' goals. If the goal is to understand the complete ADME of a single molecule, determine its distribution, and identify intermediates in the metabolic pathway, then choosing a single molecule or a radiolabeled version can help in the elucidation of the polyphenol's pharmacokinetics.³⁵ If, on the other hand, the goal is to understand the ADME or broader health effects of a food (or mixture of foods) containing polyphenols, then models that allow for interactions with the entire food matrix and the exposure to the complex milieu of polyphenols are appropriate.

The *model system* refers to the specific *in vitro* or *in vivo* technique chosen to evaluate the polyphenol. Common *in vitro* techniques include *in vitro* digestions for measuring the bioaccessibility of polyphenols through digestion with different food matrices present, cell culture models to examine polyphenol bioavailability (e.g., Caco-2 cells) or tissue effects (e.g., HepG2 cells), and microbial/fecal fermentations to identify colonic metabolites.³⁶ These techniques help isolate and identify different steps in the digestion and absorption of polyphenols, giving researchers a "snapshot" of what is happening at different steps along the process. By contrast, most *in vivo* techniques aim to get a better understanding of the broader effects of polyphenols in living systems. Common *in vivo* methods, including animal and human models, allow for the elucidation of pharmacokinetics, polyphenol metabolism, and possible mechanisms for health effects.

In the context of colonic metabolism, two of the most useful models are microbial fermentations and human clinical trials, in which ileostomists are compared to healthy individuals with an intact GI tract. These models allow for the identification of colonic metabolites, while also isolating the specific contribution of the large intestine to polyphenol ADME.³⁶ As discussed below, this is critical in studies of polyphenols because such a large portion of the ingested dose reaches, and is absorbed in, the colon. The remainder of this review summarizes current understanding in the colonic catabolism of polyphenols, while relying on studies using the techniques discussed here.

1.2.2 Overview of polyphenol ADME

Summary of intestinal absorption and metabolism

As illustrated in Figure 1.3, orally ingested polyphenols undergo extensive modifications throughout the gastrointestinal (GI) tract. Upon reaching the small intestine, polyphenols are viewed by the body as xenobiotic agents and the goal of the body is to excrete them as rapidly as possible. This leads to very low levels of absorption (< 2% for many classes of polyphenols),^{36, 37} as evidenced by low plasma and urine concentrations (low C_{\max} values) and rapid excretion (T_{\max} 0.5-2h).³⁸ Polyphenol metabolism typically occurs in three steps in the small intestine: 1) cleaving polymeric forms to monomeric forms, 2) cleaving glycosidic side chains to isolate the aglycone (via lactase phlorizin hydrolase or β -glucosidase),³⁹ and 3) phase I and II metabolism that conjugate the aglycone with sulfated, glucuronidated, or methylated moieties (via sulfotransferase (SULT), UDP-glucuronosyltransferase, or catechol-*O*-methyltransferase (COMT), respectively).³⁹ Epithelial cells lining the small intestine are a key part of this process, as they can absorb polyphenols via several transporters (e.g., SGLT-1), subject them to phase I and II metabolism within the epithelial cells before either effluxing them back into the intestinal lumen (via ABC transporters) or transporting them to the portal vein.^{12, 21} Because a large portion of polyphenols undergo this metabolism in the small intestinal epithelial cells, this is thought to be a protective adaptation of these cells to prevent the xenobiotic polyphenols from entering the bloodstream. Unlike many nutrients that are extensively absorbed in the small intestine, studies in ileostomists confirm that a majority of ingested polyphenols reach the colon intact.⁴⁰

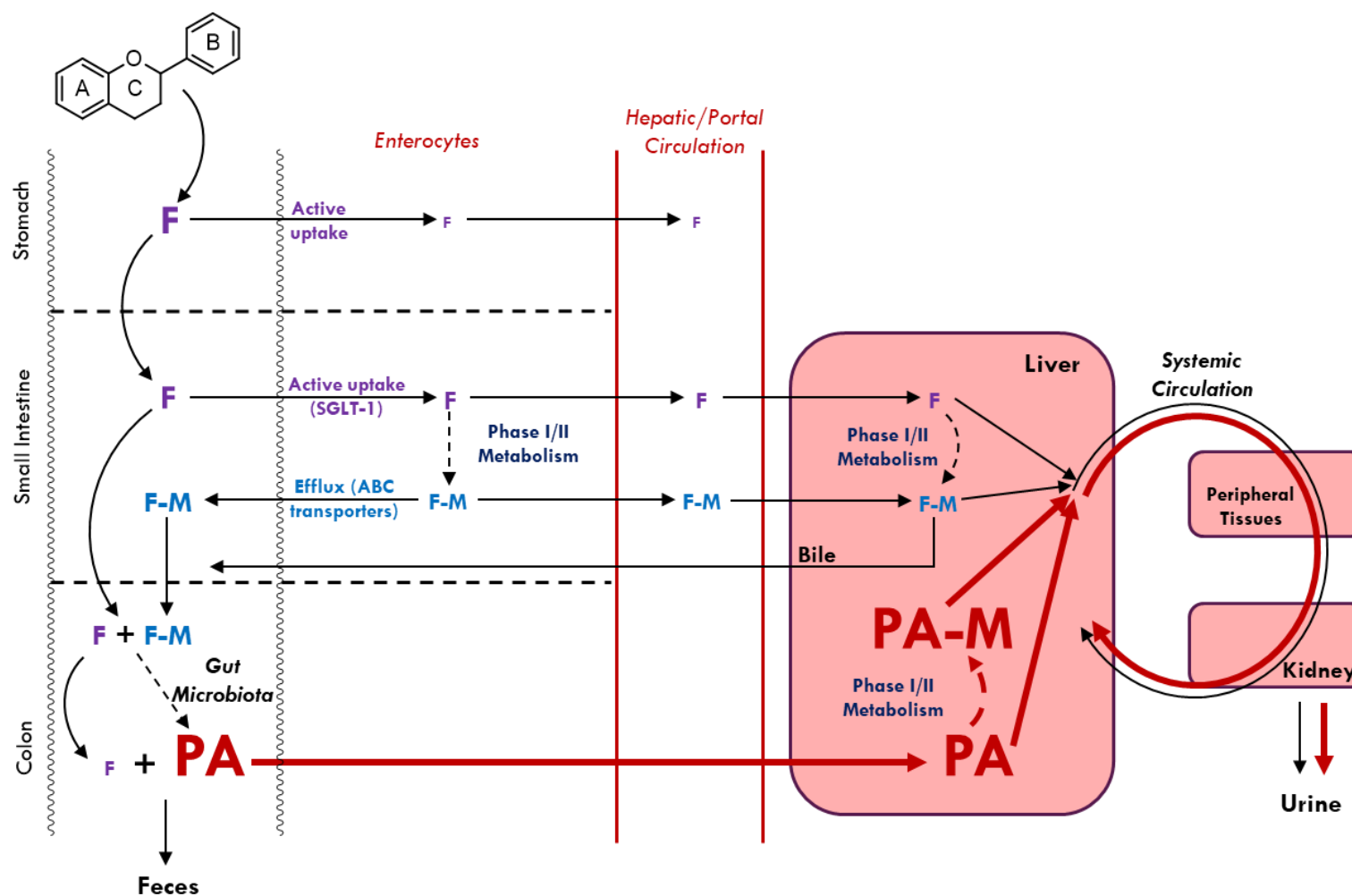


Figure 1.3 – Summary of polyphenol ADME. Orally ingested polyphenols may be absorbed at any point along the GI tract. Extensive metabolism is observed within the small intestinal epithelial cells, with only a small portion of the ingested dose absorbed into systemic circulation. In the colon, the gut microbiota catabolizes polyphenols into smaller molecular weight phenolic acids that are more extensively absorbed than their small intestinal counterparts. All absorbed metabolites may be further metabolized in the liver before being excreted in the urine or recycled to the GI tract via enterohepatic circulation.

The small portion of polyphenols that do enter systemic circulation from the small intestine are subject to further metabolism and may accumulate in some tissues. After entering small intestinal epithelial cells, polyphenols (and their metabolites) can be transported to the portal vein.¹² They are then shuttled to the liver where they can be further metabolized, before being either circulated throughout the body or recycled into the GI tract via enterohepatic circulation.²² After circulating through the body, these metabolites are then cleared by the kidneys and excreted in the urine.⁷

After passing through the small intestine, the remaining polyphenols pass to the colon. Because small intestinal absorption is low, a majority of the ingested polyphenol dose reaches the colon intact.⁴¹ Once in the colon, polyphenols have a symbiotic relationship with the gut microbiota. There is a well-recognized reciprocal reaction between the polyphenols and the gut microbiota: as polyphenols continually enter the colon, they shift the gut microbial populations to a putatively healthier state, while at the same time being more efficiently catabolized and absorbed as phenolic acid derivatives.⁴² Importantly, these colonic catabolites are absorbed to a much greater extent (up to 10-fold higher) than in the small intestine, persist in systemic circulation longer (i.e., have a greater AUC), and have a higher C_{\max} with a later T_{\max} .⁴³ Much of this evidence has been accumulated in the last 10 years, leading to a shift in paradigms, as many now believe that these colonic catabolites may be responsible for the health benefits associated with polyphenol intake.³⁶

Tissue distribution of polyphenols

Most studies have examined the absorption and metabolism of polyphenols and their metabolites by measuring plasma and urinary concentrations of these molecules and their metabolites. This strategy has defined the current understanding of the bioavailability of various polyphenols, but in order to elucidate the mechanisms associated with the health benefits attributed to polyphenols, observing phenolics in peripheral tissues may be necessary. Of the few studies that have examined polyphenol accumulation in tissues, virtually all have been performed in rodents and pigs.⁴⁴ Translating these studies to humans may be problematic because rodents show much more extensive methylation than humans, meaning that the metabolism and subsequent tissue distribution observed in animal studies must be interpreted cautiously.⁴⁵

Tissue distribution of a number of polyphenols, including radiolabeled quercetin, grape seed extract, resveratrol, grape juice, and radiolabeled pelargonidin, has been measured in studies

specifically targeting a single tissue (especially brain) as well as in studies targeting multiple tissues.⁴⁶⁻⁵⁶ These studies demonstrate several key variables that must be taken into account when measuring the tissue distribution of polyphenols: 1) the structure of the polyphenol, 2) the length of dose administration (i.e., acute or repeated dosing), and 3) the timing of sacrifice in relationship to most recent polyphenol exposure. In general, these investigators observed that polyphenols were primarily found in key metabolic tissues, including all segments of the GI tract, the liver, and the kidney,⁵²⁻⁵⁴ though a few studies also found quantifiable levels in heart, lung, spleen, and muscle.^{46, 55, 56} In addition, a number of studies focused on the ability of polyphenols to cross the blood brain barrier and accumulate in brain tissue. These studies typically found that repeated dosing was necessary to find quantifiable levels in the brain and that sacrificing within 6h of the most recent dose gives the greatest likelihood of detecting phenolics, especially in rodents.^{50, 51, 55, 57, 58}

Excretion and elimination of polyphenols

The excretion of polyphenols and their metabolites from systemic circulation has not been investigated as the primary aim in many studies, though it is often reported in studies quantitating the bioavailability or pharmacokinetics of ingested polyphenols. The main routes of excretion are in the feces, urine, breath, and bile.⁵⁴ The landmark study summarizing polyphenol excretion pathways used a ¹³C isotopically labeled cyanidin-3-glucoside in humans and measured fecal, urinary, and breath excretion for 48h. This investigation found that a total of 44% of the ingested dose was recovered, with a majority (32%) found in the feces, 6.9% exhaled as CO₂ in the breath, and 5.4% in the urine.³⁵ This is one of the only studies to measure exhalation of CO₂, but this indicates that polyphenols may be extensively catabolized to single carbon atoms and that exhaled breath may be an important route of excretion. However, beyond this study, most of the work on polyphenol excretion pathways has been described by measuring urinary or fecal excretion while targeting other aspects of polyphenol bioavailability or metabolism.⁷ The other route of excretion, biliary excretion, was originally described via intraperitoneal injections of polyphenols.⁵⁹ More recent pharmacokinetic studies support this notion by noting the appearance of a second peak in phenolic absorption several hours after the initial T_{max} was observed.^{54, 60}

1.2.3 Colonic Catabolism of Polyphenols: A Symbiotic Relationship

Over the past decade, polyphenol ADME research has shifted focus. With the emergence and recognition of the gut microbiome as a metabolically active portion of the GI tract, research into colonic metabolism and its contribution to overall polyphenol bioavailability has expanded rapidly. And, as stated earlier, with the knowledge that many polyphenols reach the colon intact, understanding their metabolic fate in the colon, as well as subsequent health effects, is becoming an important and “hot” topic of research. Although beyond the scope and focus of this review, many studies have investigated the ability of polyphenols to modify gut microbial populations. These studies demonstrate that consistent exposure to polyphenols induces a shift in microbial populations to a composition associated with better overall health, longer life expectancies, and improved qualities of life.⁶¹ Polyphenols modulate the gut microbiome by increasing microbes associated with beneficial health outcomes (e.g., *Lactobacillus* spp. and *Bifidobacterium* spp., which produce β -glucosidase to metabolize phenolics) and inhibiting those associated with negative health outcomes (e.g., *Bacteroides* spp., *Staphylococcus* spp., and certain species of *Clostridium*).^{42, 62, 63} These topics are reviewed in detail elsewhere;^{25, 42} the current focus is on the metabolic byproducts of colonic fermentation of polyphenols. As the parent polyphenol structure determines which metabolites may be formed, the following sections summarize the similarities and differences in colonic metabolism between different classes of polyphenols. Because flavonoids are the dominant polyphenols in blueberries and are the most heavily studied in relation to colonic metabolism, this section will focus primarily on their colonic metabolism, though brief descriptions of phenolic acids, stilbenes, and lignans are also given.

1.2.4 Colonic metabolism of flavonoids

Flavonoids entering the colon are subjected to an array of metabolic fates and many catabolic intermediates are observed in their breakdown, which are described for the main classes of flavonoids below. The main catabolic step for flavonoids is cleavage of the C-ring to form smaller molecular weight phenolic acids.^{61, 64} Although the initial structure of these phenolic acids is dependent upon the location of the cleavage in the C-ring as well as the substitutions on the A- and B-rings,²⁵ after undergoing this major catabolic step, the gut microbiota can efficiently perform hydrogenation, α - and β -oxidation, demethylation, and dehydroxylation reactions, allowing

interconversion between many of the phenolic acids.^{61, 64} This means that although a large number of phenolic acid metabolites may be generated initially, these interconversions may limit the number of metabolites observed in different studies because they converge on the same phenolic acid products. This is summarized in Figure 1.4.

Beyond the individual catabolites formed via the colonic breakdown of flavonoids, there are a series of reactions common to these molecules that account for most of the products observed. Upon entering the colon, most flavonoids remain glycosylated. The first step in catabolism is cleaving these moieties (similar to what occurs in the small intestine).³⁹ Then, the main catabolic step occurs: cleavage of the heterocyclic C-ring in the flavonoid backbone.^{42, 62} This results in two smaller phenolic acid molecules that can then be further metabolized by a variety of reactions to form many measurable products. Colonic metabolites can then be absorbed and are transported to the liver via the portal vein. Once in the liver they can be subjected to further metabolism via phase I and phase II metabolism.^{39, 43} Additionally, benzoic acids may be conjugated to glycine, forming hippuric acids, a common product of hepatic metabolism that may result from many different dietary inputs.⁶⁵

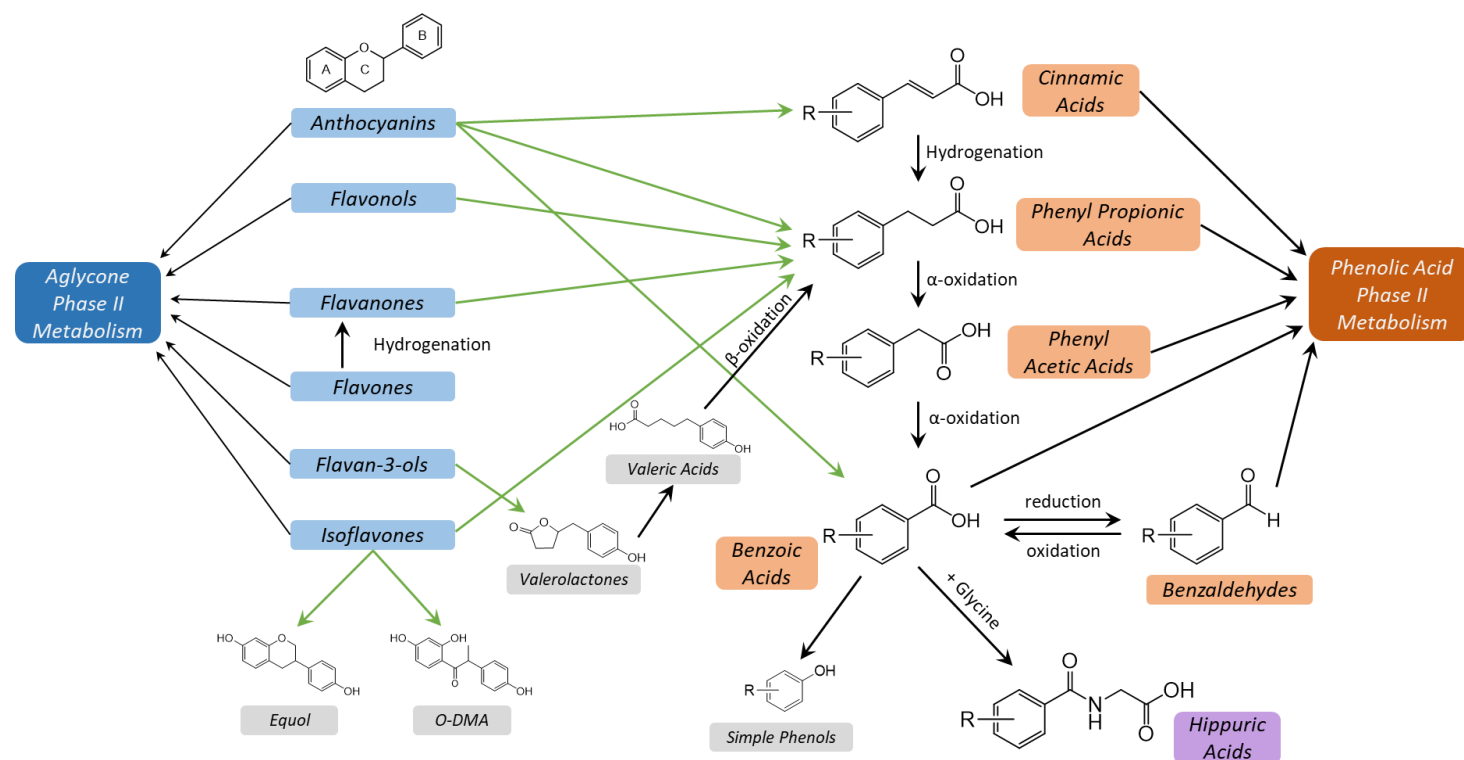


Figure 1.4 – Summary of flavonoid metabolism. In the upper GI tract, flavonoid aglycones may be absorbed and undergo phase II metabolism, resulting in glucuronidated, sulfated, and methylated metabolites (blue boxes represent intact aglycones). A majority of the ingested flavonoid dose passes to the colon, where catabolism (indicated by green arrows) is mediated by the gut microbiota to form smaller molecular weight phenolic acids containing the B-ring (orange boxes). Anthocyanins, flavonols, and flavanones undergo C-ring cleavage, forming primarily phenyl propionic and benzoic acids. Products of A-ring cleavage, including phloroglucinol, may also be observed from these flavonoids. Flavones are hydrogenated at the C2=C3 bond to flavanones and then follow the same catabolic pathways as flavanones. Flavan-3-ols undergo A-ring cleavage, resulting in valerolactones (containing the B-ring) that can be converted to valeric acids and subsequently undergo β -oxidation to form phenylpropionic acids. Isoflavones follow a different pathway, forming primarily equol and *O*-DMA metabolites. After the formation of phenolic acid catabolites, interconversions within and between different forms of the phenolic acids are possible as well as subsequent phase II metabolism. Finally, benzoic acid may conjugate with glycine in the liver forming hippuric acids (purple box). R-groups represent different substitutions that may be present on the B-ring. *O*-DMA = *O*-demethylangolensin.

Anthocyanins

Anthocyanins are perhaps the most well-studied class of polyphenols in colonic catabolism. Given their prevalence in nature, extremely low absorbance in the small intestine ($< 1\%$),⁶⁶ and proclivity for reaching the colon intact, perhaps it is not surprising that this class of molecules has received so much attention.³⁶ Additionally, their colonic catabolism is relatively straight-forward and the mechanisms involved in colonic breakdown of anthocyanins is well-described, relative to other polyphenols.

There are several steps in anthocyanin catabolism. As nearly all anthocyanins exist as glycosylated moieties in nature, the first step is cleaving side chains to isolate the parent aglycone. Then, the heterocyclic C-ring is cleaved, leaving the A- and B-rings intact for further metabolism. Anthocyanins are differentiated by substitutions on the B-ring, thus the A-rings follow the same metabolic path (forming phloroglucinol and resorcinol as end products),⁶¹ while products of B-ring metabolism depend on the parent anthocyanin structure. B-ring derived catabolites include protocatechuic acid (from cyanidin), hydroxybenzoic acid (from pelargonidin), vanillic acid (from peonidin), syringic acid (from malvidin), gallic acid (from delphinidin), and methyl-gallic acid (from petunidin).⁴² These products have been observed in studies of anthocyanin microbial metabolism, though additional products (including trans-cinnamic acids and phenyl propionic acids) have also been observed.^{25, 42} This has led to the hypothesis that anthocyanins are metabolized into either phenyl propionic acids or benzoic acids, depending upon the location of C-ring cleavage.²⁵

Flavonols, Flavanones, and Flavones

Flavonols, flavanones, and flavones share similar catabolic pathways and are discussed collectively. These flavonoids are structurally similar, with a ketone group at the 4-position of the C-ring, and follow the same pattern of C-ring cleavage as a result. These compounds may be absorbed at higher levels in the small intestine than anthocyanins, reducing the amount available for colonic fermentation.⁶⁷

The portion of flavonols, flavanones, and flavones remaining after small intestinal absorption enter the colon and follow similar catabolic pathways. First, any remaining glycosidic side chains are cleaved from the aglycone. Then, flavonols and flavones undergo hydrogenation of the C2-C3 double bond, creating several flavonol intermediates and flavanones (from

flavones).⁶¹ These products are then subjected to C-ring cleavage, creating a set of phenyl propionic acid molecules (from the B-ring of the parent flavonoid) that have been detected in various models.^{25, 68} As with the B-ring products of anthocyanin metabolism, the phenyl propionic acid products may be further converted to phenyl acetic acids and benzoic acids. Additionally, although less commonly observed, the A-ring may undergo metabolism similar to that observed with anthocyanins, forming phloroglucinol.⁶⁸

Flavan-3-ols

Due to the high levels of catechins in tea, the colonic metabolism of flavan-3-ols has been studied rather extensively.²⁵ In contrast to other flavonoids, flavan-3-ols are typically present in the aglycone form in nature, meaning they do not have to undergo deglycosylation before being absorbed in the GI tract,^{39, 69} though proanthocyanidins must be broken down into monomers before absorption. Curiously, despite the lack of need for this first metabolic step (deglycosylation), flavan-3-ols exhibit extremely low levels of absorption in the small intestine, similar to anthocyanins.³⁷ This leaves a large portion of the ingested dose intact for colonic catabolism.

Once in the colon, flavan-3-ols are metabolized in a slightly different fashion than other flavonoids: instead of cleaving the C-ring, the A-ring is cleaved, with C5-C8 being removed.²⁵ This results in a series of detectable intermediates called valerolactones. The valerolactones contain the B-ring and a 5-membered heterocyclic ester ring and are routinely quantified in studies of polyphenol colonic metabolism.²⁵ These intermediates are further catabolized by opening the valerolactone ring and shortening the resulting carbon chain.⁷⁰ The resulting molecule is phenyl propionic acid, which can be further metabolized to phenyl acetic and benzoic acids. One unique feature of the gallate ester forms of flavan-3-ols (ECG and EGCG) is that the gallated moieties can be hydrolyzed to form gallic acid, a trihydroxylated benzoic acid that can be further metabolized by the gut microbiota.⁴¹

Isoflavones

Isoflavones form unique products in the colon. After cleavage of the glycosidic side chains, isoflavones aglycones may remain intact and are metabolized to either equol (from daizein) and *O*-demethylangolensins (from daizein and genistein).^{61, 71} Equol, in particular, has been the subject

of many studies because of its impact on human health, though only about 1/3 of humans have the necessary microbes to produce it in the colon.⁷² In addition to these unique colonic metabolites of isoflavones, the aglycone may also undergo C-ring cleavage to form phenylpropionic acids and subsequent metabolites.²⁵

1.2.5 Colonic metabolism of phenolic acids

Phenolic acids are commonly found as free, bound, and dimerized forms in nature. The first step in colonic metabolism is to free the bound and dimerized phenolic acids, which occurs with esterases and hydrolysis reactions, respectively.²⁵ Once free, phenolic acids may be interconverted between forms, subjected to further phase II metabolism, or conjugated to glycine to form hippuric acid following the same pathways as B-ring catabolites of flavonoids (see Figure 1.4).^{25, 61}

Most of the work on the colonic metabolism has been done with chlorogenic acid, owing to the high amounts present in coffee.¹² Chlorogenic acid is a hydroxycinnamic acid consisting of caffeic acid bound to quinic acid. Chlorogenic may be absorbed in the small intestine, though studies comparing ileostomists to those with an intact colon indicate that the colon accounts for most of the total absorption (8% vs 29% total absorption, respectively).^{73, 74} Although chlorogenic is initially cleaved to form caffeic acid (a cinnamic acid), this is only observed transiently in systemic circulation, with hydrogenation to phenylpropionic acids accounting for the bulk of the observed metabolites.^{75, 76} As the conversion of cinnamic acids to phenylpropionic acids occurs primarily in the colon, this adds further support to the notion that colonic metabolism plays a major role in the absorption and metabolism of chlorogenic acids.

1.2.6 Colonic metabolism of stilbenes

Stilbenes may be present in a variety of foods, though by far the most heavily studied stilbene is resveratrol, present in grapes and red wine. Several studies have investigated the metabolism of resveratrol, reporting the presence of a number of metabolites that contain the characteristic C6-C2-C6 backbone still intact.²⁵ The primary steps in colonic metabolism of stilbenes are hydrogenation and dihydroxylation.⁶¹ Metabolites observed from both upper and lower intestinal metabolism include glucuronidated and sulfated forms of resveratrol and

dihydroresveratrol, though the rate and extent to which each metabolite is produced varies greatly between people.⁷⁷ Upper and lower GI metabolism is typically determined by observed T_{\max} values for each compound, with different metabolites appearing within 2-3h after ingestion (indicating small intestinal absorption) or peaking 8-24h after ingestion (indicating colonic absorption). Additionally, lunularin, a hydrogenated and dehydroxylated form of resveratrol, has the latest appearance, with a T_{\max} of 24-48h after ingestion, and appears to be the terminal metabolite formed after resveratrol ingestion.⁷⁸

1.2.7 Colonic metabolism of lignans

Lignan colonic metabolism is less well-studied than that of other major classes of polyphenols, though the studies that have been done consistently indicate that, regardless of the starting materials, lignans are almost exclusively metabolized to enterolactones and enterodiols.^{12, 61} These products are formed by a succession of deglycosylation, demethylation, oxidation, and dehydroxylation reactions by the gut microbiota.⁷⁹ Further phase II metabolism of these products is also observed, as glucuronidated forms of enterolactones and enterodiols have been characterized following the consumption of lignan-rich foods.⁸⁰

1.3 Safety of dietary polyphenols

Although there have been countless investigations into the health benefits of polyphenols and the amounts needed to observe meaningful impacts on health, there are very few investigations into the potential effects (positive or negative) of polyphenols at high doses. This section addresses this issue from a number of different vantage points. First, common sources of dietary polyphenols and average daily intake are addressed, as are new consumption modalities that are gaining popularity and have the potential to significantly increase exposure to polyphenols. Then, the available literature regarding polyphenol safety and toxicity is reviewed. Finally, current regulatory guidance on the safety of polyphenols as well as efforts to establish dietary recommendations for polyphenols are discussed.

1.3.1 Polyphenol consumption

Daily polyphenol intake

Given their ubiquity in fruits and vegetables as well as their presence in coffee and tea (two of the three most commonly consumed beverages globally),⁸¹ virtually all individuals consume quantifiable amounts of polyphenols on a daily basis. There have been several estimations of total polyphenol intakes in recent years, with most studies using food frequency questionnaires and/or 24h dietary recalls to collect dietary data. Polyphenol intake is then calculated using a variety of tools, most commonly the United States Department of Agriculture (USDA) Phenolic Database or Phenol Explorer.⁸²⁻⁸⁴ Regardless of the specific methods used, most estimates from the last 20 years put estimates of total polyphenol intakes at ~1 g/d in adults.^{23, 84-87} A majority of estimates have been performed in European countries, with many estimates calculated as part of the EPIC study (European Prospective Investigation into Cancer and Nutrition), though many other regions of the world have been studied as well.⁸⁴ These estimates suggest that total polyphenol intake varies from 300-1800 mg/d in different countries, with flavonoids and phenolic acids accounting for approximately 95% of total polyphenolic intakes, while stilbenes and lignans account for < 1% of total intake.^{84, 87} Overall, the main contributors to total polyphenol intake are tea, coffee, red wine, fruits, and vegetables, though regional and cultural differences in consumption patterns vary greatly and lead to vastly different major dietary sources in different countries.^{84, 87-90} In the United States, total polyphenol consumption is ~800 mg/d, with flavonoids accounting for ~200 mg/d and phenolic acids ~300 mg/d.^{21, 84}

Differing consumption patterns can dramatically alter the dietary polyphenols to which different individuals are exposed. For example, coffee drinkers will consume large quantities of chlorogenic acids (i.e., phenolic acids), green tea drinkers will consume large amounts of catechins and proanthocyanidins (i.e., flavan-3-ols), and fruit and vegetable consumers will have widely varying intakes based on the fruits and vegetables in their diets (e.g., fruit-derived anthocyanins or soya derived isoflavones). Because some polyphenols are more bioactive than others, with different polyphenols offering different health benefits, it is exceedingly difficult to translate estimates of total polyphenol consumption to specific health outcomes.

Polyphenol consumption modalities

One of the biggest challenges for researchers attempting to accurately estimate total polyphenol intakes is the constantly shifting and ever-expanding availability of polyphenol-rich options for consumers. In recent years, there has been a significant increase in the number of polyphenol-rich dietary supplements, “natural” colorants, and functional foods (e.g., nutraceutical foods incorporating polyphenols⁹¹) available to consumers, which may serve to rapidly increase polyphenol consumption. However, when combined, these consumption modalities significantly increase the dose and frequency of polyphenol ingestion.

Increased popularity of polyphenol-rich dietary supplements

Dietary supplements are frequently used by consumers to address deficiencies of essential nutrients in their diets or to incorporate putatively health-promoting substances into their diets. As shown in Figure 1.5, the consumption of dietary supplements has risen significantly over the past few decades. In particular, consumption of polyphenol-rich botanical supplements has increased nearly 20-fold over the past quarter century.⁹²⁻⁹⁵ This was catalyzed by the passage of the Dietary Supplements Health and Education Act (DSHEA) in 1994. DSHEA expanded the definition of dietary supplements beyond vitamins and minerals, allowing botanical and herbal supplements (among others) to be regulated in a different manner than drugs and food additives.^{95, 96} This effectively loosened the regulation of dietary supplements and has given rise to a plethora of new supplement manufacturers marketing their products to consumers, significantly increasing polyphenol consumption in comparison to current dietary intake levels.

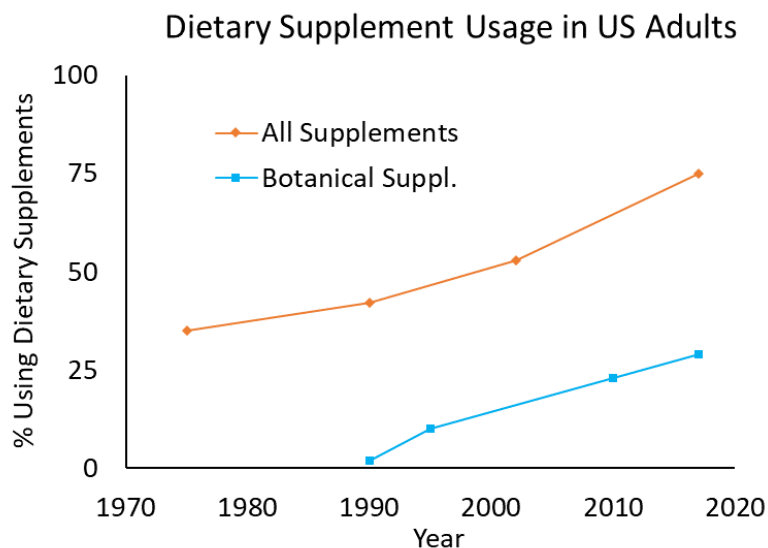


Figure 1.5 – Increased use of dietary supplements among US adults from 1970 to present. The passage of DSHEA in 1994 catalyzed the consumption of non-vitamin, non-mineral supplements, as evidenced by the increase in botanical supplement usage, a category that includes polyphenol-rich dietary supplements.⁹²⁻⁹⁵

Consuming higher levels of polyphenols in purified forms like dietary supplements presents a variety of challenges that must be investigated to fully understand their impacts. Principally, separating plant-derived polyphenols from their food matrix may significantly alter their bioavailability, especially with the removal of carbohydrates, including mono- and disaccharides as well as fiber. Because fiber is an important modulator of the gut microbiome,⁹⁷ colonic metabolism of polyphenols may be altered when they are consumed as purified extracts. Additionally, removing the food matrix allows for a more concentrated dose to be consumed at a single time. This may prove to be the biggest driver of increased polyphenol consumption, as the estimated daily consumption of ~1 g/d can easily be obtained with a single pill and much higher levels of consumption are possible with repeated intake of dietary supplements.

Increased use of polyphenols as food colorants

Artificial colorants have faced scrutiny recently, and many consumers have advocated for their removal from the food supply. This has been fueled by several highly cited studies that observed better health outcomes when artificial colorants were removed from the diet. In particular, these studies found that removing artificial colorants from the diet limited hyperactivity in adults

and children.⁹⁸⁻¹⁰⁰ Despite the fact that the vast majority of the literature regarding artificial colorants has failed to produce similar results, many consumers have cited these studies as evidence of the negative consequences of artificial colorants and demanded their replacement with “natural” alternatives.¹⁰¹

Over the past decade, many food companies have removed or pledged to remove artificial colorants from their products.¹⁰² To achieve this, extracts of highly pigmented molecules from plant and algae sources have been identified as sources of “natural” colorants. Many polyphenol-rich plants are popular choices because of their water solubility and ability to impart vibrant colors to foods.¹⁰³ Additionally, extracts of polyphenol-rich plants are generally considered “exempt from certification” by the Food and Drug Administration (FDA)¹⁰¹ and, depending on how they are extracted, can be considered a “coloring foodstuff” by the European Food Safety Administration (EFSA), meaning they do not have to meet the same standards as a “color additive”.¹⁰² Thus, in both the US and Europe, “natural” colorants derived from polyphenol-rich plants are not subjected to the same rigorous safety testing as their artificial counterparts.

Taken together, polyphenol-rich dietary supplements and “natural” colorants may increase both the dose and frequency of polyphenol consumption. The tacit assumption amongst consumers is that because polyphenol-rich dietary supplements and “natural” colorants are derived from plant-based sources, they are safe to consume;¹⁰¹ to date, however, this has not been adequately vetted and more research is needed to fully understand the safety and efficacy of polyphenols under these new consumption modalities.^{85, 104}

1.3.2 Current knowledge on polyphenol safety and toxicity

Green tea extracts

Green tea is one of the most frequently studied sources of polyphenols, owing to its global popularity and noted health benefits in numerous studies. In light of this, green tea extracts have been marketed as dietary supplements (especially as weight loss supplements) and, while they have been studied more than any other polyphenol or polyphenol-rich food for potential toxicity, there are still relatively few publications regarding their safety. There are no known cases of toxicity with green tea infusions (i.e., traditional beverage preparations), but adverse effects have been noted with the consumption of concentrated green tea extracts (GTE) in pill form.¹⁰⁵

A number of cases of side effects and/or toxicity have been reported in humans after consuming GTE. The most common side effects are GI issues (e.g., stomach ache, bloating, abdominal pain) and hepatotoxicities.¹⁰⁶ As of 2009, a total 216 known reports of side effects resulting from GTE were known, of which 34 involved liver damage.¹⁰⁵ The hepatotoxic side effects reported were, in general, the most severe side effects observed.¹⁰⁵ The most well-known incidence of hepatotoxicity was observed in Europe related to a GTE dietary supplement marketed for weight loss (Exolise). There were 13 reported cases of hepatotoxicity resulting from the consumption of this supplement, which provided 375 mg EGCG/d and 75-150 mg caffeine/d when taken as recommended.¹⁰⁵

In a recent review of 159 adverse events in humans related to GTE, the authors confirmed the liver as the target organ for toxicity.¹⁰⁷ Hepatotoxicity was dependent upon a number of factors, including sex, fed vs. fasting state, and acute vs. chronic dosing.¹⁰⁷ Females are the primary consumers of botanical supplements (especially those marketed as weight-loss supplements)¹⁰⁸ and are more susceptible to potential side effects than males in both human and animal studies.¹⁰⁸ Many dieticians recommend consuming green tea and GTE apart from meals because they interfere with iron bioavailability.¹⁰⁹ However, consuming GTE supplements in the fasted state increases the risk of hepatotoxicity, as the bioavailability of catechins can increase more than fivefold than when consumed in the fed state in humans,¹¹⁰ an effect that has also been observed in animals.¹¹¹ In addition, repeated exposure to GTE catechins increases their bioavailability.¹¹² This correlates with the typical onset of symptoms, which typically appear between 1-3 months after initiating the supplement regimen.¹⁰⁸ The causal link between GTE and the noted cases of hepatotoxicity is still being developed, though in nearly all cases where the GTE supplement was discontinued, liver issues resolved. Additionally, in all cases of rechallenge (i.e., the subject voluntarily restarted GTE supplementation), the same symptoms of hepatotoxicity resurfaced.¹⁰⁸

Taken together, these studies seem to indicate that GTE may induce side effects, particularly hepatotoxicity, in humans. However, it remains challenging to definitively attribute the observed toxicities to the polyphenols in GTE because green tea contains significant amounts of caffeine that may confound the results. Although no adverse effects from traditional beverage preparations of green tea, when consuming > 5 cups of tea per day, restlessness and trouble sleeping were noted, most likely due to the caffeine content of the tea.¹⁰⁵ In high doses, caffeine can cause severe toxicity,¹¹³ making it difficult to assign the toxic causality in GTE to either the

polyphenols or the caffeine content.¹⁰⁵ It is possible that the hepatotoxicity of GTE may be due to the combination of high doses of both catechins and caffeine. This was demonstrated in a recent animal study, where the administration of 1200 mg GTE/kg bw/d to rats for 6 months increased activity levels and decreased body weight, but these effects were not replicated in a dose-matched group treated with decaffeinated GTE.¹¹⁴

Other animal investigations into GTE have found varying results. In a 14-week study in rats and mice using doses up to 1000 mg GTE/kg bw/d, adverse effects were observed. In the highest dose group, animals had significantly lower body weight as well as histopathological changes in the liver, nose, lymph nodes, and thymus. Several mice (but not rats) in the highest dose group died as a result of treatment.¹¹⁵ Other rat studies on GTE have not observed toxicity at equivalent doses.^{114, 116} Several studies in dogs have demonstrated that, when administered doses of ≥ 400 mg/kg bw/d under fasting conditions, significant toxicity is noted, including extensive histopathological changes to major organs and mortality, though these symptoms were markedly decreased when GTE was administered with food.^{111, 117}

Grape-derived polyphenols

Grape skins and seeds are rich sources of polyphenols and their extracts can be used as colorants in a number of food applications. The safety of grape color was evaluated in dogs over 90d, with a diet of 15% (w/w) grape color resulting in decreased weight gain but no other histopathological, hematological, or serum chemistry changes.¹¹⁸ A follow up study with grape color examined its effects on reproductive performance through two generations of SD rats. While no issues with reproductive performance were noted, rats consuming the 15% (w/w) diet had lower body weights as did their offspring. However, this was attributed to the lack of isocaloricity in the diets and not to the grape color itself.¹¹⁹ In a more recent 90d investigation of grape seed and grape skin extracts incorporated into the chow of SD rats at doses up to 2.5% (w/w) did not result in any observable differences in body weight, histopathology, hematology or serum chemistry.¹²⁰

Anthocyanin-rich extracts

The toxicity of anthocyanin-rich extracts was first examined over 50 years ago, using anthocyanins derived from currants, blueberries, and/or elderberries in mice, rats, guinea pigs,

rabbits, and dogs.¹²¹ No overt toxicity was noted at the highest doses when consumed orally, though a mild sedative effect was observed. No teratogenicity was observed over three generations in rodents or rabbits. However, when administered at lower doses intraperitoneally or intravenously, rodents exhibited seizures and death.¹²¹ This reinforces the importance of considering the route of administration, as the limited bioavailability of orally-ingested anthocyanins is key in preventing toxicity.¹²² Another study investigated the safety of an anthocyanin-rich purple corn derived colorant in F344 rats over 90d, finding no significant effects when incorporated in the diet 0-5% (w/w), though significant darkening of the urine and feces were noted.¹²³ Finally, a study of an anthocyanin-rich bilberry extract showed no toxic effects when administered acutely at doses > 2 g/kg bw in rats and > 3 g/kg bw in dogs. When chronically dosed for 6 months at lower doses (500 mg/kg bw in rats and 320 mg/kg bw in dogs), no toxic effects were observed, though darkening of excreta was noted.¹²⁴

Other polyphenols and polyphenol-rich extracts

Several other investigations into the potential toxicity of polyphenol-rich extracts and isolated phenolics have been performed. In a study of apple-derived polyphenols over 90d in SD rats at doses up to 2 g/kg bw/d, no adverse effects were noted.¹²⁵ Resveratrol caused side effects (predominantly related to GI distress) in humans over 28d at doses of 5 g/d,¹²⁶ while in rats and dogs, resveratrol induced small changes in body weight over 90d at doses \geq 1000 mg/kg bw/d, though no other signs of toxicity were observed.¹²⁷ Quercetin administered to rats at 0-4% (w/w) in the diet over two years showed no significant differences were observed between groups at 6 or 15 months, though the development of neoplastic lesions in the highest dose group were observed in the kidney after 24 months.¹²⁸

One unique case that bears mentioning here is soy-derived isoflavones. As noted earlier, they can act as SERMs and, at high doses, can reduce fertility.¹²⁹ Isoflavones are phytoestrogens that bind to estrogen receptors, which alters the transcription of multiple estrogen-related genes; these effects have been studied in detail and are reviewed elsewhere.¹³⁰ Concerning classic safety and toxicity, few other adverse effects have been observed, though the full implications of their phytoestrogenicity are still being evaluated and may be an area of future research into their potential for toxicity.

1.3.3 Regulatory guidance for polyphenol safety

As the popularity and prevalence of dietary supplements grows, there is a growing awareness of the need to establish safety guidelines for polyphenol consumption, especially at higher doses as may be present in dietary supplements. Based on the potential for adverse events and toxicity observed in connection with consuming high doses of polyphenols (as summarized in the previous section), there is a growing awareness of the need to establish regulatory guidance surrounding polyphenol-rich dietary supplements.

The European Food Safety Administration (EFSA) has produced the bulk of the specific regulatory guidelines regarding polyphenols. EFSA has approved several health claims for polyphenol-rich foods (e.g., cocoa flavan-3-ols and endothelial function¹³¹ or olive oil polyphenols and protection of blood lipids from oxidative damage¹³²). In the context of safety, they have produced the only known guidance to specifically govern the safety assessment of botanical supplements. The guidance was issued in 2009, and recommended a two-tiered approach in the safety assessment of botanicals intended for use in food supplements.¹³³ In the first tier, the safety assessment is based on available knowledge, while in the second tier, newly generated data are required to adequately assess the safety of the botanical in question. They emphasize that this guidance is intended to provide an approach to assessing safety, but specifically avoids creating a list of safe and unsafe products. This guidance also resulted in the creation and continual maintenance of a Compendium of Botanicals that contains all known toxic, addictive, and psychotropic effects of botanicals.¹³³ The Compendium is publicly available and details the plant species, which part(s) of the plant induced the adverse effects, and the bioactive agent responsible for the toxicity.¹³⁴ The guidance provided by EFSA as well as the Compendium provide a framework to begin evaluating botanicals, though much work is still needed to fully elucidate the safety of botanical supplements.

Outside of Europe, there is little official guidance on the regulation and safety of high doses of polyphenols. In the US, the passage of DSHEA in 1994 expanded the definition of dietary supplements to include non-vitamin and non-mineral supplements (e.g., botanicals), while also effectively loosening regulations on the industry.¹³⁵ Since that time, the presence of polyphenol-rich supplements has grown, but there have been no specific guidelines issued for polyphenols and botanicals. That may change in the near future, as the FDA recently partnered with the National Institutes of Health's National Institute of Environmental Health Sciences (NIH-NIEHS) and the

Health and Environmental Sciences Institute (HESI), signing a Memorandum of Agreement in October 2019 to create the Botanical Safety Consortium (BSC).¹³⁶ The BSC will be composed of scientists from all sectors and is tasked with developing scientific approaches to assess the safety of botanicals used in dietary applications.

As noted by the 1st International Conference on Polyphenols and Health in 2003, adverse effects from high doses of polyphenol have been noted, but there was little evidence upon which to determine a dose threshold for different phenolics and/or food sources of complex mixtures of phenolics.¹⁰⁴ To properly evaluate the safety of such doses, they stated that “toxicologic testing may be required to ensure safe levels of intake.”¹⁰⁴ And, though the toxicity of polyphenols has remained a priority agenda item for discussion at subsequent annual meetings, the most recent meetings did not contain a single presentation addressing this issue, though it remains a featured track in the preliminary agenda for the upcoming conference in 2020.¹³⁷⁻¹⁴⁰ Thus, there remains a dearth of information addressing the potential for adverse effects of high doses of polyphenols. The initial conference rightly pointed out that “we must take the results of the experimental (toxicity) studies seriously, as seriously as we take the (studies of) beneficial effects.”¹⁰⁴ To date, however, this challenge has not been met.

1.3.4 Establishing dietary recommendations for polyphenols

In light of the numerous health benefits attributed to polyphenols, there is great interest in developing dietary recommendations. Establishing recommendations will require the development of an appropriate model through which dietary polyphenols may be evaluated. As this model is developed, understanding the minimum doses needed to elicit a beneficial health outcome as well as maximum doses that may be safely consumed is necessary. Although many in the field are enthusiastic about the potential for developing dietary recommendations, there are many hurdles that must be cleared before dietary recommendations can be created for polyphenols.^{141, 142}

Hormesis

The concept of hormesis has been gaining in popularity over the past 15 years, and is now accepted as a probable mechanism of action for many physical, chemical, and biological conditions that impact health.¹⁴³ Hormesis was originally defined in a toxicological context as a dose-response

relationship that provided stimulation at low doses, but was inhibitory at high doses.¹⁴⁴ This definition has been expanded to encapsulate the idea that small quantities/doses have the opposite effect of large quantities/doses (Figure 1.6).¹⁴⁵ It is in this context that many hormetins, defined as any stress condition capable of producing an hormetic response, have been evaluated.¹⁴³ One classic hormetin is exercise, which promotes good health when repeatedly performed at moderate levels but can be debilitating at excessive levels. The beneficial effect of exercise is driven by mild cellular stresses that improve cellular resiliency. However, as exercise intensity and/or duration is increased, the stress becomes counterproductive and outweighs any potential health benefit.¹⁴⁶

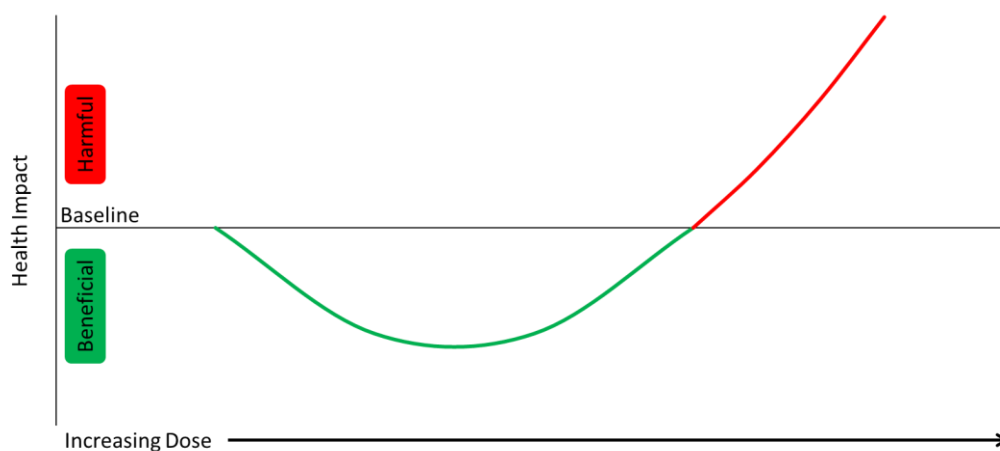


Figure 1.6 – Illustration of hormesis. Low doses of hormetins provide a health benefit by exerting a mild stress that in turn elicits an adaptive response that improves overall health. However, at high doses, the stress is counterproductive and becomes detrimental to health.

One of the challenges to hormesis gaining widespread acceptance is the fact that hormetic effect sizes are often small, with most documented cases providing only 30-60% improvement in the endpoint measured.¹⁴⁴ This has made it difficult to clearly observe hormetic effects in different models, especially when studied in short-term models that only consider specific endpoints. However, when examined over longer periods of time and considered in the context of overall health, the effect sizes may be much larger, exhibiting synergistic and pleiotropic effects.¹⁴³ Because of this, a single hormetin can affect multiple different endpoints and multiple hormetins can affect a single endpoint in biological systems. While adding to the challenge and complexity of fully understanding hormesis, it also helps explain why hormesis has been applied to many different disciplines, including toxicology, pharmacology, aging, and nutrition.

When considering the impacts of diet on overall health, the short-term effects are often minimal, but the long-term effects significantly impact longevity, cognitive function, and quality of life. This has been noted in many studies, and, in the context of hormesis, moderate calorie restriction and intermittent fasting have repeatedly demonstrated the potential for beneficial effects derived from diet. Provided the diet maintains nutritional adequacy for all essential macro- and micronutrients, the mild cellular stresses provided over long periods of time with calorie restriction or intermittent fasting may lead to increased longevity.^{143, 146}

Many different components of the diet have also been examined for their ability to impact health in an hormetic manner. In particular, many vitamins and minerals are known to benefit health when consumed at low doses, but cause toxicity at higher doses. This is typically modeled by a U-shaped curve and has been the defining model for the establishment of Dietary Reference Intakes (DRI, Figure 1.7).¹⁴⁷ And, though usually not described in terms of hormesis, the U-shaped DRI curve is analogous to the U- or J-shaped hormesis curve and the concepts are typically discussed using a similar conceptual framework (i.e., low dose health benefit and high dose health detriment).

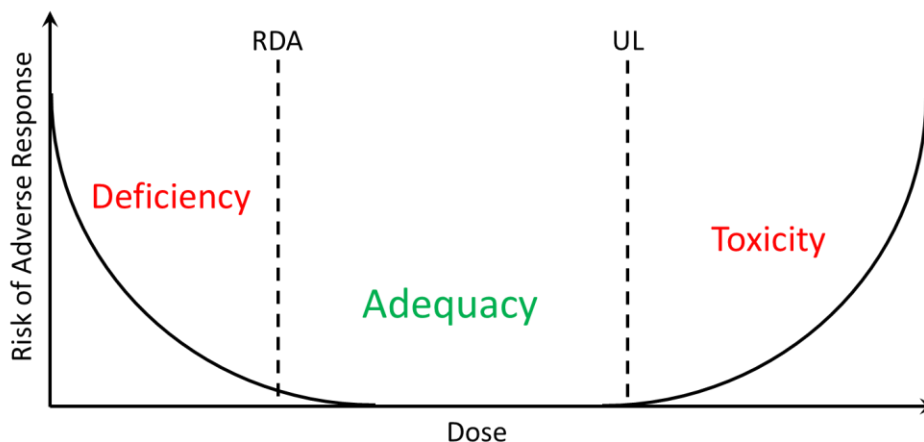


Figure 1.7 – U-shaped DRI curve. At low doses (i.e., below the Recommended Dietary Allowance (RDA)), there is a risk of nutrient deficiency, while at high doses (i.e., above the Upper Limit (UL)), there is a risk of toxicity from excessive intakes. The goal is to maintain adequate nutrient intake. This is analogous to the U- or J-shaped hormesis curve, where a certain level of consumption is needed to optimize health, but excessive consumption is detrimental.

Dietary polyphenols, are increasingly being recognized as hormetins.^{5, 148} Given the numerous health benefits ascribed to consistent fruit and vegetable consumption throughout the lifespan, many have adopted the hormesis framework as a model for describing the health effects of polyphenols.¹⁴⁶ The basis for polyphenols acting in an hormetic manner was initially described in cellular models, where they exhibited an antioxidant effect (either directly or indirectly through signaling pathways) at low doses, but a prooxidant effect at higher doses.¹⁴⁸ When this concept is applied *in vivo*, the effects are more difficult to tease out, especially because of the low bioavailability and extensive metabolism of dietary polyphenols, as discussed earlier. Many dietary intervention studies employing polyphenols are relatively short in duration and the effect sizes (when observed) are small, making it difficult to draw definitive mechanistic links between polyphenols and health benefits. However, as discussed earlier, most hormetic effect sizes are small and typically only accumulate over long periods of time. And, when these small effect sizes are considered alongside the epidemiological evidence of the long-term health benefits of polyphenols, the hypothesis of hormesis is a plausible explanation for the manner in which polyphenols affect human health.

Moving forward: Establishing dietary recommendations for polyphenols

Despite the health benefits attributed to polyphenols, they have been precluded from inclusion in traditional dietary recommendations because they are not considered nutrients essential for biological functioning and survival.¹⁴¹ Until recently, this was considered an impassable barrier. However, in 2017, the National Academies of Medicine published guidance to expand the use of the DRI-development process and allowed for the setting of DRIs on basis of preventing chronic diseases.¹⁴⁹ These guidelines acknowledged that certain dietary components are not essential for the maintenance of normal physiological function, but may increase the health span and delay or prevent the development of chronic diseases later in life.¹⁴⁹ The first application of this new guidance was produced in 2019 and updated the DRIs for sodium and potassium on the basis of cardiovascular diseases.¹⁵⁰

Given the accumulating health benefits of polyphenols, specifically their ability to delay or prevent chronic diseases, there is now a path forward for establishing dietary recommendations for polyphenols. Establishing these guidelines will be challenging for a number of reasons. First, polyphenols are often discussed collectively because they typically exist as complex mixtures *in*

planta and in the diet, making it difficult to elucidate the specific effects of individual polyphenols. Second, more conclusive dose-response data are needed to specifically define the dose-range that both elicits the beneficial effects and is safe for consumption. To date, virtually all polyphenol research has focused on the minimum dose needed to demonstrate a beneficial health effect, while there are scant evidences regarding the potential for toxicity from polyphenols. This is a major challenge that will need to be addressed before DRIs can be established. Finally, there are few validated surrogate endpoints exist for evaluating chronic disease risk,¹⁴² which makes the establishment of definitive connections between polyphenols and the endpoint of chronic disease difficult. Taken together, there are a number of issues that must be addressed and clarified before DRIs can be established for polyphenols.

1.4 Conclusion and thesis overview

Polyphenols are ubiquitously present throughout the diet, as they are present in all fruits and vegetables as well as coffee and tea, and they may have significant impacts on human health. As our understanding of their metabolism and bioavailability continues to improve, there are many gaps in knowledge that have yet to be addressed. In particular, the safety, metabolism, and health effects of polyphenols at high doses has not been adequately addressed. With the increased prevalence of botanical dietary supplements, evaluating and understanding these effects is a critical step. In light of these challenges, the research herein seeks to address metabolic changes that occur both acutely and chronically when high doses of blueberry polyphenols are consumed, while also addressing the potential for toxicity. To assess this, three specific aims are evaluated:

1. Acute dose-response and pharmacokinetics of colonic metabolism of blueberry polyphenols over 48h. The *working hypothesis* is that colonic metabolites will be absorbed more extensively and appear in the plasma and urine at later times than small intestinal metabolites. In this experiment, ovariectomized Sprague-Dawley (OVX-SD) rats will be acutely gavaged with purified blueberry polyphenols and plasma and urinary excretion of phenolic metabolites measured.
2. Safety and systemic effects of 90d of repeated dosing with elevated levels of blueberry polyphenols. The *working hypothesis* is that repeated administration of high doses of blueberry polyphenols will induce adverse effects in OVX-SD rats, especially in the GI tract. Following the Organisation for Economic and Cooperative Development (OECD)

guidelines for sub-chronic toxicity tests, rats will be gavaged with a concentrated extract of blueberry polyphenols for 90d. Upon completion of the study, a complete necropsy, including urinalysis, hematology, serum biochemistry, and histopathology of key metabolic tissues, will be performed to assess toxicity that may result from repeated administration of high doses of blueberry polyphenols.

3. Shifts in polyphenol metabolism with repeated dosing of high levels of blueberry polyphenols over 90d. The *working hypothesis* is that dose-dependent shifts will be seen in polyphenol metabolism early in the study, but that these changes will plateau and remain consistent throughout the rest of the study period. Using the same model as aim 2, urinary metabolites will be collected at six different times over 90d to assess metabolism.

The data obtained from these studies will help to clarify the safety and metabolism of blueberry polyphenols at elevated doses. This will not only advance our understanding of the colonic metabolism and safety of dietary polyphenols, but will also aid in the creation of dietary guidance for polyphenols.

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CHAPTER 2. INCREASING DOSES OF BLUEBERRY POLYPHENOLS ALTERS COLONIC METABOLISM AND CALCIUM ABSORPTION IN OVARECTOMIZED RATS

A version of this manuscript will be submitted for publication.

Dennis P. Cladis,¹ Hawi Debelo,² Pam J. Lachcik,³ Mario G. Ferruzzi,² Connie M. Weaver^{1*}

¹ Dept. of Food Science, Purdue University, 745 Agriculture Mall Dr, W Lafayette, IN 47907, USA

² Plants for Human Health Institute, North Carolina State University, 600 Laureate Way, Kannapolis, NC 28081, USA

³ Dept. of Nutrition Science, Purdue University, 700 W State St, W Lafayette, IN 47907, USA

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*Corresponding author email: weaverconnie1995@gmail.com

2.1 Abstract

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 polyphenol, as may be present in dietary supplements, are unknown, especially when considering the colonic metabolites formed. To evaluate the pharmacokinetics of this consumption modality in an animal model of postmenopausal women, 5-mo old, ovariectomized Sprague-Dawley rats were acutely dosed with purified blueberry polyphenols (0, 75, 350, and 1000 mg total polyphenols/kg bw) and ⁴⁵Ca 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 cinnamic and hippuric acids, with smaller amounts other phenolic acids, flavonols, and anthocyanins also detected. Colonically generated metabolites were observed at levels up to 10-

fold higher than their small intestinal counterparts. Most metabolites followed a dose-response relationship, exhibiting significant differences in total excretion ($p < 0.05$), though several showed saturated absorption at the medium dose. Maximal metabolite concentrations were reached within 12h for a majority of compounds measured, though some (e.g., hippuric acid) peaked up to 24h post-dosing. Calcium absorption was significantly increased in the highest dose group ($p = 0.03$). These results indicate that increased doses of blueberry polyphenols may induce changes in intestinal metabolism, which may alter GI function.

2.2 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

Recently, new avenues linking blueberry polyphenols to health endpoints has explored the connection between blueberries and bone health. These reports suggest that blueberry polyphenols may be able to mitigate menopause-associated bone loss and reduce the incidence of osteoporosis in aging females.^{7, 8}

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.⁹⁻¹¹ Small intestine-derived phase II metabolites are poorly absorbed (<2% for many classes of polyphenols),^{12, 13} as evidenced by low plasma and urine concentrations (i.e., low C_{\max} values) and rapid excretion (T_{\max} 0.5-2h).¹⁴ 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.¹⁵

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.^{11, 16} 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.¹⁷ 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}).^{10, 18} This not only increases the overall bioavailability of polyphenols, but raises the possibility that the colonic catabolites may be driving the observed health benefits.¹² 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.¹⁹ 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.²⁰ 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,²¹⁻²⁴ expanding the range of doses studied is critical to understand the full scope of the metabolism of these compounds. Thus, we sought to evaluate the metabolism and dose-response of blueberry polyphenols in a rat model by quantitating the urinary and plasma metabolites of an acute dose for 48h after dosing over a large range of doses. Additionally, we sought to link this to bone health by assessing the impact of blueberry polyphenols on calcium absorption. We hypothesized that colonic catabolites would be absorbed much later than and to a much greater extent than small intestinal metabolites.

2.3 Materials and Methods

2.3.1 Chemicals/materials and vendors

Commercial standards of cyanidin-3-O-glucoside chloride, delphinidin-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, acetonitrile, 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).

2.3.2 Animal protocols

Study overview

The study design is illustrated in Figure 2.1. Upon arrival, animals were maintained on a polyphenol-free diet during a 1-week stabilization. Animals were then randomized to treatment groups ($n = 8/\text{gp}$), 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.

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 (OVX), 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.

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.

Preparation of blueberry phenolics for oral gavage

VitaBlue Pure American Blueberry Extract, containing 28.8% total phenolics (w/w), was donated by FutureCeuticals (Mokena, 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. Immediately prior to receiving the oral gavage, each animal underwent a one-time, 8h fast.

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.²⁵ Animals were anesthetized with isoflurane and given buprenex (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 6000 rpm (3500g) 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, 2x12h 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, 4x12h 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.

⁴⁵Ca absorption

Immediately after gavaging animals with blueberry phenolics, six animals per group were given a second oral gavage containing 20 µCi ⁴⁵Ca 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 ⁴⁵Ca 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.

2.3.3 Polyphenol analyses

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.²⁶ 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.²⁶

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, Torrance, 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 x 200 μL 0.1% formic acid in water, and then dried under nitrogen for 30 minutes. Finally, 100 μL 0.1% formic acid in methanol was loaded and wells soaked 5 minutes before elution into a 96-well plate (350 μL 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.

Quantification of total phenolics in starting materials

Total phenolics were quantified in crude extracts via the Folin method and corrected for vitamin C content as described elsewhere.²⁷

Quantification 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 μm, 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 0.001-100 μM. 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.²⁸ A complete list of phenolic

compounds and metabolites measured, including corresponding MRMs and standards used for quantitation, is shown in Table A.1.

2.3.4 Fractional calcium absorption

Total femoral calcium deposition was used to determine fractional calcium absorption, as described elsewhere.^{29, 30} 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 ⁴⁵Ca 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. ⁴⁵Ca in femurs as previously described.^{30, 31}

2.3.5 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.

2.4 Results and Discussion

2.4.1 Phenolic profiles of raw materials, rat diets, and gavage doses

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 2.1). 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 2.1, 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.

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 2.1). 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.

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 2.1, 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.

2.4.2 Urinary excretion of phenolic metabolites.

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 2.2). 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 2.2). These metabolites exhibited a dose-dependent shift in excretion, with higher doses showing increased proportions of cinnamic and phenyl propanoic acids and a concomitant decrease in the proportion of hippuric acids.

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 2.3a. For the other phenolic acids, 4 exhibited similar excretion in both the 0-12h and 12-24h urine collections (exemplified in Figure 2.3c), while the 3 remaining metabolites did not have a clear peak excretion time. (C_{max} and T_{max} values can be found in Table 2.2. Pharmacokinetic curves for all phenolic acids can be found in the Supporting Information, Figures A.1-A.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 anthocyanins¹¹ and chlorogenic acids.^{32, 33} 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.³⁴ Similar results were observed after blueberry intake in healthy adults, as cinnamic acid derivatives (especially

glucuronidated and sulfated forms) were prominent metabolites.^{35, 36} 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.¹⁰

Other phenolic acids, including benzoic acids, phenyl acetic acids (PAA), and phenyl propionic 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,^{35, 37} PAA,³⁶ or benzaldehydes and PAA³⁸ 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.

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 excretions in the 0-12h and 12-24h urine collections (Figures A.15-A.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 2.3b), 3 had similar excretions during the 0-12h and 12-24h time points (exemplified in Figure 2.3d), and 2 did not exhibit a discernable peak excretion. (See Table 2.2 for C_{max} and T_{max} as well as Figures A.19-A.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 A.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,^{35, 38} 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.³⁹

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 2.3e and A.37-A.38). Additionally, 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,⁴⁰ anthocyanins,^{11, 41} and chlorogenic acids.^{32, 33} 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.^{36, 42-44} We found hippuric acid to be a major metabolite, but as shown in Figures 2.3e and A.37-A.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 2.2). 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, there may be too high a content of polyphenols for the colonic microbes to fully metabolize prior to fecal excretion (see next section for details).

Metabolic sinks in colonic metabolism of blueberry polyphenols

A summary of colonic metabolism of polyphenols is shown in Figure 2.4. 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 2.2, 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.

2.4.3 Plasma pharmacokinetics

A total of 16 metabolites (6 anthocyanins, 6 phenolic acids, 3 flavonols, and hippuric acid) were detected in the plasma (Table 2.3). 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 2.3 and Figures A.39-A.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.

2.4.4 Calcium absorption

To link phenolic absorption to a functional outcome, fractional calcium absorption was measured using ^{45}Ca , an isotopic tracer. As shown in Figure 2.5, fractional calcium absorption was

significantly higher in the highest dose group as compared to control. This relationship was demonstrated for both the right and left femurs when analyzed separately and together.

Maintaining bone health throughout the lifespan is key to preventing osteoporosis, and this can be accomplished through either increasing calcium absorption and bone formation or inhibiting bone resorption. 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%).^{30, 45, 46} Additionally, these 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.⁴⁷ Taken together, these studies may indicate that polyphenols consumed at dietary doses do not acutely alter calcium absorption, though long term effects may emerge later.

Several other studies employed the ^{45}Ca technique to measure calcium absorption, though these studies were focused on honey³¹ and whey protein concentrates.⁴⁵ In contrast to our results with blueberry polyphenols, these studies observed an hormetic response to the test article, wherein calcium absorption was increased at low doses but no different than control at higher doses.^{31, 45} Importantly, these studies note that the acute increase in calcium absorption was not observed after 8 weeks of treatment, indicating that the effect may be transient or that an adaptation occurs, meaning the treatment may not have long-term benefits.^{31, 45} Following up with a long-term study may help clarify the differences in acute and chronic effects of blueberry polyphenols on calcium absorption and bone health.

2.4.5 Limitations, Conclusions, and Future Work

There were 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.¹⁰ 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 in the lab. 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 ($T_{\max} < 2\text{h}$).¹⁴ 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.

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2.6 Author contributions

DPC, MGF, and CMW designed research; DPC and PJL performed all animal procedures and experiments; DPC, HD, and MGF conducted phenolic analyses; DPC, PJL, and CMW performed ⁴⁵Ca analysis. DPC wrote manuscript. All authors reviewed and approved the final version of the manuscript.

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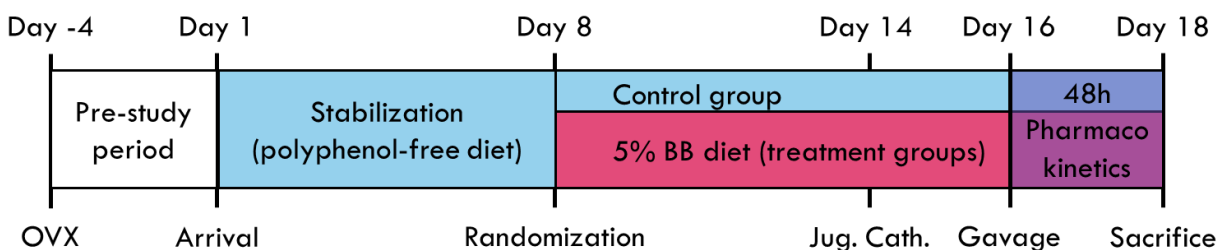


Figure 2.1 – Study design and timeline. All animals were ovariectomized (OVX) by the vendor prior to shipping (day -4). Upon arrival (day 1), animals were stabilized for one week on a polyphenol-free diet. Randomization took place on day 8, with control animals remaining on the polyphenol-free diet and 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.

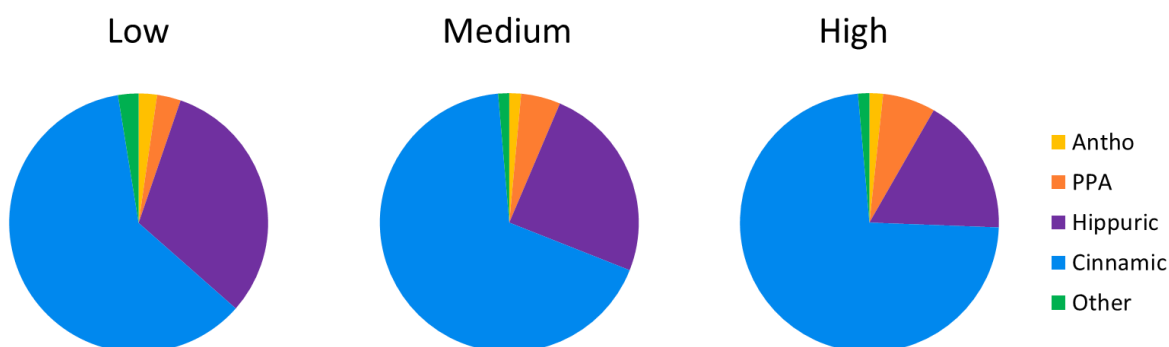


Figure 2.2 – Dose dependent excretion of phenolics over 48h. For all doses, cinnamic acids were the predominant metabolite found in urine, followed by hippuric acids. As dose increased, hippuric acids decreased as a percentage of total metabolites excreted, while cinnamic acids and phenyl propionic acids increased. (Antho = sum of all anthocyanins excreted; PPA = sum of phenyl propionic 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, phenyl acetic acids, and flavonols excreted.)

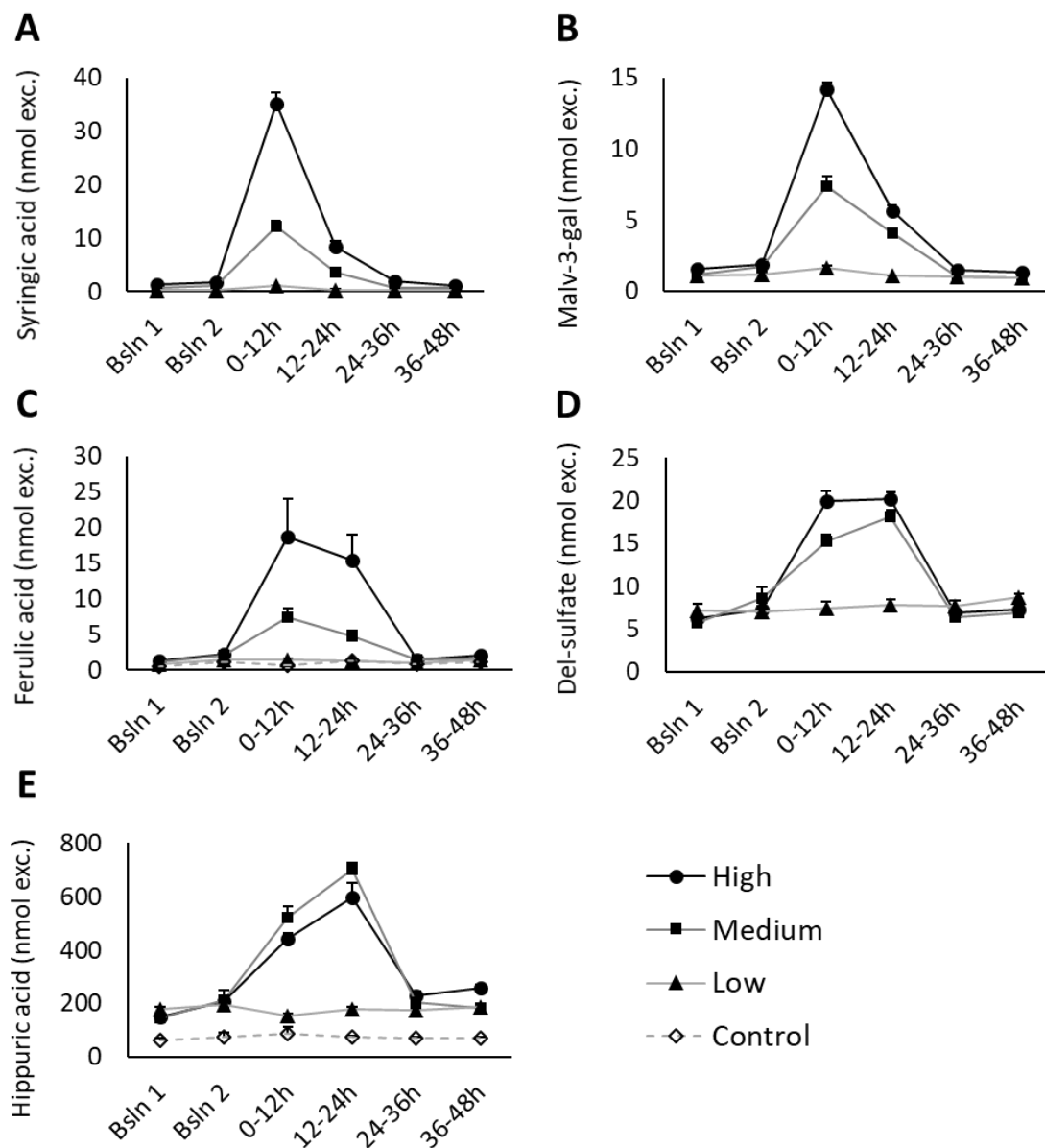


Figure 2.3 – Urinary excretion pharmacokinetics of selected metabolites. Most phenolic acid, flavonol, and anthocyanin metabolites demonstrated a dose-response relationship and were maximally excreted within 12h of dosing (exemplified by syringic acid (A) and malvidin-3-O-galactoside (B)), while others 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 \pm SEM; n = 6-8/gp; see Appendix A for statistics. Bsln 1 = Baseline 0-12h; Bsln 2 = Baseline 12-24h.

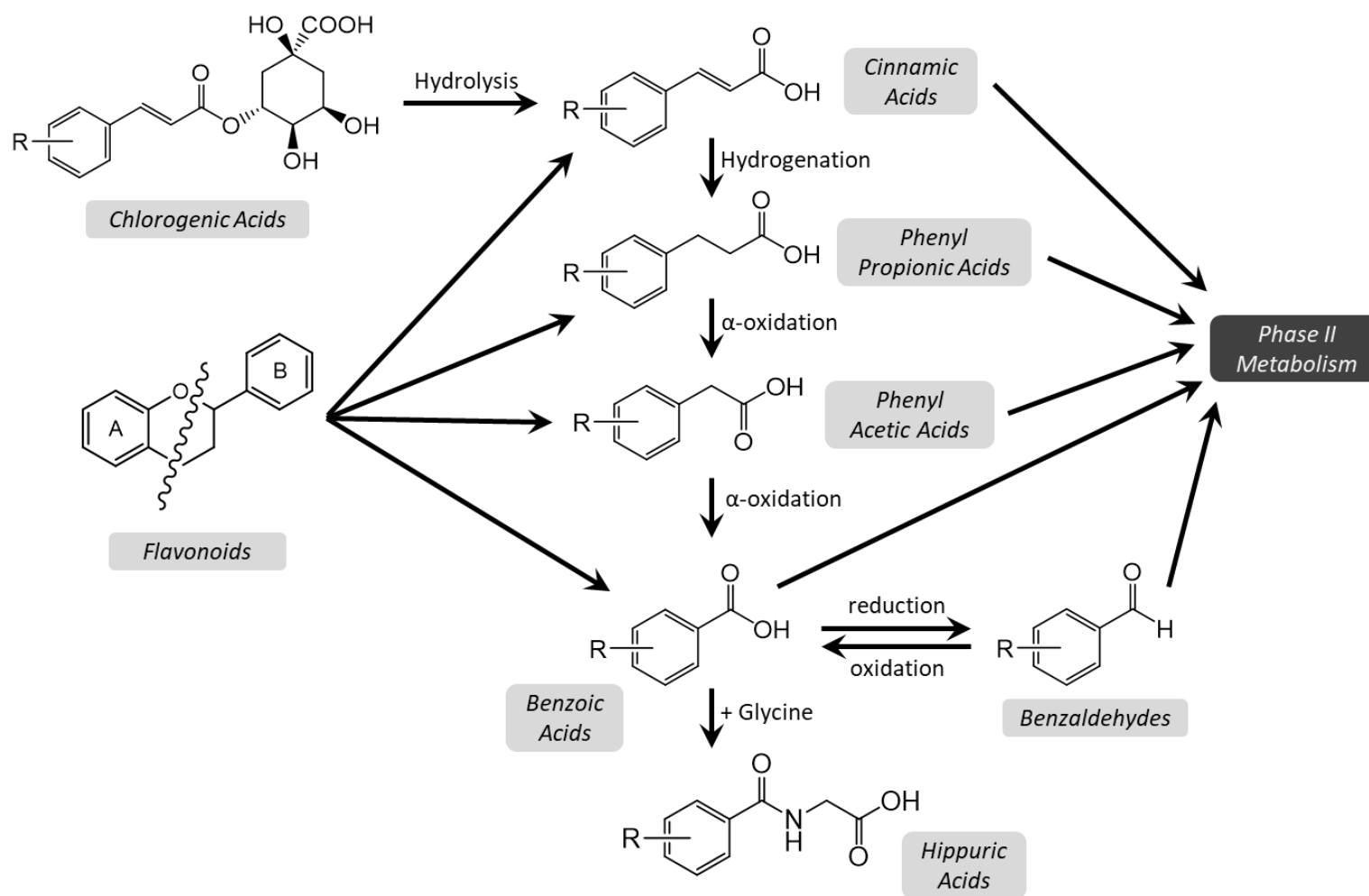


Figure 2.4 – Summary of colonic catabolism of major blueberry polyphenols. Chlorogenic acids are hydrolyzed to cinnamic acids, while the heterocyclic ring of flavonoids (anthocyanins and flavonols in blueberries) is cleaved, producing two, smaller molecular weight phenolic acids from the A and B rings. 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).

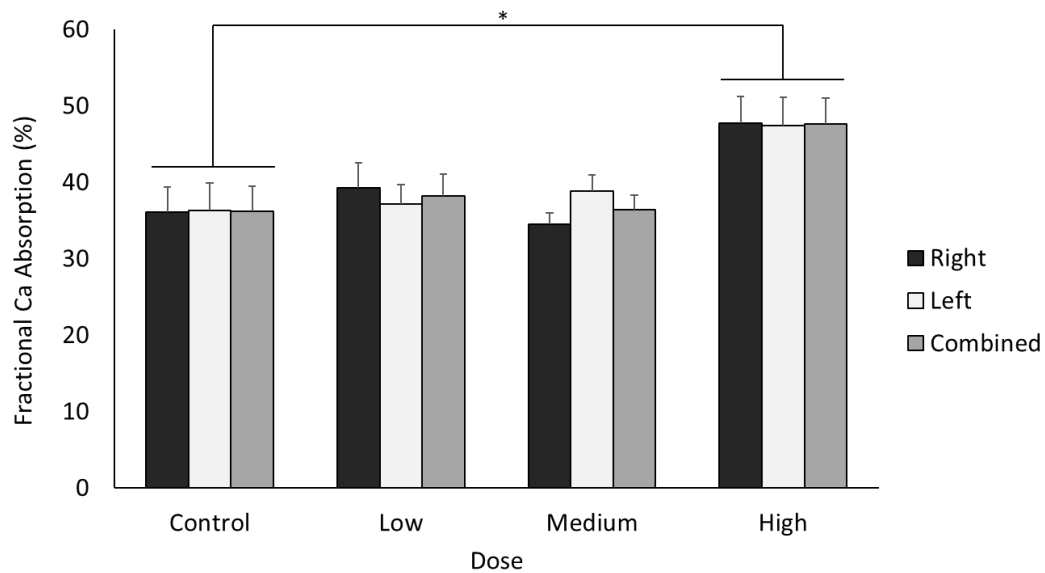


Figure 2.5 – Fractional ^{45}Ca absorption. Fractional calcium absorption was significantly higher in the high dose group for both the right and left femurs separately and when analyzed together. * $p < 0.05$ significantly different from respective control, using Dunnett's test. Data shown as mean \pm SEM.

Table 2.1 – Phenolic content of raw materials, rat diets, and gavage doses.*

Polyphenol	Raw Materials		Rat Diets		Control	Gavage Doses		
	FD	CE	PPF	5% BB		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
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

Table 2.1 continued

<i>Flavonols</i>									
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	
Kaempferol	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.

* 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 2.2 – Total urinary excretion of phenolic metabolites for 48h after gavage. *

	Control	Total Urinary Excretion (nmol)			Control	Qualitative T _{max} ^			Control	C _{max} (nmol excreted)			
		Low	Medium	High		Low	Medium	High		Low	Medium	High	
Anthocyanins													
Cyanidin													
Galactoside	nd	0.19 ± 0.04 ^a	0.41 ± 0.08 ^b	0.68 ± 0.08 ^c	-	np	0-12h	0-12h	-	np	0.19 ± 0.04	0.46 ± 0.09	
Glucoside	nd	0.17 ± 0.03 ^a	0.31 ± 0.08 ^b	0.41 ± 0.04 ^c	-	np	0-24h	0-12h	-	np	0.11 ± 0.05	0.22 ± 0.04	
Glucuronide	nd	nd	0.05 ± 0.02 ^a	0.13 ± 0.05 ^b	-	-	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 ^a	2.08 ± 0.21 ^b	2.81 ± 0.23 ^c	-	np	0-12h	0-12h	-	np	0.98 ± 0.20	1.74 ± 0.22	
Glucoside	nd	0.79 ± 0.07 ^a	1.94 ± 0.19 ^b	2.00 ± 0.29 ^b	-	np	0-24h	0-24h	-	np	0.81 ± 0.14	0.94 ± 0.22	
Glucuronide	nd	4.21 ± 0.92 ^a	12.7 ± 2.18 ^b	31.4 ± 1.33 ^c	-	np	0-12h	0-12h	-	np	6.35 ± 1.60	20.8 ± 5.1	
Sulfate	nd	31.8 ± 5.83 ^a	46.9 ± 6.01 ^b	54.4 ± 4.23 ^b	-	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 ^a	13.5 ± 2.51 ^b	22.7 ± 1.30 ^c	-	np	0-12h	0-12h	-	np	7.36 ± 2.00	14.2 ± 1.3	
Glucoside	nd	6.06 ± 0.66 ^a	14.3 ± 0.88 ^b	19.7 ± 1.02 ^c	-	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 ^a	0.08 ± 0.01 ^b	0.14 ± 0.02 ^c	-	np	0-12h	0-12h	-	np	0.04 ± 0.01	0.09 ± 0.02	
Glucoside	nd	0.05 ± 0.01 ^a	0.08 ± 0.02 ^b	0.14 ± 0.02 ^c	-	np	0-12h	0-12h	-	np	0.03 ± 0.01	0.09 ± 0.02	
Glucuronide	nd	2.12 ± 0.83 ^a	3.01 ± 1.01 ^{ab}	3.38 ± 1.27 ^b	-	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 ^a	1.05 ± 0.30 ^b	2.49 ± 0.32 ^c	-	np	0-12h	0-12h	-	np	0.49 ± 0.23	1.73 ± 0.38	
Glucoside	nd	0.44 ± 0.08 ^a	0.90 ± 0.25 ^b	1.47 ± 0.17 ^c	-	np	0-12h	0-12h	-	np	0.37 ± 0.14	0.94 ± 0.19	
Glucuronide	nd	0.69 ± 0.25 ^a	1.81 ± 0.47 ^b	5.63 ± 0.56 ^c	-	np	0-12h	0-12h	-	np	0.92 ± 0.24	3.49 ± 0.46	
Sulfate	nd	0.32 ± 0.09 ^a	0.43 ± 0.08 ^b	0.44 ± 0.09 ^{ab}	-	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 ^a	17.0 ± 2.89 ^b	48.2 ± 4.49 ^c	-	0-12h	0-12h	0-12h	-	1.03 ± 0.30	12.2 ± 3.0	35.1 ± 6.0	
BzA sulfate	11.2 ± 1.72 ^{ab}	7.96 ± 1.20 ^c	12.6 ± 1.75 ^b	9.62 ± 1.40 ^{ac}	np	np	np	np	np	np	np	np	
BzA glucuronide	1.45 ± 0.30 ^a	1.14 ± 0.16 ^b	2.25 ± 0.21 ^c	1.95 ± 0.31 ^c	np	np	12-24h	12-24h	np	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 ^a	0.95 ± 0.20 ^b	1.87 ± 0.52 ^c	1.22 ± 0.23 ^b	np	0-24h	0-24h	0-24h	np	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 ^a	34.4 ± 6.10 ^a	28.6 ± 3.82 ^a	-	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 ^b	328 ± 49.6 ^c	579 ± 67.1 ^d	-	0-12h	0-12h	12-24h	np	25.4 ± 6.9	177 ± 24	275 ± 34	
3-OH-4-OME-PPA	nd	2.00 ± 1.01 ^a	4.77 ± 1.99 ^b	6.76 ± 5.20 ^b	-	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 ^a	8.78 ± 3.77 ^b	8.52 ± 1.73 ^b	np	np	0-12h	0-12h	np	np	3.18 ± 1.64	3.97 ± 1.38	
Caffeic acid sulfate	1.30 ± 0.03 ^a	9.55 ± 4.01 ^b	15.4 ± 6.56 ^b	18.1 ± 2.69 ^b	np	np	0-12h	0-12h	np	np	6.43 ± 4.57	9.74 ± 0.78	

Table 2.2 continued

Caffeic acid glucuronide	443 ± 28.4 ^a	402 ± 61.8 ^a	677 ± 97.7 ^b	897 ± 163 ^c	-	np	0-12h	0-12h	-	np	261 ± 50	379 ± 134
Ferulic acid	nd	5.76 ± 1.26 ^a	22.6 ± 4.34 ^b	38.8 ± 0.81 ^c	np	np	0-24h	0-24h	np	np	11.7 ± 3.0	29.5 ± 5.9
Ferulic acid sulfate	4.01 ± 1.03 ^a	5.17 ± 1.70 ^a	15.2 ± 3.47 ^b	37.8 ± 13.7 ^c	np	np	0-12h	0-12h	np	np	7.41 ± 3.52	18.7 ± 14.9
Ferulic acid glucuronide	469 ± 82.8 ^a	936 ± 147 ^b	3611 ± 450 ^c	4772 ± 674 ^c	-	np	0-12h	0-12h	-	np	2069 ± 354	2943 ± 302
Chlorogenic acid	nd	40.1 ± 10.4 ^a	256 ± 13.5 ^b	360 ± 61.1 ^c	-	np	0-24h	0-12h	-	np	66.1 ± 23.4	238 ± 67
<i>Total Cinnamic Acids</i>	<i>917</i>	<i>1403</i>	<i>4606</i>	<i>6122</i>								
<i>Total Phenolic Acids</i>	<i>935</i>	<i>1507</i>	<i>5007</i>	<i>6798</i>								
Hippuric Acids												
Hippuric acid	304 ± 70.2 ^a	706 ± 66.8 ^b	1611 ± 154 ^c	1528 ± 193 ^c	np	np	12-24h	12-24h	np	np	700 ± 78	598 ± 146
3-OH-hippuric acid	3.94 ± 1.01 ^a	18.4 ± 3.14 ^b	64.2 ± 13.4 ^c	42.6 ± 9.1 ^d	np	np	12-24h	12-24h	np	np	31.8 ± 8.1	26.3 ± 7.6
<i>Total Hippuric Acids</i>	<i>308</i>	<i>724</i>	<i>1675</i>	<i>1571</i>								
Flavonols												
Myricetin	nd	trace	trace	trace	-	-	-	-	-	-	-	-
Quercetin	nd	11.5 ± 4.47 ^a	19.7 ± 7.26 ^b	26.9 ± 2.24 ^b	-	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 ^a	1.48 ± 0.18 ^b	2.70 ± 0.19 ^c	-	np	0-12h	0-12h	-	np	0.72 ± 0.14	1.83 ± 0.28
4-OMe-quercetin	nd	0.60 ± 0.25 ^a	1.39 ± 0.32 ^b	2.49 ± 0.61 ^c	-	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 ^a	2.93 ± 0.58 ^b	7.17 ± 1.31 ^c	-	np	0-12h	0-12h	-	np	1.61 ± 0.43	4.16 ± 0.58
<i>Total Flavonols</i>	<i>nd</i>	<i>13.4</i>	<i>25.5</i>	<i>39.3</i>								
<i>Total Phenolics Excreted</i>	<i>1243</i>	<i>2298</i>	<i>6808</i>	<i>8557</i>								

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

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

^ 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 2.3 – Phenolics detected in plasma. *

	Plasma AUC (uM*h)				Qualitative T _{max} ^				C _{max} (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 ^a	8.00 ± 2.22 ^b	-	-	12h	12h	-	-	0.161 ± 0.092	0.507 ± 0.107
Caffeic gcnd	nd	0.36 ± 0.06 ^a	0.55 ± 0.11 ^b	0.92 ± 0.20 ^c	-	np	6h	6h	-	np	0.021 ± 0.008	0.084 ± 0.020
Ferulic acid	nd	nd	nd	trace	-	-	-	-	-	-	-	-
Ferulic sulf	1.88 ± 0.26 ^a	6.16 ± 1.50 ^b	6.33 ± 3.04 ^b	9.12 ± 2.89 ^b	np	np	6h	6h	np	np	0.262 ± 0.169	0.825 ± 0.317
Ferulic gcnd	nd	5.78 ± 0.75 ^a	7.17 ± 1.05 ^b	11.3 ± 0.72 ^c	-	np	6h	6h	-	np	0.217 ± 0.073	0.797 ± 0.141
Hippuric acid	3.19 ± 0.36 ^a	25.8 ± 3.08 ^b	47.7 ± 5.13 ^c	74.3 ± 7.54 ^d	np	np	12h	24-36h	np	np	1.535 ± 0.510	2.065 ± 0.806
Flavonols												
Quer gcnd	nd	0.32 ± 0.01 ^a	0.34 ± 0.05 ^a	0.57 ± 0.14 ^b	-	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 ^a	3.56 ± 0.73 ^a	4.31 ± 0.20 ^b	-	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.

* 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/gp) and presented as mean ± SD. Superscript letters indicate significant differences between doses (p < 0.05 with Tukey's HSD test).

^ Qualitative T_{max} represents the blood collection during which plasma levels of the metabolite was greatest; np indicates no peak observed; time ranges demonstrate similar amounts quantitated at multiple time points.

CHAPTER 3. A 90 DAY ORAL TOXICITY STUDY OF BLUEBERRY POLYPHENOLS IN OVARIECTOMIZED SPRAGUE-DAWLEY RATS

A version of this manuscript has been submitted to Food and Chemical Toxicology.

Dennis P. Cladis,^a Shiyu Li,^a Lavanya Reddavari,^a Abigail Cox,^b Mario G. Ferruzzi,^c Connie M. Weaver^{a*}

^a Dept. of Food Science, Purdue University, 745 Agriculture Mall Dr, W Lafayette, IN 47907, USA

^b Dept. of Comparative Pathobiology, Purdue University, 625 Harrison St, W Lafayette, IN 47907, USA

^c Plants for Human Health Institute, North Carolina State University, 600 Laureate Way, Kannapolis, NC 28081, USA

*Corresponding author email: weaverconnie1995@gmail.com

3.1 Abstract

Regular consumption of polyphenol-rich fruits and vegetables is associated with beneficial health outcomes. To increase polyphenol intakes, consumers are increasingly using herbal and botanical dietary supplements containing concentrated polyphenol extracts. However, the safety of this consumption modality has not been vetted. To address this, ovariectomized Sprague-Dawley (OVX-SD) rats were orally gavaged with purified blueberry polyphenols at 0-1000 mg total polyphenols/kg bw/d for 90d. No differences in behavior, body weight, or food consumption were observed. No tumors or macroscopic changes were observed, and histopathological analyses showed no differences among groups. Although several statistically significant differences between treatment and control groups were observed in urine (color and pH) and blood (monocyte count, total cholesterol, and chloride ion concentration) analyses, these parameters were within normal ranges and not considered biologically significant. Intestinal permeability assessed via FITC-dextran showed increased intestinal permeability in the highest dose, though no

morphological differences were found throughout the gastrointestinal tract. Given the lack of other systemic changes, this finding is likely of minimal physiological importance. These results indicate a NOAEL for blueberry polyphenols in OVX-SD rats is ≥ 1000 mg total polyphenols/kg bw/d, which translates to a 70 kg human consuming ~10 g polyphenols. Keywords: Blueberry, Polyphenol, Sub-chronic toxicity

3.2 Abbreviations

BB	Lyophilized blueberry dose group
BMD	Bone mineral density
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
FER	Food efficiency ratio
FITC	Fluorescein isothiocyanate
GI	Gastrointestinal
GTE	Green tea extract
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
OVX-SD	Ovariectomized Sprague Dawley

3.3 Introduction

Regular fruit and vegetable consumption is part of a healthy diet to promote adequate intakes of essential nutrients and to increase exposure to health-promoting polyphenols. When consumed regularly, polyphenols have been linked to improvements in numerous health endpoints, including cardiovascular and neurocognitive health, while also lowering the potential for developing chronic diseases.¹⁻⁴

Blueberries are a rich source of polyphenols, containing large amounts of anthocyanins, chlorogenic acid, and quercetin (Figure 3.1).⁵ Both blueberries and their constituent polyphenols have been linked to the health benefits described above.⁶ In addition, the popularity of blueberries in the US has grown dramatically in the past few decades and growth is expected to continue.⁷

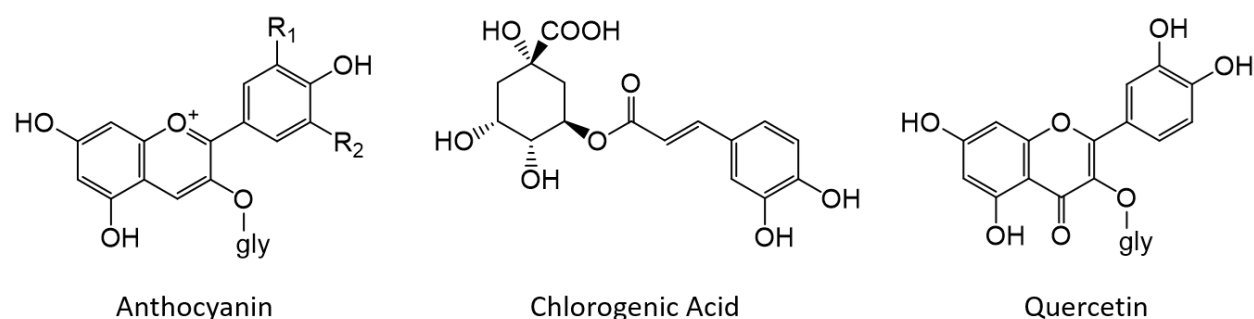


Figure 3.1 – Structure of common polyphenols in blueberries. Anthocyanins account for approximately half of all polyphenols in blueberries and can take different forms, depending on substitutions at the R1 and R2 positions. *In planta*, anthocyanins and quercetins are generally found in their glycosylated form, with linkages to glucose, galactose, and arabinose moieties (indicated by “gly”) being most commonly found in nature.

In light of these benefits, many consumers have sought to increase their polyphenol consumption, often turning to dietary supplements to meet this need. Recent estimates show that 75% of US adults consume at least one dietary supplement and 30% consume polyphenol-rich herbal and botanical supplements.⁸⁻¹¹ The tacit assumption amongst consumers is that because these supplements are derived from natural sources, they must be safe to consume. However, this hypothesis has not been adequately vetted, and the scientific literature lacks sufficient evidence to validate or nullify this assumption of safety.^{12, 13} And, given clinical cases of hepatotoxicity resulting from consuming dietary supplements containing high levels of polyphenol-rich green tea extracts,¹⁴ the presumption of safety for this consumption modality cannot be assumed. In light of this, many regulatory agencies (e.g., Food and Drug Administration (FDA), National Toxicology Program, and National Cancer Institute), are increasing efforts to test herbal and botanical supplement safety.¹⁵

Most clinical studies regarding the health benefits of polyphenols report that they are well-tolerated at normal to even higher dietary doses, though there are several reports of adverse events and side effects occurring after repeatedly consuming higher doses more in line with supplemental paradigms. These adverse events are dose-dependent and most often associated with some form of gastrointestinal distress (e.g., nausea, abdominal pain, diarrhea).^{13, 16-18} These effects are often more pronounced in animal models¹⁸ and extend to other organ systems, with indications of altered function in the liver, kidneys, and reproductive systems after oral ingestion of high doses of green

tea catechins, quercetin, and soy isoflavones.^{17, 19} However, few of these investigations exist in the literature, despite a call for more studies regarding the safety of polyphenols at high doses.²⁰

To address the paucity of available evidence surrounding the safety of blueberry polyphenols at elevated doses and the fact that women ages 51-70y are the most frequent consumers of herbal and botanical dietary supplements,⁸ we employed an ovariectomized (OVX) rat model to test the safety of increasing doses of blueberry polyphenols, following OECD 408 guidelines for sub-chronic toxicity testing.²¹

3.4 Materials and Methods

3.4.1 Chemicals/Materials and vendors

Gallic acid, sodium carbonate, fluorescein isothiocyanate-dextran (FITC), and Folin and Ciocalteu's reagent (2N) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Extraction and LC-MS grade solvents, including methanol, water, acetonitrile, and formic acid, as well as 10% neutral buffered formalin and 0.9% aqueous sodium chloride solution were purchased from Thermo Fisher Scientific (Waltham, MA, USA). VitaBlue Pure American Blueberry Extract was obtained from FutureCeuticals (Mokena, IL, USA); freeze-dried blueberries were obtained from the Wild Blueberry Association of North America (WBANA, Old Towne, ME, USA).

3.4.2 Animal protocols

Study Overview

Fifty-four 5-month old, virgin, ovariectomized (OVX), female Sprague Dawley rats were used in this study. Upon arrival, rats were randomized to treatment groups and given one month to stabilize from OVX surgery. Animals then underwent 90d of dosing via oral gavage, following OECD guidelines for sub-chronic toxicity studies,²¹ and were monitored daily. A total of 5 treatment groups plus one non-gavaged group were used in the study. Animals were monitored daily for signs of overt toxicity; body weight and food consumption were monitored weekly throughout the study. Upon completion of the 90d dosing regimen, gut permeability was assessed using FITC, and animals were euthanized via CO₂ asphyxiation and necropsied.

Animal Care

Animal experiments were conducted in adherence to Purdue University Animal Care and Use Committee guidelines, following an approved protocol (1808001790). 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.

Rat chow diet

All animals were maintained on a polyphenol-free diet throughout the stabilization and study periods. Polyphenol-free diets were based on the AIN-93M diet, using corn oil in place of soybean oil to remove isoflavones and prevent confounding by polyphenols unrelated to blueberry treatment. Diets were prepared by Research Diets (New Brunswick, NJ, USA).

Treatment groups

A total of 5 treatment groups were used for this study. Because there are no data upon which to reliably base power calculations, OECD 408 guidelines specify using 10 animals per group.²¹ Animals were randomized to treatment groups upon arrival and maintained in these groups throughout the study. The first four treatment groups received an oral gavage of purified blueberry polyphenols (see below for details), containing 0, 50, 250, or 1000 mg total polyphenols/kg bw/d (designated as “water”, “low”, “medium”, and “high”, respectively). The low 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 factor²²). The medium and high dose groups were 5- and 20-fold higher, respectively, to mimic higher concentrations as may be present in dietary supplements. The fifth group was dose-matched to the low-dose group but was dosed with lyophilized, whole blueberries (designated as “BB”) rather than the purified extract to monitor potential differences between whole foods vs. isolated polyphenolics.

Non-gavage control group

In addition to the 5 treatment groups, a smaller group of animals (n = 4) was maintained throughout the study and subjected to all study procedures with the exception of oral gavage. These animals were monitored throughout the experiment to detect potential adverse effects of the daily oral gavage distinct from polyphenol treatment-related effects.

Oral gavage dose preparation and administration

Concentrated blueberry phenolic extracts, containing 26.7% total phenolics (w/w), were used to prepare oral gavage slurries for the water, low, medium, and high dose groups, respectively, while lyophilized blueberries, containing 3.52% total phenolics (w/w), were used to prepare an oral gavage slurry for the BB group. All gavage doses were administered shortly after “lights on” each day (approximately 0800-1000), with the gavage needle dipped in a sugar solution (0.5 g/mL) to minimize stress and induce swallowing.²³

3.4.3 Polyphenol analysis

Lyophilized whole blueberries, purified blueberry extract, animal diets, and gavage doses were extracted and analyzed in triplicate, as described elsewhere.²⁴ Samples 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).²⁴ All samples were analyzed for total and individual phenolics via the Folin method and UPLC-MS/MS, respectively, as described previously.^{5, 25, 26} The polyphenolic composition of the starting materials is shown in Table 3.1.

3.4.4 Gut permeability

On the last day of oral gavage, gut permeability was assessed using the FITC method.^{27, 28} After receiving their prescribed dose of phenolics, animals were fasted for 4h before receiving FITC via oral gavage (50 mg/100 g bw). After an additional 4h fast, blood was drawn from the jugular vein and coagulated at room temperature for 30 min. Serum was obtained via centrifugation at 2200 g for 90s. The FITC-dextran concentration of serum was determined at 490, 520 nm with BioTek™ Cytation™ 1 Cell Imaging Multi-Mode Reader (Thermo Fisher Scientific Inc., Winooski, VT, USA) to evaluate the intestinal permeability.

3.4.5 Necropsy and terminal measures

Urinalysis

During the last week of the study, urine was collected for 24h using metabolic cages and submitted to the Clinical Pathology lab at Purdue University for analysis. The following parameters were measured: total volume, color, specific gravity, pH, protein, glucose, ketones, bilirubin, blood, urobilirubin, epithelial cells, and triphosphate crystals.

Serum biochemistry and hematology

Immediately after euthanasia, blood was collected from the abdominal aorta and submitted to the Clinical Pathology lab at Purdue University for analysis. Samples for hematology were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, centrifuged to separate plasma, and analyzed for the following: total protein (plasma), red blood cell count, hematocrit, hemoglobin concentration, mean corpuscular volume, mean corpuscular hemoglobin concentration, red blood cell distribution width, white blood cell count, segmented neutrophils, lymphocyte count, lymphocyte morphology, monocytes, eosinophils, platelet count (or estimate if too clumped to count), mean platelet volume, anisocytosis, poikilocytosis, polychromasia, target cells, and reticulocyte number. Samples for serum biochemical analyses were collected in tubes without any anticoagulant and allowed to clot for at least 30 minutes prior to separating serum via centrifugation. Serum was then analyzed for the following: glucose, blood urea nitrogen, creatinine, phosphorus, calcium, sodium, potassium, chloride, carbon dioxide, anion gap, total protein (serum), albumin, globulin, albumin:globulin ratio, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, total bilirubin, cholesterol, amylase, and lipase.

Necropsy

After drawing blood from the abdominal aorta, animals were flushed with 200 mL saline. Major tissues, including liver, kidney, pancreas, spleen, brain, stomach, small intestine, cecum, large intestine, lungs, heart, femur, tibia, and vertebrae were harvested, patted dry, and weighed. Intestinal contents were removed and the tissues flushed with 3x10 mL saline. To obtain representative samples of the major sections of the intestines, 1 cm segments of tissue were taken from the following locations: 1 cm distal to stomach (duodenum), midpoint of small intestine

(jejunum), 1 cm proximal to cecum (ileum), and midpoint of large intestine (colon).²⁹ Finally, ovariectomy was checked by visual inspection.

Histopathology

Harvested tissues were fixed in 10% neutral buffered formalin for ≥ 1 week. Tissues were embedded in paraffin and sectioned into 4 μm thick slices prior to staining with hematoxylin and eosin. All slides were prepared by the Histology Research Laboratory at Purdue University. Slides of kidney, heart, liver, spleen, lung, and pancreas were analyzed for the water and high dose groups only, while slides of stomach, duodenum, jejunum, ileum, cecum, and colon were evaluated for all dose groups. Histopathology of GI tissues was scored on a histomorphological scale from 0-3 (normal to severe abnormality) for each of the following: goblet cell numbers, mucosal hyperplasia, crypt cell death, erosion, mononuclear infiltrate, polymorphonuclear leukocyte infiltrate, crypt architectural distortion, and involvement of submucosa. Slides were read and interpreted by a board-certified veterinary pathologist.

Bone mineral density

Bones from euthanized animals, including right femur, right tibia, and L1-L4 vertebrae were removed and manually cleaned before analysis of bone mineral density using a PIXImus 2 mouse densitometer (GE Lunar PIXImus).

3.4.6 Statistics

Statistics were completed using SAS (SAS Institute, Raleigh, NC). When data were not normal, appropriate transformations were performed before analysis to ensure normality. Differences between dose groups were analyzed via one-way ANOVA. Post hoc analyses were carried out with Dunnett's test (to compare doses to water control) and Tukey's HSD test (to compare all dose groups to each other), with significance defined as $p < 0.05$ unless otherwise noted. Guidance in SAS coding was provided by the Statistical Consulting Service at Purdue University.

3.5 Results

3.5.1 Clinical findings and survival rate

No observable change in behavior was noted in any animal throughout the study. Two animals were euthanized prior to study completion due to issues related to oral gavage (e.g., aspiration of dose into lungs). These events were deemed to be related to dose administration and not the phenolic treatment itself. All other animals survived until scheduled necropsy, with no observed signs or symptoms of adverse, treatment-related effects.

3.5.2 Body weight and food consumption

No significant differences in body weight were noted between any of the treatment groups (Figure 3.2). Due to the large variations in body weight within each group, we also examined the percent change in body weight on a weekly basis as well as total change over 90 days (Figure 3.3a,b). While no significant differences were noted week to week, the high dose group gained significantly more weight than the lyophilized blueberry group over 90d. Food consumption among groups was similar throughout the study, though the lyophilized blueberry group tended to eat less than the water control and high dose groups (Figure 3.3c,d). These differences in body weight change and food consumption were more pronounced over 90 days and were reflected in the food efficiency ratio (FER, defined as weight gain/food intake), with the high dose group having a significantly higher FER than the lyophilized blueberry group (Figure 3.3e,f).

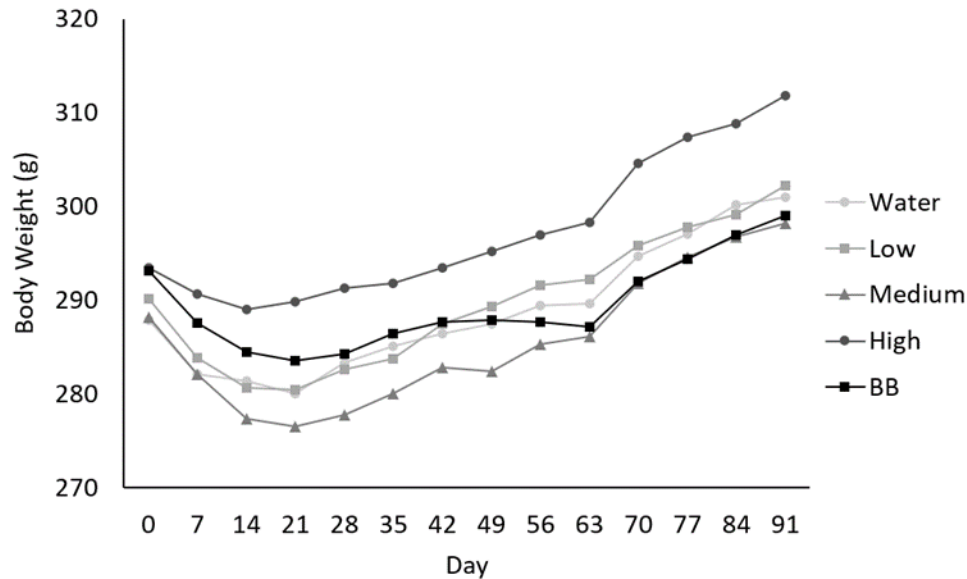


Figure 3.2 – Body weight over 90d. No significant differences in absolute body weight were observed throughout the study. BB = lyophilized blueberry dose group.

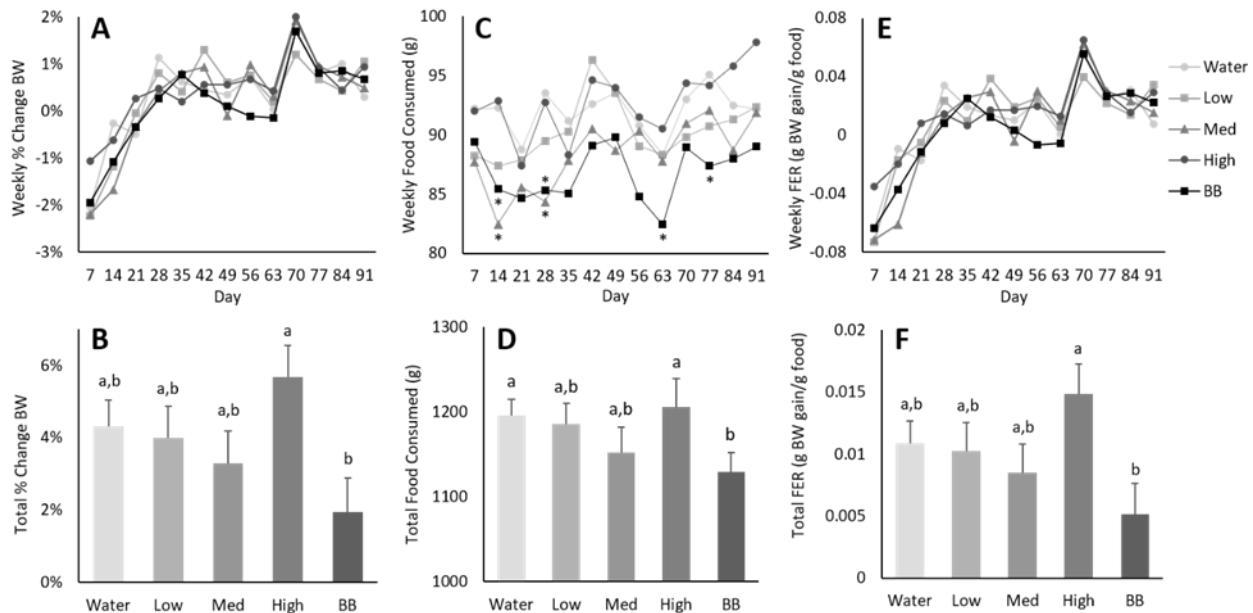


Figure 3.3 – Week-to-week and total body weight change (a,b), food consumption (c,d), and food efficiency ratio (FER, defined as g body weight gain/g food intake; e,f). In week-to-week comparisons, no significant differences from water control were observed in body weight change (a) or FER (e), though several significant differences in food consumption were observed. Over the course of 90d, the high dose group gained significantly more weight (b), ate more food (d), and had a higher FER (f) than the BB dose group. Data shown as mean \pm SEM. Significant differences detected using one-way ANOVA with either Dunnett's (a,c,e) or Tukey's HSD (b,d,f) post hoc test ($\alpha=0.05$) and are indicated with (*) or lower case letters, respectively. BB = lyophilized blueberry dose group; Med = medium dose group

3.5.3 Gross necropsy and organ weights

Upon completion of the study, animals were euthanized and a full necropsy was performed. During necropsy, no tumors or other abnormalities were noted in any of the animals. Harvested organs were weighed and, with the exception of the pancreas, showed no differences in absolute weight, as a percentage of whole-body weight (organ wt/bw), or as a percentage of lean mass (organ wt/brain wt)³⁰ (Table 3.2). The differences in pancreatic weights most likely resulted from challenges associated with harvest, as it has a diffuse structure within the mesentery and abdominal fat of rats,³¹ and is likely not physiologically significant.

3.5.4 Histopathological evaluation

Histopathological analyses were performed by a board-certified veterinary pathologist who was blinded to treatment groups. Analysis compared the water and high dose groups for all tissues (Figure B.1). No significant histopathological lesions were noted. Several mild lesions that were confined to a focal area were observed and were likely the result of normal biological variability between animals.

3.5.5 Clinical pathology

Hematology

Results from hematological analyses are shown in Table 3.3. Of all parameters measured, only one (monocyte count for lyophilized blueberries) was significantly different from the water control. All measured values were within the normal range for rats, and the statistically significant difference observed was not considered biologically significant.

Serum biochemistry

Results from serum biochemistry are shown in Table 3.4. The only statistically significant differences observed were decreased total cholesterol and chloride ion concentration in the high dose group compared to water control. Despite attaining statistical significance, these values are within normal ranges for rats and not considered physiologically significant.

Urinalysis

Results from urinalysis are shown in Table 3.5. Urine from the high dose group was significantly more acidic than the water control. A dose-dependent darkening of urine and feces was observed shortly after the administration of the first oral gavage dose and persisted throughout the study (Figure B.2). This was most likely due to the high concentration of blueberry polyphenols, as confirmed by the dose-response appearance of blueberry polyphenol metabolites in urine (Table 3.5). In preliminary experiments, similar results were observed, though within 48h after discontinuing treatment, urine and fecal color returned to normal and urinary polyphenol metabolites were no longer observed (data not shown).

3.5.6 Intestinal permeability

FITC test

Intestinal permeability was analyzed using the FITC technique. As shown in Figure 3.4a, intestinal permeability was significantly higher in the high dose group as compared to other treatment groups ($p < 0.05$).

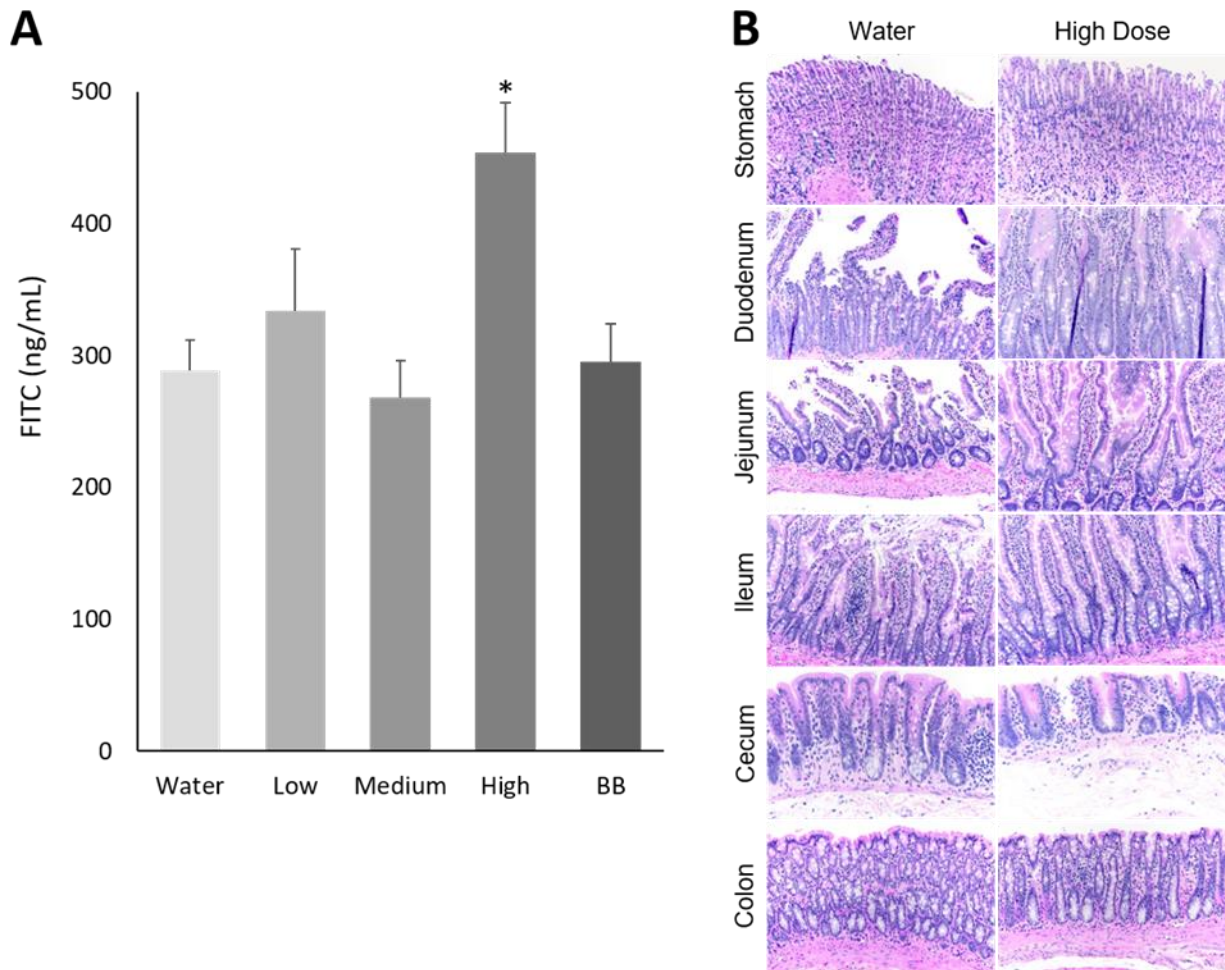


Figure 3.4 – Intestinal barrier function. The integrity of the GI tract was measured using the FITC-dextran method (a). Despite differences in FITC uptake, no histologically significant differences were found (b). Representative histology slides shown at 20x magnification. Data shown as mean \pm SEM. Significant differences detected using one-way ANOVA with Tukey's HSD post hoc test ($\alpha=0.05$) and indicated with (*). BB = lyophilized blueberry dose group; FITC = fluorescein isothiocyanate-dextran. Intestinal histopathology

Based on the results of the FITC test, histopathological analyses of each segment of the GI tract were performed for all animals in all dose groups. Using a histomorphological scale for eight potential markers of toxicity (see Methods), all intestinal segments were scored as “normal” for all dose groups (Figures 3.4b and B.3). Although several prominent lymphoid bodies and gastric submucosal edemas were observed in several rats, these were observed in rats from all groups and did not trend towards a specific treatment group, thus, they were considered to be due to biological variation.

3.5.7 Bone mineral density

Bone mineral density was measured in the femur, tibia, and lumbar spine (Figure B.4). No significant differences were observed between the water and treatment groups.

3.5.8 Non-gavaged control group

Non-gavaged animals were no different from water controls for any of the endpoints measured (Tables B.1-B.4 and Figures B.3-B.6). There was a non-significant trend towards higher body weights during the first few weeks of the study, though this effect resolved later in the study and was most likely due to the initial stress of daily gavage in treatment groups and unrelated to the blueberry polyphenols. Food consumption patterns were significantly different early in the study, though differences disappeared after the first month of the study. This was most likely due to the combined effects of stress associated with and the additional stomach volume from daily gavage. These factors may have initially suppressed appetite in gavage-fed animals, though they did not alter body weight or FER significantly over the course of 90d. The only statistically significant difference was in serum CO₂ concentration, though this difference was not considered biologically significant.

3.6 Discussion

In the current study, few differences were observed among treatment groups, with almost all measured parameters showing no significant differences between blueberry polyphenol treatments and water control. Although slight differences were observed in 90d body weight change, food consumption, and FER between the high and BB dose groups, these findings did not lead to other significant changes in histopathology. Organ weights were similar among all treatment groups, even after adjustments for body size (organ wt/bw) or brain weight (organ wt/brain wt). Adjusting for brain weight is a surrogate measurement that adjusts for lean body mass, which typically is not affected by xenobiotics like blueberry polyphenols.³⁰ Clinical pathology measures were within normal ranges, though several statistically significant differences were observed between treatment groups and the water control.

Postmenopausal women are the most frequent consumers of herbal and botanical dietary supplements and also the most vulnerable to rapid bone loss, as they lose ~20% of their total bone

mass in the first 5 years after menopause, leaving them susceptible to osteoporosis.³² Recent investigations suggest that polyphenols may help slow or even reverse age-related bone loss.³³ However, using the OVX rat model to mimic this life stage, we did not see any significant differences in BMD among treatment groups.

The most notable difference was observed in the darkened color of urinary and fecal output for animals treated with blueberry polyphenols. There was a dose-dependent darkening of the urine, and the high dose group had significantly more acidic urine. Additionally, the FITC-dextran test showed evidence of increased intestinal permeability in the high dose group. And, though we did not observe histopathological differences in any portion of the GI tract among groups, this increase in intestinal permeability may indicate mildly damaged gut barrier function, which could lead to increased levels of blueberry polyphenols in systemic circulation. This may be significant because native polyphenols typically demonstrate low levels of bioavailability *in vivo*.³⁴ If gut barrier function, especially in the small intestine, is damaged and increasing levels of polyphenols are found in circulation, this may explain the decrease in urinary pH in the highest dose group, as fruit-derived polyphenols are acidic. Additionally, colonic metabolism of polyphenols produces large quantities of low molecular weight phenolic acids. Colonic metabolites are acidic and may be absorbed at levels 10-fold greater than small intestinal phenolic metabolites.³⁵ After entering systemic circulation, phenolic acids are excreted in the urine and may also contribute to decreasing urine pH. If this persists over longer periods of time, it may lead to systemic acidosis in the animals. In the current study, however, we did not observe any other systemic changes in the animals, indicating that if systemic acidosis did eventually develop, it would require longer than 90d.

Several other safety studies of fruit and vegetable extracts are available in the literature. One of the oldest and most frequently cited studies was performed by Pourrat et al³⁶ and laid the foundation for toxicity studies of anthocyanin-rich extracts. In this study, extracts of currants, blueberries, and elderberries were tested over varying time periods and found that repeated doses of up to 9 g/kg bw/d did not show evidence of toxicity over three successive generations of rats, mice, or rabbits.³⁶ In the 1980s, investigations of purple color from grape extracts showed no evidence of toxicity up to 15% (w/w) of the diet in beagle dogs over 90d or Sprague-Dawley rats over two successive generations. The only difference noted in these studies was decreased weight gain in the highest dose group, most likely due to the lack of isocaloricity among diets.^{37, 38} A more recent study of grape skin and grape seed extracts in SD rats over 90d at 0-2.5% (w/w) of the diet

showed no differences among treatment groups.³⁹ Other investigations on the safety of anthocyanins showed that extracts of purple corn⁴⁰ and bilberry⁴¹ administered for 3-6 months in various animal models showed no evidence of toxicity, apart from discolored urine and feces. Safety studies of other polyphenol-rich extracts, like apple polyphenols and green tea extract (GTE), are also found in the literature, with varying results in rodents. Apple polyphenols showed no toxicity at up to 2000 mg/kg bw/d over 90d of oral gavage in rats,⁴² while GTE showed minimal impact at doses up to 1200 mg/kg bw/d over 6-months in one study⁴³ but significant toxicity in a second study over 14-weeks at 1000 mg/kg bw/d.⁴⁴

Setting safety guidelines for polyphenols and polyphenol-rich extracts is challenging because there is significant heterogeneity in polyphenol content between extracts and standardizing them is challenging. Additionally, the paucity of studies and their varied results add to this challenge. In the present study, we chose to base our doses off total polyphenol content and incorporate them into a gavage-fed preparation to minimize study variability and maximize the translatability of our results. Despite the small, though statistically significant, differences in gut permeability and urine pH in the high dose group, all measured parameters remained in the normal range for this animal model and we, therefore, propose that the NOAEL for blueberry polyphenols is ≥ 1000 mg/kg bw/d for OVX-SD rats. This translates to a 70 kg human consuming ~10 g blueberry polyphenols per day, an amount higher than is currently available in dietary supplements. Thus, we conclude that regular consumption of blueberry polyphenols in foods and supplements is likely safe for consumers.

3.7 Declaration of competing interest

The authors have no competing financial interests to declare.

3.8 Acknowledgments

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Table 3.1 – Polyphenol content of blueberries and extract used in doses.^a

(Poly)phenol	Raw Materials (mg/100 g)		Water	Gavage Doses (mg/kg bw)			
	FD	CE		Low	Medium	High	BB
Total Phenolics^b	3517 ± 54.8	26715 ± 384	nd	50.0 ± 3.77	253 ± 6.07	1000 ± 18.4	50.8 ± 7.47
<i>Anthocyanins</i>							
Cyanidins							
Arabinoside	21.4 ± 0.30	93.8 ± 14.9	nd	0.11 ± 0.01	0.54 ± 0.04	2.27 ± 0.23	0.23 ± 0.07
Galactoside + Glucoside	46.3 ± 0.50	126 ± 12.2	nd	0.44 ± 0.04	2.09 ± 0.17	8.98 ± 0.82	1.00 ± 0.30
Delphinidins							
Arabinoside	20.8 ± 0.37	301 ± 50.4	nd	0.35 ± 0.05	1.80 ± 0.17	7.68 ± 0.84	0.15 ± 0.06
Galactoside + Glucoside	100 ± 2.02	960 ± 281	nd	0.81 ± 0.15	3.62 ± 0.71	11.6 ± 2.67	0.54 ± 0.28
Malvidins							
Arabinoside	12.6 ± 0.30	209 ± 31.5	nd	0.32 ± 0.03	1.59 ± 0.14	6.67 ± 0.57	0.16 ± 0.04
Galactoside	23.9 ± 1.48	529 ± 66.8	nd	0.78 ± 0.09	3.62 ± 0.49	12.9 ± 2.21	0.15 ± 0.08
Glucoside	34.5 ± 0.69	376 ± 50.0	nd	0.45 ± 0.04	2.17 ± 0.28	7.20 ± 1.27	0.22 ± 0.11
Peonidins							
Arabinoside	13.9 ± 0.10	56.6 ± 9.61	nd	0.08 ± 0.01	0.39 ± 0.03	1.66 ± 0.15	0.18 ± 0.05
Galactoside	40.2 ± 0.34	227 ± 22.8	nd	0.35 ± 0.04	1.69 ± 0.14	7.18 ± 0.69	0.58 ± 0.17
Glucoside	75.0 ± 1.40	249 ± 28.5	nd	0.40 ± 0.02	1.85 ± 0.15	8.06 ± 0.55	1.02 ± 0.29
Petunidins							
Arabinoside	28.4 ± 0.30	486 ± 71.0	nd	0.72 ± 0.07	3.47 ± 0.27	14.8 ± 1.30	0.35 ± 0.12
Galactoside	59.0 ± 1.42	1121 ± 108	nd	1.86 ± 0.16	8.60 ± 0.57	37.3 ± 3.49	0.68 ± 0.22
Glucoside	74.5 ± 0.86	598 ± 62.8	nd	0.90 ± 0.09	4.28 ± 0.32	18.7 ± 1.79	0.86 ± 0.27
<i>Phenolic Acids</i>							
Benzoic Acids							
Gallic acid	nd	18.6 ± 2.99	nd	0.029 ± 0.006	0.168 ± 2.00	0.62 ± 0.11	nd
Protocatechuic acid	0.57 ± 0.12	8.82 ± 1.90	nd	0.017 ± 0.003	0.070 ± 0.020	0.35 ± 0.10	0.013 ± 0.004
Cinnamic Acids							
Caffeic acid	trace	47.7 ± 11.7	nd	0.064 ± 0.006	36.7 ± 0.035	1.18 ± 0.06	0.007 ± 0.004
Chlorogenic acid	621 ± 21.7	1738 ± 322	nd	2.64 ± 0.17	13.0 ± 1.30	48.9 ± 3.85	4.86 ± 0.68
Ferulic acid	2.01 ± 0.22	43.7 ± 7.17	nd	0.088 ± 0.005	0.41 ± 0.032	16.0 ± 0.18	0.044 ± 0.008
Feruloylquinic acid	8.28 ± 0.45	33.4 ± 5.33	nd	0.074 ± 0.007	0.35 ± 0.040	14.3 ± 0.13	0.143 ± 0.014
<i>Flavan-3-ols</i>							
Catechin	10.7 ± 0.17	39.9 ± 12.3	nd	0.092 ± 0.020	0.37 ± 0.15	13.9 ± 0.22	0.089 ± 0.030
Epicatechin	7.78 ± 0.44	11.3 ± 4.04	nd	0.023 ± 0.008	0.10 ± 0.042	0.39 ± 0.05	0.031 ± 0.013
Epigallocatechin	nd	20.9 ± 2.79	nd	0.048 ± 0.004	0.25 ± 0.012	0.78 ± 0.07	nd
<i>Flavonols</i>							
Myricetin	1.78 ± 0.15	46.3 ± 12.0	nd	0.062 ± 0.014	0.32 ± 0.046	1.09 ± 0.30	0.031 ± 0.001
Kaempferol	nd	10.8 ± 1.12	nd	0.028 ± 0.001	0.14 ± 0.001	0.69 ± 0.01	nd

Table 3.1 continued

Galactoside + Glucoside	96.5 ± 5.26	360 ± 52.8	nd	0.49 ± 0.064	2.37 ± 0.29	9.16 ± 0.99	1.05 ± 0.40
Quercetin	1.80 ± 0.18	242 ± 48.6	nd	0.32 ± 0.050	1.60 ± 0.14	5.02 ± 1.45	0.041 ± 0.009
Galactoside + Glucoside	309 ± 9.78	1165 ± 117	nd	19.3 ± 0.12	9.25 ± 0.099	35.4 ± 2.61	4.45 ± 1.18
Rutin	32.6 ± 0.93	74.6 ± 16.3	nd	0.12 ± 0.011	0.56 ± 0.064	2.51 ± 0.21	0.431 ± 0.068

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

^a Data are comprised of three analytical replicates and presented as mean ± SD.

^b Measured via Folin assay.

Table 3.2 – Absolute and relative tissue weights.

		Water	Low	Dose Medium	High	BB
Final body wt	(g)	301 ± 17	302 ± 22	298 ± 25	312 ± 37	299 ± 21
Brain	(g)	1.67 ± 0.071	1.64 ± 0.101	1.68 ± 0.135	1.62 ± 0.118	1.60 ± 0.071
	(g/100 g BW)	0.56 ± 0.026	0.54 ± 0.029	0.57 ± 0.039	0.52 ± 0.067	0.54 ± 0.047
Colon	(g)	0.84 ± 0.146	0.78 ± 0.201	0.77 ± 0.099	0.86 ± 0.219	0.78 ± 0.083
	(g/100 g BW)	0.28 ± 0.048	0.26 ± 0.058	0.26 ± 0.021	0.28 ± 0.062	0.26 ± 0.028
	(g/100 g brain)	50.1 ± 8.9	47.3 ± 9.87	45.8 ± 5.62	54.2 ± 17.92	48.5 ± 4.83
Heart	(g)	1.09 ± 0.079	1.17 ± 0.177	1.09 ± 0.086	1.13 ± 0.088	1.10 ± 0.064
	(g/100 g BW)	0.36 ± 0.032	0.39 ± 0.065	0.37 ± 0.026	0.36 ± 0.019	0.37 ± 0.018
	(g/100 g brain)	65.5 ± 5.66	71.7 ± 11.74	65.2 ± 6.82	70.6 ± 8.80	68.6 ± 5.26
Liver	(g)	8.08 ± 0.514	7.76 ± 1.223	7.68 ± 1.199	8.17 ± 1.039	7.68 ± 0.925
	(g/100 g BW)	2.69 ± 0.185	2.56 ± 0.264	2.54 ± 0.237	2.62 ± 0.192	2.56 ± 0.164
	(g/100 g brain)	484 ± 40.6	473 ± 54.3	457 ± 66.5	510 ± 92.1	481 ± 69.4
Lungs	(g)	3.21 ± 0.792	2.70 ± 1.014	2.90 ± 0.971	2.81 ± 0.974	2.45 ± 1.081
	(g/100 g BW)	1.07 ± 0.258	0.91 ± 0.377	0.97 ± 0.318	0.90 ± 0.288	0.81 ± 0.34
	(g/100 g brain)	192 ± 44.5	165 ± 63.1	174 ± 61.9	177 ± 71.4	154 ± 72.7
Kidneys	(g)	1.77 ± 0.151	1.78 ± 0.170	1.75 ± 0.201	1.87 ± 0.230	1.72 ± 0.163
	(g/100 g BW)	0.59 ± 0.028	0.59 ± 0.028	0.59 ± 0.025	0.60 ± 0.026	0.58 ± 0.033
	(g/100 g brain)	106 ± 8.3	109 ± 9.4	104 ± 9.4	117 ± 18.4	108 ± 12.9
R Kidney	(g)	0.89 ± 0.075	0.90 ± 0.082	0.89 ± 0.102	0.95 ± 0.103	0.88 ± 0.087
	(g/100 g BW)	0.30 ± 0.015	0.30 ± 0.012	0.30 ± 0.013	0.31 ± 0.013	0.29 ± 0.016
	(g/100 g brain)	53.4 ± 3.82	54.9 ± 3.97	53.0 ± 4.40	59.2 ± 8.42	54.8 ± 6.84
L Kidney	(g)	0.88 ± 0.081	0.88 ± 0.098	0.86 ± 0.102	0.92 ± 0.129	0.85 ± 0.081
	(g/100 g BW)	0.29 ± 0.016	0.29 ± 0.020	0.29 ± 0.015	0.30 ± 0.016	0.28 ± 0.019
	(g/100 g brain)	52.8 ± 4.8	53.7 ± 5.9	51.1 ± 5.2	57.6 ± 10.1	53.1 ± 6.3
Pancreas	(g)	2.30 ± 0.456	2.09 ± 0.413	2.71 ± 0.488	3.02 ± 0.495*	2.08 ± 0.412
	(g/100 g BW)	0.76 ± 0.143	0.69 ± 0.122	0.91 ± 0.169	1.00 ± 0.196*	0.71 ± 0.133
	(g/100 g brain)	138 ± 28.4	127 ± 23.9	161 ± 28.6	185 ± 35.4*	129 ± 26.3
Small Intestine	(g)	4.67 ± 0.601	4.75 ± 0.481	4.43 ± 0.728	5.27 ± 0.594	4.67 ± 0.598
	(g/100 g BW)	1.55 ± 0.196	1.57 ± 0.107	1.48 ± 0.164	1.70 ± 0.162	1.56 ± 0.100
	(g/100 g brain)	280 ± 36.4	291 ± 23.9	264 ± 41.0	328 ± 44.8	292 ± 43.6
Spleen	(g)	0.64 ± 0.086	0.64 ± 0.064	0.65 ± 0.117	0.68 ± 0.090	0.65 ± 0.073
	(g/100 g BW)	0.21 ± 0.027	0.21 ± 0.020	0.22 ± 0.028	0.22 ± 0.022	0.22 ± 0.021
	(g/100 g brain)	38.3 ± 4.76	39.5 ± 4.24	38.6 ± 7.38	42.2 ± 6.93	40.4 ± 5.17

Values are mean ± SD for 9-10 animals per group.

BB = whole freeze-dried blueberries.

* Significantly different from water control ($p < 0.05$).

Table 3.3 – Hematology. ^a

	Dose				
	Water	Low	Medium	High	BB
<i>n</i> ^b	9	7	8	10	6
RBC (10 ⁶ /uL)	7.64 ± 0.12	7.58 ± 0.25	7.65 ± 0.13	7.75 ± 0.30	7.53 ± 0.62
HCT (%)	44.1 ± 1.71	45.0 ± 2.28	44.0 ± 0.93	44.6 ± 2.24	42.5 ± 2.74
HGB (g/dL)	14.0 ± 0.48	14.3 ± 0.52	14.1 ± 0.31	14.1 ± 0.62	13.4 ± 1.28
MCV (fL)	61.6 ± 0.98	63.7 ± 1.49	61.8 ± 1.65	61.5 ± 2.24	60.4 ± 3.78
MCHC (g/dL)	31.6 ± 1.09	31.9 ± 0.93	32.0 ± 0.39	31.3 ± 0.76	31.5 ± 1.38
RDW (%)	13.8 ± 1.06	13.8 ± 0.47	13.5 ± 0.70	13.5 ± 0.74	14.8 ± 2.96
WBC (10 ³ /uL)	11.3 ± 2.41	10.3 ± 1.18	11.5 ± 1.59	12.1 ± 2.62	11.7 ± 2.20
SEG (10 ³ /uL)	0.62 ± 0.22	0.78 ± 0.41	1.01 ± 0.36	1.06 ± 0.46†	0.95 ± 0.38
LYMPH (10 ³ /uL)	10.2 ± 2.44	8.9 ± 0.97	10.1 ± 1.64	10.6 ± 2.55	10.1 ± 2.41
MONO (10 ³ /uL)	0.32 ± 0.16	0.49 ± 0.17	0.28 ± 0.15	0.38 ± 0.18	0.57 ± 0.21*
EOS (10 ³ /uL)	0.23 ± 0.16	0.18 ± 0.14	0.21 ± 0.13	0.17 ± 0.09	0.17 ± 0.10
RETIC # (10 ³ /uL)	420 ± 95	475 ± 56	430 ± 86	408 ± 89	423 ± 134

Values are mean ± SD.

^a Several additional parameters (polychromasia, target cells, and anisocytosis) were examined and scored qualitatively, with all results considered normal; thus, they are not shown in the chart. Platelets were too clumped to count in most samples, so not shown in the chart.

^b Several blood samples were unable to be analyzed due to improper clotting. The total number of samples analyzed for each group is given by *n*.

BB = whole freeze-dried blueberries; RBC = Red Blood Cell count; HCT = Hematocrit; HGB = Hemoglobin; MCV = mean corpuscular volume; MCHC = mean corpuscular hemoglobin concentration; RDW = red blood cell distribution width; WBC = white blood cell count; SEG = segmented neutrophils; LYMPH = lymphocyte count; MONO = monocyte count; EOS = eosinophils; RETIC # = reticulocyte number.

* Significantly different from water control ($p < 0.05$).

† Near significant difference from water control ($p = 0.072$).

Table 3.4 – Serum biochemistry.

	Water	Low	Dose Medium	High	BB
Total protein (g/dL)	6.01 ± 0.21	6.03 ± 0.24	6.03 ± 0.21	5.99 ± 0.17	5.99 ± 0.17
Albumin (g/dL)	3.39 ± 0.14	3.39 ± 0.18	3.42 ± 0.14	3.42 ± 0.16	3.40 ± 0.15
Globulin (g/dL)	2.62 ± 0.10	2.64 ± 0.11	2.61 ± 0.11	2.57 ± 0.07	2.59 ± 0.11
A/G ratio	1.29 ± 0.06	1.29 ± 0.09	1.32 ± 0.04	1.32 ± 0.06	1.32 ± 0.10
BUN (mg/dL)	14.9 ± 2.42	17.6 ± 3.10	16.8 ± 2.49	14.8 ± 1.32	16.3 ± 3.50
Creatinine (mg/dL)	0.67 ± 0.149	0.69 ± 0.088	0.74 ± 0.088	0.70 ± 0.115	0.68 ± 0.097
ALKP (U/L)	123 ± 30.7	112 ± 18.0	111 ± 25.1	117 ± 19.9	114 ± 15.7
ALT (U/L)	50.3 ± 8.3	48.9 ± 9.1	45.7 ± 7.7	53.8 ± 10.7	44.0 ± 5.9
Total bilirubin (mg/dL)	0.12 ± 0.044	0.17 ± 0.048	0.15 ± 0.053	0.14 ± 0.052	0.17 ± 0.071
Amylase (U/L)	1662 ± 318	1517 ± 333	1614 ± 393	1702 ± 304	1673 ± 362
Lipase (U/L)	97.0 ± 23.3	100 ± 35.8	105 ± 27.2	101 ± 15.9	120 ± 35.0
Cholesterol (mg/dL)	135 ± 10.3	128 ± 11.2	123 ± 14.8	118 ± 8.9*	131 ± 20.0
Calcium (mg/dL)	11.6 ± 0.20	11.6 ± 0.30	11.5 ± 0.35	11.6 ± 0.13	11.5 ± 0.26
Chloride (mmol/L)	99.4 ± 1.2	99 ± 1.3	98.6 ± 1.2	97.3 ± 0.9*	99.8 ± 1.6
Phosphorus (mg/dL)	7.53 ± 0.61	7.96 ± 0.83	7.52 ± 0.81	7.40 ± 0.52	7.41 ± 0.35
Potassium (mmol/L)	7.02 ± 0.80	7.27 ± 0.89	7.36 ± 0.47	7.06 ± 0.72	7.10 ± 0.83
Sodium (mmol/L)	141 ± 1.5	141 ± 1.0	142 ± 1.1	141 ± 0.9	141 ± 0.8
Anion gap (mmol/L)	16.1 ± 2.40	16.2 ± 2.41	16.6 ± 1.65	17.4 ± 2.17	16.5 ± 2.80
CO ₂ (mmol/L)	32.7 ± 1.70	33.2 ± 1.62	33.8 ± 2.28	33.2 ± 1.69	32.0 ± 3.91

Values are mean ± SD for 9-10 animals per group.

BB = whole freeze-dried blueberries; A/G = Albumin:Globulin ratio; BUN = Blood Urea Nitrogen; ALKP = Alkaline Phosphatase; ALT = Alanine Transaminase; CO₂ = Carbon Dioxide.

* Significantly different from water control (p < 0.05).

Table 3.5 – Urinalysis.

	Water	Low	Dose Medium	High	BB
<i>Quantitative measures</i>					
Volume (mL)	11.2 ± 4.5	12.4 ± 6.6	10.6 ± 3.2	10.1 ± 3.6	8.2 ± 3.0
Specific Gravity	1.025 ± 0.010	1.056 ± 0.088	1.028 ± 0.016	1.032 ± 0.020	1.031 ± 0.010
Protein (g/L)	0.34 ± 0.37	0.36 ± 0.36	0.41 ± 0.56	0.49 ± 0.36	0.46 ± 0.42
pH	7.6 ± 0.6	7.6 ± 0.7	7.5 ± 0.6	6.6 ± 0.4*	7.4 ± 0.8
24h metab. (umol exc.) ^a	4.50 ± 0.53 ^w	12.3 ± 4.87 ^x	42.7 ± 16.7 ^y	91.2 ± 34.6 ^z	11.4 ± 3.40 ^x
<i>Qualitative measures^b</i>					
Color ^c					
Normal	10	10	6	4	9
Darkened	0	0	3	6	0
Glucose					
Negative	8	9	7	10	5
Trace	2	1	2	0	4
Triphosphate crystals					
None	7	4	2	2	4
Few	1	3	5	5	4
Moderate	0	1	1	2	0
Many	2	2	1	1	1

Values are mean ± SD for 9-10 animals per group.

BB = whole freeze-dried blueberries.

^a Quantitated as sum 24h urinary excretion of phenolic metabolites via LC-MS/MS. Letters indicate significant differences between dose groups using Tukey's HSD test ($p < 0.05$).

^b Qualitative measures also included ketones, bilirubin, and blood in the urine, none of which were detected in samples, so they are not included in the table.

^c Color was independently graded as pale yellow, yellow, dark yellow, or brown. Samples categorized as "normal" if color was yellow or pale yellow and "darkened" if dark yellow or brown.

* Significantly different from water control ($p < 0.05$).

CHAPTER 4. CHANGES IN BLUEBERRY POLYPHENOL METABOLISM ARE DEPENDENT ON DOSE AND FOOD MATRIX OVER 90 DAYS IN OVARIECTOMIZED SPRAGUE-DAWLEY RATS

A version of this manuscript will be submitted for publication.

Dennis P. Cladis,¹ Kaitlyn J. Cooper,² Mario G. Ferruzzi,³ Connie M. Weaver^{1*}

¹ Dept. of Food Science, Purdue University, 745 Agriculture Mall Dr, W Lafayette, IN 47907, USA

² Dept. of Biological Sciences, Purdue University, 915 W State St, W Lafayette, IN 47907, USA

³ Plants for Human Health Institute, North Carolina State University, 600 Laureate Way, Kannapolis, NC 28081, USA

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*Corresponding author email: weaverconnie1995@gmail.com

4.1 Abstract

Fruit and vegetable derived polyphenols are associated with many health benefits when consumed regularly. Blueberries are a rich source of polyphenols, leading to their increased marketing as dietary supplements. However, the metabolic changes associated with repeated consumption of high doses of purified blueberry polyphenols, especially colonically generated metabolites, is poorly understood. To evaluate this, 5-month-old, ovariectomized, Sprague-Dawley rats were gavaged for 90 days with either a purified extract of blueberry polyphenols (0, 50, 250, or 1000 mg total polyphenols/kg bw/d) or lyophilized blueberries (50 mg total polyphenols/kg bw/d, equivalent to 1-2 cups/d in humans). Polyphenol metabolism was assessed via 24h urine collections on days 0, 7, 15, 30, 60, and 90 as well as collecting GI tissues, brain, liver, and kidney at sacrifice. Phenolic metabolites were analyzed via UPLC-MS/MS. A total of 38 out of 52 possible urinary metabolites were observed, with 15 also found in at least one of the tissues analyzed. A clear dose response was observed for most metabolites, with significantly

different excretion profiles between the lyophilized blueberry and purified phenolic extract treatments indicating a food matrix effect. The majority of excreted metabolites were phenolic acids (64-91% of all metabolites quantitated), followed by hippuric acids (7-36%). These results indicate that ingested blueberry polyphenols are efficiently metabolized over a large range of doses, though there are clear metabolic shifts as the dose and food matrix change. Thus, purified polyphenol extracts, as may be found in dietary supplements, are metabolized differently than those found in whole fruits and vegetables, which may alter their biological effects *in vivo*.

4.2 Introduction

Polyphenols, secondary plant metabolites present in fruits and vegetables, may have many health benefits when consumed regularly. As a regular component of the diet, they are able to reduce oxidative stress and inflammation,¹ leading to improved cardiovascular and neurocognitive function^{2, 3} as well as decreased incidence of many chronic diseases.^{4, 5} Blueberries are a rich source of polyphenols – including flavonoids and chlorogenic acid – and have been associated with numerous health benefits in both human and animal studies.⁶⁻⁸

To elucidate the connection between blueberry polyphenols and their purported health benefits, their metabolism and bioavailability must be understood. Polyphenols are xenobiotic molecules that typically exhibit low bioavailability. In the small intestine, they are absorbed into epithelial cells, undergoing phase-II metabolism before being effluxed back into the intestinal lumen or absorbed into systemic circulation.^{9, 10} Only a small portion of the ingested dose (< 2%) is absorbed into systemic circulation from the small intestine and is quickly cleared from the body.^{11, 12} The remainder of the ingested dose and effluxed contents pass to the colon, where the gut microbiome catabolizes polyphenols to smaller molecular weight phenolic acids.¹³ These phenolic acids are absorbed at significantly higher levels than their small intestinal counterparts and are hypothesized to be responsible for a number of the health benefits associated with consuming polyphenols.^{10, 14}

The majority of studies regarding the metabolism and bioavailability of polyphenols in both preclinical and clinical studies employ acute doses that may not accurately mimic typical consumption patterns. In studies comparing the bioavailability of polyphenols in single and repeated dosing paradigms, significant changes in metabolism and bioavailability were observed, often indicating increased levels of absorption with repeated exposure.^{15, 16} This is attributed to an

adaptive effect in the upper GI tract, allowing polyphenols to be absorbed more efficiently with repeated exposure, while in the colon, repeated exposure to polyphenols shifts gut microbial populations to more efficiently catabolize flavonoids and absorb their phenolic acid byproducts.^{13,17}

In recent years, there has been a rapid rise in the consumption of herbal and botanical dietary supplements, as consumers seek to maximize the health benefits attributed to fruit and vegetable derived polyphenols.^{18,19} Dietary supplements contain high doses of purified polyphenol extracts, which increases the complexity of understanding polyphenol metabolism. By increasing the ingested dose and removing the food matrix, polyphenol absorption and metabolism may be altered throughout the GI tract.

In a recent study, we examined the acute pharmacokinetics associated with consuming escalating doses of purified blueberry polyphenols as may be found in dietary supplements.²⁰ Using a rat model, we observed dose-dependent shifts in metabolism, especially for colonically generated phenolic acid metabolites. To address whether these metabolic shifts are transient or persist with repeated dosing, the present study monitors changes in the metabolism of both lyophilized whole blueberries and increasing doses of a purified blueberry polyphenol extract over 90 days in a rat model. We hypothesized that significant, dose-dependent shifts in metabolism would be observed early in the study, but that these changes would plateau and remain constant through the remainder of the study.

4.3 Materials and methods

4.3.1 Chemicals/Materials and vendors

Commercial standards of cyanidin-3-O-glucoside chloride, malvidin-3-O-glucoside chloride, peonidin-3-O-glucoside chloride, petunidin-3-O-glucoside chloride, gallic acid, caffeic acid, ferulic acid, ethyl gallate, taxifolin, chlorogenic acid, 4-hydroxybenzoic acid, 3-hydroxybenzaldehyde, hippuric acid, 3-hydroxyhippuric acid, isovanillin, 4-hydroxyphenylacetic acid, 3-hydroxyphenylpropionic acid, 3-methoxyphenylacetic acid, vanillic acid, homovanillic acid, syringic acid, quercetin, myricetin, quercetin-3-O-glucuronide, 4-methoxyquercetin, p-coumaric acid, epicatechin, kaempferol, and quercetin-3-O-rutinoside as well as sodium carbonate and Folin and Ciocalteu's reagent (2N) were purchased from Sigma-Aldrich (St. Louis, MO,

USA). Extraction and LC-MS grade solvents, including methanol, water, acetonitrile, hexanes, and formic acid were purchased from Thermo Fisher Scientific (Waltham, MA, USA). VitaBlue Pure American Blueberry Extract was obtained from FutureCeuticals (Mokena, IL, USA); lyophilized blueberry powder was obtained from the Wild Blueberry Association of North America (Old Towne, ME, USA).

4.3.2 Animal protocols

Study overview

Five-month old, virgin, ovariectomized (OVX), female Sprague-Dawley rats were used in this study. Upon arrival, rats were randomized to treatment groups and given one month to stabilize from ovariectomy. Animals then underwent 90d of dosing via oral gavage. A total of 5 treatment groups plus one non-gavaged group were used in the study. Urine and feces were collected throughout the study to monitor phenolic metabolism. 24h after the last dose was administered, animals were euthanized and tissues collected for analysis of phenolic tissue distribution.

Animal care

Animal experiments were conducted in adherence to Purdue University Animal Care and Use Committee (PACUC) guidelines, following an approved protocol (1808001790). 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.

Treatment groups

A total of 5 treatment groups were used for this study. Because there are no data upon which to reliably base power calculations, we followed Organisation for Economic and Co-operative Development (OECD) 408 guidelines, which specify using 10 animals per group in 90d escalating dose studies.²¹ Animals were randomized to treatment groups upon arrival and maintained in these groups throughout the study. The first four treatment groups received an oral gavage of purified blueberry polyphenols (see below for details), containing 0, 50, 250, or 1000 mg total polyphenols/kg bw/d (designated water, low, medium, and high, respectively). The low

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 factor²²), with the medium and high dose groups 5- and 20-fold higher, respectively, to mimic higher concentrations as may be present in dietary supplements. The fifth group was dose-matched to the low-dose group, but was dosed with lyophilized, whole blueberries (designated BB) rather than the purified extract to monitor potential differences between whole blueberries and isolated polyphenols.

Non-gavage control group

In addition to the 5 treatment groups, a smaller group of animals (n = 4) was maintained throughout the study and subjected to all study procedures with the exception of oral gavage. These animals were monitored throughout the experiment to detect potential adverse effects of daily oral gavage distinct from blueberry polyphenol treatment-related effects.

Diets

For the duration of the study, all animals were fed polyphenol-free diets. These diets were based on the AIN-93M diet, using corn oil in place of soybean oil to remove isoflavones and minimize confounding by polyphenols unrelated to blueberry treatment. Diets were prepared by Research Diets (New Brunswick, NJ, USA).

Oral gavage dose preparation and administration

Concentrated blueberry phenolic extracts, containing 26.7% total phenolics (w/w), were used to prepare oral gavage slurries for the water, low, medium, and high dose groups, while lyophilized blueberries, containing 3.52% total phenolics (w/w), were used to prepare an oral gavage slurry for the BB group. All gavage doses were administered shortly after “lights on” each day (approximately 0800-1000), with the gavage needle dipped in a sugar solution (0.5 g/mL) to minimize stress and induce swallowing.²³

Urine and fecal collection

On days 0, 7, 15, 30, 60, and 90, urine and feces were collected. On each of these days, animals were given their daily oral gavage dose and then placed in metabolic cages for 24h to

collect excreta. Urine was centrifuged to remove food spill and debris, acidified to a final concentration of 0.1% formic acid, blanketed with nitrogen, and frozen at -80°C until analysis. Feces were collected in centrifuge tubes and frozen at -80°C.

Tissue collection

Animals were euthanized via CO₂ asphyxiation 24h after receiving the final oral gavage dose. Immediately after sacrifice, animals were perfused with 200 mL saline to clear blood from tissues and eliminate possible confounding of polyphenol analyses. Then, small intestine and colon were harvested and flushed with 3x10mL saline before being snap frozen in liquid nitrogen and stored at -80°C until analysis, while brain, liver and kidney were snap frozen immediately and stored at -80°C until analysis.

4.3.3 Polyphenol analyses

Extraction and purification of phenolics in starting materials, animal chow, and oral gavage preparations

Lyophilized whole blueberries, purified blueberry extract, animal diets, and all gavage doses were extracted in triplicate, as described elsewhere.²⁴ Samples 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.²⁴ Random samples of oral gavage doses were taken approximately once every 10d throughout the study to monitor for potential polyphenol degradation. These samples were analyzed for total phenolics and, when necessary, adjustments made to ensure accurate dosing throughout the study (Figure C.1).

Quantification of total and individual phenolics in starting materials

Total phenolics were quantified in crude extracts via the Folin method and corrected for vitamin C interferences as described elsewhere.²⁵ Individual phenolics were quantified via UPLC-MS/MS using a Waters UPLC Acquity I Class system equipped with a TQD detector. Samples were injected (5 µL) and phenolics separated using an Acquity BEH C18 column (2.1 µm, 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

anthocyanins) or 0.1% formic acid in water (for non-anthocyanins)) 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: ESI+ (to quantify anthocyanins): 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. The same conditions were used in ESI- to quantitate non-anthocyanins. Phenolic content of starting materials, diets, and gavage doses are shown in Table 4.1.

Identification and quantification of each compound was based on authentic standards, using calibration curves ranging from 0.001-100 μ M. 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.²⁶ A complete list of compounds detected, including corresponding MRMs and standards used for quantification are shown in Table C.1.

Extraction and purification of urine polyphenol metabolites

Phenolics were extracted and purified via SPE using strataX, polymeric reversed phase microelution 96 well plate with a capacity of 2 mg/well (Phenomenex, Torrance, CA, USA). The SPE cartridges were preconditioned with 200 μ L 1% formic acid in methanol followed by 200 μ L 1% formic acid in water. Urine samples were diluted 1:1 with 1% formic acid in water, and 100 μ L of this mixture loaded onto SPE cartridges. Samples were washed with 2 x 200 μ L 0.1% formic acid in water and dried under nitrogen for 15 minutes. Samples were eluted with 100 μ L 0.1% formic acid in methanol. All steps were aided by gentle, positive pressure nitrogen gas delivered via Waters Positive Pressure-96 Processor. To the eluate was added 20 μ L of 50 μ M taxifolin as a volume control. Eluted samples were capped with a pre-slit silicon mat (Cap-mat 96 well 7 mm round plug pre-slit silicone/PTFE, Waters) and analyzed immediately.

Quantification of urine polyphenol metabolites

Phenolic metabolites were quantified using the same UPLC-MS/MS conditions listed above. However, to capture metabolites that were not present in the starting materials, different MRM transitions were measured (Table C.1). As before, samples were quantified by authentic standards or, when standards were not available, structurally related compounds were used.

Extraction, purification, and analysis of tissue polyphenol metabolites

Polyphenols and their metabolites were extracted from 0.5-1 g samples of brain, liver, kidney, small intestine, and colon, following previously described methods.²⁷ Briefly, tissues were defatted by homogenizing with 5 mL hexanes and vortex mixed 10 min. The resulting slurry was centrifuged for 10 min at 3000 g, and the supernatant discarded. Samples were then extracted with 5 mL 1% formic acid in methanol, being vortexed 10 min and centrifuged 10 min at 3000 g. The resulting supernatant was transferred to a clean test tube and the extraction repeated; supernatants were combined, dried under nitrogen, and stored at -80°C until analysis.

Tissue extracts were resolubilized with 2 mL 2% formic acid in water. This mixture was diluted 1:1 with 1% formic acid in water, before loading 200 µL onto preconditioned SPE plate. Samples were washed, dried, eluted, and analyzed following the same procedures used for urine samples.

4.3.4 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. Two-way ANOVA was used to determine differences within dose groups (across different time points) and between dose groups (at the same time point). Post hoc analyses were carried out with Tukey's HSD test, with significance defined as $p < 0.05$. Guidance in SAS coding was provided by the Statistical Consulting Service at Purdue University.

4.4 Results and Discussion

4.4.1 Urinary excretion of phenolic metabolites: excretion patterns of individual metabolites.

A total of 52 metabolites were analyzed in this study. Of these, 36 were quantifiable in at least one treatment group, with two additional compounds being observed in trace amounts (isovanillin in high dose group and homovanillic acid in medium and high dose groups). The remaining compounds were either not detected or could not be separated from background

interferences in the chromatographic spectra and thus could not be reliably identified or quantitated.

There were several common patterns observed in the excretion of metabolites, as illustrated in Figure 4.1. Nearly all measured metabolites exhibited a dose response relationship, though the urinary excretion within this dose-response differed over 90 days between metabolites. About half of the metabolites quantitated reached maximal levels of excretion within 7-15 days and then plateaued, exhibiting consistent excretion for the remainder of the study (Figure 4.1A). Several other metabolites showed increasing levels of urinary excretion throughout the 90d study, especially in the high dose group, indicating that their production increases throughout the study period (Figure 4.1B). Another set of metabolites (most notably anthocyanin metabolites) showed peak levels of excretion early in the study, but then were less prominent later in the study (Figure 4.1C). The few remaining metabolites exhibited similar levels of absorption between the medium and high dose groups (including hippuric acid metabolites), indicating that the production and/or absorption of these compounds may be saturable at high levels of blueberry polyphenol intake (Figure 4.1D). A complete list of the qualitative category for each quantitated metabolite is in Table 4.2, while graphical illustrations, quantitation, and statistical comparisons between time points and doses can be found in the Supporting Information (Figures C.2-C.37). These data correlate with another study feeding blueberries to rats and measuring phenolic metabolism via 24h urine collections. In that study, several metabolites reached steady-state production in 4 weeks, while others showed continual increases in production up to 8 weeks.²⁸

4.4.2 Tissue distribution of phenolic metabolites.

Phenolic metabolites in GI tissues

A number of phenolic metabolites were observed in the small intestine and colon (Table 4.3), though only a few of these metabolites were quantifiable. In the small intestine, a total of 12 metabolites were observed in at least one of the treatment groups, with 5 being quantifiable. Of the metabolites that were quantifiable, 2 were observed in all dose groups (hippuric acid and ferulic acid sulfate). No significant differences in tissue concentration were observed between treatment groups, though ferulic acid sulfate was found in a higher percentage of animals as dose increased, which indicates a dose-response relationship. The remaining metabolites also showed a dose-

dependent relationship, as they were primarily observed in the higher dose groups. In the colon, a total of 9 metabolites were observed in at least one treatment group, with 3 being quantifiable. Similar to the small intestine, a dose-dependent response was observed, with metabolites detected with greater frequency in higher dose groups, though there were no statistically significant differences in metabolites quantified in all treatment groups.

Polyphenols and their metabolites are observed throughout the GI tract following treatment with a variety of polyphenols.²⁹⁻³¹ This is not surprising, as the intestinal epithelial cells interact extensively with polyphenols. However, it is difficult to completely clear luminal contents from the GI tract, which may confound observation of phenolic metabolites in the GI tissues. In the present study, we observed darkened cecal and colonic contents at sacrifice, indicating that the blueberry phenolics had not yet been cleared from the system.

Phenolic metabolites in peripheral tissues

To assess tissue distribution of phenolic metabolites, brain, kidney, and liver were analyzed (Table 4.4). In the brain, three metabolites were observed, with only one (dihydrocaffeic acid sulfate) observed in all animals. In previous animal studies, quantifiable levels of catechin metabolites were observed in the brain after repeated dosing with grape seed polyphenol extract¹⁵ and other treatments high in catechins,³² but not after 4-8 weeks on a wild blueberry diet.²⁸ Along with the current study, these results seem to indicate that catechin metabolites are more likely to cross the blood brain than blueberry phenolic metabolites, though the accumulation of polyphenols in the brain may not be necessary for the neurocognitive benefits associated with them.³²

In both the kidney and liver, 6 metabolites were observed, with 3 being quantifiable in the kidney and 2 in the liver. In both tissues, metabolites followed a dose-response relationship. Additionally, hippuric acid was a prominent metabolite in both tissues, demonstrating a statistically significant dose-response relationship. The presence of hippuric acid in both tissues is not surprising, as it can arise from many different dietary precursors and is formed in the liver before being filtered in the kidney and excreted in the urine.¹⁷ However, given the lack of quantifiable phenolic metabolites in other tissues, the significant dose-response relationship between treatment groups indicates that even 24h after receiving the final dose, animals are still clearing phenolic metabolites from that dose. As many studies report the rapid clearance of most phenolic metabolites from systemic circulation,³³ the fact that hippuric acid is still being produced

in the liver 24h after receiving the final dose lends further credence to the hypothesis that phenolic metabolites may persist in circulation longer than originally thought.³³⁻³⁵ These results mirror our acute study, where hippuric acid metabolites exhibited maximal levels of formation and excretion 12-24h post-dosing.²⁰ Finally, as discussed below, hippuric acid is one of the last products formed from ingested polyphenols. The fact that hippuric acid was observed in quantifiable levels and still exhibited a significant dose-response relationship, while other quantifiable tissue metabolites of blueberry polyphenols did not, indicates that phenolic acid is still being formed long after other metabolites have been cleared from circulation.

4.4.3 Urinary excretion of phenolic metabolites: excretion of total and structurally related families of metabolites.

Urinary excretion of total metabolites, total phenolic acids, total flavonoids, and total hippuric acids is shown in Figure 4.2, with statistical comparisons in Table 4.5. For total metabolites excreted (Figure 4.2A), all animals had similar levels of detectable metabolites at baseline and these stayed consistent in the water and non-gavaged groups throughout the study. Treatment groups showed a dose-response relationship, with the low, medium, and BB groups reaching peak levels of metabolite excretion in the first week of the study before remaining constant throughout the rest of the study, while the high dose group showed increased levels of excretion throughout the study. Interestingly, the medium and high dose groups showed similar levels of metabolite excretion early in the study, but were different at later time points. This may indicate a dose-dependent maximum threshold of metabolite production early in the study, but with continued dosing at high levels, additional metabolic changes may occur, and higher levels of metabolites may be produced. These results mirror those found in a dose-response trial of resveratrol with human volunteers. When comparing acute dosing versus repeat daily dosing for 29 days, plasma AUC values for resveratrol and its phase-II metabolites after 29d were found to be decreased at the lowest dose, similar in concentration in the medium doses, and increased at the highest dose.³⁶ The authors hypothesized that this may be due to the increased $T_{1/2}$ and delayed T_{max} observed, though all groups above the lowest dose group had similar $T_{1/2}$ and T_{max} values.³⁶ Regardless of the cause, it appears that there may be a dose-dependent effect on the absorption and metabolism of polyphenols. Total phenolic acid metabolite excretion (Figure 4.2B) accounted for most of the total metabolites excreted and mirrored the excretion trends noted for total metabolites.

This indicates that the phenolic acids and their phase-II metabolites are driving the relationships observed for total metabolite excretion.

Flavonoid excretion showed a clear dose-response relationship that peaks within the first week of dosing before plateauing (Figure 4.2C). As expected, no flavonoids are observed in the water and non-gavaged groups. In contrast to other families of metabolites, however, the flavonoids excreted mimic the ratio of total polyphenols in the original doses, i.e., the medium and high dose metabolites are observed at approximately 5x and 20x the amounts found in the low and BB dose groups. This indicates that the production of these metabolites is not limited by dose, whereas other metabolites appear to be produced less efficiently at higher doses (i.e., ratio of low:medium:high is less than 1:5:20, as observed here for flavonoids).

Hippuric acid metabolites are formed by the conjugation of benzoic acid and glycine, and their production can be initiated by a number of different dietary inputs, though polyphenols are prominent precursors in the production of hippuric acid metabolites.³⁴ As shown in Figure 4.2D, hippuric acids were present in all groups at baseline, and showed increased production in treatment groups. However, in contrast to other metabolites, hippuric acids showed similar levels of excretion between BB, medium, and high doses throughout the study, indicating that there may be maximal levels of production and excretion (i.e., a “saturation effect”), and that the influence of having the whole food matrix present in the BB group may increase the production of hippuric acid metabolites. This saturation effect for hippuric acids was also observed in our acute study;²⁰ the fact that this effect is consistent throughout the 90d study period may indicate that hippuric acid metabolite production has an upper limit of production and cannot be produced beyond that threshold, even with prolonged treatment.

4.4.4 Ratio of structurally related metabolites is stable within, but not between, doses over time and is dependent upon the food matrix.

Figure 4.3 shows relative amounts of the most prominent metabolite subclasses generated. Cinnamic acids (caffeic, ferulic, and isoferulic acids and their phase-II metabolites) comprised over half of all metabolites (56-87%); hippuric acids (hippuric acid and 3-hydroxyhippuric acid) and phenyl propionic acids (PPA, including 3-hydroxy PPA, 3-hydroxy-4-methoxy PPA, and dihydrocaffeic acid sulfate) were also prominent, accounting for 6-39% and 4-15%, respectively,

of total metabolites. All other metabolites quantitated in this study accounted for $\leq 2\%$ of total metabolites.

Metabolite production is consistent within each dose throughout 90d treatment

Despite shifts in the production of individual and total phenolic metabolites, the relative proportion of excreted metabolites is consistent throughout the study (Figure 4.3). If total phenolic metabolite excretion increases throughout the study (as it does in the high dose group), but the relative proportion of phenolic metabolite subclasses is relatively consistent, this indicates that while the relative composition of the gut microbiome is not changing throughout the study, the microbes are more efficiently metabolizing and absorbing the ingested phenolics. Over longer periods of time, this may result in significant health impacts for the host.

Differences in metabolite production between doses

As the dose increases, there is a shift away from hippuric acid production and a rise in PPA and other non-cinnamic acid metabolites. This supports the hypothesis that hippuric acid metabolism reaches a saturation point at higher doses, while also indicating that the shift away from hippuric acid production results in higher quantities of metabolites formed earlier in the metabolic chain.

Food matrix effects

When comparing the dose-matched low and BB doses, there are clear differences in the relative amounts of phenolic subclasses excreted (Figure 4.3). Most notably, higher relative levels of hippuric acid excretion are observed in the BB group as compared to the low dose extract group. Because total phenolic metabolite excretion for both the low and BB dose groups is the same, this shift in relative amounts of hippuric acids implies that other components of the whole fruit are likely impacting gut microbial metabolism of phenolics. Berry fruits contain significant amounts of fiber and simple carbohydrates, which are known to impact the gut microbiome.³⁷ The results of the current study suggest this is important to consider, especially in the context of health benefits derived from whole foods versus dietary supplements. As noted in other studies, the health benefits derived from whole fruits and vegetables may not be derived from consuming purified phenolic

extracts of these fruits and vegetables.³⁸ Based on the results presented here, we posit that this difference may be due to shifts in the gut microbiome and the preferential production of different metabolites with different food matrices.

4.4.5 Summary of phenolic metabolism and metabolic sinks observed in the current study.

A summary of the colonic metabolism of major blueberry polyphenols is shown in Figure 4.4. From the flavonoids and chlorogenic acid comprising the phenolic load of the lyophilized blueberries and purified phenolic extract administered to the rats, extensive metabolism to smaller molecular weight phenolic acids was observed. Although several phase II flavonoid metabolites were observed, virtually the entire bioavailable portion of the dose was converted to smaller molecular weight phenolic acids. And, of the phenolic acids produced, over 80% were present in their glucuronidated or sulfated forms (Figure C.38), indicating the efficient conversion of flavonoids → phenolic acids → phenolic acid phase II metabolites + hippuric acids. This observation is consistent with many other studies showing that flavonoid bioavailability in the small intestine is low, but that when the ingested dose reaches the colon, the gut microbiome is able to efficiently catabolize flavonoids into phenolic acids (and their phase-II metabolites), resulting in significantly higher bioavailability.⁹ In addition, blueberry phenolic metabolites in the current study accumulated at the cinnamic acid and PPA intermediates (Figure 4.4). We posit that these phenolic acids are the favored products of blueberry polyphenol metabolism (as opposed to phenylacetic and benzoic acids) and that once these phenolic acids are formed, they are either further metabolized via phase-II metabolism or efficiently converted all the way through the metabolic chain and conjugated with glycine to form hippuric acids. Finally, based on the dose-dependent shift from hippuric acids to cinnamic acids and PPAs at higher doses, it appears that these phenolic acids may be metabolic sinks and, when high enough doses are present, are rate limiting steps in the production of other phenolic acid metabolites.

4.4.6 Limitations, conclusions and future directions.

There were several limitations present in this study. First, collecting urine samples from animals in metabolic cages introduces the possibility of oxidation and contamination. Because urine was collected over 24h, samples were exposed to oxygen for several hours before collection

and small food particles and debris were observed upon collection. Given the relative stability of phenolic acid metabolites, the use of a polyphenol-free chow, and the challenges associated with other collection methodologies, we judged this to be the best method for collecting a complete 24h urine sample from the animals. Second, the lack of authentic standards for some of the metabolites quantitated in this study may have led to an over or under estimation of their actual amounts. Phase II metabolites, in particular, are rarely available as commercial standards and are difficult to synthesize in the lab; however, given that they generally ionize more easily than their commercially available parent compounds, they are often over estimated by LCMS analyses.¹⁰ To test this, future work comparing intact urine samples to those treated with glucuronidase and sulfatase enzymes would ensure differentiation between and accurate quantitation of phenolic acids versus their phase II metabolites. The disadvantage of this approach is that the distinction between the parent phenolic acid, the glucuronidated metabolites, and the sulfated metabolites is no longer possible.

In conclusion, we have shown that blueberry polyphenols are extensively metabolized, even at high doses as may be present in dietary supplements. The relative amounts of phenolic metabolites are dependent upon the total phenolic load ingested and the presence or absence of the food matrix. This may have important implications for the efficacy of whole foods versus dietary supplements in contributing to the health status of the host. Because most of the metabolites observed in this study are products of colonic metabolism, future investigations should also analyze changes in the gut microbiome and correlate them to dose- and food matrix-dependent changes in phenolic metabolism.

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4.6 Author Contributions

DPC, MGF, and CMW designed research; DPC performed all animal procedures and experiments; DPC, KJC, and MGF conducted phenolic analyses. DPC wrote manuscript. All authors reviewed and approved the final version of the manuscript.

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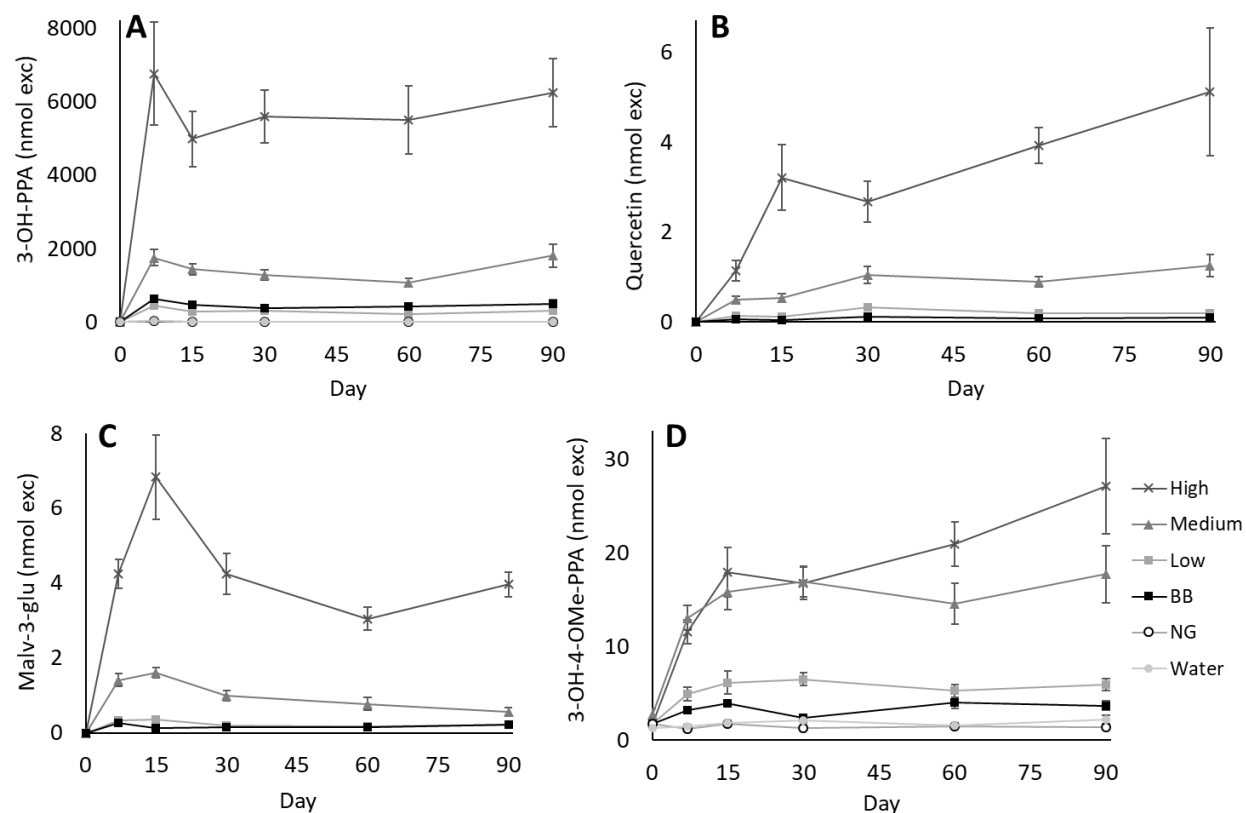


Figure 4.1 – Urinary excretion of individual phenolic metabolites over time, illustrating common examples of urinary excretion patterns. A) 3-hydroxy-PPA, an example of a metabolite that reached maximal excretion levels within 7-15 days before remaining constant throughout the remainder of the study. B) Quercetin, an example of a metabolite that showed increasing levels of excretion throughout the 90d study. C) Malvidin-3-glucoside, typical of several anthocyanins that exhibited peak excretion levels during the first few collection points before decreasing to lower levels of excretion at later time points. D) 3-hydroxy-4-methoxy-PPA, an example of a metabolite that showed similar levels of excretion between the medium and high dose groups, indicating saturated production of the metabolite. Data shown as mean \pm SEM. A complete list of metabolites and which excretion pattern they follow are found in Table 4.2. For graphs of all quantitated metabolites and statistical comparisons, see Supporting Information (Figures C.2-C.37).

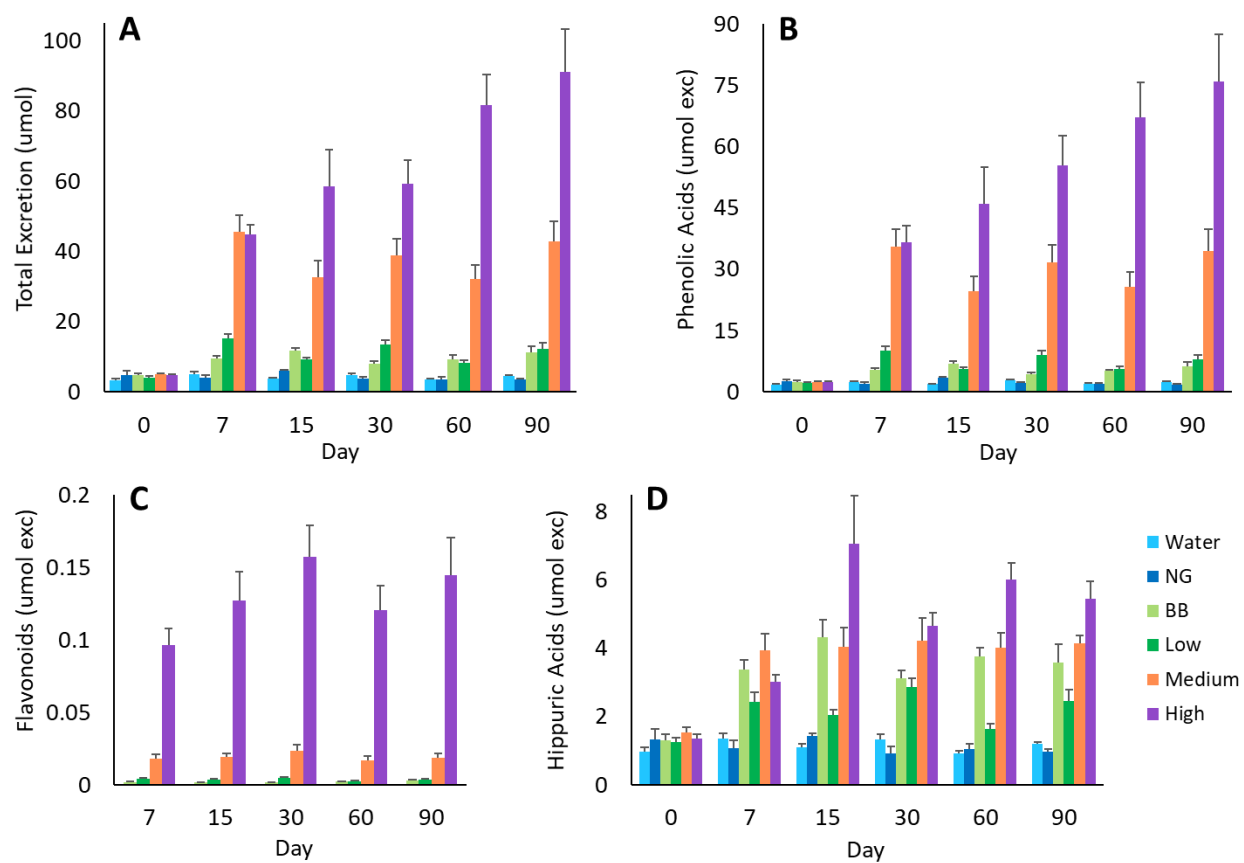


Figure 4.2 – Urinary excretion of A) total metabolites, B) phenolic acid metabolites, C) flavonoid metabolites, and D) hippuric acid metabolites. A clear dose-response was noted for all detected products except hippuric acids. Data shown as mean \pm SEM. Statistical comparisons shown in Table 4.5.

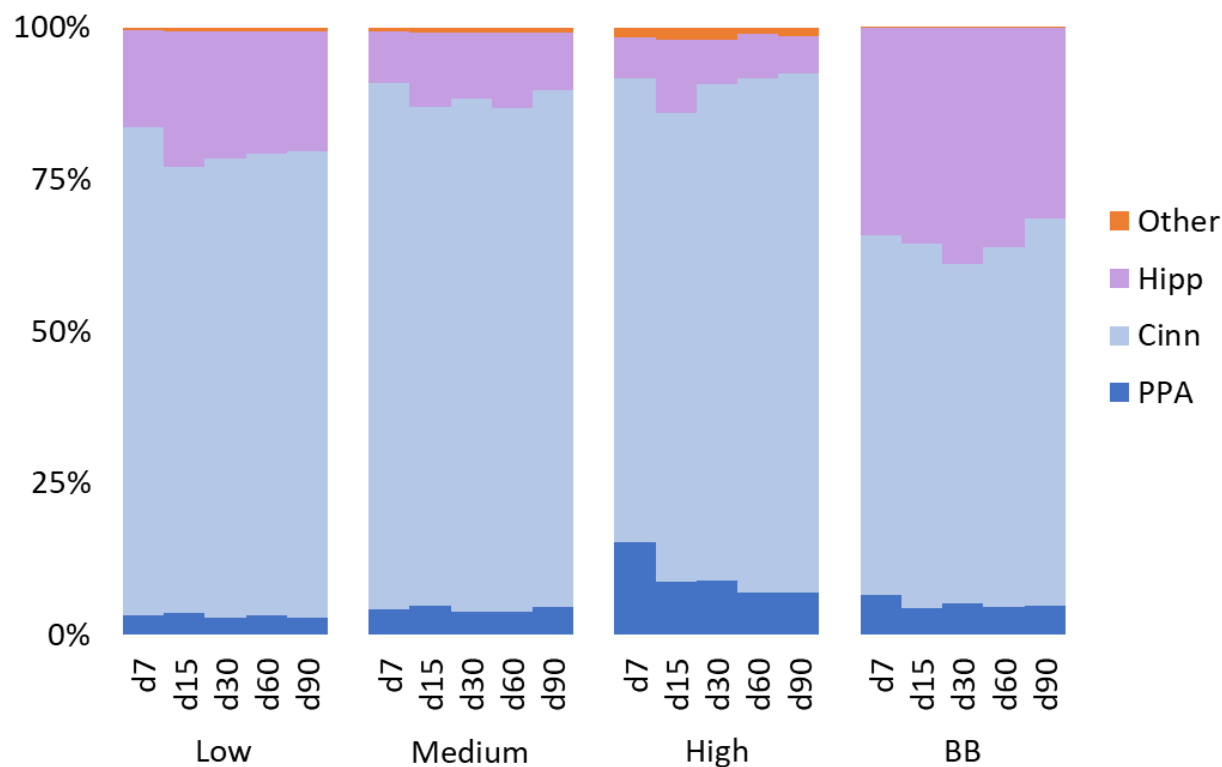


Figure 4.3 – Relative amounts of urinary phenolic metabolites. From low to high dose, the relative amounts of phenylpropionic acids (PPA) and “other” compounds increased, while hippuric acids decreased. When comparing the dose-matched low and BB groups, the relative amounts of hippuric acids increased while cinnamic acids decreased. Data shown as mean \pm SEM. PPA = sum of PPA (3-hydroxy-PPA, 3-hydroxy-4-methoxy-PPA, and 3,4-dihydroxy-PPA sulfate); Cinn = sum of *trans*-cinnamic acids (caffeic, ferulic, and isoferulic acid in parent, glucuronidated, or sulfated form); Hipp = sum of hippuric acids (hippuric acid and 3-hydroxy-hippuric acid); Other = sum of all other phenolic metabolites quantitated (flavonoids, phenylacetic and benzoic acids, and benzaldehydes in parent, glucuronidated, or sulfated form).

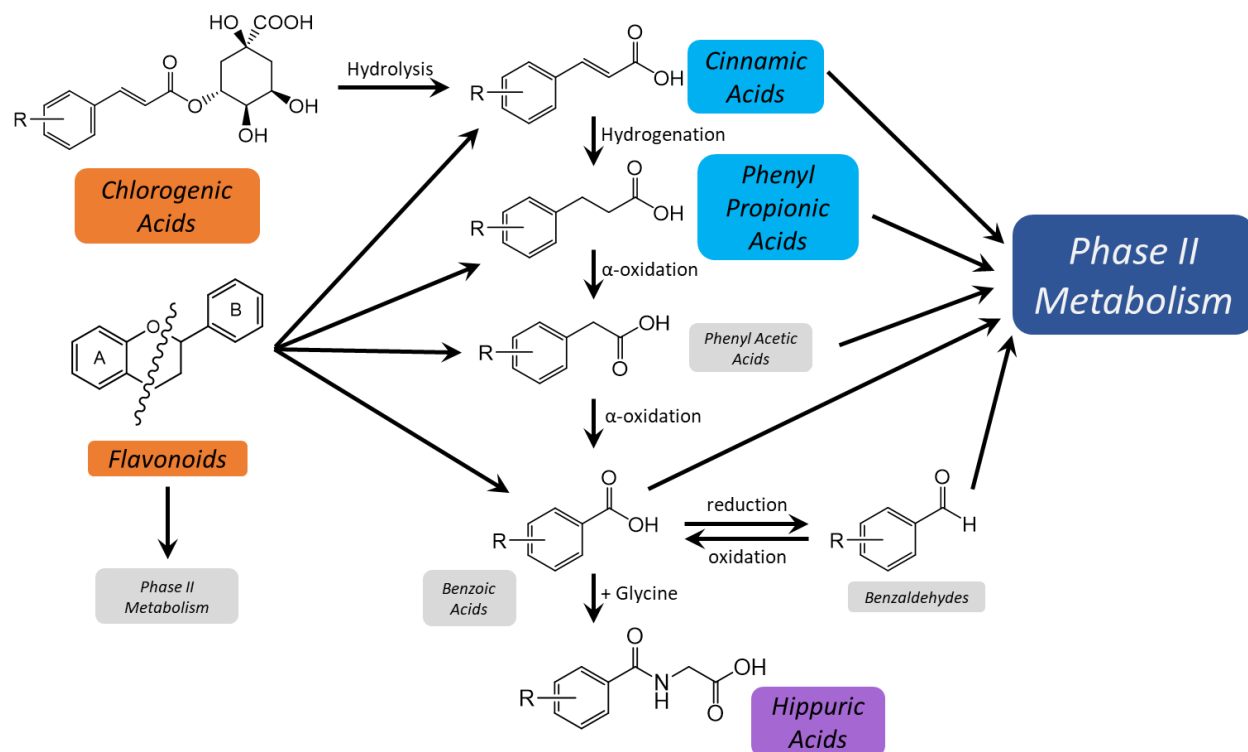


Figure 4.4 – Summary of colonic catabolism of major blueberry (poly)phenols. Flavonoids and chlorogenic acid are the predominant polyphenols in blueberries. After ingestion, several metabolic products are observed. In the upper GI tract, flavonoids may undergo phase II metabolism (though this accounts for a very small portion of all bioavailable metabolites). The major metabolic step occurs in the colon, where the heterocyclic ring in flavonoids and the caffeic-quinic bond in chlorogenic acid are cleaved to form a series of smaller molecular weight phenolic acids. These phenolic acids can be converted to other phenolic acids or diverted to phase II metabolism. The terminal step in the chain is the formation of hippuric acid, a conjugation of benzoic acids with glycine. Various metabolites within each phenolic acid subclass are formed by different substitutions on the benzene ring (represented by –R). Colored boxes indicate the most prominent starting materials (orange) and subsequent metabolites (blue and purple), while box sizes highlight relative contributions to total metabolism.

Table 4.1 – Polyphenol content of starting materials and oral gavage doses.^a

(Poly)phenol	Raw Materials (mg/100 g)		Water	Gavage Doses (mg/kg bw)			
	FD	CE		Low	Medium	High	BB
Total Phenolics^b	3517 ± 54.8	26715 ± 384	nd	50.0 ± 3.77	253 ± 6.07	1000 ± 18.4	50.8 ± 7.47
<i>Anthocyanins</i>							
Cyanidins							
Arabinoside	21.4 ± 0.30	93.8 ± 14.9	nd	0.11 ± 0.01	0.54 ± 0.04	2.27 ± 0.23	0.23 ± 0.07
Galactoside + Glucoside	46.3 ± 0.50	126 ± 12.2	nd	0.44 ± 0.04	2.09 ± 0.17	8.98 ± 0.82	1.00 ± 0.30
Delphinidins							
Arabinoside	20.8 ± 0.37	301 ± 50.4	nd	0.35 ± 0.05	1.80 ± 0.17	7.68 ± 0.84	0.15 ± 0.06
Galactoside + Glucoside	100 ± 2.02	960 ± 281	nd	0.81 ± 0.15	3.62 ± 0.71	11.6 ± 2.67	0.54 ± 0.28
Malvidins							
Arabinoside	12.6 ± 0.30	209 ± 31.5	nd	0.32 ± 0.03	1.59 ± 0.14	6.67 ± 0.57	0.16 ± 0.04
Galactoside	23.9 ± 1.48	529 ± 66.8	nd	0.78 ± 0.09	3.62 ± 0.49	12.9 ± 2.21	0.15 ± 0.08
Glucoside	34.5 ± 0.69	376 ± 50.0	nd	0.45 ± 0.04	2.17 ± 0.28	7.20 ± 1.27	0.22 ± 0.11
Peonidins							
Arabinoside	13.9 ± 0.10	56.6 ± 9.61	nd	0.08 ± 0.01	0.39 ± 0.03	1.66 ± 0.15	0.18 ± 0.05
Galactoside	40.2 ± 0.34	227 ± 22.8	nd	0.35 ± 0.04	1.69 ± 0.14	7.18 ± 0.69	0.58 ± 0.17
Glucoside	75.0 ± 1.40	249 ± 28.5	nd	0.40 ± 0.02	1.85 ± 0.15	8.06 ± 0.55	1.02 ± 0.29
Petunidins							
Arabinoside	28.4 ± 0.30	486 ± 71.0	nd	0.72 ± 0.07	3.47 ± 0.27	14.8 ± 1.30	0.35 ± 0.12
Galactoside	59.0 ± 1.42	1121 ± 108	nd	1.86 ± 0.16	8.60 ± 0.57	37.3 ± 3.49	0.68 ± 0.22
Glucoside	74.5 ± 0.86	598 ± 62.8	nd	0.90 ± 0.09	4.28 ± 0.32	18.7 ± 1.79	0.86 ± 0.27
<i>Phenolic Acids</i>							
Benzoic Acids							
Gallic acid	nd	18.6 ± 2.99	nd	0.029 ± 0.006	0.168 ± 2.00	0.62 ± 0.11	nd
Protocatechuic acid	0.57 ± 0.12	8.82 ± 1.90	nd	0.017 ± 0.003	0.070 ± 0.020	0.35 ± 0.10	0.013 ± 0.004
Cinnamic Acids							
Caffeic acid	trace	47.7 ± 11.7	nd	0.064 ± 0.006	36.7 ± 0.035	1.18 ± 0.06	0.007 ± 0.004
Chlorogenic acid	621 ± 21.7	1738 ± 322	nd	2.64 ± 0.17	13.0 ± 1.30	48.9 ± 3.85	4.86 ± 0.68
Ferulic acid	2.01 ± 0.22	43.7 ± 7.17	nd	0.088 ± 0.005	0.41 ± 0.032	16.0 ± 0.18	0.044 ± 0.008
Feruloylquinic acid	8.28 ± 0.45	33.4 ± 5.33	nd	0.074 ± 0.007	0.35 ± 0.040	14.3 ± 0.13	0.143 ± 0.014
<i>Flavan-3-ols</i>							
Catechin	10.7 ± 0.17	39.9 ± 12.3	nd	0.092 ± 0.020	0.37 ± 0.15	13.9 ± 0.22	0.089 ± 0.030
Epicatechin	7.78 ± 0.44	11.3 ± 4.04	nd	0.023 ± 0.008	0.10 ± 0.042	0.39 ± 0.05	0.031 ± 0.013
Epigallocatechin	nd	20.9 ± 2.79	nd	0.048 ± 0.004	0.25 ± 0.012	0.78 ± 0.07	nd
<i>Flavonols</i>							
Myricetin	1.78 ± 0.15	46.3 ± 12.0	nd	0.062 ± 0.014	0.32 ± 0.046	1.09 ± 0.30	0.031 ± 0.001
Kaempferol	nd	10.8 ± 1.12	nd	0.028 ± 0.001	0.14 ± 0.001	0.69 ± 0.01	nd

Table 4.1 continued

Galactoside + Glucoside	96.5 ± 5.26	360 ± 52.8	nd	0.49 ± 0.064	2.37 ± 0.29	9.16 ± 0.99	1.05 ± 0.40
Quercetin	1.80 ± 0.18	242 ± 48.6	nd	0.32 ± 0.050	1.60 ± 0.14	5.02 ± 1.45	0.041 ± 0.009
Galactoside + Glucoside	309 ± 9.78	1165 ± 117	nd	19.3 ± 0.12	9.25 ± 0.099	35.4 ± 2.61	4.45 ± 1.18
Rutin	32.6 ± 0.93	74.6 ± 16.3	nd	0.12 ± 0.011	0.56 ± 0.064	2.51 ± 0.21	0.431 ± 0.068

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

^a Data are comprised of three analytical replicates and presented as mean ± SD.

^b Measured via Folin assay.

Table 4.2 – Patterns of metabolite excretion, corresponding to graphs in Figure 4.1.

Metabolites with excretion patterns similar to:			
Fig. 1A	Fig. 1B	Fig. 1C	Fig. 1D
3-OH-PPA	Caffeic acid	Del-3-gal	3-OH-4-OMe-PPA
4-OMe-quercetin	Caffeic acid sulfs	Del-3-glu	3-OH-hippuric acid
Caffeic acid gcnd	Ferulic acid	Del gcnd	Dihydrocaffeic acid sulf
Chlorogenic acid	Myricetin	Malv-3-gal	Hippuric acid
Cyn-3-gal	Peon-3-gal	Malv-3-glu	
Cyn-3-glu	Pet gcnd	Myricetin gcnd	
Ferulic acid gcnds	Quercetin	Pet-3-gal	
Isoferulic acid		Pet-3-glu	
Isoferulic acid gcnd			
Isoferulic acid sulfs			
Peon-3-glu			
Quercetin gcnds			
Syringic acid			
Vanillic acid			

OH = hydroxy; PPA = phenylpropionic acid; OMe = methoxy; gcnd(s) = glucuronide(s); Cyn = cyanidin; gal = galactoside; glu = glucoside; sulf(s) = sulfate(s); Peon = peonidin; Pet = petunidin; Del = delphinidin; Malv = malvidin.

Table 4.3 – Polyphenol levels in gastrointestinal tissues.

<i>n</i>	Tissue Polyphenols (ng polyphenol / g tissue)					
	Water	NG	BB	Low	Medium	High
	9	4	8	9	8	9
Small Intestine						
Delphinidin-3-galactoside	nd	nd	nd	nd	nd	trace (4)
Malvidin-3-galactoside	nd	nd	nd	nd	nd	trace (5)
Petunidin-3-galactoside	nd	nd	nd	nd	nd	trace (7)
Syringic Acid	nd	nd	nd	nd	nd	trace (2)
3-methoxy-PAA	nd	nd	nd	nd	nd	trace (4)
3-hydroxy-PPA	nd	nd	nd	trace (3)	trace (2)	20.0 - 78.8 (6)
Dihydrocaffeic acid sulfate	nd	nd	trace (3)	nd	trace (3)	trace (5)
Ferulic acid glucuronide	nd	nd	nd	nd	0.48 - 8.26 (5)	1.73 - 8.87 (3)
Ferulic acid sulfate	3.94 - 58.2 (4)	3.59 - 7.03 (2)	3.29 - 44.5 (7)	3.44 - 55.0 (4)	5.84 - 135 (7)	1.88 - 58.9 (9)
Hippuric acid	9.86 - 796 (7)	20.2 - 41.7 (4)	9.96 - 1396 (9)	21.6 - 430 (9)	42.7 - 678 (8)	36.5 - 900 (9)
3-hydroxy-hippuric acid	nd	nd	trace (3)	nd	trace (6)	trace (3)
Quercetin glucuronide	nd	nd	nd	nd	nd	0.23 - 1.58 (6)
Colon						
Delphinidin-3-galactoside	nd	nd	nd	nd	nd	trace (5)
Malvidin-3-galactoside	nd	nd	nd	nd	nd	trace (7)
Petunidin-3-galactoside	nd	nd	nd	nd	nd	trace (6)
Syringic Acid	nd	nd	nd	nd	nd	trace (3)
3-methoxy-PAA	nd	nd	trace (2)	trace (2)	trace (5)	trace (4)
3-hydroxy-PPA	16.8 - 37.1 (3)	nd	23.9 - 459 (2)	49.0 - 52.4 (3)	104 - 4549 (3)	27 - 5897 (5)
Ferulic acid glucuronide	nd	nd	nd	nd	2.52 - 28.5 (5)	trace (2)
Ferulic acid sulfate	nd	nd	nd	nd	trace (2)	trace (2)
Hippuric acid	64.1 - 131 (2)	nd	26.3 - 64.4 (2)	15.6 - 59.9 (4)	94.8 - 874 (3)	10.1 - 112 (6)

Data shown as range of concentrations observed, with the total number of samples the metabolite was observed in indicated in parentheses. No statistically significant differences were noted between groups using Tukey's HSD ($p < 0.05$). NG = non-gavaged group; nd = not detected; trace = metabolite observed, but below the LOQ.

Table 4.4 – Polyphenol levels in peripheral tissues.

<i>n</i>	Tissue Polyphenols (ng polyphenol / g tissue)					
	Water	NG	BB	Low	Medium	High
Brain						
Isovanillin	nd	nd	trace (3)	nd	nd	trace (2)
3-hydroxy-PPA	nd	nd	nd	nd	trace (1)	11.7 - 67.3 (4)
Dihydrocaffeic acid sulfate	12.9 - 137 (10)	36.9 - 129 (4)	26.6 - 96.7 (9)	6.88 - 106 (10)	14.1 - 128 (9)	24.0 - 102 (10)
Kidney						
3-hydroxy-PPA	trace (2)	nd	trace (1)	nd	19.9 - 309 (3)	48.4 - 915 (5)
Caffeic acid sulfate	nd	nd	nd	nd	nd	trace (4)
Ferulic acid glucuronide	nd	nd	nd	nd	trace (5)	trace (4)
Hippuric acid	17.7 - 311 (10) ^b	29.7 - 93.2 (4) _b	10.8 - 223 (9) ^b	29.3 - 177 (10) ^b	116 - 716 (9) ^{ab}	79.6 - 1661 (10) ^a
3-hydroxy hippuric acid	nd	nd	nd	nd	trace (6)	trace (6)
Quercetin glucuronide	nd	nd	nd	nd	nd	0.05 - 0.90 (5)
Liver						
Isovanillin	trace (2)	nd	trace (1)	trace (2)	trace (2)	nd
3-hydroxy-PPA	trace (2)	nd	trace (1)	trace (2)	0.76 - 12.3 (5)	0.35 - 118 (5)
Caffeic acid sulfate	nd	nd	nd	nd	nd	trace (4)
Ferulic acid glucuronide	nd	nd	nd	nd	nd	trace (2)
Hippuric acid	4.51 - 17.2 (6) ^b	9.4 (1) ^{ab}	4.04 - 21.7 (7) _{ab}	3.94 - 14.5 (9) ^b	8.50 - 46.9 (7) ^{ab}	10.0 - 89.4 (10) ^a
Quercetin glucuronide	nd	nd	nd	nd	nd	trace (2)

Data shown as range of concentrations observed, with the total number of samples the metabolite was observed in indicated in parentheses. Statistically significant differences between groups denoted by lower case letters using Tukey's HSD test ($p < 0.05$). NG = non-gavaged group; nd = not detected; trace = metabolite observed, but below the LOQ.

Table 4.5 – Summary of urinary phenolic excretion (μmol excreted over 24h).

		Day					
		0	7	15	30	60	90
<i>Flavonoids</i>							
Water		nd	nd	nd	nd	nd	nd
NG		nd	nd	nd	nd	nd	nd
BB		nd	0.002 ± 0.001 ^{d,AB}	0.002 ± 0.001 ^{d,B}	0.002 ± 0.001 ^{d,AB}	0.002 ± 0.001 ^{c,A}	0.003 ± 0.002 ^{c,A}
Low		nd	0.004 ± 0.002 ^{c,A}	0.003 ± 0.002 ^{c,AB}	0.004 ± 0.003 ^{c,A}	0.003 ± 0.001 ^{c,B}	0.004 ± 0.002 ^{c,AB}
Medium		nd	0.018 ± 0.009 ^b	0.019 ± 0.008 ^b	0.023 ± 0.013 ^b	0.017 ± 0.009 ^b	0.019 ± 0.008 ^b
High		nd	0.096 ± 0.035 ^{a,B}	0.127 ± 0.060 ^{a,AB}	0.157 ± 0.065 ^{a,A}	0.121 ± 0.054 ^{a,AB}	0.144 ± 0.073 ^{a,AB}
<i>Hippuric Acids</i>							
Water		0.97 ± 0.45	1.35 ± 0.50 ^b	1.10 ± 0.30 ^c	1.33 ± 0.50 ^c	0.92 ± 0.29 ^d	1.19 ± 0.21 ^c
NG		1.33 ± 0.61	1.08 ± 0.44 ^b	1.43 ± 0.18 ^{bc}	0.93 ± 0.36 ^c	1.04 ± 0.31 ^d	0.97 ± 0.13 ^c
BB		1.31 ± 0.49 ^B	3.38 ± 0.88 ^{a,A}	4.34 ± 1.51 ^{b,A}	3.11 ± 0.73 ^{ab,A}	3.75 ± 0.82 ^{ab,A}	3.59 ± 1.28 ^{ab,A}
Low		1.25 ± 0.42 ^C	2.43 ± 0.90 ^{a,AB}	2.04 ± 0.54 ^{a,AB}	2.87 ± 0.83 ^{b,A}	1.65 ± 0.49 ^{c,BC}	2.45 ± 1.06 ^{b,AB}
Medium		1.52 ± 0.50 ^B	3.96 ± 1.42 ^{a,A}	4.05 ± 1.59 ^{a,A}	4.23 ± 1.94 ^{ab,A}	4.01 ± 1.23 ^{b,A}	4.15 ± 0.65 ^{a,A}
High		1.36 ± 0.38 ^C	3.01 ± 0.64 ^{a,B}	7.07 ± 4.24 ^{a,A}	4.67 ± 1.10 ^{a,A}	6.01 ± 1.53 ^{a,A}	5.45 ± 1.45 ^{a,A}
<i>Phenolic Acids</i>							
Water		1.63 ± 0.73	2.30 ± 0.81 ^d	1.84 ± 0.51 ^b	2.77 ± 0.64 ^c	1.92 ± 0.37 ^d	2.36 ± 0.41 ^d
NG		2.46 ± 1.09	1.99 ± 0.70 ^d	3.36 ± 0.62 ^b	2.09 ± 0.49 ^c	1.84 ± 0.63 ^d	1.80 ± 0.32 ^d
BB		2.39 ± 0.93 ^B	5.38 ± 1.45 ^{c,A}	6.81 ± 2.31 ^{b,A}	4.21 ± 1.36 ^{c,AB}	5.10 ± 0.71 ^{c,A}	6.22 ± 2.44 ^{c,A}
Low		2.07 ± 0.73 ^D	9.97 ± 3.34 ^{b,A}	5.51 ± 1.59 ^{b,BC}	9.08 ± 3.16 ^{b,AB}	5.58 ± 1.94 ^{c,C}	7.87 ± 3.29 ^{c,ABC}
Medium		2.31 ± 0.68 ^B	35.6 ± 12.6 ^{a,A}	24.5 ± 10.6 ^{a,A}	31.6 ± 13.1 ^{a,A}	25.8 ± 10.2 ^{b,A}	34.4 ± 15.3 ^{b,A}
High		2.32 ± 0.55 ^C	36.7 ± 11.9 ^{a,B}	46.0 ± 26.9 ^{a,B}	55.4 ± 21.9 ^{a,AB}	67.2 ± 26.8 ^{a,A}	75.9 ± 33.1 ^{a,A}
<i>Total Excretion</i>							
Water		3.34 ± 1.45	5.11 ± 1.67 ^c	3.75 ± 1.04 ^e	4.76 ± 1.39 ^d	3.42 ± 0.72 ^d	4.50 ± 0.53 ^d
NG		4.86 ± 2.08	3.98 ± 1.41 ^c	5.98 ± 0.62 ^{de}	3.67 ± 0.96 ^d	3.62 ± 1.16 ^d	3.58 ± 0.46 ^d
BB		4.75 ± 1.76 ^B	9.49 ± 1.90 ^{b,A}	11.7 ± 2.34 ^{cd,A}	7.98 ± 2.17 ^{c,A}	9.22 ± 3.53 ^{c,A}	11.4 ± 3.88 ^{c,A}
Low		4.12 ± 1.29 ^D	15.2 ± 4.32 ^{b,A}	9.13 ± 2.23 ^{c,BC}	13.6 ± 3.71 ^{b,AB}	8.19 ± 2.46 ^{c,C}	12.3 ± 4.87 ^{c,ABC}
Medium		4.89 ± 1.43 ^B	45.5 ± 14.4 ^{a,A}	32.8 ± 13.2 ^{b,A}	38.8 ± 14.8 ^{a,A}	32.1 ± 11.5 ^{b,A}	42.7 ± 16.7 ^{b,A}
High		4.74 ± 1.11 ^C	44.9 ± 7.92 ^{a,B}	58.6 ± 31.4 ^{a,B}	59.3 ± 20.2 ^{a,AB}	81.8 ± 27.7 ^{a,A}	91.2 ± 34.6 ^{a,A}

Lower case letters indicate differences between doses at the same time point (i.e., down a column), while upper case letters indicate differences between time points and within dose (i.e., across a row). Two-way ANOVA with Tukey's HSD ($p < 0.05$) used for all comparisons. NG = non-gavaged group; nd = not detected; trace = metabolite observed, but below the LOQ.

CHAPTER 5. SUMMARY AND CONCLUSIONS

Fruit and vegetable derived polyphenols may have a number of impactful health benefits. Given their ubiquity in nature, prevalence in the human diet, and the growth in the number of health-conscious consumers seeking herbal and botanical dietary supplements, the consumption of polyphenols is increasing. As polyphenols become more prevalent in the diet in a number of ways, researchers are actively seeking to better understand them. One of the biggest challenges in polyphenol research is understanding how they exert their purported health benefits. In light of this, many have focused on the bioavailability of polyphenols and their metabolites, while others have sought to better understand the bioavailable forms of polyphenols and which cellular mechanisms they use to induce their effects. In addition, much effort has been expended in an attempt to determine the dose of polyphenols necessary to obtain significant biological activity *in vivo*. This is a challenging task because polyphenols are heterogeneous mixtures in all plants and their bioavailability varies greatly depending on their food matrix.

In addition to these challenges, consumption patterns are changing and polyphenols are becoming much more prevalent in the diet. There are a number of ways this is happening, but the primary drivers are the increased use of botanical supplements, the use of polyphenol-rich extracts as coloring agents in food products, and the growth and interest consumers have in functional foods. As these consumption methodologies become more prevalent and polyphenol intakes increase, there are a several key questions that must be addressed. First, the safety of increased doses of polyphenols must be considered. Second, the metabolic changes that may occur as increased doses of polyphenols and their purified extracts are consumed must be examined. Finally, the efficacy of this paradigm shift must be evaluated to determine if increased doses of polyphenols are able to meaningfully impact health.

The goal of the research presented here was to address both the safety and metabolic changes that may occur with the consumption of increasing doses of polyphenols. Because dietary polyphenols are thought to act in an hormetic manner, we hypothesized that significant changes in in polyphenol metabolism would be observed with increasing doses. Additionally, we hypothesized that toxicity would occur with repeated dosing at high levels. To test this, we performed several experiments using lyophilized whole blueberries and extracted blueberry polyphenols in rat models. Blueberries were chosen for these investigations on the basis of their

high polyphenol content, their ability to beneficially affect health via a number of different signaling pathways, and their popularity amongst consumers.

In the first study, the pharmacokinetics of blueberry polyphenol metabolites and ^{45}Ca absorption were measured in an acute model. Using ovariectomized Sprague-Dawley (OVX-SD) rats, purified blueberry polyphenols were administered in an escalating dose fashion to mimic high dose intakes of polyphenols as may be present in dietary supplements. Blood and urine were collected for 48h after dosing and analyzed using a targeted metabolomics approach to quantitate the presence of key phenolic metabolites. The results of this study showed extensive metabolism of blueberry polyphenols. Nearly all of the observed metabolites were phenolic and hippuric acids, indicating extensive colonic metabolism of blueberry polyphenols. Cinnamic acids and their phase-II metabolites were the most prominent phenolic metabolites observed. Most metabolites were excreted within 12h of dosing and exhibited a significant dose-response relationship. However, several metabolites were excreted at later time points and showed saturated absorption. Finally, calcium absorption and femoral deposition was significantly increased in the highest dose group.

When comparing these results to other studies in the literature, a few trends are observed. Most other investigators employing polyphenol-rich berries in human and animal models often find hippuric acids to be the dominant metabolite, as opposed to cinnamic acids as found in our work. This may be due to several factors, though as shown in chapter 4, there is a significantly higher production of hippuric acid metabolites when consuming a lyophilized blueberry than there is with a purified phenolic extract. Thus, in studies using whole foods, the differences in metabolism may be significantly impacted by the food matrix as well as the polyphenols. Additionally, authentic standards for phase II metabolites of phenolic acids are rarely available and over-estimation of these metabolites is common because of the ease with which they ionize. In regards to calcium absorption, our results for the lower dose groups match up well with other studies, as calcium absorption is not significantly altered at dietary doses. However, when consumed in larger doses, we showed a significant increase in calcium absorption, which has not been tested in previous investigations.

Taken together, the results of the first study indicate that blueberry polyphenols are extensively metabolized to colonically-generated phenolic acid metabolites. Additionally, there was a dose-dependent shift in metabolite excretion that corresponded to the saturated production

of several metabolites in this study, most notably the hippuric acids. When considering this alongside the increase in calcium absorption in the highest dose group, these results indicate that there are significant dose-dependent changes in the metabolism of blueberry polyphenols, most likely stemming from alterations in intestinal processing of blueberry phenolics. And, although no signs of adverse effects were noted in this study, we hypothesized that repeated dosing over prolonged periods of time may induce negative outcomes and toxicity.

To test this second hypothesis, we performed a 90d repeated dosing study. Similar to the acute study, OVX-SD rats received purified blueberry polyphenols in an escalating dose fashion. To study the possibility of a food matrix effect, an additional group was included that was dose-matched to the low dose group and received lyophilized blueberries instead of the purified extract. During the study, urine was collected to monitor changes in polyphenol metabolism and evaluate if the changes observed in the acute study remained over longer periods of time. At the end of the study, a complete necropsy and a standard battery of toxicology tests were performed to determine if any adverse effects occurred as a result of blueberry polyphenols. Additionally, key metabolic tissues were collected to measure tissue distribution of polyphenols.

When examining the results of the classical toxicology outcomes, including changes in body weight, food consumption, tissue weights, hematology, urinalysis, and gut permeability, minimal differences were observed between the groups. There were no histopathologically significant changes in any of the tissues examined, nor were there any abnormal results in hematology or urinalysis. There were significant differences in the food consumption and body weight gain over 90d between the high dose and lyophilized blueberry groups, as well as a small but significant increase in intestinal permeability in the high dose group, though these were not considered to be biologically meaningful. Taken together, these results indicated that blueberry polyphenols are safe up to 1 g/kg bw/d in these rats, an amount that translates to ~10 g polyphenols/kg bw/d in a 70 kg human.

Although there are relatively few investigations into the potential for polyphenol toxicity (especially in comparison to the number of studies reporting health benefits), there have been a few related studies that serve as good comparators for the work presented here. In investigations using high doses of berries and grapes (i.e., anthocyanin-rich fruits similar to blueberries), no observations of toxicity were noted. However, these investigators noted a dose-dependent darkening of the urine and feces, similar to what was observed in our work. The majority of the

toxicity observed with polyphenols in human or animal studies have been found with either green tea extracts (GTE) or other polyphenol-rich extracts that were mixed with other components that may have confounded toxicity (e.g., caffeine). In studies of GTE, hepatotoxicity is the most commonly reported adverse effect. Overall, given the dearth of information related to polyphenol toxicity, much work is still needed to establish the safety of polyphenols at high doses.

When examining changes in metabolism over 90d, several interesting trends emerged. First, most compounds exhibited a significant dose-response relationship, with cinnamic and hippuric acids being the most prominent metabolites. Second, the relative proportion of each class of metabolites was stable over 90d, indicating that any metabolic changes that may be occurring happen within the first week of exposure to polyphenols and then remain steady over 90d. However, there was a steady increase in the production of metabolites in the highest dose group throughout the 90d study period, indicating that phenolic metabolites are more efficiently absorbed over long-term exposure. Third, there was a dose-dependent shift in the metabolites produced in this study; as the dose increased, the relative production of phenylpropionic acids (PPA) metabolites increased while hippuric acid production decreased. This is likely due to the saturation of hippuric acid production. Fourth, a relatively small number of metabolites were detected in the tissues, though the metabolites that were detected demonstrated a significant dose-response relationship. Finally, there appears to be a significant food matrix effect, as the lyophilized blueberries produced significantly more hippuric acid metabolites than the purified extract at the same dose of total polyphenols. Taken together, these results indicate that the metabolism of blueberry polyphenols is relatively stable over 90d, though there is a dose-dependent shift in metabolite production as well as a significant food matrix effect.

As compared to other investigations of repeated dosing with polyphenols, our results highlight some interesting trends. Other investigators have noted a significant dose-response with repeated consumption of polyphenols, though the higher doses are typically less efficiently metabolized and absorbed than lower doses. This matches up well with our results, as we observed a significant dose-response for most metabolites, but the metabolism was less efficient as the dose increased. The increased absorption of phenolic metabolites in the high dose group is consistent with another study of blueberry polyphenols in humans, where increased levels of metabolites were observed with repeated dosing. This was hypothesized to come from an adaptive effect in the gut, where the phenolics are more efficiently metabolized with repeated exposure. As mentioned

previously, our results differ from other studies with berries, as we found cinnamic acids to be the most extensively produced metabolites, while other studies found hippuric acids to be the most prominent metabolites. This may be due to a number of factors, but is likely derived from the fact that whole berries contain significant amounts of fiber and simple carbohydrates that may result in significant metabolic differences when compared to polyphenol-rich extracts. This was clearly observed in our work and, when coupled with the fact that phase-II metabolites of phenolic acids are often over-estimated in the absence of authentic standards, our results may line up well with the results of other studies. Although further experiments are needed to clarify the genesis of these differences (e.g., comparing our results to similar analyses using glucuronidases and sulfatases), there may be significant similarities between investigations.

Tissue distribution of polyphenols is not extensively reported in the literature, though the results reported here reflect several trends observed in the literature and serve to help expand our knowledge of tissue distribution and storage of phenolic metabolites. In general, polyphenols are only observed in tissues shortly after dosing (i.e., within 6h) and cleared rapidly. In one study, the investigators reported the presence of several phenolic metabolites in the tissues after 2-6h, but not after 18h, indicating that tissues clear phenolic metabolites relatively quickly. Of the few investigators that do demonstrate tissue storage of phenolic metabolites for longer than 24h, virtually all observed metabolites are derivatives of catechins. Investigators have found catechin metabolites from green tea and grape seed extracts stored in tissues for several days, but, outside of these specific metabolites, tissue storage of phenolics appears to be extremely limited.

When looking at the results of both experiments side-by-side, the same trends in metabolism appear in both acute and repeat-dosing models. The main metabolites observed in both studies were cinnamic and hippuric acids. Both studies demonstrated a dose-dependent shift in the relative contribution of metabolites produced with increased doses, with PPA increasing and hippuric acids decreasing as a percentage of total metabolite production. And, because no significant adverse effects were observed from the classical toxicology measures over 90d, it appears that high doses of blueberry polyphenols are safe to consume.

These results have several important implications for consumers. First and foremost, the safety of high doses of blueberry polyphenols was confirmed, meaning that even in a dietary supplement paradigm that presents the consumer with a high dose of a concentrated extract of polyphenols, there is a very low risk of toxicity. Second, there are dose- and food matrix-dependent

effects of blueberry polyphenols on metabolism. These shifts may have an impact on health outcomes, as both the quantity and composition of the absorbed metabolites can differentially impact the health status of the host. Thus, these results provide an intriguing platform to begin examining how differences in metabolite production impact health. Finally, the theory of hormesis would suggest that there is an ideal dose that maximizes the health benefits obtained from polyphenols. Based on this, future experiments comparing the health effects of increasing doses of polyphenols with and without the food matrix, as well as other food components that may affect metabolism (e.g., fiber), will help identify both what the ideal dose is and what the relative composition of metabolites is at that dose. This would help to not only clarify health benefits, but would also help dieticians and researchers tailor polyphenol consumption strategies to optimize health.

The gut microbiome is an important part of phenolic metabolism. Although we did not monitor changes in microbial species in this work, the differences in colonically-produced metabolites suggests that there are significant shifts in the gut microbiota that are driving these differences. Given the recent connections between the gut microbiome, metabolism, and overall health status, it is likely that any changes in overall health will be driven by the adaptation of the gut microbiome to different phenolic loads. Future work exploring the effects of polyphenols on the gut microbiome may take advantage of a number of different models to understand both the contributions of the gut microbiome to phenolic metabolism as well as the impacts of different disease states that affect the gut microbiome and, consequently, phenolic metabolism. For example, using a germ-free model or treating animals with antibiotics would eliminate colonic metabolism by the gut microbiota and increase understanding of which phenolic metabolites are formed in the colon versus other metabolic organs (e.g., liver). This approach would clarify the specific contributions of the gut microbiota to phenolic metabolism and further our understanding of the importance of colonic metabolism to overall phenolic metabolism. This approach would be especially helpful with certain metabolites, like hippuric acid, that can be formed from many different dietary inputs and may be formed in either the liver or the colon. By using an antibiotic model, hippuric acid would no longer be produced in the colon and none of the phenolic acid precursors necessary for generating it would be produced either. Systemically, this means that hippuric acid would be generated entirely from the liver without colonic feedstocks, which may significantly alter the total production of hippuric acids. Using different animal models that focus

on animals at different life stages or comparing phenolic metabolism in different disease states associated with dysbiosis (e.g., obesity or diabetes) will shed light on the efficiency of gut microbiota to metabolize polyphenolics in different health conditions and which metabolites are preferentially formed under these specific conditions. By furthering our understanding of differences in metabolism with different compositions of gut microbiota (and how the polyphenols can shift microbial status), we will better understand the connections between phenolic metabolites and health outcomes, especially in different disease states. As interest in the gut microbiome continues to grow, continuing to investigate the metabolic consequences of increased doses of polyphenols under different health statuses is key to filling in knowledge gaps and connecting phenolic metabolism to health outcomes.

Although much work remains to be done, the work described in this dissertation demonstrate significant, dose-dependent shifts in the metabolism of blueberry polyphenols. These differences appear immediately and are sustained at over 90d. Future work examining the impact of these metabolites on overall health status as well as changes to the gut microbiome will help further clarify the ramifications of ingesting increased doses of polyphenols.

APPENDIX A. SUPPLEMENTAL INFORMATION FOR CHAPTER 2: INCREASING DOSES OF BLUEBERRY POLYPHENOLS ALTERS COLONIC METABOLISM IN OVARIECTOMIZED RATS

Table A.1 – Mass spectrometry parameters for compounds quantified via MS².

Figure A.1-A.14 – Urinary excretion of phenolic acids.*

Figure A.15-A.18 – Urinary excretion of flavonols.*

Figure A.19-A.35 – Urinary excretion of anthocyanins.*

Figure A.36 – Total urinary excretion of anthocyanins over 48h.

Figure A.37-A.38 – Urinary excretion of hippuric acids.*

Figure A.39-A.45 – Plasma pharmacokinetics.*

* Graphs in figures A.1-A.35 and A.37-A.38 depict urinary excretion of polyphenol metabolites, while graphs in figures A.39-A.45 show plasma concentrations of polyphenol metabolites. For clarity, statistical comparisons are shown in tables below each figure. Lower case letters indicate differences between doses and the same time point (i.e., down a column), while upper case letters indicate differences between time points and within dose (i.e., across a row). Two-way ANOVA with Tukey's HSD ($p < 0.05$) used for all comparisons. nd = not detected; trace = metabolite detected but below LOQ. Data shown as mean \pm SEM in graphs; mean \pm SD in tables.

Table A. 1 – Mass spectrometry parameters for compounds quantified via MS².

Compound	MRM mode	RT (min)	MW	Precursor ion (m/z)	MS ² fragments (m/z) ^a	CV	CE (eV)	Quantitation Standard
Anthocyanins								
Cyanidin								
Arabinoside	+	1.77	419.1	419	287	46	20	Cyanidin-3-glucoside
Galactoside	+	1.41	449.1	449	137, 287	46	54, 20	
Glucoside	+	1.62	449.1	449	137, 287	46	54, 20	
Glucuronide	+	1.8	463.1	464	287	35	20	
Delphinidin								
Arabinoside	+	1.41	435.4	435	303	100	18	Delphinidin-3-glucoside
Galactoside	+	1.12	465.4	465	69, 303	100	74, 18	
Glucoside	+	1.24	465.4	465	69, 303	100	74, 18	
Glucuronide	+	3.2	479.4	479	303	100	18	
Sulfate	+	4.11	383.4	383	153, 303	100	54, 18	
Malvidin								
Arabinoside	+	2.79	463.4	463	331	100	34	Malvidin-3-glucoside
Galactoside	+	2.4	493.4	493	315, 331	100	50, 34	
Glucoside	+	2.63	493.4	493	315, 331	100	50, 34	
Peonidin								
Arabinoside	+	2.44	433.4	433	301	35	20	Peonidin-3-glucoside
Galactoside	+	2.11	463.4	464	301	35	20	
Glucoside	+	2.4	463.4	464	301	35	20	
Glucuronide	+	4.28	477.4	477	301	35	20	
Sulfate	+	4.24	381.4	381	301	35	20	
Petunidin								
Arabinoside	+	2.13	449.4	449	317	40	22	Petunidin-3-glucoside
Galactoside	+	1.69	479.4	479	317	40	22	
Glucoside	+	1.91	479.4	479	317	40	22	
Glucuronide	+	3.77	493.4	493	317	40	22	
Sulfate	+	4.26	397.4	397	317	40	22	

Table A.1 continued

Phenolic Acids								
Benzaldehydes (BALD)								
4-OH-BALD	-	1.70	126	121	65, 92	44	22, 24	4-OH-BALD
3-OH-4-OMe-BALD	-	2.30	150.2	151	92, 136	24	22, 12	3-OH-4-OMe-BALD
Benzoic acids (BzA)								
Protocatechuic acid	-	0.86	154.1	153	81, 91	28	18, 24	Protocatechuic acid
Gallic acid	-	0.61	170.1	169	79, 97	32	22, 20	Gallic acid
3-OH-4-OMe-BzA	-	1.64	168.1	167	108, 152	28	20, 12	3-OH-4-OMe-BzA
Syringic acid	-	1.91	198.2	197	123, 182	36	24, 14	Syringic acid
BzA sulfate	-	3.93	202.1	201	121	28	12	4-OH-BALD
BzA glucuronide	-	3.81	298.2	297	121	30	22	4-OH-BALD
Phenyl Acetic Acids (PAA)								
4-OH-PAA	-	1.52	152.2	151	107, 133	28	10	4-OH-PAA
3-OMe-PAA	-	3.62	166.2	165	106, 121	28	24, 6	3-OMe-PAA
3-OH-4-OMe-PAA	-	2.35	182.2	181	79, 122	14	16, 12	3-OH-4-OMe-PAA
Phenyl Propionic Acids (PPA)								
3-OH-PPA	-	2.82	166.2	165	121, 147	24	16, 12	3-OH-PPA
3-OH-4-OMe-PPA	-	3.24	196.2	195	135, 136	32	26, 12	3-OH-4-OMe-PPA
<i>trans</i> -Cinnamic Acids								
<i>p</i> -Coumaric acid	-	2.41	164	163	93, 120	28	28, 32	<i>p</i> -Coumaric acid
Caffeic acid	-	1.62	180.2	179	107, 117	32	22, 24	Caffeic acid
Caffeic acid sulfate	-	4.35	260.2	259	179	32	12	Caffeic acid
Caffeic acid glucuronide	-	1.46	356.2	355	179	30	18	Caffeic acid glucuronide
Ferulic acid	-	3.12	194.2	193	134, 178	30	18, 12	Ferulic acid
Ferulic acid sulfate	-	1.82	274.2	273	134, 193	30	18, 12	Ferulic acid
Ferulic acid glucuronide	-	3.51	370.2	369	193	30	22	Ferulic acid
Chlorogenic acid	-	1.49	354.3	353	85, 191	26	40, 18	Chlorogenic acid
Feruloylquinic acid	-	2.64	368.3	367	134, 191	30	40, 20	Ferulic acid
Ethyl gallate	-	2.62	198.2	197	124, 125	30	30	Ethyl gallate
Taxifolin	-	3.19	304.3	303	125, 177	34	22, 14	Taxifolin

Table A.1 continued

Catechins								
Catechin	-	1.40	290.3	289	<i>109, 123</i>	33	24, 26	Catechin
Epicatechin	-	2.11	290.3	289	<i>109, 123</i>	33	24, 26	Epicatechin
(Epi)gallocatechin peak 1	-	0.80	306.3	305	<i>125, 179</i>	40	20, 14	Catechin
(Epi)gallocatechin peak 2	-	1.00	306.3	305	<i>125, 179</i>	40	20, 14	Catechin
Flavonols								
Quercetin	-	4.19	302.2	301	<i>151, 179</i>	38	20, 18	Quercetin
Kaempferol	-	4.52	286.2	285	<i>146, 151</i>	58	30, 18	Quercetin
Myricetin	-	3.79	318.2	317	<i>151, 179</i>	46	26, 20	Myricetin
Kaempferol-3-glycs ^b	-	1.40	448.4	447	<i>284</i>	42	18	Quercetin-3-glucoside
Quercetin-3-glycs	-	3.39	464.1	463	<i>271, 300</i>	42	40, 24	Quercetin-3-glucoside
Quercetin-3-rutinoside	-	3.41	610.2	609	<i>300</i>	42	24	Quercetin
Quercetin glucuronide	-	3.46	478.1	477	<i>151, 300</i>	42	38, 22	Quercetin glucuronide
4-OMe-quercetin	-	4.62	316.2	315	<i>151, 300</i>	28	32, 20	4-OMe-quercetin
Me-quercetin glucuronide	-	3.80	492.1	491	<i>151, 315</i>	42	38, 22	Quercetin glucuronide
Hippuric Acids								
Hippuric acid	-	1.58	179.2	178	<i>56, 77</i>	28	12, 16	Hippuric acid
3-OH-hippuric acid	-	1.04	195.2	194	<i>93, 121</i>	32	22, 24	3-OH-hippuric acid

MRM = Multiple Reaction Monitoring; RT = retention time; MW = molecular weight; CV = cone voltage; CE = collision energy.

^a Daughter fragments monitored; fragment listed in italics used for quantitation.

^b Galactoside and glucoside peaks overlapped and were not distinguishable, so quantitated as sum of both peaks.

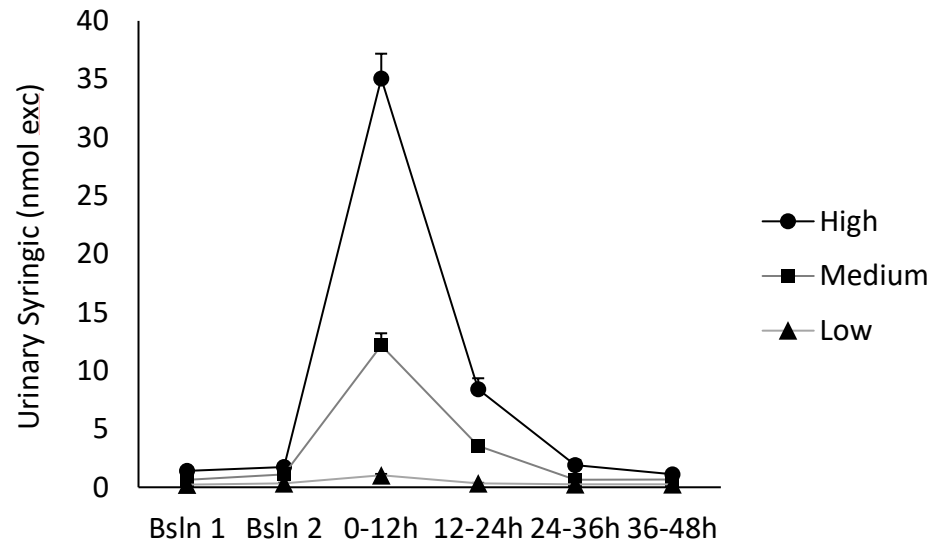


Figure A. 1 – Urinary excretion of syringic acid over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.23 ± 0.09^{aB}	0.35 ± 0.11^{aB}	1.03 ± 0.3^{aA}	0.35 ± 0.09^{aB}	0.25 ± 0.06^{aB}	0.27 ± 0.09^{aB}
Medium	0.65 ± 0.34^{bC}	1.12 ± 0.59^{bC}	12.16 ± 2.95^{bA}	3.55 ± 0.92^{bB}	0.65 ± 0.4^{bC}	0.65 ± 0.29^{bC}
High	1.39 ± 0.58^{cC}	1.74 ± 0.59^{cC}	35.06 ± 6^{cA}	8.41 ± 2.65^{cB}	1.9 ± 1.25^{cC}	1.12 ± 0.28^{cC}

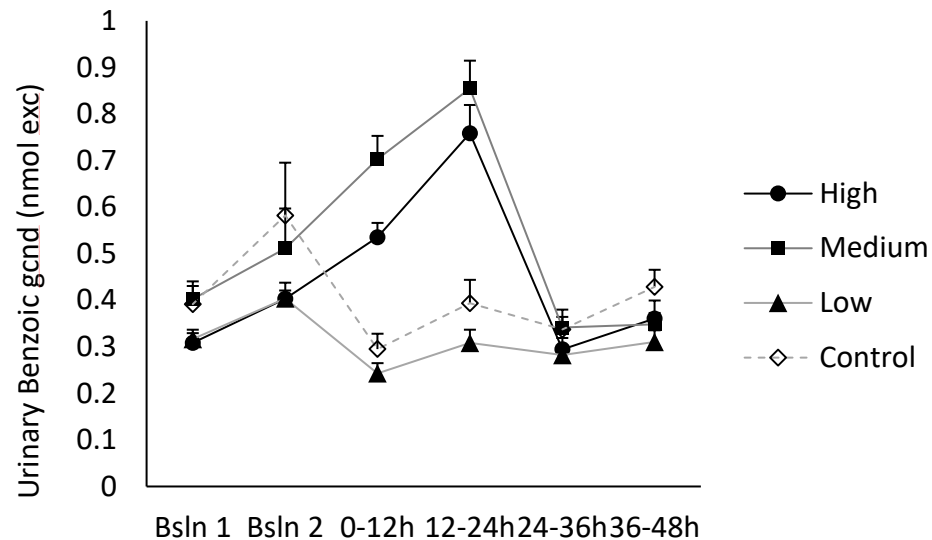


Figure A. 2 – Urinary excretion of benzoic acid glucuronide over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	0.39 ± 0.14 ^A	0.58 ± 0.32 ^A	0.3 ± 0.09 ^{bC}	0.39 ± 0.14 ^{bABC}	0.34 ± 0.12 ^{BC}	0.43 ± 0.1 ^{AB}
Low	0.32 ± 0.04 ^A	0.4 ± 0.05 ^A	0.24 ± 0.06 ^{bB}	0.31 ± 0.08 ^{bAB}	0.28 ± 0.05 ^{AB}	0.31 ± 0.07 ^{AB}
Medium	0.4 ± 0.08 ^B	0.51 ± 0.24 ^B	0.7 ± 0.14 ^{aA}	0.85 ± 0.17 ^{aA}	0.34 ± 0.06 ^B	0.35 ± 0.07 ^B
High	0.31 ± 0.08 ^C	0.4 ± 0.1 ^C	0.54 ± 0.09 ^{aB}	0.76 ± 0.17 ^{aA}	0.29 ± 0.07 ^C	0.36 ± 0.1 ^C

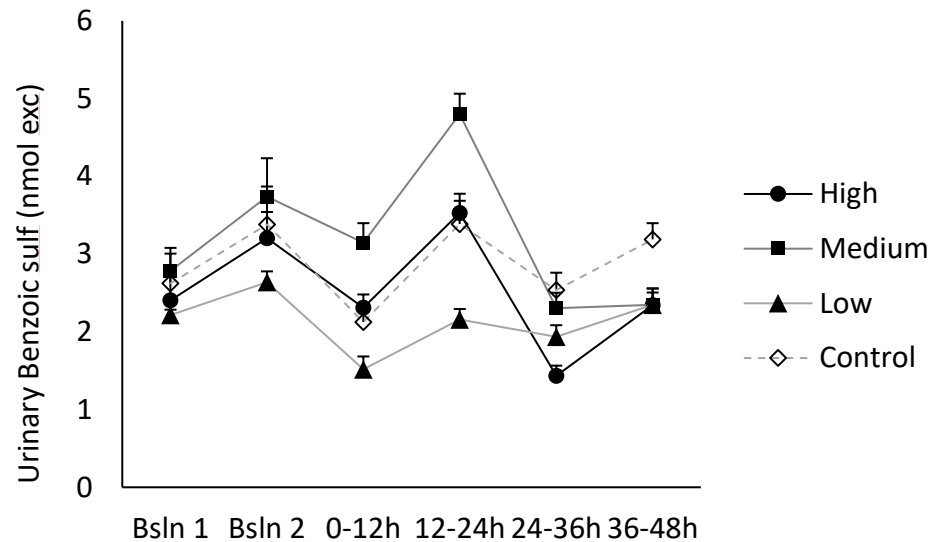


Figure A. 3 – Urinary excretion of benzoic acid sulfate over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	2.62 ± 1.09 ^{AB}	3.38 ± 1.3 ^{AB}	2.13 ± 0.59 ^{bA}	3.39 ± 0.85 ^{bB}	2.54 ± 0.63 ^{aAB}	3.19 ± 0.59 ^B
Low	2.21 ± 0.2 ^A	2.64 ± 0.39 ^A	1.52 ± 0.44 ^{cB}	2.16 ± 0.38 ^{cA}	1.93 ± 0.42 ^{abAB}	2.35 ± 0.6 ^A
Medium	2.78 ± 0.83 ^B	3.74 ± 1.39 ^B	3.15 ± 0.71 ^{Abc}	4.8 ± 0.74 ^{aA}	2.3 ± 0.56 ^{aD}	2.35 ± 0.59 ^{CD}
High	2.41 ± 0.87 ^{AB}	3.21 ± 0.95 ^{AB}	2.31 ± 0.48 ^{abB}	3.53 ± 0.7 ^{abA}	1.43 ± 0.37 ^{bC}	2.34 ± 0.43 ^B

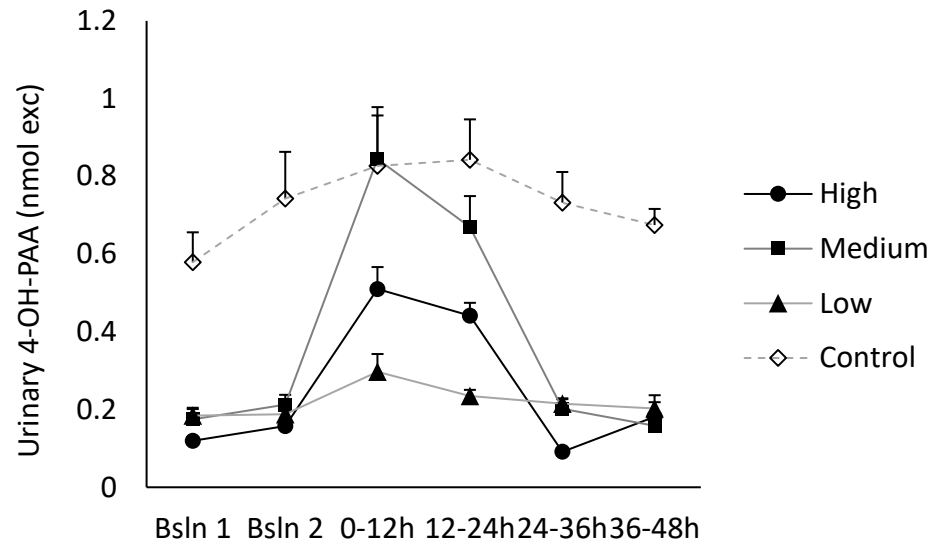


Figure A. 4 – Urinary excretion of 4-hydroxyphenyl acetic acid over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	0.58 ± 0.22 ^a	0.74 ± 0.34 ^a	0.83 ± 0.37 ^{ab}	0.84 ± 0.29 ^a	0.73 ± 0.22 ^a	0.67 ± 0.12 ^a
Low	0.18 ± 0.05 ^b	0.19 ± 0.04 ^b	0.3 ± 0.12 ^c	0.23 ± 0.04 ^c	0.21 ± 0.04 ^b	0.2 ± 0.05 ^b
Medium	0.17 ± 0.08 ^{bB}	0.21 ± 0.07 ^{bB}	0.84 ± 0.38 ^{aA}	0.67 ± 0.23 ^{abA}	0.2 ± 0.07 ^{bB}	0.16 ± 0.08 ^{bB}
High	0.12 ± 0.03 ^{bBC}	0.16 ± 0.05 ^{bBC}	0.51 ± 0.16 ^{bA}	0.44 ± 0.09 ^{bA}	0.09 ± 0.02 ^{cC}	0.18 ± 0.15 ^{bB}

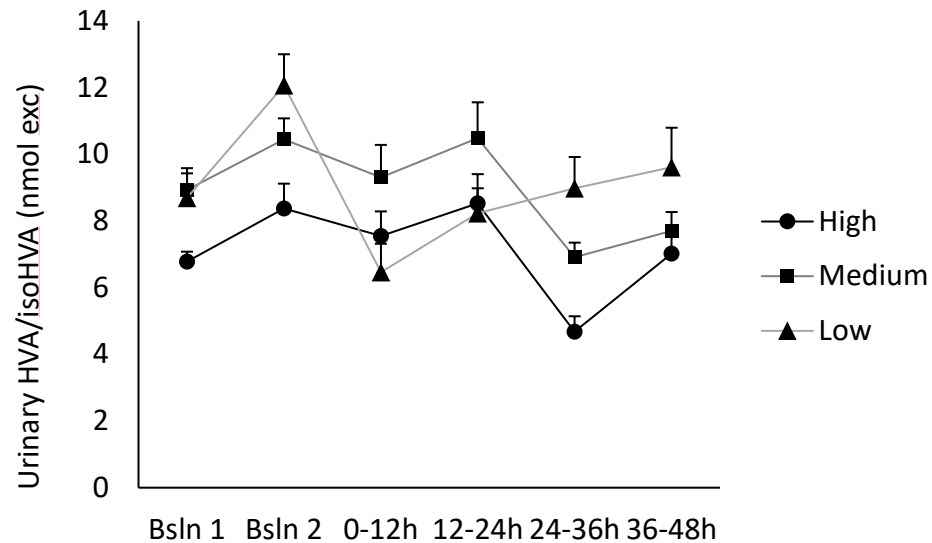


Figure A. 5 – Urinary excretion of (iso)homovanillic acid over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	8.69 ± 2.07 ^{aA}	12.07 ± 2.64 ^{aA}	6.46 ± 2.25 ^{bB}	8.23 ± 2.12 ^{aAB}	8.98 ± 2.66 ^{AB}	9.61 ± 3.34 ^A
Medium	8.93 ± 1.83 ^{Aab}	10.45 ± 1.66 ^{abA}	9.31 ± 2.75 ^{aAB}	10.48 ± 3.04 ^{aA}	6.91 ± 1.24 ^B	7.7 ± 1.61 ^{AB}
High	6.78 ± 0.84 ^{bA}	8.37 ± 1.97 ^{bA}	7.55 ± 2.08 ^{abA}	8.53 ± 2.48 ^{bA}	4.68 ± 1.31 ^B	7.02 ± 1.77 ^A

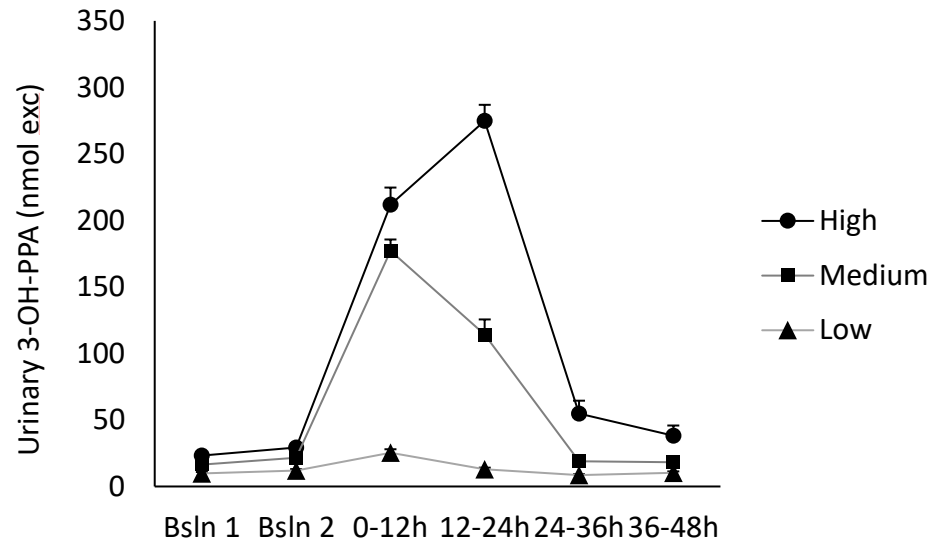


Figure A. 6 – Urinary excretion of 3-hydroxyphenyl propionic acid over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	9.69 ± 3.33 ^{cB}	12.08 ± 2.85 ^{cB}	25.37 ± 6.93 ^{bA}	12.91 ± 3.53 ^{cB}	8.54 ± 1.81 ^{cB}	10.4 ± 2.64 ^{cB}
Medium	16.45 ± 5.88 ^{bC}	21.53 ± 6.99 ^{bC}	177.12 ± 24.15 ^{aA}	114.11 ± 32.43 ^{bB}	18.96 ± 5.23 ^{bC}	18.17 ± 5.44 ^{bC}
High	23.24 ± 7.67 ^{aC}	29.18 ± 9.16 ^{aC}	211.81 ± 36.43 ^{aA}	274.82 ± 34.15 ^{aA}	54.68 ± 27.76 ^{aB}	38.07 ± 20.5 ^{aBC}

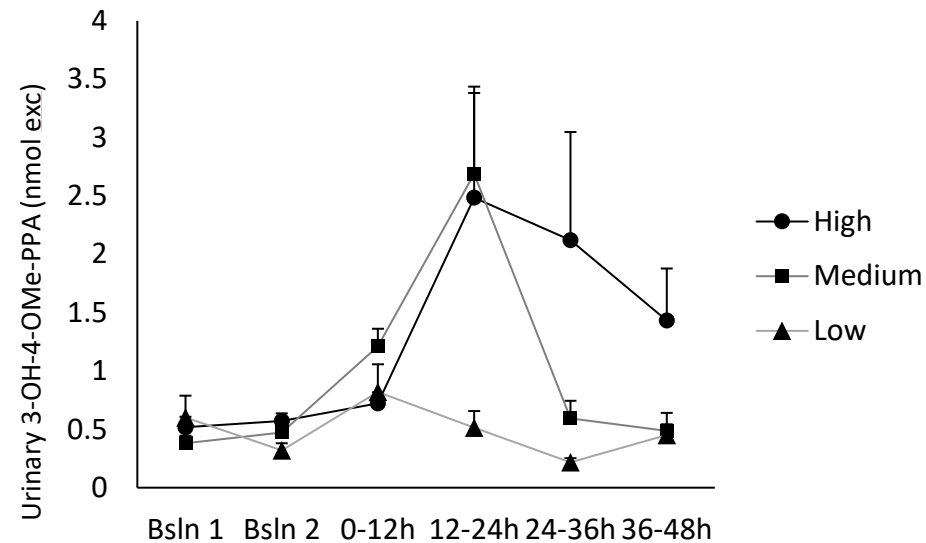


Figure A. 7 – Urinary excretion of 3-hydroxy-4-methoxyphenyl propionic acid over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.6 ± 0.42^{AB}	0.32 ± 0.17^{AB}	0.82 ± 0.58^A	0.51 ± 0.38^{bAB}	0.22 ± 0.09^b	0.45 ± 0.46^b
Medium	0.38 ± 0.18^B	0.47 ± 0.22^B	1.21 ± 0.43^A	2.69 ± 1.83^{aA}	0.59 ± 0.42^{bB}	0.49 ± 0.16^{bB}
High	0.52 ± 0.25^B	0.57 ± 0.19^B	0.72 ± 0.27^{AB}	2.48 ± 2.69^{aA}	2.12 ± 2.45^{aAB}	1.43 ± 1.09^{aAB}

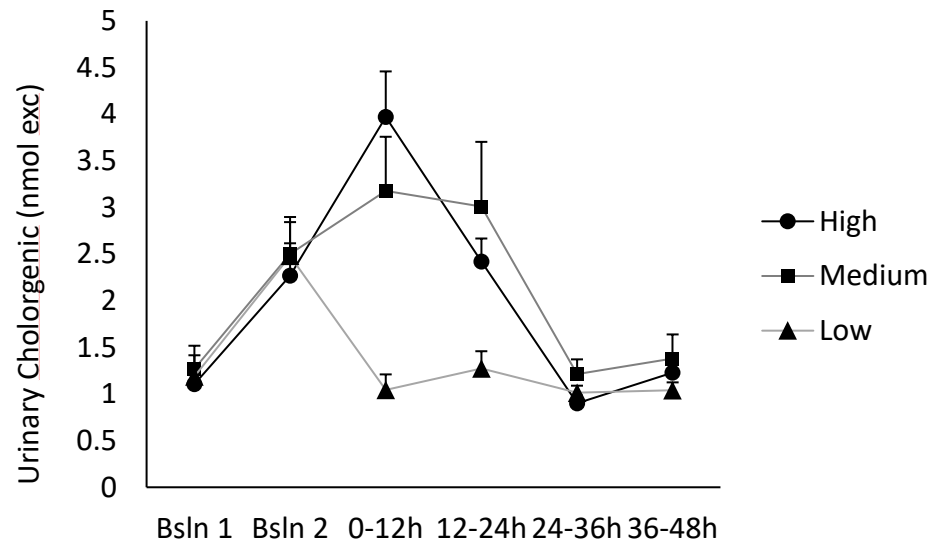


Figure A. 8 – Urinary excretion of chlorogenic acid over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	1.19 ± 0.64 ^B	2.48 ± 1.03 ^A	1.04 ± 0.44 ^{bB}	1.27 ± 0.52 ^{bB}	1.01 ± 0.21 ^B	1.04 ± 0.24 ^B
Medium	1.27 ± 0.7 ^B	2.5 ± 1.12 ^A	3.18 ± 1.64 ^{aA}	3.01 ± 1.97 ^{aA}	1.21 ± 0.45 ^B	1.38 ± 0.73 ^B
High	1.11 ± 0.27 ^C	2.27 ± 0.98 ^B	3.97 ± 1.38 ^{aA}	2.42 ± 0.7 ^{aAB}	0.9 ± 0.24 ^D	1.23 ± 0.34 ^{CD}

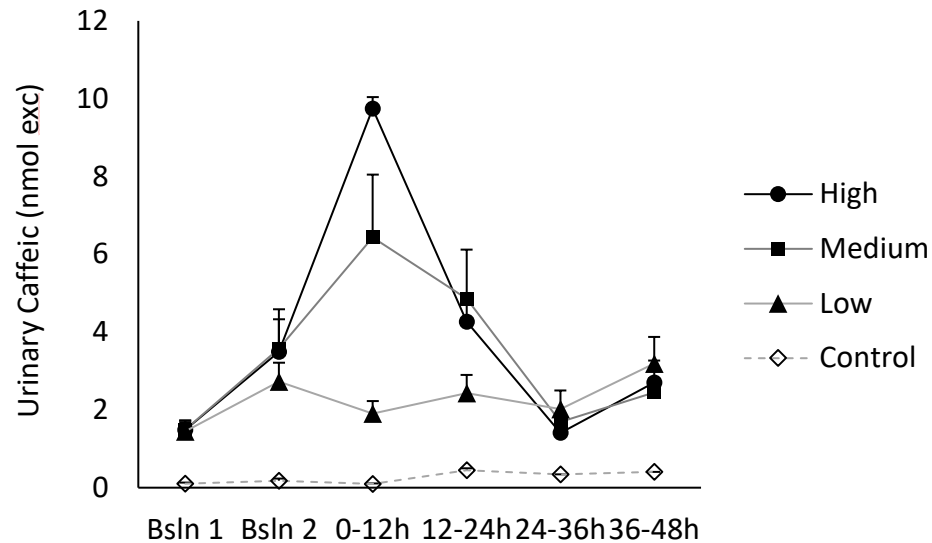


Figure A. 9 – Urinary excretion of caffeic acid over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	0.11 ± 0.06 ^b	0.18 ± 0.11 ^b	0.1 ± 0.02 ^c	0.45 ± 0.07 ^b	0.34 ± 0.06 ^b	0.41 ± 0.07 ^b
Low	1.45 ± 0.78 ^a	2.73 ± 1.37 ^a	1.9 ± 0.85 ^b	2.43 ± 1.32 ^a	2.02 ± 1.35 ^a	3.19 ± 1.94 ^a
Medium	1.49 ± 0.67 ^{aC}	3.57 ± 2.13 ^{aBC}	6.43 ± 4.57 ^{aA}	4.86 ± 3.57 ^{aAB}	1.69 ± 0.59 ^{aC}	2.44 ± 1.05 ^{aBC}
High	1.48 ± 0.55 ^{aC}	3.49 ± 3.09 ^{aBC}	9.74 ± 0.78 ^{aA}	4.27 ± 1.66 ^{aB}	1.41 ± 0.88 ^{aC}	2.7 ± 1.49 ^{aBC}

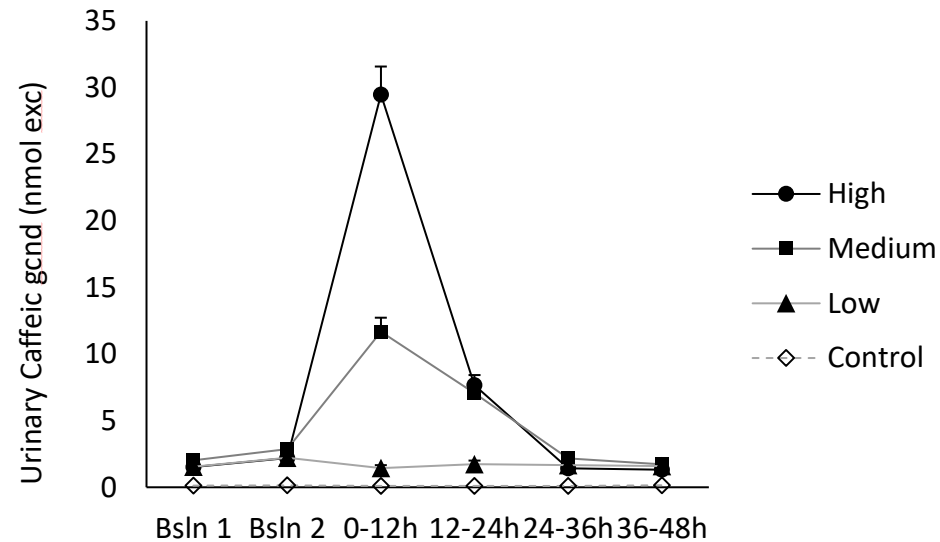


Figure A. 10 – Urinary excretion of caffeic acid glucuronide over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	0.12 ± 0.03 ^b	0.14 ± 0.04 ^b	0.1 ± 0.03 ^d	0.11 ± 0.05 ^c	0.1 ± 0.03 ^b	0.14 ± 0.05 ^b
Low	1.55 ± 0.42 ^a	2.22 ± 0.95 ^a	1.44 ± 0.59 ^c	1.73 ± 0.78 ^b	1.67 ± 0.6 ^a	1.59 ± 0.76 ^a
Medium	2.03 ± 0.27 ^{aC}	2.86 ± 1.29 ^{aC}	11.67 ± 2.98 ^{bA}	7.07 ± 1.73 ^{aB}	2.17 ± 0.7 ^{aC}	1.72 ± 0.32 ^{aC}
High	1.52 ± 0.49 ^{aC}	2.19 ± 0.59 ^{aC}	29.48 ± 5.93 ^{aA}	7.67 ± 2.13 ^{aB}	1.42 ± 0.48 ^{aC}	1.32 ± 0.63 ^{aC}

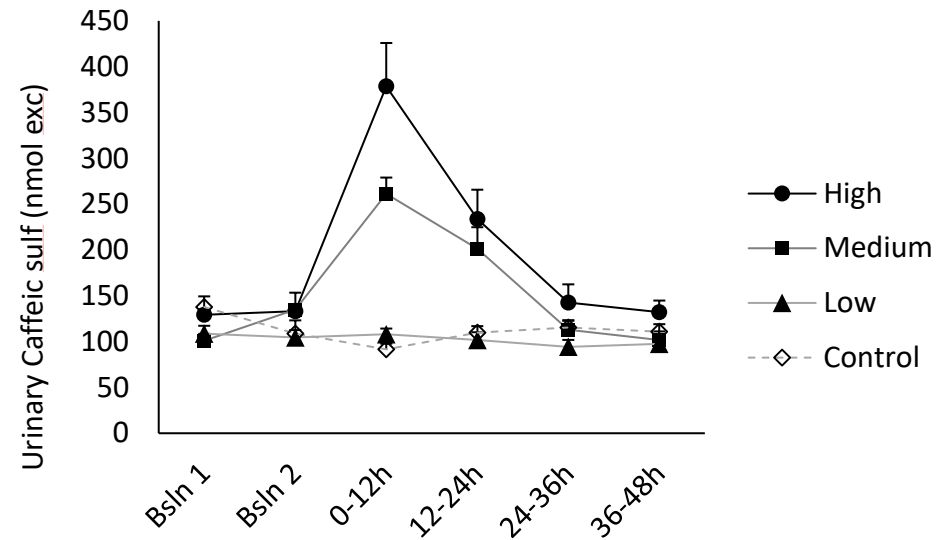


Figure A. 11 – Urinary excretion of caffeic acid sulfate over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	137.8 ± 32.9	108.9 ± 40.4	91.6 ± 17.5	109.9 ± 19.8	115.5 ± 22.2 ^{ab}	110.8 ± 23.2
Low	108.7 ± 24.3	104.4 ± 23.1	108 ± 16.6	101.8 ± 23.8	94.3 ± 21.5 ^b	97.6 ± 16.9
Medium	101 ± 21.2 ^B	134.8 ± 52.6 ^B	261.3 ± 50.1 ^{aA}	201.2 ± 66.8 ^{aA}	113 ± 27.6 ^{abB}	101.7 ± 26.7 ^B
High	129.2 ± 17.3 ^C	133.1 ± 18.9 ^C	378.5 ± 133.9 ^{aA}	233.7 ± 90.6 ^{aB}	142.6 ± 56.2 ^{aC}	132.2 ± 33.2 ^C

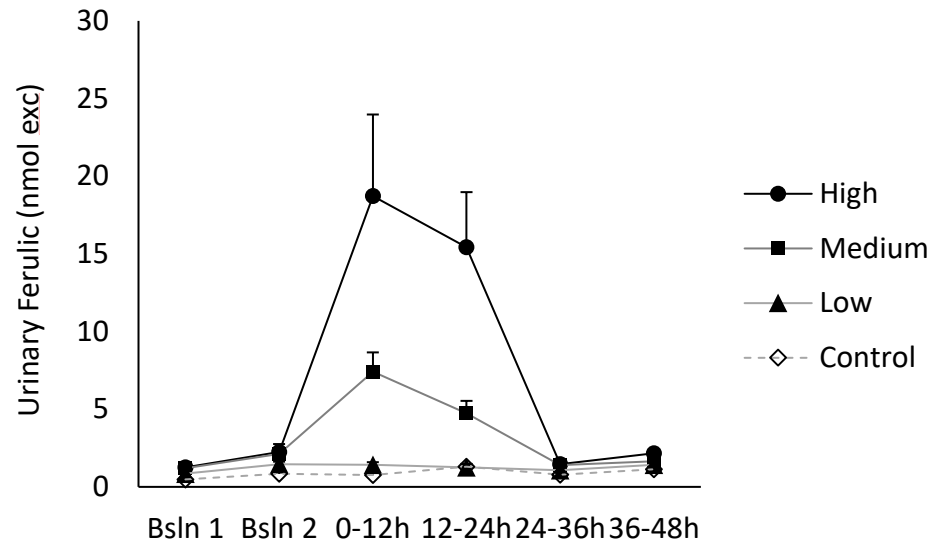


Figure A. 12 – Urinary excretion of ferulic acid over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	0.48 ± 0.18 ^{bB}	0.85 ± 0.27 ^{bAB}	0.77 ± 0.43 ^{dAB}	1.3 ± 0.42 ^{cA}	0.79 ± 0.14 ^{AB}	1.14 ± 0.39 ^A
Low	0.86 ± 0.42 ^a	1.46 ± 0.46 ^a	1.43 ± 0.43 ^c	1.25 ± 0.65 ^c	1.07 ± 0.59	1.42 ± 0.74
Medium	1.2 ± 0.42 ^{aB}	2.13 ± 1.07 ^{aB}	7.41 ± 3.52 ^{aA}	4.75 ± 2.24 ^{bA}	1.41 ± 0.33 ^B	1.64 ± 0.56 ^B
High	1.26 ± 0.47 ^{aB}	2.24 ± 1.46 ^{aB}	18.72 ± 14.86 ^{aA}	15.43 ± 10.03 ^{aA}	1.47 ± 0.62 ^B	2.16 ± 0.6 ^B

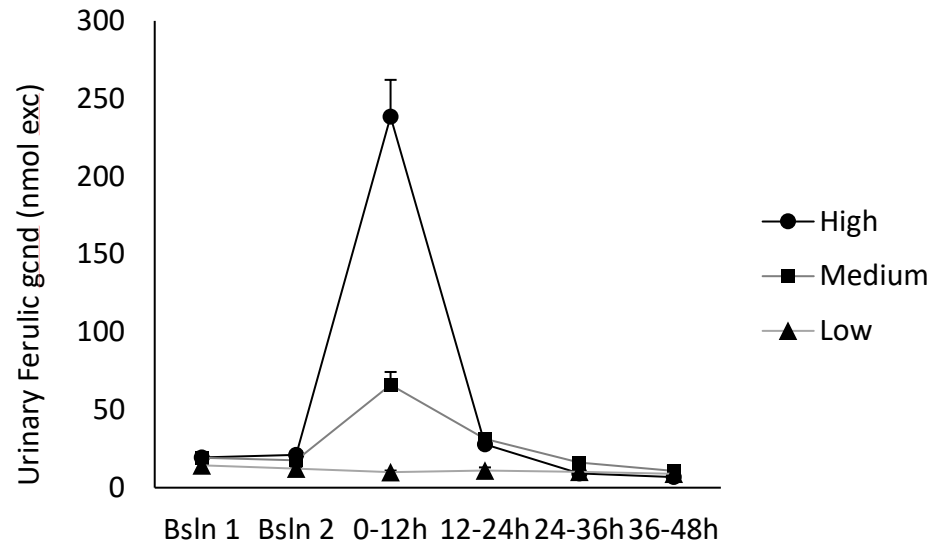


Figure A. 13 – Urinary excretion of ferulic acid glucuronide over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	14.4 ± 5.7	12.3 ± 2.5	9.9 ± 2.9 ^c	11.1 ± 5.5 ^b	10.2 ± 3.3 ^{ab}	8.9 ± 2.7 ^a
Medium	19.2 ± 4.2 ^C	17.6 ± 4.4 ^C	66.1 ± 23.4 ^{bA}	31.3 ± 10.5 ^{aB}	16.2 ± 6.5 ^{aCD}	10.8 ± 3 ^{aD}
High	19.5 ± 2.4 ^B	21.2 ± 6.2 ^B	238.4 ± 66.9 ^{aA}	27.8 ± 6.6 ^{aB}	9.2 ± 4 ^{bC}	6.8 ± 4.1 ^{bC}

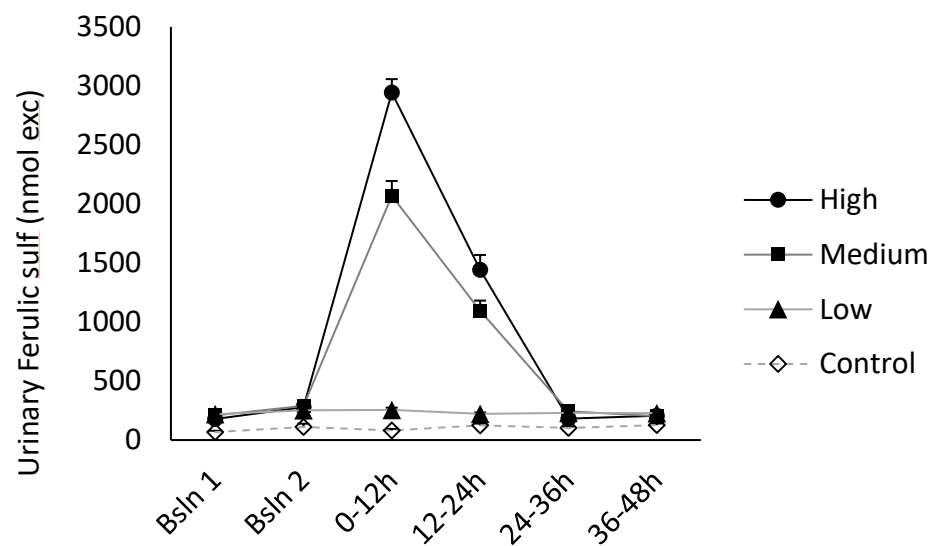


Figure A. 14 – Urinary excretion of ferulic acid sulfate over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	67.6 ± 31.7 ^{bB}	110.8 ± 78.2 ^{bAB}	80.9 ± 37.8 ^{cB}	125 ± 41.1 ^{cA}	102.5 ± 33.7 ^{bAB}	126.4 ± 39 ^{bA}
Low	219.1 ± 31.5 ^a	250.8 ± 52.9 ^a	255.2 ± 50.7 ^b	221.3 ± 34.2 ^b	230.9 ± 53.3 ^a	228.3 ± 49 ^a
Medium	209.8 ± 59.3 ^{aC}	289.7 ± 105.6 ^{aC}	2069.4 ± 353.8 ^{aA}	1095.8 ± 242.1 ^{aB}	243.4 ± 55.7 ^{aC}	202.3 ± 30.7 ^{aC}
High	177.9 ± 23.6 ^{aC}	278.7 ± 82.7 ^{aC}	2943.2 ± 301.9 ^{aA}	1440.6 ± 359.1 ^{aB}	182.3 ± 60.4 ^{aC}	205.7 ± 41.3 ^{aC}

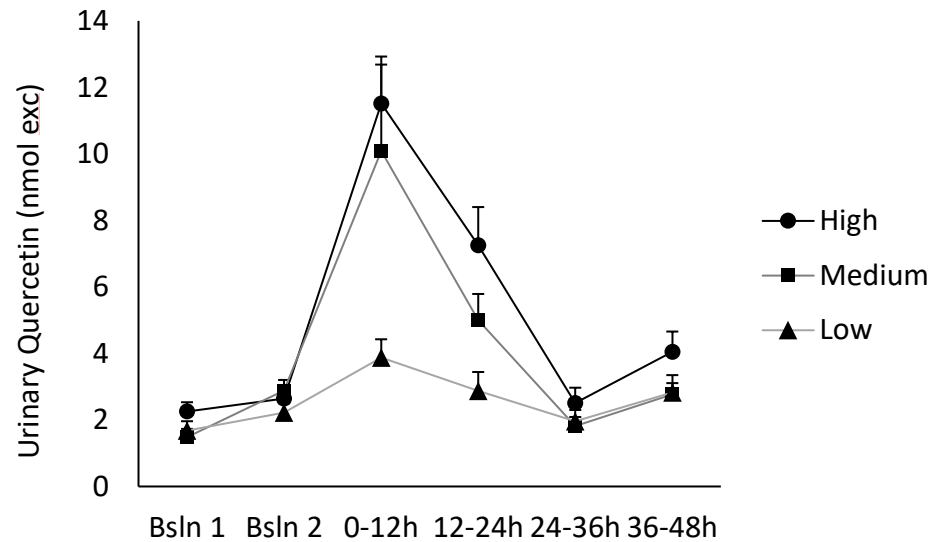


Figure A. 15 – Urinary excretion of quercetin over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	1.68 ± 0.8 ^B	2.22 ± 0.74 ^B	3.88 ± 1.46 ^{bA}	2.88 ± 1.59 ^{bAB}	1.96 ± 0.97 ^B	2.83 ± 1.47 ^{AB}
Medium	1.48 ± 0.7 ^C	2.89 ± 0.84 ^C	10.09 ± 7.35 ^{aA}	5 ± 2.22 ^{abAB}	1.82 ± 0.77 ^C	2.77 ± 0.97 ^{BC}
High	2.25 ± 0.79 ^C	2.64 ± 1.01 ^C	11.52 ± 3.99 ^{aA}	7.25 ± 3.26 ^{aAB}	2.5 ± 1.32 ^C	4.05 ± 1.61 ^{BC}

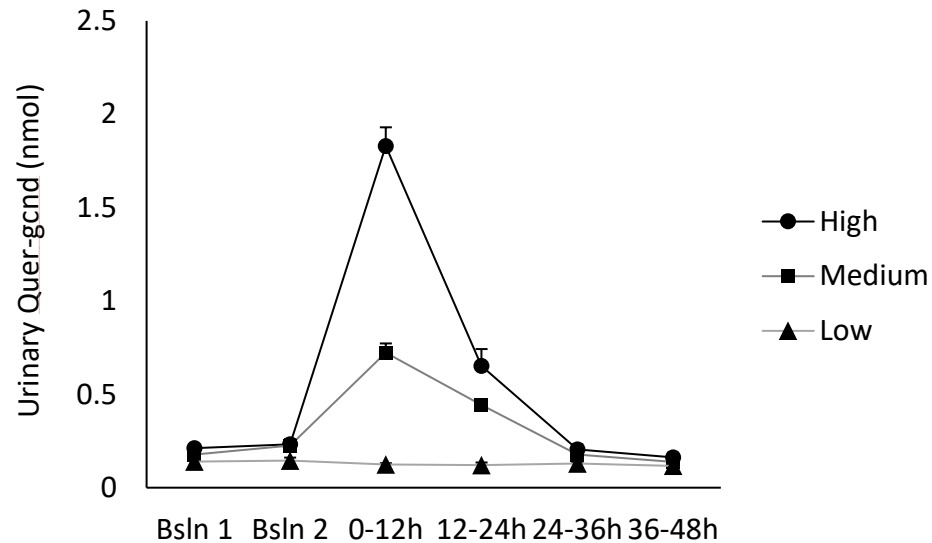


Figure A. 16 – Urinary excretion of quercetin-glucuronide over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.14 ± 0.02 ^b	0.15 ± 0.05 ^b	0.12 ± 0.02 ^c	0.12 ± 0.04 ^c	0.13 ± 0.02 ^b	0.12 ± 0.03
Medium	0.18 ± 0.03 ^{aCD}	0.23 ± 0.1 ^{aC}	0.72 ± 0.14 ^{bA}	0.44 ± 0.06 ^{bB}	0.18 ± 0.04 ^{aCD}	0.14 ± 0.03 ^D
High	0.21 ± 0.06 ^{aCD}	0.23 ± 0.06 ^{aC}	1.83 ± 0.28 ^{aA}	0.65 ± 0.25 ^{aB}	0.21 ± 0.08 ^{aCD}	0.16 ± 0.07 ^D

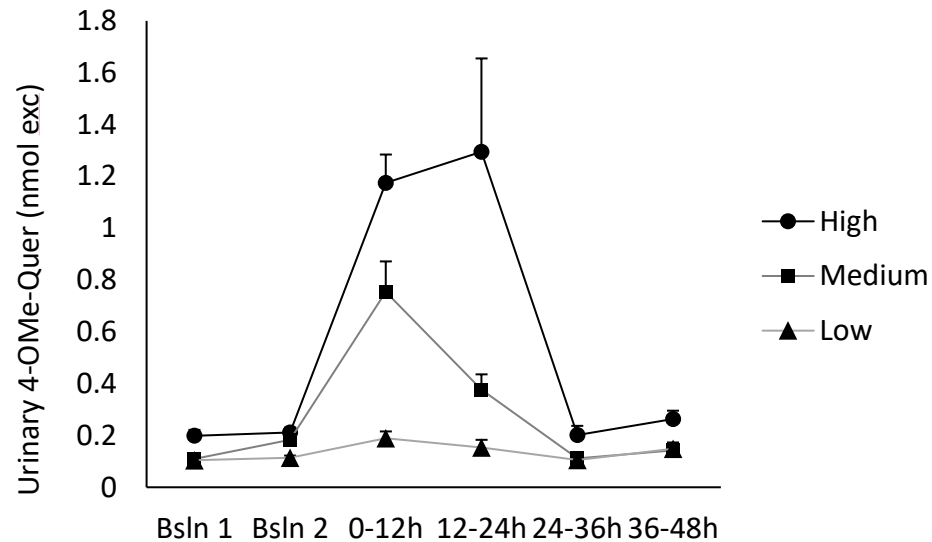


Figure A. 17 – Urinary excretion of 4-methoxy quercetin over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.1 ± 0.05 ^{bB}	0.11 ± 0.02 ^{bB}	0.19 ± 0.08 ^{bA}	0.15 ± 0.08 ^{cAB}	0.11 ± 0.05 ^{bB}	0.15 ± 0.07 ^{bAB}
Medium	0.11 ± 0.04 ^{bC}	0.18 ± 0.04 ^{aC}	0.75 ± 0.33 ^{aA}	0.38 ± 0.17 ^{bB}	0.11 ± 0.04 ^{abC}	0.14 ± 0.05 ^{bC}
High	0.2 ± 0.06 ^{aB}	0.21 ± 0.06 ^{aB}	1.17 ± 0.31 ^{aA}	1.29 ± 1.02 ^{aA}	0.2 ± 0.11 ^{aB}	0.26 ± 0.1 ^{aB}

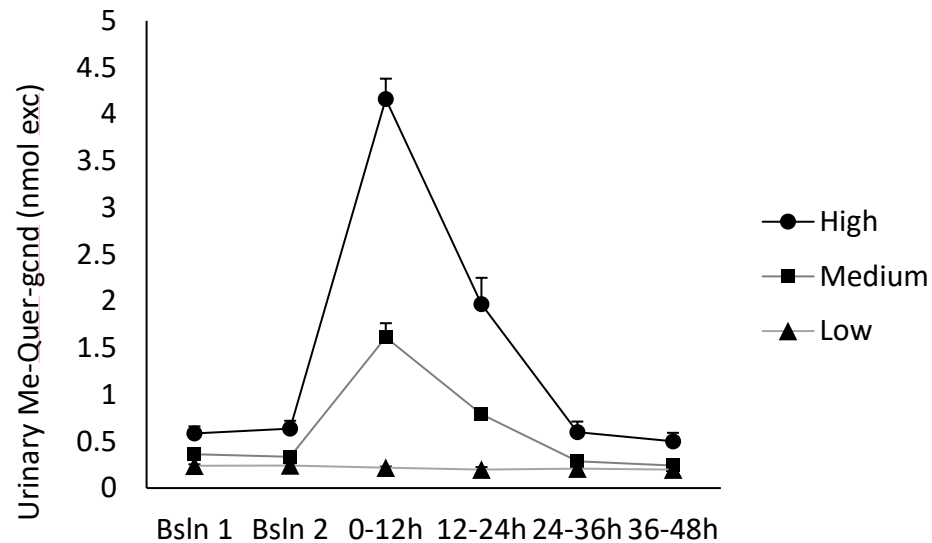


Figure A. 18 – Urinary excretion of methyl-quercetin-glucuronide over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.24 ± 0.04 ^b	0.24 ± 0.07 ^b	0.22 ± 0.04 ^c	0.2 ± 0.08 ^c	0.21 ± 0.05 ^b	0.2 ± 0.09 ^b
Medium	0.36 ± 0.16 ^{bC}	0.34 ± 0.1 ^{bC}	1.61 ± 0.43 ^{bA}	0.79 ± 0.18 ^{bB}	0.29 ± 0.09 ^{bC}	0.24 ± 0.09 ^{bC}
High	0.59 ± 0.21 ^{aC}	0.64 ± 0.24 ^{aC}	4.16 ± 0.58 ^{aA}	1.97 ± 0.75 ^{aB}	0.6 ± 0.3 ^{aC}	0.5 ± 0.24 ^{aC}

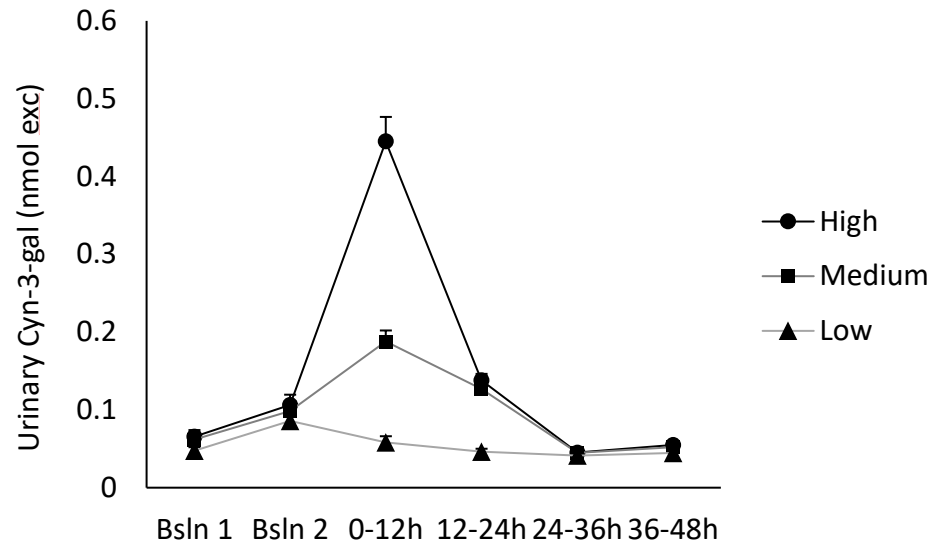


Figure A. 19 – Urinary excretion of cyanidin-3-galactoside over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.0473 ± 0.0133 ^A	0.0858 ± 0.022 ^A	0.0583 ± 0.0206 ^{cAB}	0.0463 ± 0.0108 ^{bAB}	0.0413 ± 0.0134 ^B	0.0446 ± 0.0159 ^B
Medium	0.062 ± 0.034 ^C	0.0984 ± 0.0373 ^C	0.1879 ± 0.0399 ^{bA}	0.1267 ± 0.0489 ^{aB}	0.0448 ± 0.0145 ^D	0.0521 ± 0.0175 ^D
High	0.0656 ± 0.0163 ^C	0.1065 ± 0.0369 ^C	0.445 ± 0.0891 ^{aA}	0.138 ± 0.0234 ^{aB}	0.0452 ± 0.0085 ^D	0.0548 ± 0.0057 ^D

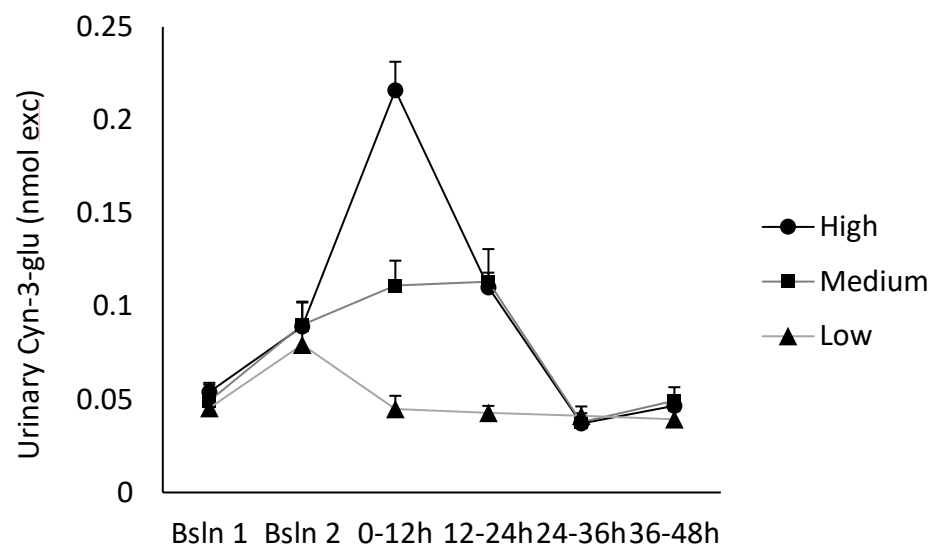


Figure A. 20 – Urinary excretion of cyanidin-3-glucoside over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.0453 ± 0.0151 ^B	0.0794 ± 0.0239 ^A	0.0448 ± 0.0188 ^{cB}	0.0427 ± 0.0107 ^{bB}	0.0411 ± 0.0143 ^B	0.0393 ± 0.013 ^B
Medium	0.0492 ± 0.0247 ^{BC}	0.0901 ± 0.0337 ^B	0.1111 ± 0.0379 ^{bA}	0.1132 ± 0.0495 ^{aA}	0.0378 ± 0.0136 ^D	0.0493 ± 0.0205 ^{CD}
High	0.0539 ± 0.0142 ^{CD}	0.0892 ± 0.0375 ^C	0.216 ± 0.0433 ^{aA}	0.11 ± 0.0226 ^{aB}	0.037 ± 0.0078 ^E	0.0466 ± 0.0089 ^{DE}

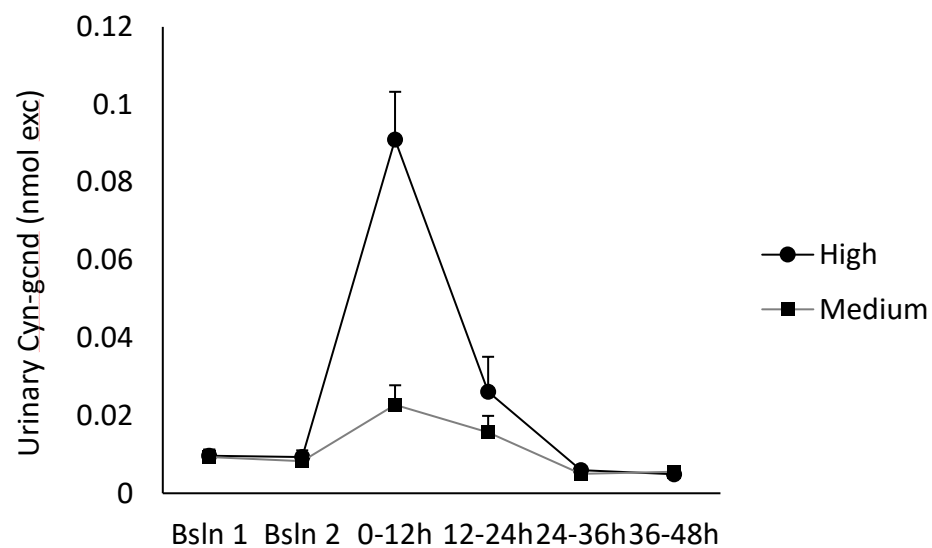


Figure A. 21 – Urinary excretion of cyanidin-glucuronide over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	nd	nd	nd	nd	nd	nd
Medium	0.0093 ± 0.0045 ^{BC}	0.0083 ± 0.0027 ^{BC}	0.0228 ± 0.0142 ^b	0.0157 ± 0.012 ^B	0.005 ± 0.0016 ^C	0.0055 ± 0.0003 ^C
High	0.0097 ± 0.0045 ^{BC}	0.0093 ± 0.0049 ^{BC}	0.091 ± 0.0348 ^a	0.0262 ± 0.0253 ^B	0.006 ± 0.0019 ^C	0.0049 ± 0.0014 ^C

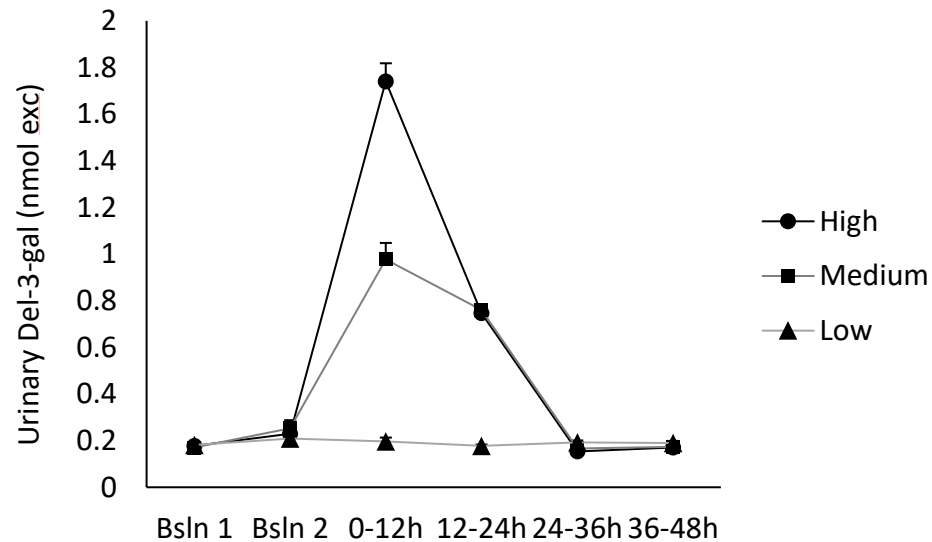


Figure A. 22 – Urinary excretion of delphinidin-3-galactoside over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.1821 ± 0.0096	0.2089 ± 0.0251	0.1968 ± 0.043 ^c	0.1777 ± 0.0163 ^b	0.1924 ± 0.0222	0.1904 ± 0.0226
Medium	0.1705 ± 0.0297 ^B	0.2522 ± 0.1005 ^B	0.9769 ± 0.2007 ^{bA}	0.7607 ± 0.0642 ^{aA}	0.1664 ± 0.0359 ^B	0.173 ± 0.0269 ^B
High	0.178 ± 0.0256 ^C	0.2291 ± 0.0856 ^C	1.7408 ± 0.2185 ^{aA}	0.7474 ± 0.0909 ^{aB}	0.1536 ± 0.0192 ^C	0.1701 ± 0.0184 ^C

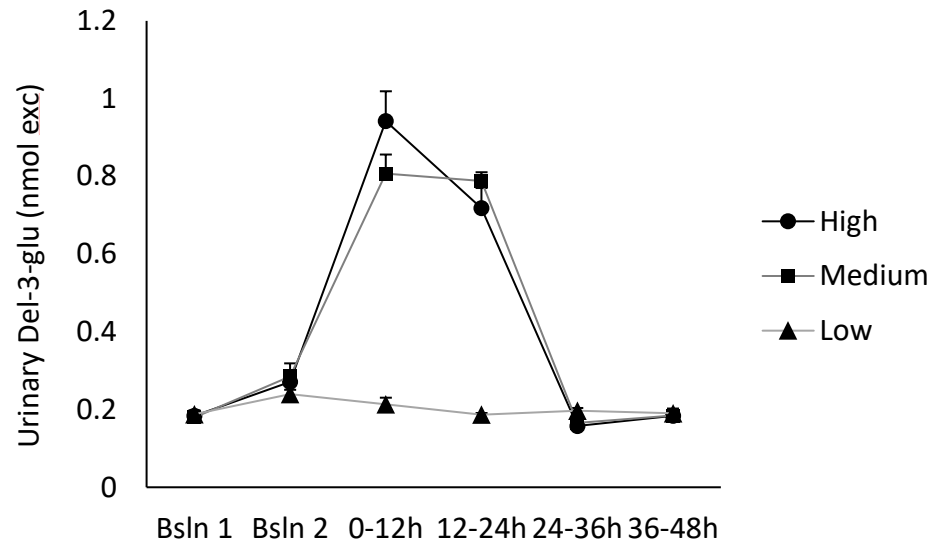


Figure A. 23 – Urinary excretion of delphinidin-3-glucoside over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.1883 ± 0.0202	0.2397 ± 0.0316	0.2137 ± 0.0445 ^b	0.1867 ± 0.0124 ^b	0.197 ± 0.0199 ^a	0.1906 ± 0.0185
Medium	0.1795 ± 0.049 ^{BC}	0.2849 ± 0.096 ^B	0.807 ± 0.1387 ^{aA}	0.7878 ± 0.0644 ^{aA}	0.1656 ± 0.0317 ^{bC}	0.1844 ± 0.0514 ^{BC}
High	0.184 ± 0.0255 ^{CD}	0.271 ± 0.0838 ^C	0.9421 ± 0.217 ^{aA}	0.7179 ± 0.1472 ^{aB}	0.1578 ± 0.0188 ^{bD}	0.1839 ± 0.0166 ^{CD}

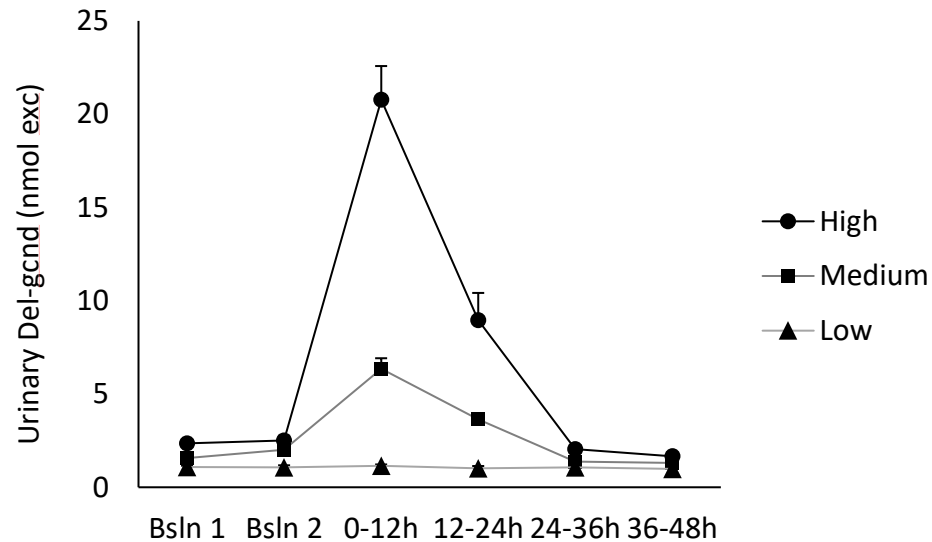


Figure A. 24 – Urinary excretion of delphinidin glucuronide over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	1.08 ± 0.22 ^b	1.07 ± 0.31 ^b	1.15 ± 0.19 ^c	1.01 ± 0.37 ^c	1.06 ± 0.29 ^b	0.99 ± 0.43 ^b
Medium	1.57 ± 0.39 ^{bC}	2.01 ± 0.80 ^{aC}	6.35 ± 1.60 ^{bA}	3.63 ± 0.59 ^{bB}	1.37 ± 0.41 ^{bC}	1.31 ± 0.39 ^{abC}
High	2.36 ± 0.56 ^{aC}	2.50 ± 0.75 ^{aC}	20.77 ± 5.12 ^{aA}	8.96 ± 4.11 ^{aB}	2.05 ± 0.54 ^{aCD}	1.66 ± 0.55 ^{aD}

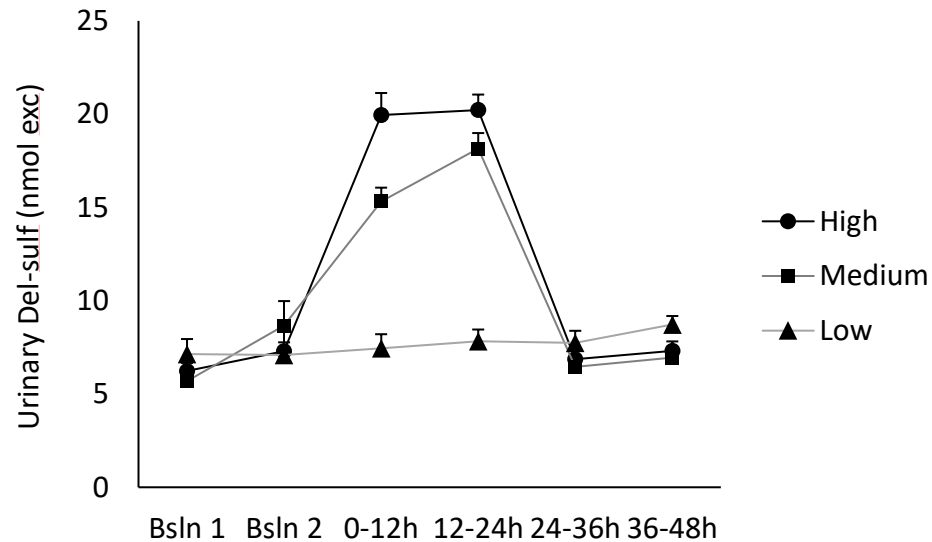


Figure A. 25 – Urinary excretion of delphinidin-sulfate over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	7.14 ± 2.28	7.09 ± 1.92	7.45 ± 1.99 ^c	7.83 ± 1.78 ^b	7.75 ± 1.81	8.72 ± 1.29
Medium	5.69 ± 0.89 ^B	8.67 ± 3.69 ^B	15.32 ± 2.11 ^{bA}	18.16 ± 2.35 ^{aA}	6.45 ± 1.2 ^B	6.95 ± 1.65 ^B
High	6.24 ± 1.22 ^B	7.29 ± 3.03 ^B	19.95 ± 3.35 ^{aA}	20.22 ± 2.35 ^{aA}	6.87 ± 1.34 ^B	7.31 ± 1.35 ^B

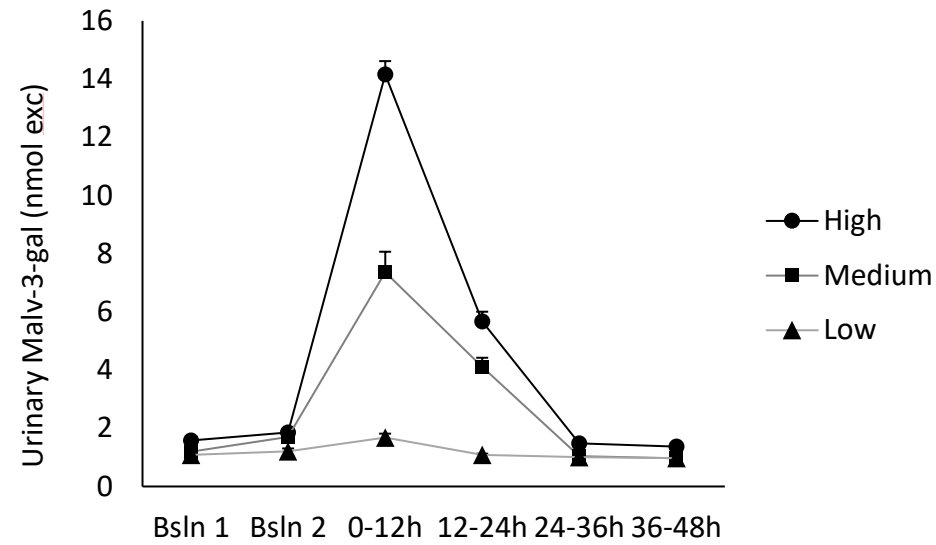


Figure A. 26 – Urinary excretion of malvidin-3-galactoside over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	1.09 ± 0.12 ^{bB}	1.21 ± 0.26 ^{bB}	1.68 ± 0.33 ^{cA}	1.09 ± 0.11 ^{cB}	1.01 ± 0.13 ^{bB}	0.97 ± 0.19 ^{bB}
Medium	1.19 ± 0.27 ^{bC}	1.70 ± 0.50 ^{aC}	7.36 ± 2.00 ^{bA}	4.11 ± 0.82 ^{bB}	1.04 ± 0.31 ^{bC}	0.97 ± 0.17 ^{bC}
High	1.58 ± 0.20 ^{aC}	1.86 ± 0.39 ^{aC}	14.16 ± 1.30 ^{aA}	5.67 ± 0.96 ^{aB}	1.48 ± 0.18 ^{aC}	1.37 ± 0.15 ^{aC}

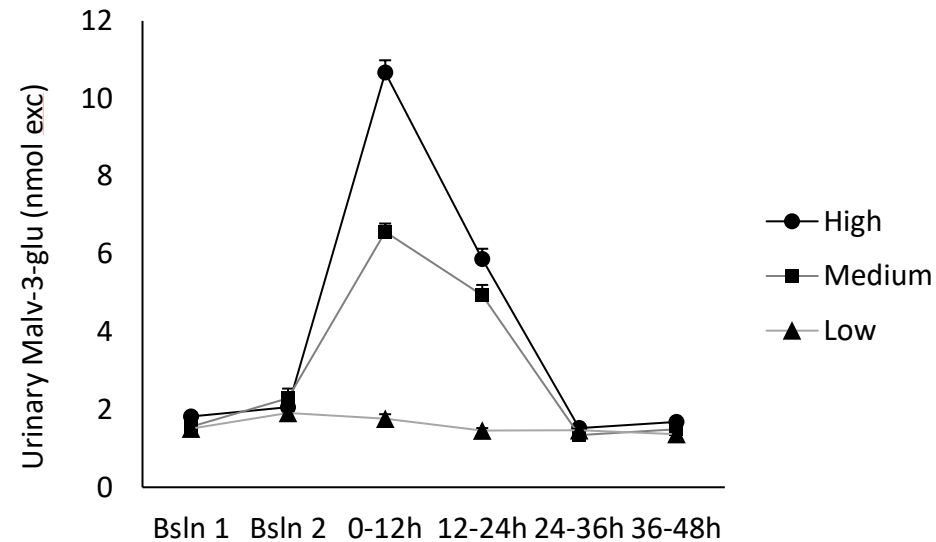


Figure A. 27 – Urinary excretion of malvidin-3-glucoside over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	1.51 ± 0.14	1.91 ± 0.25	1.76 ± 0.31 ^C	1.46 ± 0.17 ^b	1.47 ± 0.25	1.37 ± 0.25
Medium	1.56 ± 0.34 ^{CD}	2.29 ± 0.7 ^C	6.57 ± 0.57 ^{bA}	4.94 ± 0.75 ^{aB}	1.34 ± 0.27 ^D	1.49 ± 0.3 ^D
High	1.82 ± 0.21 ^C	2.06 ± 0.12 ^C	10.67 ± 0.9 ^{aA}	5.87 ± 0.76 ^{aB}	1.52 ± 0.2 ^C	1.68 ± 0.11 ^C

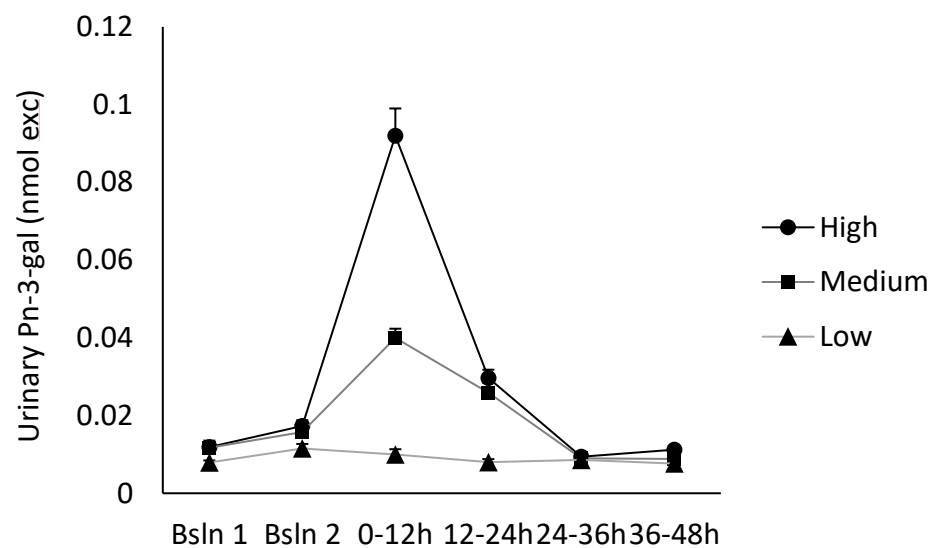


Figure A. 28 – Urinary excretion of peonidin-3-galactoside over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.0079 ± 0.0014 ^b	0.0115 ± 0.0033 ^b	0.01 ± 0.0036 ^c	0.008 ± 0.0022 ^b	0.0086 ± 0.0028	0.0076 ± 0.0026
Medium	0.0117 ± 0.0053 ^{aC}	0.0158 ± 0.0045 ^{aC}	0.04 ± 0.0067 ^{bA}	0.0259 ± 0.0071 ^{aB}	0.009 ± 0.0036 ^D	0.0088 ± 0.0031 ^D
High	0.0119 ± 0.0021 ^{aC}	0.0173 ± 0.0042 ^{aC}	0.092 ± 0.0199 ^{aA}	0.0297 ± 0.0061 ^{aB}	0.0094 ± 0.0028 ^C	0.0113 ± 0.0013 ^C

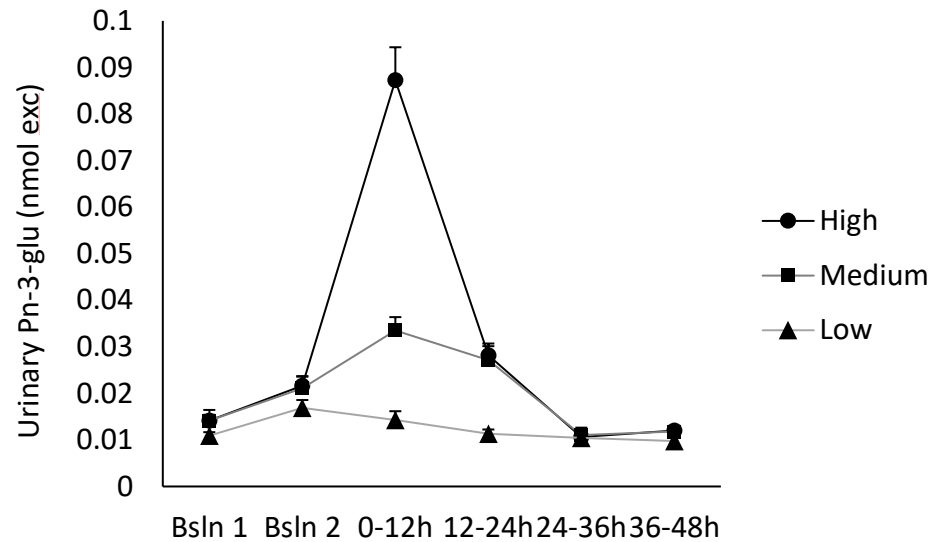


Figure A. 29 – Urinary excretion of peonidin-3-glucoside over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.0109 ± 0.0022	0.0169 ± 0.0047	0.0143 ± 0.005 ^c	0.0114 ± 0.0025 ^b	0.0105 ± 0.0035	0.0098 ± 0.0024
Medium	0.0142 ± 0.0064 ^{BC}	0.0211 ± 0.0074 ^B	0.0335 ± 0.0081 ^{bA}	0.0271 ± 0.0086 ^{aAB}	0.011 ± 0.0045 ^C	0.0118 ± 0.0023 ^C
High	0.0142 ± 0.0035 ^{CD}	0.0216 ± 0.0056 ^C	0.0872 ± 0.0201 ^{aA}	0.0282 ± 0.0071 ^{aB}	0.0106 ± 0.0023 ^D	0.012 ± 0.0028 ^D

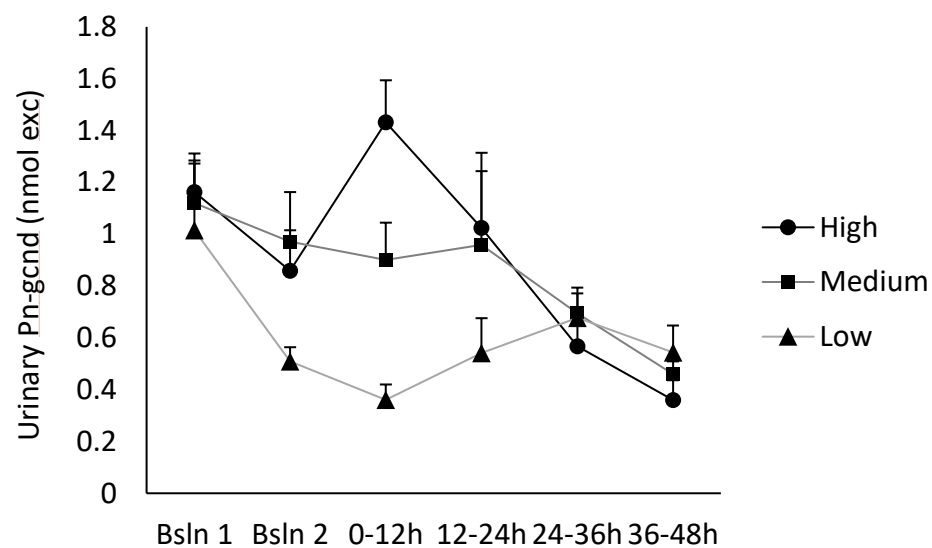


Figure A. 30 – Urinary excretion of peonidin glucuronide over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	1.01 ± 0.73^A	0.51 ± 0.14^B	0.36 ± 0.16^{bB}	0.54 ± 0.38^{bB}	0.68 ± 0.27^B	0.54 ± 0.29^B
Medium	1.12 ± 0.47^A	0.97 ± 0.54^A	0.9 ± 0.41^{aAB}	0.96 ± 0.81^{abAB}	0.69 ± 0.28^{AB}	0.46 ± 0.14^B
High	1.16 ± 0.42^{AB}	0.86 ± 0.44^B	1.43 ± 0.46^{aA}	1.02 ± 0.82^{aAB}	0.57 ± 0.24^{BC}	0.36 ± 0.2^C

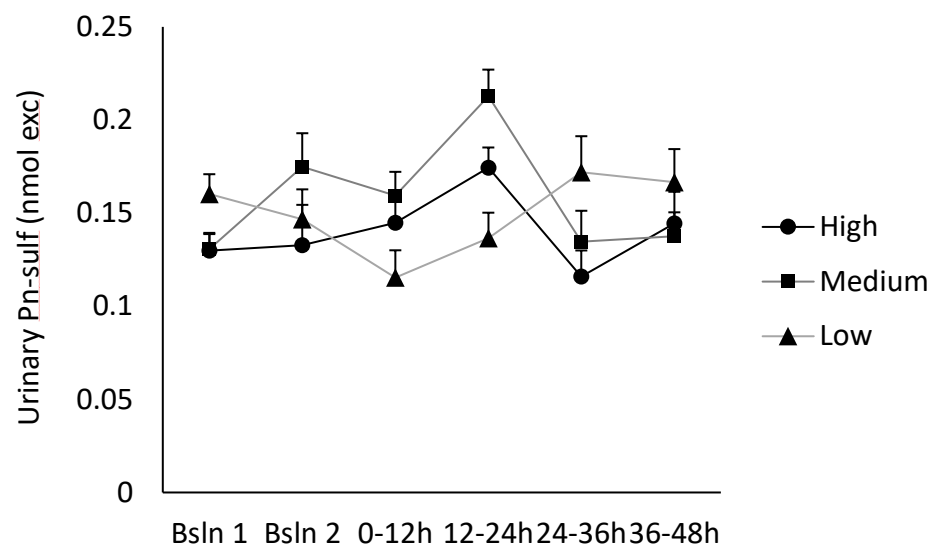


Figure A. 31 – Urinary excretion of peonidin sulfate over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.1603 ± 0.03 ^A	0.1468 ± 0.0452 ^{AB}	0.1154 ± 0.0388 ^B	0.1365 ± 0.0388 ^{AB}	0.1719 ± 0.0547 ^A	0.1667 ± 0.0501 ^A
Medium	0.1307 ± 0.0247	0.1748 ± 0.0511	0.1594 ± 0.0361	0.2128 ± 0.0404	0.1347 ± 0.0468	0.1376 ± 0.0362
High	0.1299 ± 0.0251 ^B	0.1328 ± 0.0611 ^B	0.1448 ± 0.0368 ^{AB}	0.1744 ± 0.0306 ^A	0.1159 ± 0.0396 ^B	0.1445 ± 0.0449 ^{AB}

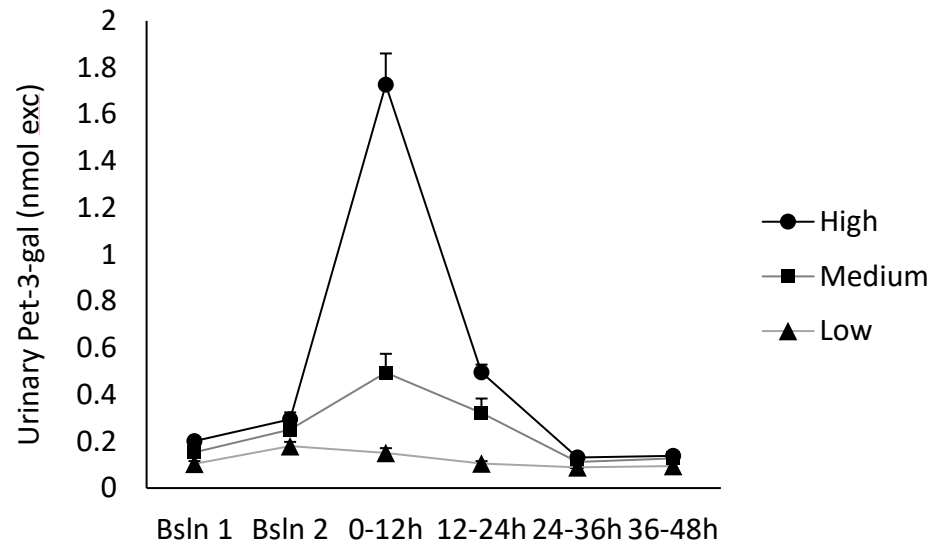


Figure A. 32 – Urinary excretion of petunidin-3-galactoside over 48h.

	Basln 1	Basln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.1 ± 0.03 ^{bB}	0.18 ± 0.05 ^{bA}	0.15 ± 0.05 ^{cA}	0.11 ± 0.03 ^{cAB}	0.09 ± 0.03 ^{bB}	0.09 ± 0.03 ^{AB}
Medium	0.15 ± 0.09 ^{abC}	0.25 ± 0.1 ^{abC}	0.49 ± 0.23 ^{bA}	0.32 ± 0.17 ^{bB}	0.11 ± 0.05 ^{abD}	0.13 ± 0.04 ^D
High	0.2 ± 0.04 ^{aC}	0.29 ± 0.09 ^{aC}	1.73 ± 0.38 ^{aA}	0.5 ± 0.09 ^{aB}	0.13 ± 0.02 ^{aD}	0.14 ± 0.02 ^D

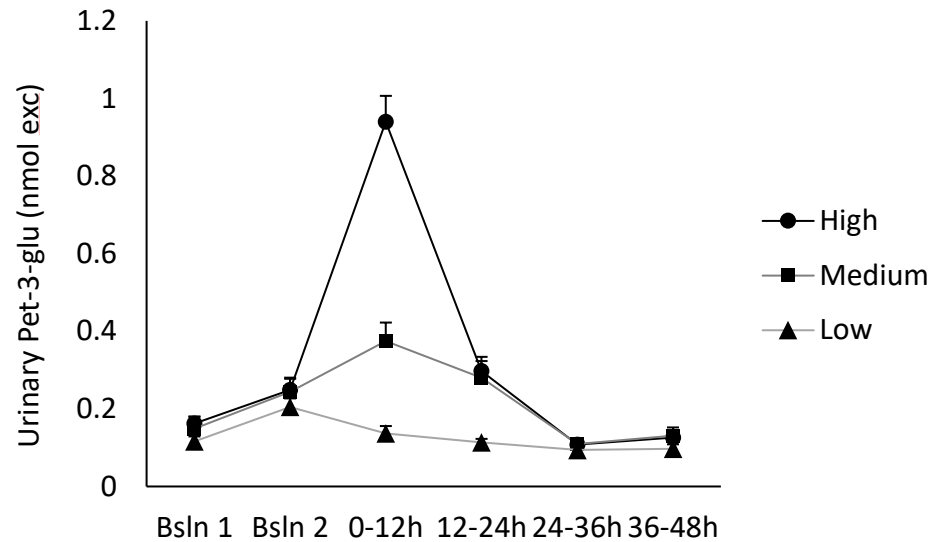


Figure A. 33 – Urinary excretion of petunidin-3-glucoside over 48h.

	Basln 1	Basln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.12 ± 0.04 ^B	0.2 ± 0.06 ^A	0.14 ± 0.05 ^{cB}	0.11 ± 0.03 ^{bB}	0.09 ± 0.03 ^B	0.1 ± 0.03 ^B
Medium	0.15 ± 0.09 ^{CD}	0.24 ± 0.1 ^{BC}	0.37 ± 0.14 ^{bA}	0.28 ± 0.15 ^{aB}	0.11 ± 0.04 ^D	0.13 ± 0.06 ^D
High	0.16 ± 0.05 ^{CD}	0.25 ± 0.09 ^{BC}	0.94 ± 0.19 ^{aA}	0.3 ± 0.07 ^{aB}	0.11 ± 0.02 ^D	0.13 ± 0.02 ^D

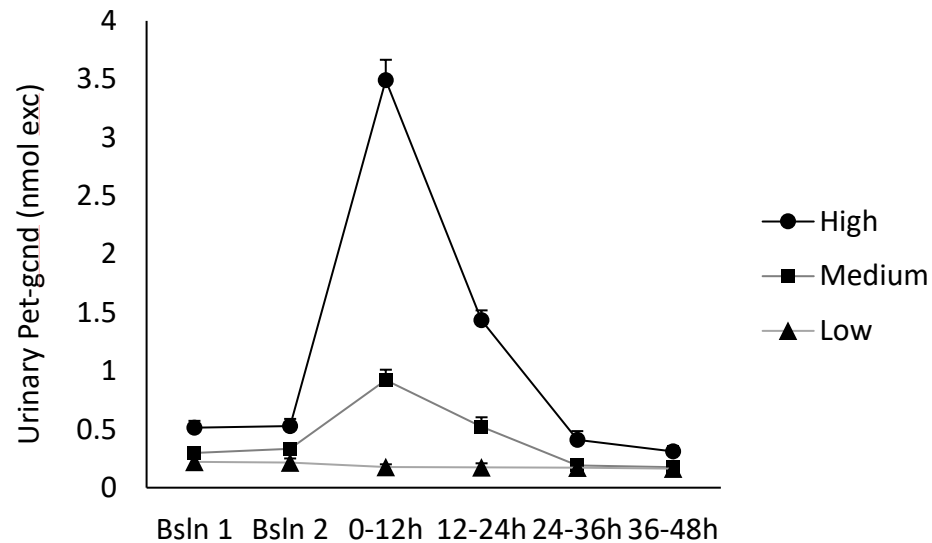


Figure A. 34 – Urinary excretion of petunidin glucuronide over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.22 ± 0.07 ^b	0.22 ± 0.1 ^b	0.18 ± 0.06 ^c	0.17 ± 0.1 ^c	0.17 ± 0.05 ^b	0.16 ± 0.12 ^b
Medium	0.3 ± 0.1 ^{bC}	0.33 ± 0.14 ^{bC}	0.92 ± 0.24 ^{bA}	0.52 ± 0.23 ^{bB}	0.19 ± 0.08 ^{bD}	0.18 ± 0.09 ^{bD}
High	0.51 ± 0.16 ^{aC}	0.53 ± 0.18 ^{aC}	3.49 ± 0.46 ^{aA}	1.44 ± 0.2 ^{aB}	0.41 ± 0.2 ^{aD}	0.31 ± 0.12 ^{aD}

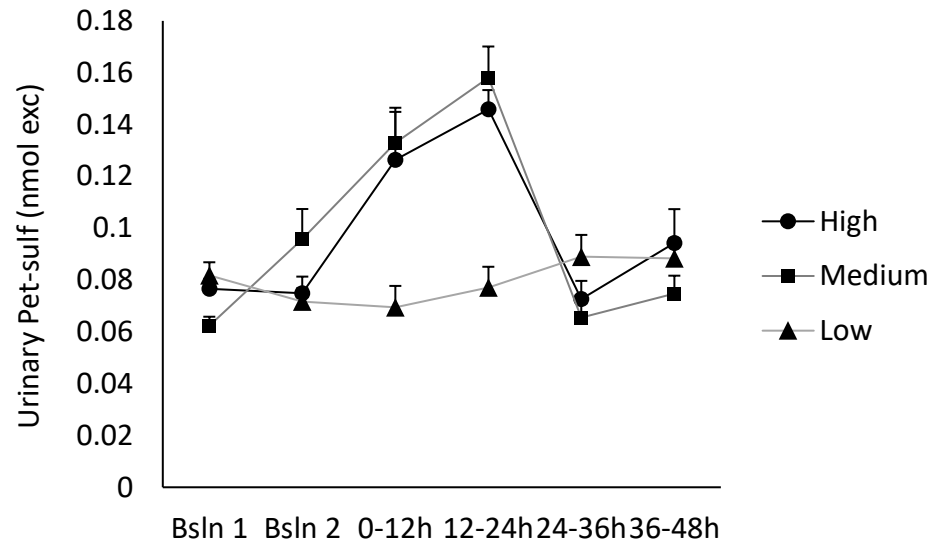


Figure A. 35 – Urinary excretion of petunidin sulfate over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	nd	nd	nd	nd	nd	nd
Low	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.02 ^b	0.08 ± 0.02 ^b	0.09 ± 0.02	0.09 ± 0.02
Medium	0.06 ± 0.01 ^B	0.1 ± 0.03 ^B	0.13 ± 0.04 ^{aA}	0.16 ± 0.03 ^{aA}	0.07 ± 0.02 ^B	0.07 ± 0.02 ^B
High	0.08 ± 0.01 ^C	0.07 ± 0.02 ^C	0.13 ± 0.05 ^{aAB}	0.15 ± 0.02 ^{aA}	0.07 ± 0.02 ^C	0.09 ± 0.03 ^{BC}

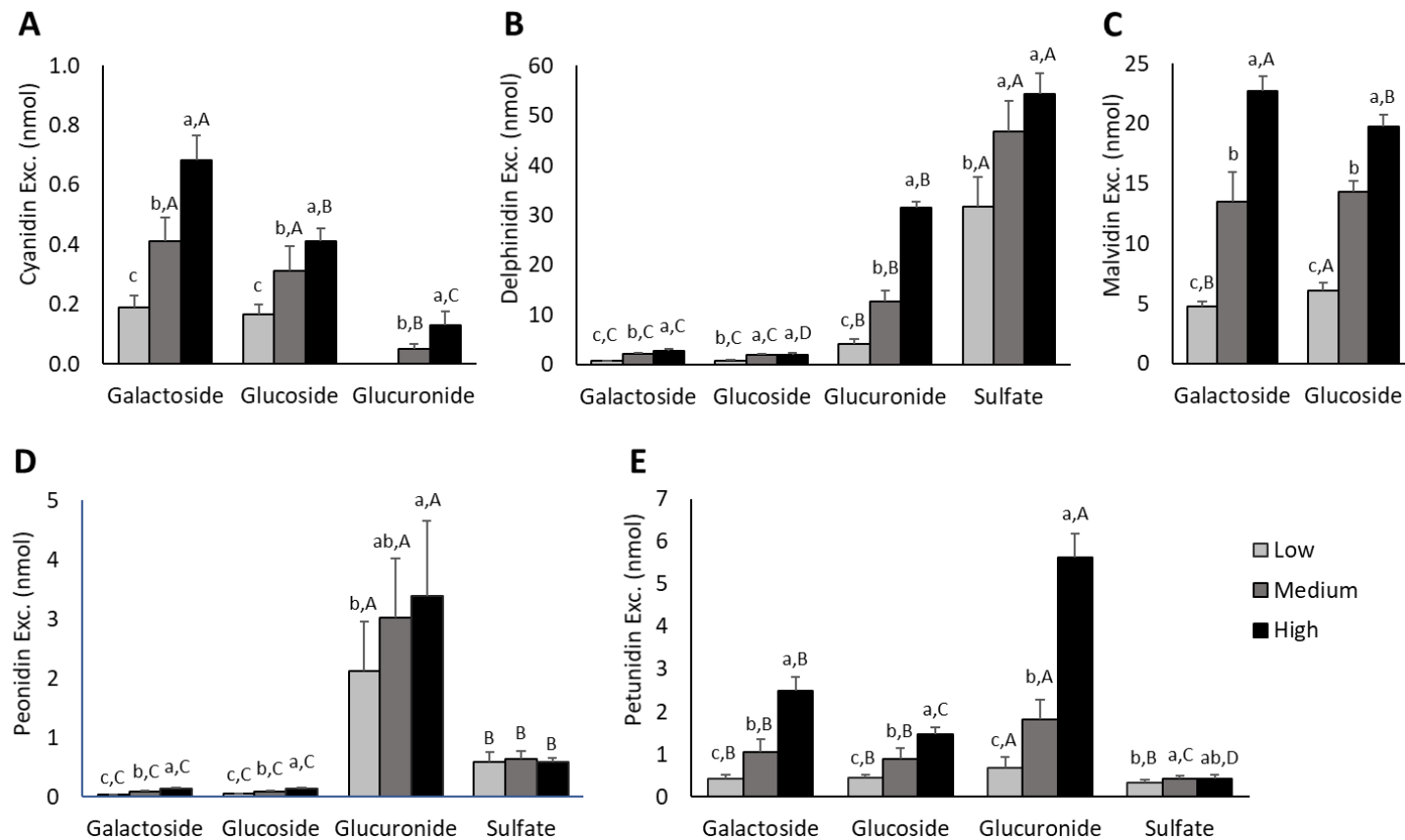


Figure A. 36 – Total urinary excretion of anthocyanins over 48h. Derivatives of A) cyanidin, B) delphinidin, C) malvidin, D) peonidin, E) petunidin. Urinary excretion of anthocyanins occurred in a dose-dependent manner. Cyanidins and malvidins were primarily excreted as parent glycosides (i.e., galactosides and glucosides), while other forms were more commonly excreted as metabolites. Data shown as mean \pm SEM. Lowercase letters indicate significant differences ($p < 0.05$) between doses; capital letters indicate differences between derivatives.

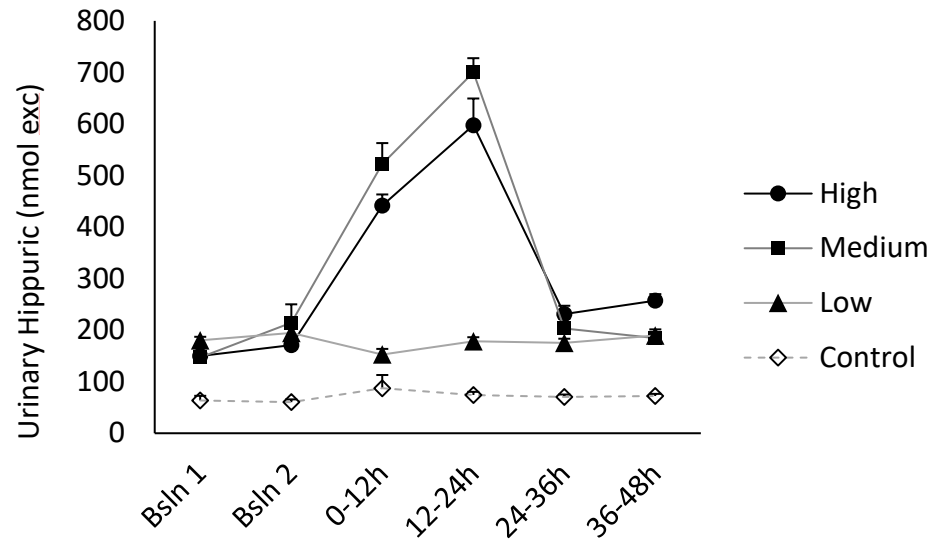


Figure A. 37 – Urinary excretion of hippuric acid over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	63.7 ± 25.2 ^b	60.3 ± 14.2 ^b	87.4 ± 72.5 ^c	74.2 ± 17.4 ^c	70.7 ± 12.7 ^b	72.2 ± 11.8 ^b
Low	180.4 ± 18.9 ^a	194.6 ± 28.8 ^a	152.7 ± 29 ^b	178.5 ± 22 ^b	175.4 ± 22.6 ^a	189.3 ± 34.4 ^a
Medium	147.1 ± 33.8 ^{aB}	214.7 ± 100.3 ^{aB}	522.2 ± 115.1 ^{aA}	700.1 ± 77.8 ^{aA}	203.5 ± 51.3 ^{aB}	184.9 ± 31 ^{aB}
High	149.9 ± 12.6 ^{aC}	170.8 ± 37 ^{aC}	441.6 ± 61.4 ^{aA}	597.7 ± 146.3 ^{aA}	231.3 ± 45.4 ^{aB}	257.2 ± 33.6 ^{aB}

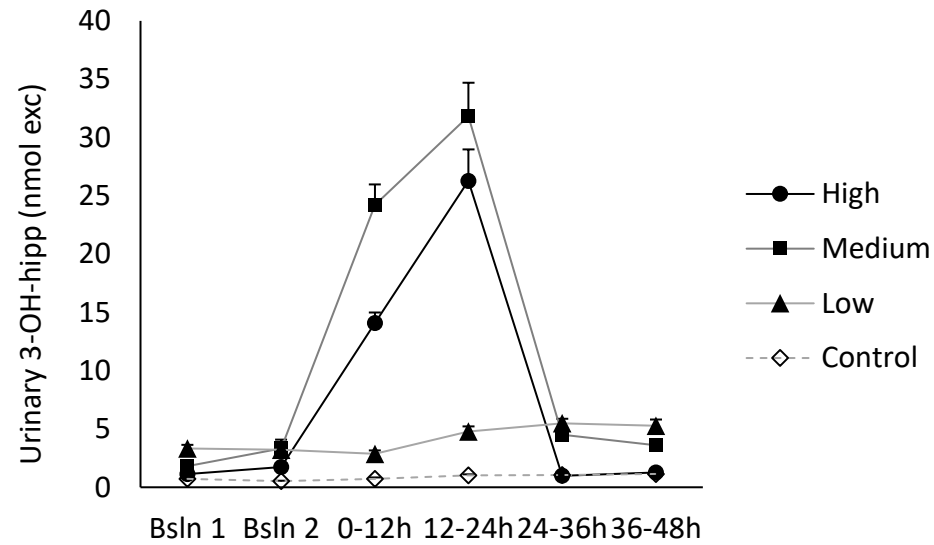


Figure A. 38 – Urinary excretion of 3-hydroxy hippuric acid over 48h.

	Bsln 1	Bsln 2	0-12h	12-24h	24-36h	36-48h
Control	0.71 ± 0.2 ^{cB}	0.53 ± 0.1 ^{cB}	0.71 ± 0.38 ^{dB}	1.03 ± 0.22 ^{cA}	1.06 ± 0.39 ^{bA}	1.14 ± 0.3 ^{bA}
Low	3.32 ± 0.9 ^{aBC}	3.22 ± 0.88 ^{aBC}	2.87 ± 0.77 ^{cC}	4.78 ± 1.25 ^{bABC}	5.49 ± 1.11 ^{aA}	5.29 ± 1.48 ^{aAB}
Medium	1.81 ± 0.5 ^{bC}	3.32 ± 2.18 ^{aBC}	24.22 ± 4.97 ^{aA}	31.82 ± 8.13 ^{aA}	4.52 ± 1.59 ^{aB}	3.6 ± 1.07 ^{aBC}
High	1.12 ± 0.24 ^{bC}	1.72 ± 0.78 ^{bC}	14.07 ± 2.59 ^{bB}	26.28 ± 7.62 ^{aA}	0.99 ± 0.42 ^{bC}	1.27 ± 0.68 ^{bC}

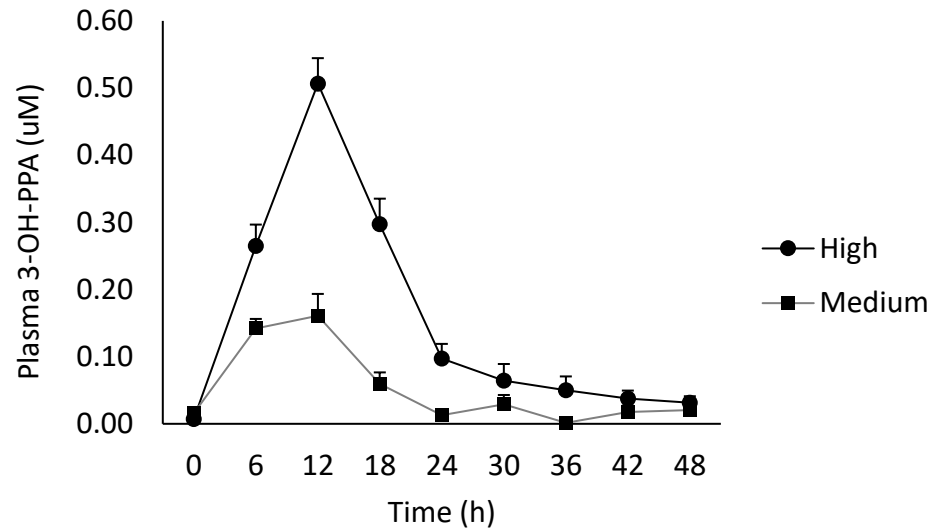


Figure A. 39 – Plasma pharmacokinetics of 3-hydroxy phenyl propionic acid over 48h.

	0h	6h	12h	18h	24h	30h	36h	42h	48h
Control	nd	nd	nd	nd	nd	nd	nd	nd	nd
Low	nd	nd	nd	nd	nd	nd	nd	nd	nd
Medium	0.007 ± 0.012 ^{BC}	0.142 ± 0.037 ^{bA}	0.161 ± 0.092 ^{bA}	0.05 ± 0.042 ^{bAB}	0.008 ± 0.01 ^{BC}	0.029 ± 0.024 ^{bAB}	0.001 ± 0.002 ^C	0.018 ± 0.019 ^{BC}	0.02 ± 0.025 ^{BC}
High	0.004 ± 0.007 ^D	0.265 ± 0.09 ^{aAB}	0.507 ± 0.107 ^{aA}	0.297 ± 0.107 ^{aAB}	0.097 ± 0.062 ^{BC}	0.065 ± 0.069 ^{aCD}	0.05 ± 0.058 ^{CD}	0.037 ± 0.034 ^{CD}	0.032 ± 0.025 ^{CD}

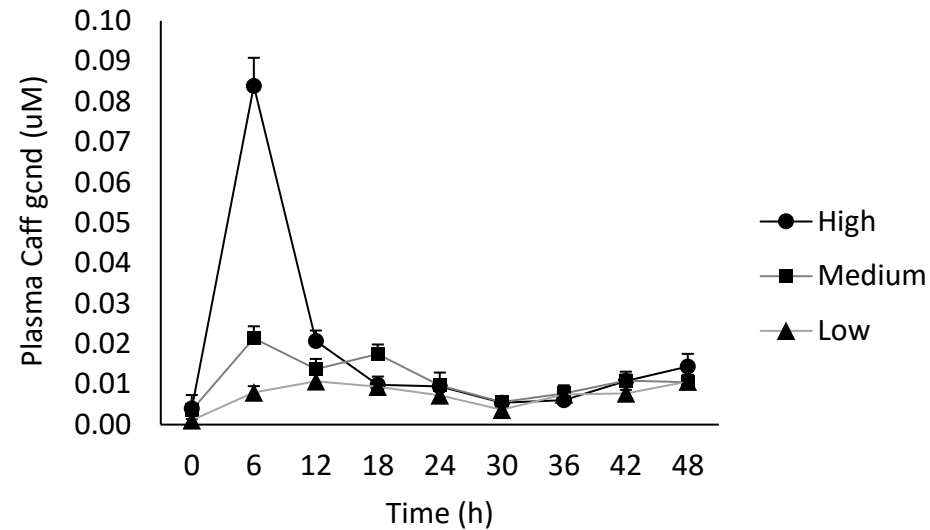


Figure A. 40 – Plasma pharmacokinetics of caffeic acid glucuronide over 48h.

	0h	6h	12h	18h	24h	30h	36h	42h	48h
Control	nd	nd	nd	nd	nd	nd	nd	nd	nd
Low	0.0003 ± 0.0005 ^{bB}	0.0079 ± 0.0039 ^{cAB}	0.0107 ± 0.007 ^A	0.0094 ± 0.0039 ^A	0.0073 ± 0.0036 ^A	0.0037 ± 0.0024 ^{AB}	0.0075 ± 0.0059 ^{AB}	0.0077 ± 0.0024 ^A	0.0107 ± 0.0044 ^A
Medium	0.0009 ± 0.0018 ^{aB}	0.0214 ± 0.0079 ^{bA}	0.0137 ± 0.0072 ^{AB}	0.0175 ± 0.0067 ^A	0.0097 ± 0.0037 ^{AB}	0.0056 ± 0.0031 ^B	0.0077 ± 0.0031 ^{AB}	0.0109 ± 0.0042 ^{AB}	0.0105 ± 0.0039 ^{AB}
High	0.0015 ± 0.0037 ^{aE}	0.0839 ± 0.0197 ^{aA}	0.0207 ± 0.0073 ^B	0.0098 ± 0.0059 ^{BCD}	0.0095 ± 0.0096 ^{BCDE}	0.0054 ± 0.0044 ^{DE}	0.006 ± 0.0031 ^{CDE}	0.0109 ± 0.0064 ^{BCD}	0.0143 ± 0.0084 ^{BC}

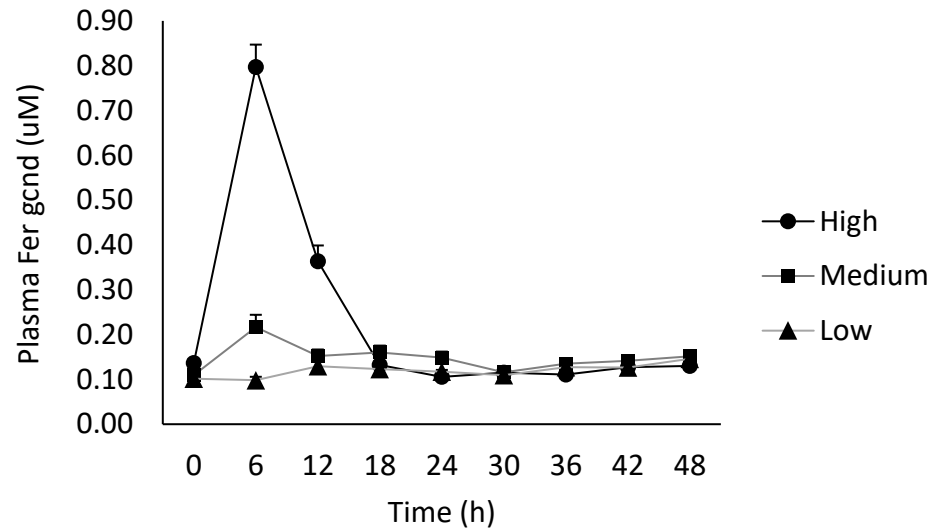


Figure A. 41 – Plasma pharmacokinetics of ferulic acid glucuronide over 48h.

	0h	6h	12h	18h	24h	30h	36h	42h	48h
Control	nd	nd	nd	nd	nd	nd	nd	nd	nd
Low	0.1 ± 0.02^b	0.1 ± 0.02^c	0.13 ± 0.04^b	0.12 ± 0.01	0.12 ± 0.01^{ab}	0.11 ± 0.02	0.13 ± 0.02	0.13 ± 0.02	0.15 ± 0.03
Medium	0.11 ± 0.02^{abD}	0.22 ± 0.07^{bA}	0.15 ± 0.04^{bBC}	0.16 ± 0.04^B	0.15 ± 0.04^{bBC}	0.12 ± 0.02^{CD}	0.14 ± 0.02^{BCD}	0.14 ± 0.02^{BCD}	0.15 ± 0.03^{BCD}
High	0.14 ± 0.03^{aC}	0.8 ± 0.14^{aA}	0.36 ± 0.1^{aB}	0.13 ± 0.03^C	0.11 ± 0.01^{aC}	0.11 ± 0.03^C	0.11 ± 0.02^C	0.13 ± 0.02^C	0.13 ± 0.03^C

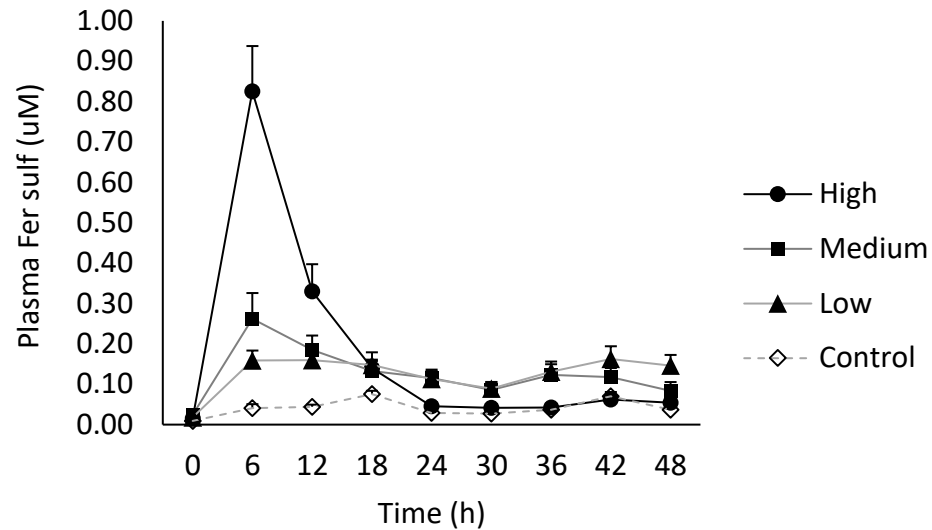


Figure A. 42 – Plasma pharmacokinetics of ferulic acid sulfate over 48h.

	0h	6h	12h	18h	24h	30h	36h	42h	48h
Control	0.007 ± 0.009 ^C	0.041 ± 0.026 ^{cAB}	0.044 ± 0.015 ^{bAB}	0.075 ± 0.022 ^A	0.029 ± 0.028 ^{bB}	0.027 ± 0.031 ^{cBC}	0.037 ± 0.02 ^{cAB}	0.07 ± 0.03 ^A	0.037 ± 0.019 ^{bAB}
Low	0.013 ± 0.013 ^B	0.159 ± 0.061 ^{bA}	0.159 ± 0.032 ^{aA}	0.148 ± 0.034 ^A	0.113 ± 0.051 ^{aA}	0.089 ± 0.044 ^{aA}	0.131 ± 0.066 ^{aA}	0.162 ± 0.084 ^A	0.146 ± 0.066 ^{aA}
Medium	0.015 ± 0.023 ^B	0.262 ± 0.169 ^{bA}	0.185 ± 0.102 ^{aA}	0.133 ± 0.071 ^A	0.116 ± 0.058 ^{aA}	0.086 ± 0.055 ^{abA}	0.123 ± 0.071 ^{abA}	0.117 ± 0.064 ^A	0.083 ± 0.058 ^{abAB}
High	0.011 ± 0.02 ^E	0.825 ± 0.317 ^{aA}	0.33 ± 0.19 ^{aAB}	0.14 ± 0.111 ^{BC}	0.046 ± 0.027 ^{abCD}	0.042 ± 0.027 ^{bcD}	0.042 ± 0.029 ^{bcdD}	0.062 ± 0.017 ^{CD}	0.054 ± 0.032 ^{bCD}

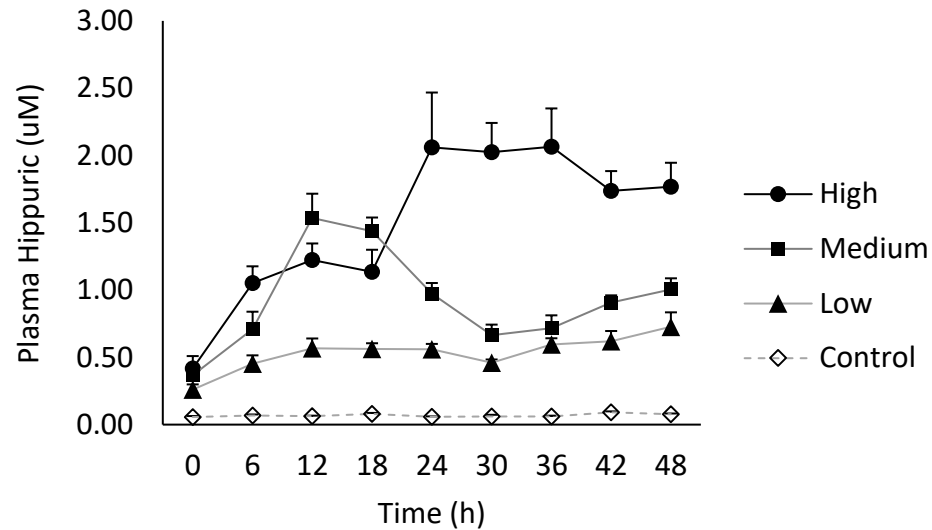


Figure A. 43 – Plasma pharmacokinetics of hippuric acid over 48h.

	0h	6h	12h	18h	24h	30h	36h	42h	48h
Control	0.06 ± 0.02^b	0.07 ± 0.02^c	0.06 ± 0.01^c	0.08 ± 0.02^c	0.06 ± 0.01^d	0.06 ± 0.03^c	0.06 ± 0.02^c	0.09 ± 0.02^c	0.08 ± 0.02^c
Low	0.26 ± 0.1^{aB}	0.45 ± 0.15^{bAB}	0.57 ± 0.18^{bA}	0.56 ± 0.11^{bA}	0.56 ± 0.11^{cA}	0.46 ± 0.07^{bA}	0.6 ± 0.12^{bA}	0.62 ± 0.2^{bA}	0.73 ± 0.28^{bA}
Medium	0.37 ± 0.18^{aC}	0.71 ± 0.34^{abB}	1.54 ± 0.51^{aA}	1.44 ± 0.29^{aA}	0.97 ± 0.23^{bAB}	0.67 ± 0.22^{bB}	0.72 ± 0.27^{bB}	0.91 ± 0.15^{bAB}	1.00 ± 0.22^{bAB}
High	0.42 ± 0.26^{aC}	1.05 ± 0.35^{aB}	1.22 ± 0.35^{aAB}	1.14 ± 0.46^{aB}	2.06 ± 1.15^{aAB}	2.02 ± 0.61^{aAB}	2.06 ± 0.81^{aA}	1.74 ± 0.41^{aAB}	1.77 ± 0.5^{aAB}

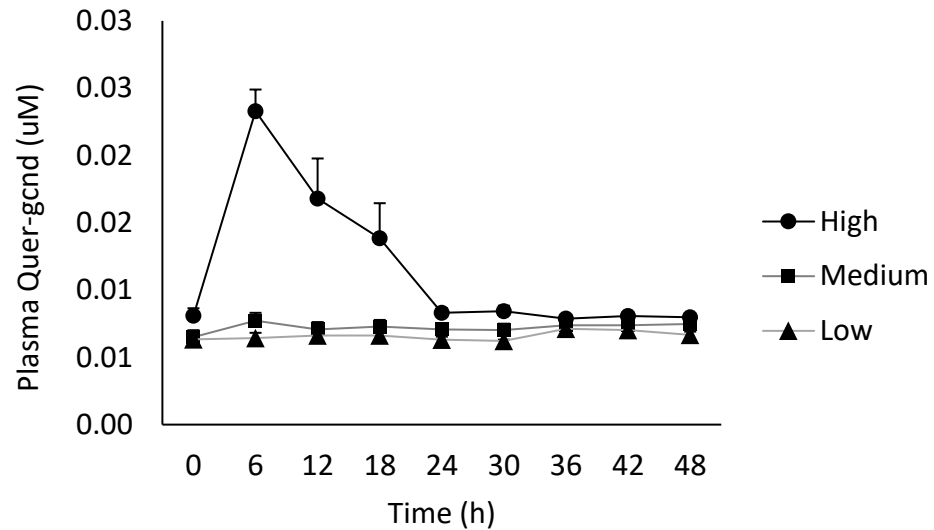


Figure A. 44 – Plasma pharmacokinetics of quercetin glucuronide over 48h.

	0h	6h	12h	18h	24h	30h	36h	42h	48h
Control	nd	nd	nd	nd	nd	nd	nd	nd	nd
Low	0.0063 ± 0.0006	0.0064 ± 0.0008 ^b	0.0066 ± 0.0004 ^b	0.0066 ± 0.0004 ^b	0.0063 ± 0.0004 ^b	0.0062 ± 0.0002	0.0071 ± 0.0008	0.007 ± 0.0009	0.0067 ± 0.0005
Medium	0.0057 ± 0.0026	0.0077 ± 0.0015 ^b	0.0071 ± 0.0016 ^b	0.0073 ± 0.0014 ^b	0.0071 ± 0.0011 ^b	0.007 ± 0.0014	0.0074 ± 0.0012	0.0074 ± 0.0015	0.0075 ± 0.0018
High	0.0061 ± 0.0039 ^D	0.0233 ± 0.0046 ^{aA}	0.0168 ± 0.0084 ^{aB}	0.0138 ± 0.0074 ^{aBC}	0.0083 ± 0.0009 ^{aCD}	0.0084 ± 0.0012 ^D	0.0079 ± 0.0005 ^D	0.0081 ± 0.0004 ^D	0.008 ± 0.0003 ^D

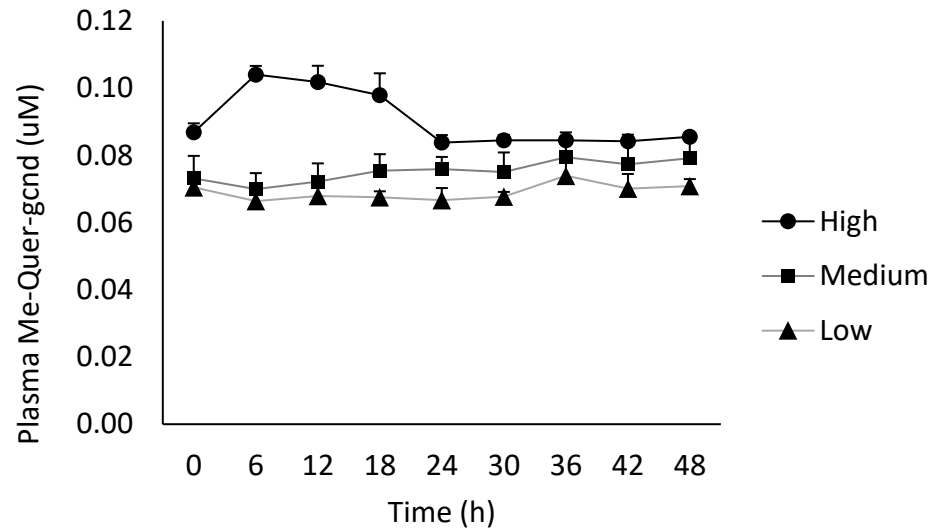


Figure A. 45 – Plasma pharmacokinetics of methyl quercetin glucuronide over 48h.

	0h	6h	12h	18h	24h	30h	36h	42h	48h
Control	nd	nd	nd	nd	nd	nd	nd	nd	nd
Low	0.053 ± 0.036 ^b	0.066 ± 0.007 ^b	0.068 ± 0.008 ^b	0.067 ± 0.004 ^b	0.067 ± 0.007 ^b	0.068 ± 0.003 ^b	0.074 ± 0.009	0.07 ± 0.01	0.071 ± 0.005
Medium	0.055 ± 0.037 ^b	0.07 ± 0.013 ^b	0.072 ± 0.015 ^b	0.075 ± 0.014 ^b	0.076 ± 0.01 ^{ab}	0.075 ± 0.016 ^{ab}	0.079 ± 0.015	0.077 ± 0.018	0.079 ± 0.018
High	0.087 ± 0.008 ^{aC}	0.104 ± 0.007 ^{aA}	0.102 ± 0.014 ^{aAB}	0.098 ± 0.018 ^{aABC}	0.084 ± 0.006 ^{aABC}	0.084 ± 0.004 ^{aBC}	0.084 ± 0.007 ^c	0.084 ± 0.005 ^c	0.086 ± 0.003 ^c

APPENDIX B. SUPPLEMENTAL INFORMATION FOR CHAPTER 3: A 90 DAY ORAL TOXICITY STUDY OF BLUBERRY POLYPHENOLS IN OVARIECTOMIZED SPRAGUE-DAWLEY RATS

Figure B.1 – Representative histopathology slides from water and high dose groups.

Figure B.2 – Urine and fecal samples demonstrating color change in response to dose.

Figure B.3 – Intestinal barrier function for all groups.

Figure B.4 – Bone mineral density.

Figure B.5 – Body weight for all groups, including non-gavaged control.

Figure B.6 – Food consumption and FER for all groups, including non-gavaged control.

Table B.1 – Absolute and relative tissue weights of water and non-gavaged groups.

Table B.2 – Hematology for water and non-gavaged groups.

Table B.3 – Serum biochemistry for water and non-gavaged groups.

Table B.4 – Urinalysis for water and non-gavaged groups.

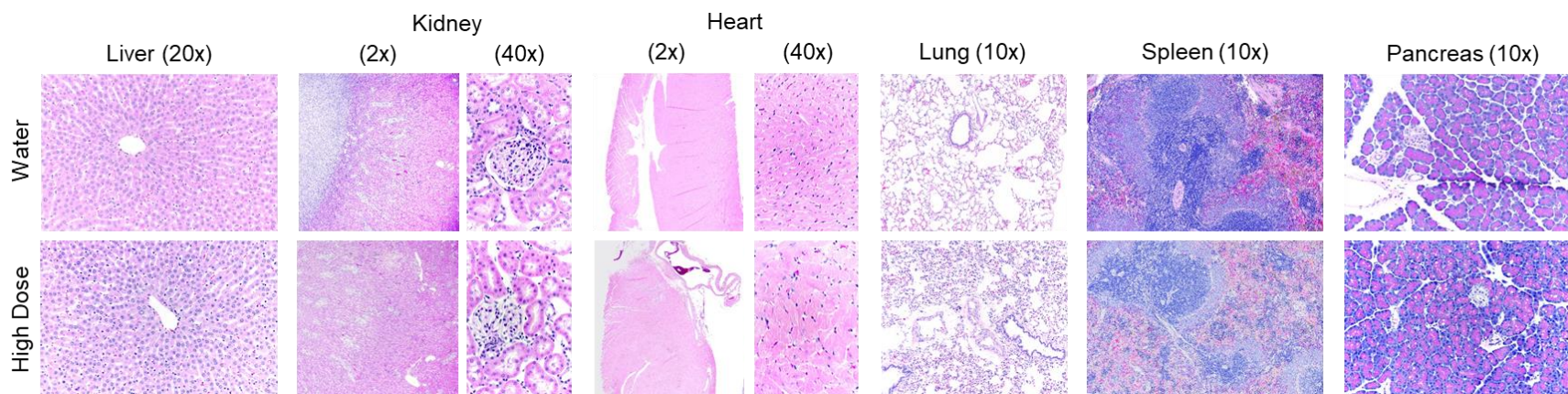


Figure B. 1 – Representative histopathology slides from water and high dose groups. Images were taken at different magnifications (shown in parentheses). No pathologically significant differences were observed between groups.

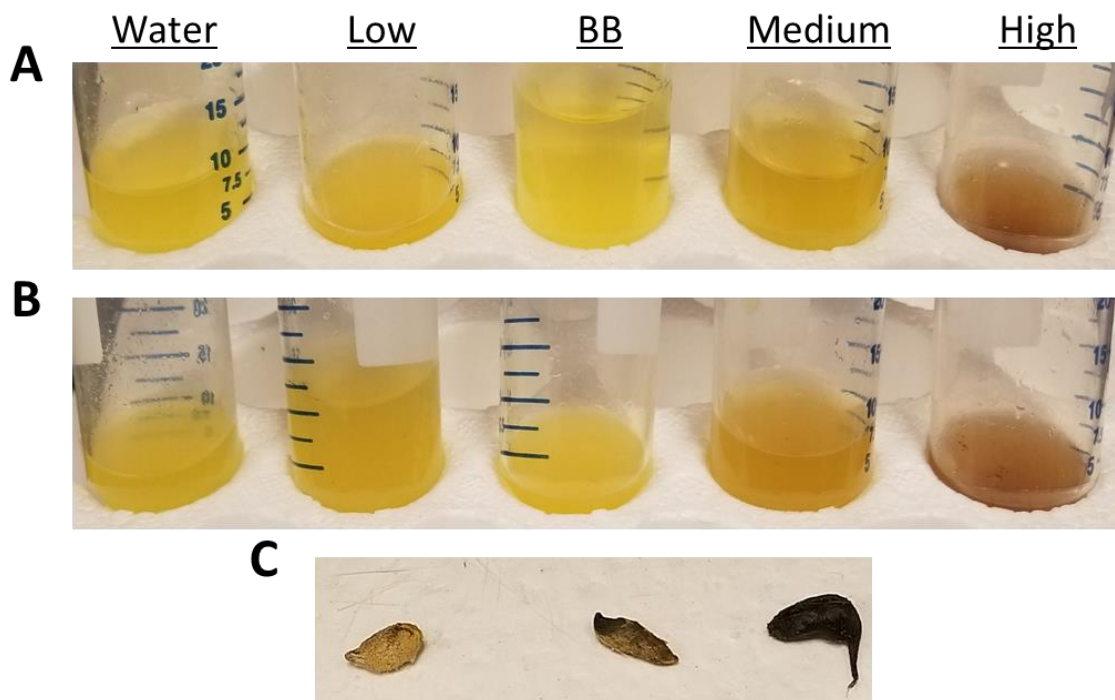


Figure B. 2 – Representative urine and fecal samples. A & B) depict 24h urine collections from animals in different dose groups after 1 week of treatment. A dose-dependent darkening of urine was observed throughout the study. C) Fecal pellets from high dose animal during first oral gavage treatment: before, ~12h, and 24h after dosing (L to R). Discoloration of fecal pellets was observed in all treatment groups throughout the study.

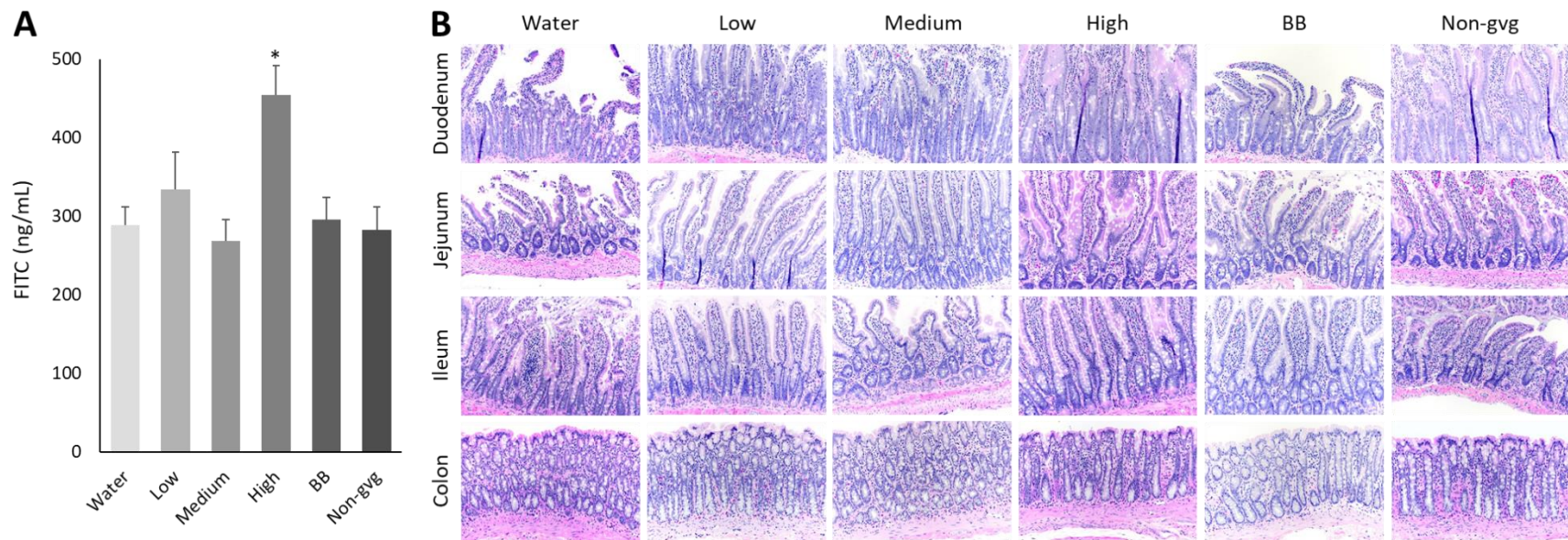


Figure B. 3 – Intestinal barrier function. The integrity of the GI tract was measured using the FITC-dextran method (a). Despite differences in FITC uptake, no histologically significant differences were found (b). Representative histology slides shown at 20x magnification. Data shown as mean \pm SEM. Significant differences detected using one-way ANOVA with Tukey's HSD post hoc test ($\alpha = 0.05$) and indicated with (*). BB = lyophilized blueberry dose group; FITC = fluorescein isothiocyanate-dextran; Non-gvg = non-gavaged group.

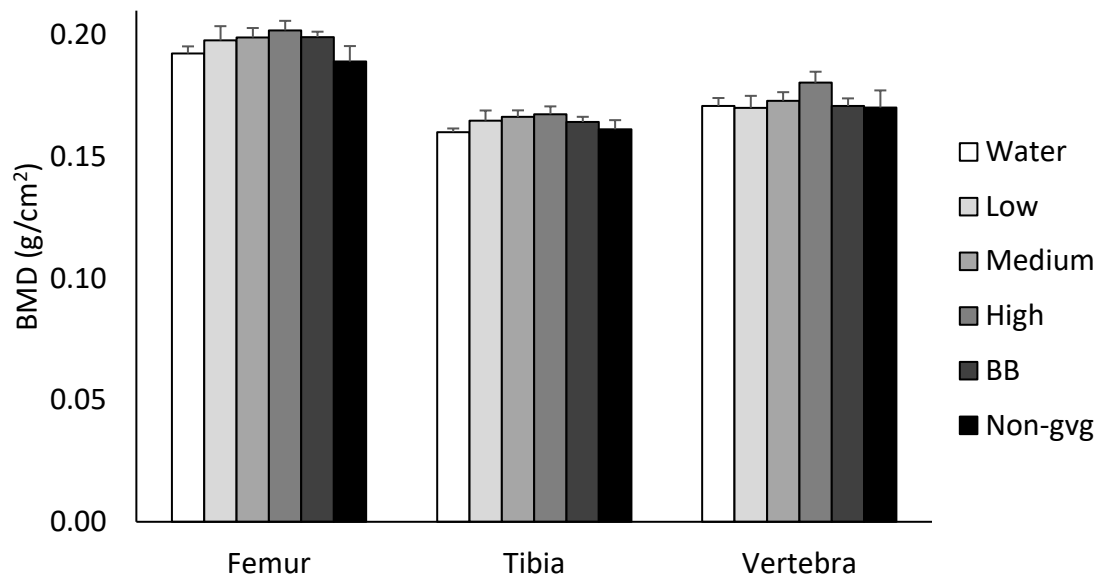


Figure B. 4 – Bone mineral density (BMD). BMD was measured in the femur, tibia, and lumbar spine of all animals. No significant differences were observed between groups. Data shown as mean \pm SEM. BB = lyophilized blueberry dose group; Non-gvg = non-gavaged control group.

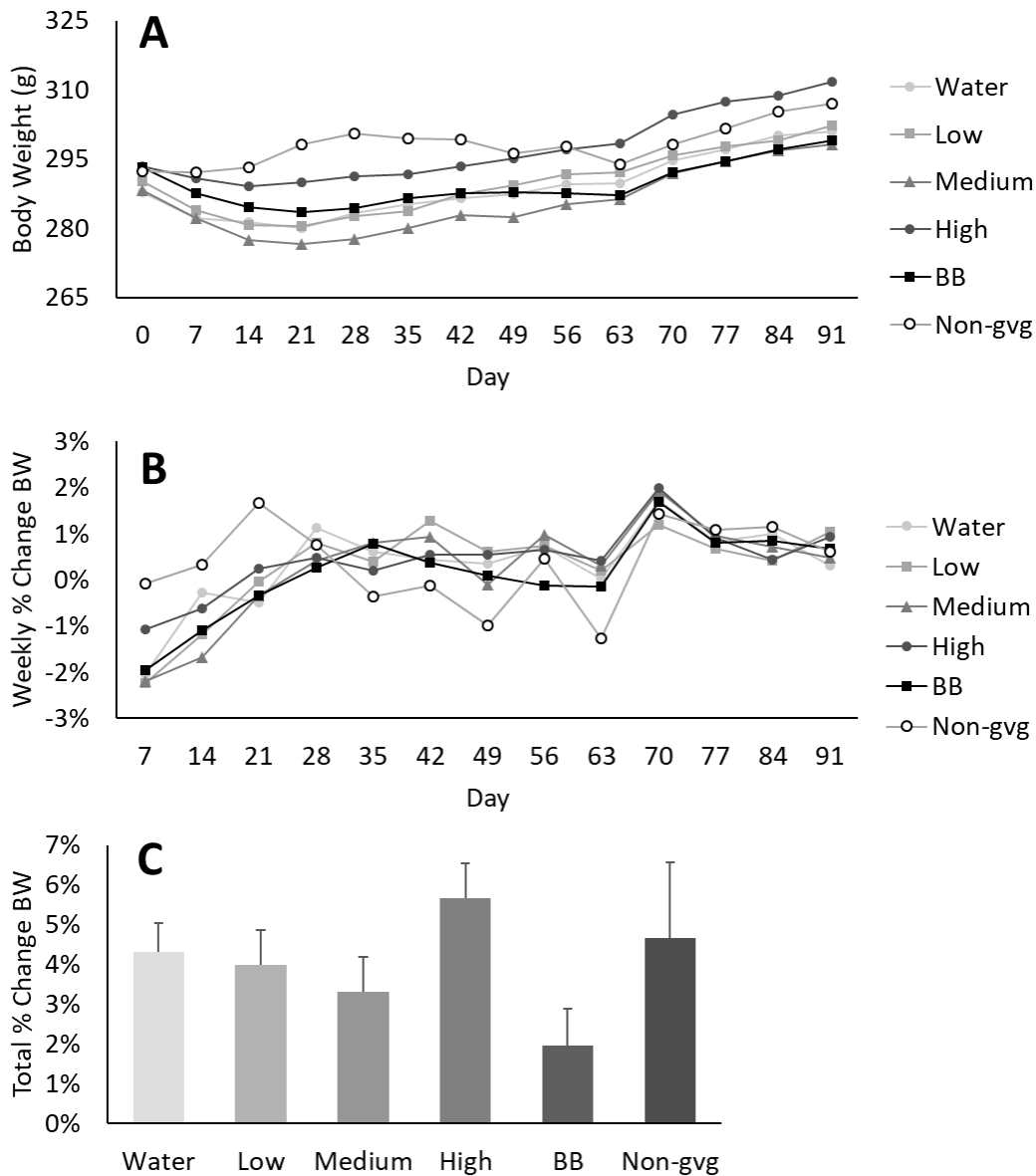


Figure B. 5 – Body weight for all groups, including non-gavaged control group. No significant differences in absolute body weight were observed throughout the study (a), and week-to-week changes in body weight were not significant (b). When incorporating the non-gavaged group, the weight gain difference between the high and BB groups is near significance ($p = 0.053$) (c). Data shown as mean \pm SEM. Significant differences detected using one-way ANOVA with Tukey's HSD post hoc test ($\alpha = 0.05$). BB = lyophilized blueberry dose group; Non-gvg = non-gavaged control group.

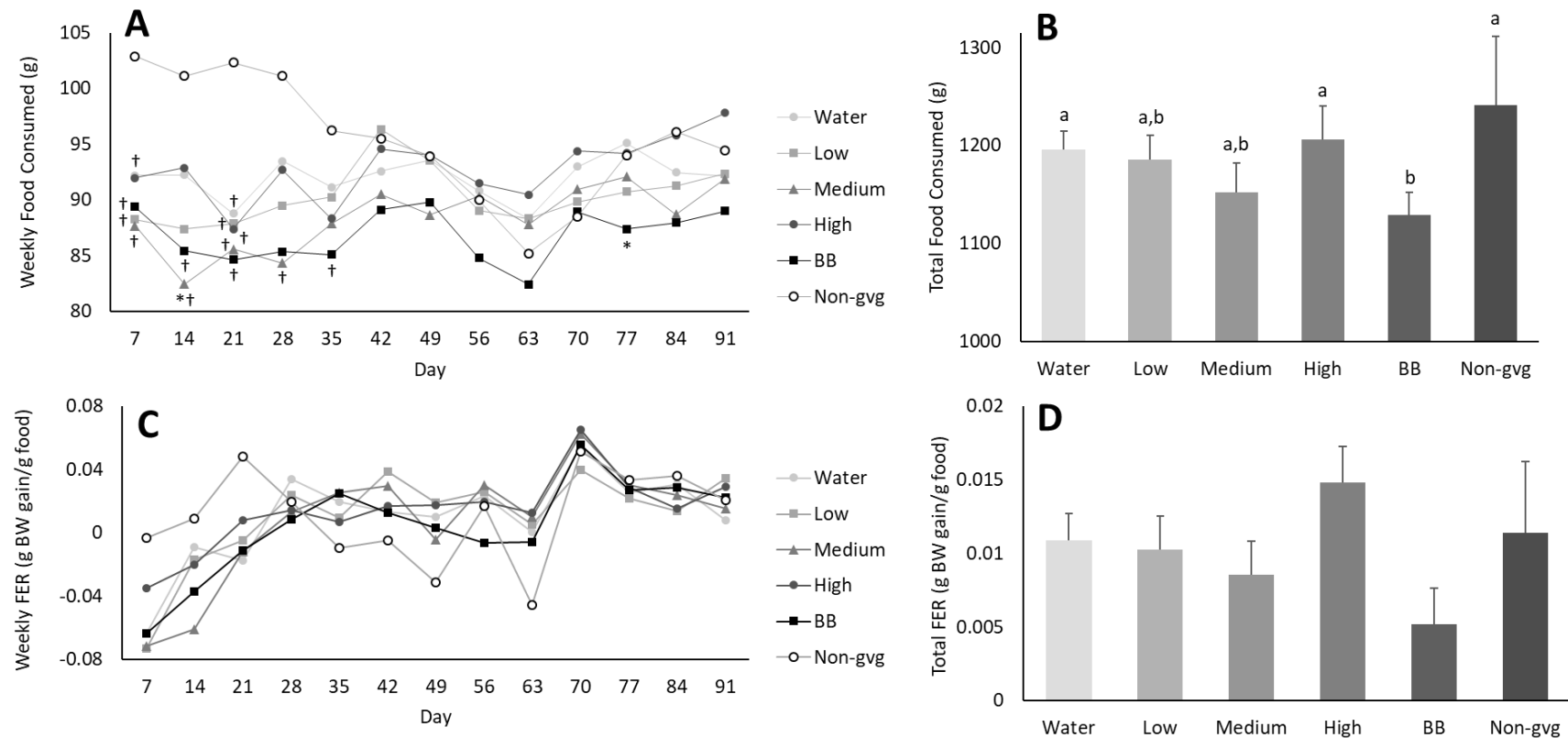


Figure B. 6 – Food consumption (a,b) and food efficiency ratio (FER, defined as g body weight gain/g food eaten; c,d) for all groups, including non-gavaged control group. Several significant differences between non-gavaged control and other treatment groups were observed in food consumption week-to-week (a), though these mostly occurred early in the study and are likely due to the addition of daily gavage. However, these differences persisted throughout the study and were observed over 90d as well (b). When incorporating body weight and food consumption, no significant differences in FER were observed week-to-week (c), though there was a near significant difference between the high and BB groups over 90d ($p = 0.057$) (d). Data shown as mean \pm SEM. Significant differences detected using one-way ANOVA with Tukey's HSD post hoc test ($\alpha = 0.05$) and are indicated as different from water (*) or non-gavaged group (†) in (a) or via lower case letters (b). BB = lyophilized blueberry dose group.

Table B. 1 – Absolute and relative tissue weights of water and non-gavaged groups.

		Group	
		Water	Non-gvg
Final body wt	(g)	301 ± 17	307 ± 27
Brain	(g)	1.67 ± 0.071	1.65 ± 0.149
	(g/100 g BW)	0.56 ± 0.026	0.54 ± 0.057
Colon	(g)	0.84 ± 0.146	0.79 ± 0.087
	(g/100 g BW)	0.28 ± 0.048	0.26 ± 0.031
	(g/100 g brain)	50.1 ± 8.9	48.2 ± 8.61
Heart	(g)	1.09 ± 0.079	1.18 ± 0.135
	(g/100 g BW)	0.36 ± 0.032	0.39 ± 0.047
	(g/100 g brain)	65.5 ± 5.66	71.4 ± 1.91
Liver	(g)	8.08 ± 0.514	7.73 ± 0.766
	(g/100 g BW)	2.69 ± 0.185	2.52 ± 0.196
	(g/100 g brain)	484 ± 40.6	471 ± 53.8
Lungs	(g)	3.21 ± 0.792	2.05 ± 0.763
	(g/100 g BW)	1.07 ± 0.258	0.70 ± 0.254
	(g/100 g brain)	192 ± 44.5	131 ± 63.6
Kidneys	(g)	1.77 ± 0.151	1.75 ± 0.127
	(g/100 g BW)	0.59 ± 0.028	0.57 ± 0.034
	(g/100 g brain)	106 ± 8.3	106 ± 4.9
R Kidney	(g)	0.89 ± 0.075	0.90 ± 0.063
	(g/100 g BW)	0.30 ± 0.015	0.29 ± 0.021
	(g/100 g brain)	53.4 ± 3.82	54.6 ± 2.03
L Kidney	(g)	0.88 ± 0.081	0.85 ± 0.066
	(g/100 g BW)	0.29 ± 0.016	0.28 ± 0.014
	(g/100 g brain)	52.8 ± 4.8	51.9 ± 3.0
Pancreas	(g)	2.30 ± 0.456	2.06 ± 0.384
	(g/100 g BW)	0.76 ± 0.143	0.68 ± 0.147
	(g/100 g brain)	138 ± 28.4	125 ± 16.9
Small Intestine	(g)	4.67 ± 0.601	4.74 ± 0.495
	(g/100 g BW)	1.55 ± 0.196	1.55 ± 0.124
	(g/100 g brain)	280 ± 36.4	290 ± 48.0
Spleen	(g)	0.64 ± 0.086	0.71 ± 0.062
	(g/100 g BW)	0.21 ± 0.027	0.23 ± 0.028
	(g/100 g brain)	38.3 ± 4.76	43.7 ± 5.94

Values are mean ± SD; water (n = 10) and non-gavage (n = 4).

Non-gvg = non-gavaged group.

* Significantly different from water control (p < 0.05). Note: statistics run as comparison of all test groups to water control, though only water and non-gavage groups are shown here for ease of comparison; for treatment groups, see Table 3.2.

Table B. 2 – Hematology for water and non-gavaged groups.

	Group	
	Water	Non-gvg
RBC ($10^6/\mu\text{L}$)	7.64 ± 0.12	7.59 ± 0.04
HCT (%)	44.1 ± 1.71	44.5 ± 0.71
HGB (g/dL)	14.0 ± 0.48	14.1 ± 0.35
MCV (fL)	61.6 ± 0.98	63.3 ± 3.54
MCHC (g/dL)	31.6 ± 1.09	31.6 ± 0.28
RDW (%)	13.8 ± 1.06	14.1 ± 0.78
WBC ($10^3/\mu\text{L}$)	11.3 ± 2.41	12 ± 0.14
SEG ($10^3/\mu\text{L}$)	0.62 ± 0.22	0.6 ± 0.14
LYMPH ($10^3/\mu\text{L}$)	10.2 ± 2.44	11.1 ± 0.28
MONO ($10^3/\mu\text{L}$)	0.32 ± 0.16	0.18 ± 0.08
RETIC # ($10^3/\mu\text{L}$)	420 ± 95	535 ± 60

Values are mean \pm SD; water (n = 9) and non-gavage (n = 2). Several blood samples were unable to be analyzed due to improper clotting, resulting in lower n/group.

Non-gvg = non-gavaged group; RBC = Red Blood Cell count; HCT = Hematocrit; HGB = Hemoglobin; MCV = mean corpuscular volume; MCHC = mean corpuscular hemoglobin concentration; RDW = red blood cell distribution width; WBC = white blood cell count; SEG = segmented neutrophils; LYMPH = lymphocyte count; MONO = monocyte count; RETIC # = reticulocyte number.

a Several additional parameters (polychromasia, target cells, and anisocytosis) were examined and scored qualitatively, thus they are not included in the chart. For all collected samples, results were considered normal and are not reported here. For most samples, platelets were too clumped to count, so they are not included in the chart.

* Significantly different from water control ($p < 0.05$). Note: statistics run as comparison of all test groups to water control, though only water and non-gavage groups are shown here for ease of comparison; for treatment groups, see Table 3.3.

Table B. 3 – Serum biochemistry for water and non-gavaged groups.

	Group	
	Water	Non-gvg
Total protein (g/dL)	6.01 ± 0.21	6.03 ± 0.26
Albumin (g/dL)	3.39 ± 0.14	3.38 ± 0.22
Globulin (g/dL)	2.62 ± 0.10	2.65 ± 0.06
A/G ratio	1.29 ± 0.06	1.28 ± 0.10
BUN (mg/dL)	14.9 ± 2.42	15.3 ± 2.06
Creatinine (mg/dL)	0.67 ± 0.149	0.60 ± 0.082
ALKP (U/L)	123 ± 30.7	102 ± 15.3
ALT (U/L)	50.3 ± 8.3	45.7 ± 0.6
Total bilirubin (mg/dL)	0.12 ± 0.044	0.15 ± 0.071
Amylase (U/L)	1662 ± 318	1583 ± 300
Lipase (U/L)	97.0 ± 23.3	102 ± 48.6
Cholesterol (mg/dL)	135 ± 10.3	145 ± 20.1
Calcium (mg/dL)	11.6 ± 0.20	11.7 ± 0.21
Chloride (mmol/L)	99.4 ± 1.2	101 ± 2.2
Phosphorus (mg/dL)	7.53 ± 0.61	7.63 ± 0.73
Potassium (mmol/L)	7.02 ± 0.8	7.13 ± 1.13
Sodium (mmol/L)	141 ± 1.5	141 ± 1.0
Anion gap (mmol/L)	16.1 ± 2.40	16.9 ± 1.61
CO ₂ (mmol/L)	32.7 ± 1.70	28.8 ± 3.86*

Values are mean ± SD; water (n = 10) and non-gavage (n = 4).

FD BB = whole freeze-dried blueberries; Non-gvg = non-gavaged group; A/G = Albumin:Globulin ratio; BUN = Blood Urea Nitrogen; ALKP = Alkaline Phosphatase; ALT = Alanine Transaminase; CO₂ = Carbon Dioxide.

* Significantly different from water control (p < 0.05). Note: statistics run as comparison of all test groups to water control, though only water and non-gavage groups are shown here for ease of comparison; for treatment groups, see Table 3.4.

Table B. 4 – Urinalysis for water and non-gavaged groups.

	Group	
	Water	Non-gvg
<i>Quantitative measures</i>		
Volume (mL)	11.2 ± 4.5	13.3 ± 4.1
Specific Gravity	1.025 ± 0.010	1.022 ± 0.006
pH	7.6 ± 0.6	7.4 ± 0.8
Protein (g/L)	0.34 ± 0.37	0.15 ± 0.17
<i>Qualitative measures</i> ^a		
Color ^b		
Normal	10	4
Darkened	0	0
Glucose		
Negative	8	3
Trace	2	1
Triphosphate crystals		
None	7	1
Few	1	2
Moderate	0	0
Many	2	1

Values are mean ± SD; water (n = 10) and non-gavage (n = 4).

FD BB = whole freeze-dried blueberries; Non-gvg = non-gavaged group.

^a Qualitative measures included ketones, bilirubin, and blood in the urine, all of which were negative and not included in the table.

^b Color was independently graded as pale yellow, yellow, dark yellow, or brown. Samples are categorized as “normal” if color was yellow or pale yellow and “darkened” if dark yellow or brown.

* Significantly different from water control (p < 0.05). Note: statistics run as comparison of all test groups to water control, though only water and non-gavage groups are shown here for ease of comparison; for treatment groups, see Table 3.5.

APPENDIX C. SUPPLEMENTAL INFORMATION FOR CHAPTER 4: CHANGES IN BLUEBERRY POLYPHENOL METABOLISM ARE DEPENDENT ON DOSE AND FOOD MATRIX OVER 90 DAYS IN OVARECTOMIZED SPRAGUE-DAWLEY RATS

Table C.1 – Mass spectrometry parameters for compounds quantified via MS².

Figure C.1 – Total polyphenols in oral gavage doses over time.

Figure C.2-C.3 – Urinary excretion of hippuric acids over 90 days.*

Figure C.4-C.19 – Urinary excretion of phenolic acids over 90 days.*

Figure C.20-C.37 – Urinary excretion of flavonoids over 90 days.*

Figure C.38 – Relative levels of phenolic acids and flavonoids in starting materials and urinary metabolites.

* All graphs in figures C.2-C.37 depict urinary excretion (in nmol) from 24h urine collections throughout the study. For clarity, statistical comparisons are shown in tables below each figure. Lower case letters indicate differences between doses and the same time point (i.e., down a column), while upper case letters indicate differences between time points and within dose (i.e., across a row). Two-way ANOVA with Tukey's HSD ($p < 0.05$) used for all comparisons. nd = not detected; trace = metabolite detected but below LOQ; BB = lyophilized whole blueberry dose; NG = non-gavaged group. Data shown as mean \pm SEM in graphs; mean \pm SD in tables.

Table C. 1 – Mass spectrometry parameters for compounds quantified via MS².

Compound	MRM mode	RT (min)	MW	Precursor ion (m/z)	MS ² fragments (m/z) ^a	CV	CE (eV)	Quantitation Standard
Anthocyanins								
Cyanidin								
Arabinoside	+	1.77	419.1	419	287	46	20	Cyanidin-3-glucoside
Galactoside	+	1.62	449.1	449	137, 287	46	54, 20	
Glucoside	+	1.86	449.1	449	137, 287	46	54, 20	
Delphinidin								
Arabinoside	+	1.41	435.4	435	303	100	18	Cyanidin-3-glucoside
Galactoside	+	1.26	465.4	465	69, 303	100	74, 18	
Glucoside	+	1.41	465.4	465	69, 303	100	74, 18	
Glucuronide	+	3.35	479.4	479	303	100	18	
Malvidin								
Arabinoside	+	2.79	463.4	463	331	100	34	Malvidin-3-glucoside
Galactoside	+	2.74	493.4	493	315, 331	100	50, 34	
Glucoside	+	2.95	493.4	493	315, 331	100	50, 34	
Peonidin								
Arabinoside	+	2.44	433.4	433	301	35	20	Peonidin-3-glucoside
Galactoside	+	2.42	463.4	464	301	35	20	
Glucoside	+	2.66	463.4	464	301	35	20	
Sulfate	+	4.28	381.4	381	301	35	20	
Petunidin								
Arabinoside	+	2.13	449.4	449	317	40	22	Petunidin-3-glucoside
Galactoside	+	2.05	479.4	479	317	40	22	
Glucoside	+	2.29	479.4	479	317	40	22	
Glucuronide	+	4.02	493.4	493	317	40	22	
Sulfate	+	4.30	397.4	397	317	40	22	
Phenolic Acids								
Benzaldehydes (BALD)								
Isovanillin	-	2.50	150.2	151	92, 136	24	22, 12	Isovanillin
Benzoic acids (BzA)								
Protocatechuic acid	-	0.86	154.1	153	81, 91	28	18, 24	Protocatechuic acid
Gallic acid	-	0.69	170.1	169	79, 97	32	22, 20	Gallic acid
Vanillic acid	-	1.82	168.1	167	108, 152	28	20, 12	Vanillic acid
Syringic acid	-	2.08	198.2	197	123, 182	36	24, 14	Syringic acid
BzA glucuronide	-	3.93	298.2	297	121	30	22	4-OH-BALD

Table C.1 continued

Phenyl Acetic Acids (PAA)								
3-OH-PAA	-	3.95	152.2	151	136, 92	34	12, 18	4-OH-PAA
4-OH-PAA	-	1.74	152.2	151	107, 133	28	10	4-OH-PAA
3-OMe-PAA	-	3.86	166.2	165	106, 121	28	24, 6	3-OMe-PAA
Homovanillic acid	-	2.12	182.2	181	122	18	14	Homovanillic acid
Phenyl Propionic Acids (PPA)								
3-OH-PPA	-	3.02	166.2	165	121, 147	24	16, 12	3-OH-PPA
3-OH-4-OMe-PPA	-	3.05	196.2	195	135, 136	32	26, 12	3-OH-4-OMe-PPA
Dihydrocaffeic acid sulfate	-	1.70	264.2	261	137, 181	28	20, 15	Caffeic acid
<i>trans</i> -Cinnamic Acids								
<i>p</i> -Coumaric acid	-	2.41	164	163	93, 120	28	28, 32	<i>p</i> -Coumaric acid
Caffeic acid	-	1.81	180.2	179	107, 117	32	22, 24	Caffeic acid
Caffeic acid sulfate ^b	-	1.68	260.2	259	135, 179	28	20, 15	Caffeic acid
Caffeic acid glucuronide	-	3.83	356.2	355	179	30	18	Caffeic acid
Ferulic acid	-	3.20	194.2	193	134, 178	30	18, 12	Ferulic acid
Ferulic acid glucuronide ^c	-	3.65, 4.18	370.2	369	193	30	22	Ferulic acid
Isoferulic acid	-	3.92	194.2	193	134, 178	30	18, 12	Ferulic acid
Isoferulic acid glucuronide	-	1.40	370.2	369	178, 193	30	18, 12	Ferulic acid
(Iso)Ferulic acid sulfate ^d	-	1.75, 2.00	274.2	273	178, 193	30	18, 12	Ferulic acid
Chlorogenic acid	-	1.70	354.3	353	85, 191	26	40, 18	Chlorogenic acid
Feruloylquinic acid	-	2.64	368.3	367	134, 191	30	40, 20	Ferulic acid
Ethyl gallate	-	2.80	198.2	197	124, 125	30	30	Ethyl gallate
Taxifolin	-	3.40	304.3	303	125, 177	34	22, 14	Taxifolin
Catechins								
Catechin	-	1.40	290.3	289	109, 123	33	24, 26	Catechin
Epicatechin	-	2.11	290.3	289	109, 123	33	24, 26	Epicatechin
(Epi)gallocatechin peak 1	-	0.80	306.3	305	125, 179	40	20, 14	Catechin
(Epi)gallocatechin peak 2	-	1.00	306.3	305	125, 179	40	20, 14	Catechin
Flavonols								
Quercetin	-	4.29	302.2	301	151, 179	38	20, 18	Quercetin
Kaempferol	-	4.52	286.2	285	146, 151	58	30, 18	Quercetin
Myricetin	-	3.84	318.2	317	151, 179	46	26, 20	Myricetin
Kaempferol-3-glycs ^e	-	1.40	448.4	447	284	42	18	Quercetin-3-glucoside
Quercetin-3-glycs	-	3.39	464.1	463	271, 300	42	40, 24	Quercetin-3-glucoside
Quercetin-3-rutinoside	-	3.41	610.2	609	300	42	24	Quercetin
Quercetin glucuronide ^f	-	3.54, 3.80, 4.01	478.1	477	151, 300	42	38, 22	Quercetin glucuronide
4-OMe-quercetin	-	4.62	316.2	315	151, 300	28	32, 20	4-OMe-quercetin
Myricetin glucuronide	-	3.58	494.4	493	151, 317	46	26, 20	Myricetin

Table C.1 continued

Hippuric Acids								
Hippuric acid	-	1.80	179.2	178	56, 77	28	12, 16	Hippuric acid
3-OH-hippuric acid	-	1.20	195.2	194	93, 121	32	22, 24	3-OH-hippuric acid

MRM = Multiple Reaction Monitoring; RT = retention time; MW = molecular weight; CV = cone voltage; CE = collision energy.

^a Daughter fragments monitored; fragment listed in italics used for quantitation.

^b Three overlapping peaks were not separable, so quantitated as sum of all three peaks.

^c Two distinct peaks were observed at this MRM, representing different isomers of ferulic acid glucuronide. They are quantitated separately as peaks 1 and 2 in the results.

^d Two distinct peaks appeared at this MRM. Based on our methods, we are unable to determine which corresponds to ferulic acid sulfate and which corresponds to isoferulic acid sulfate, thus they are reported as peak 1 and peak 2 in results.

^e Galactoside and glucoside peaks overlapped and were not distinguishable, so quantitated as sum of both peaks.

^f Three distinct peaks were observed at this MRM, representing different isomers of quercetin glucuronide. They are quantitated separately as peaks 1, 2, and 3.

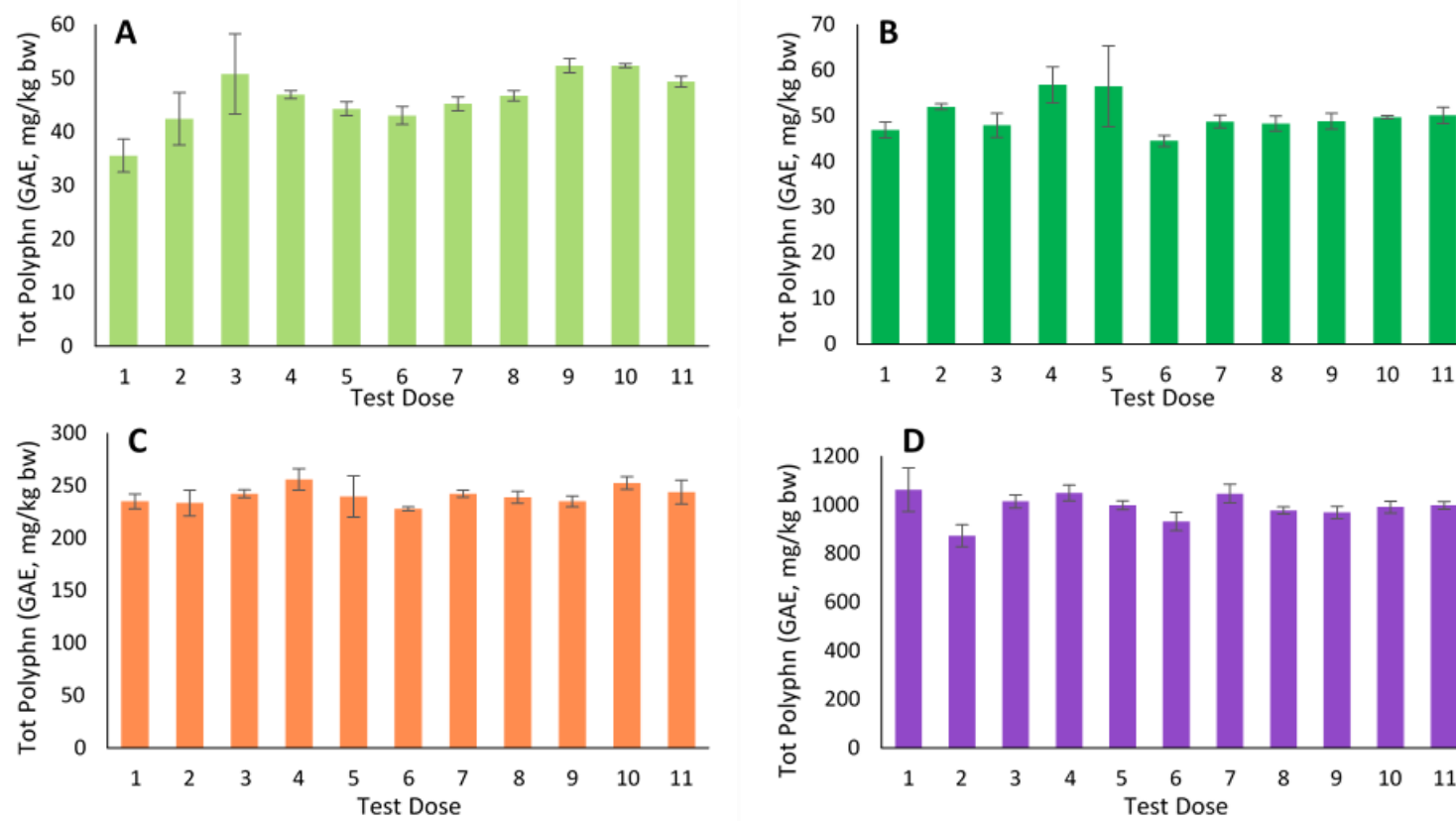


Figure C. 1 – Total polyphenols in oral gavage doses over time. Doses were randomly sampled from all doses at least once every 10 days. Results for A) lyophilized blueberry, B) low, C) medium, and D) high dose groups shown; water dose was also tested at each time point, but results confirmed the absence of polyphenols, so not shown here.

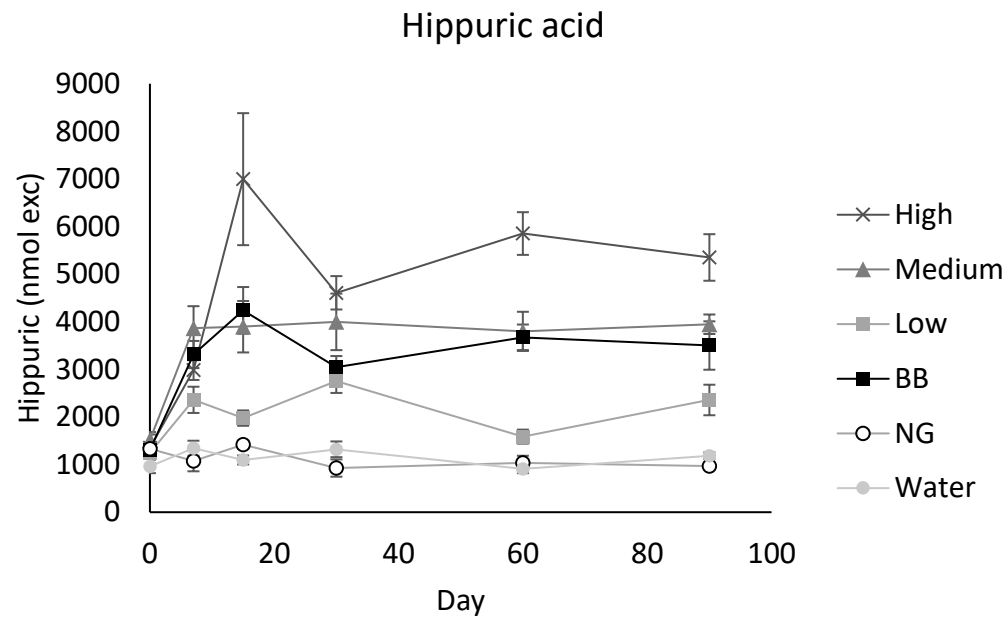


Figure C. 2 – Urinary excretion of hippuric acid over 90 days.

	Day					
	0	7	15	30	60	90
Water	963 ± 453	1345 ± 497 ^b	1098 ± 297 ^d	1322 ± 496 ^c	911 ± 286 ^d	1187 ± 211 ^c
Low	1247 ± 419 ^C	2361 ± 871 ^{a,A}	1977 ± 510 ^{c,AB}	2755 ± 788 ^{b,A}	1588 ± 465 ^{c,BC}	2358 ± 1014 ^{b,AB}
Medium	1518 ± 501 ^B	3867 ± 1383 ^{a,A}	3896 ± 1529 ^{b,A}	3998 ± 1781 ^{ab,A}	3799 ± 1164 ^{b,A}	3948 ± 579 ^{a,A}
High	1352 ± 380 ^C	2986 ± 623 ^{a,B}	6995 ± 4158 ^{a,A}	4609 ± 1052 ^{a,A}	5854 ± 1418 ^{a,A}	5351 ± 1383 ^{a,A}
BB	1310 ± 485 ^B	3312 ± 856 ^{a,A}	4239 ± 1471 ^{ab,A}	3045 ± 704 ^{ab,A}	3674 ± 805 ^{ab,A}	3501 ± 1245 ^{ab,A}
NG	1329 ± 608	1079 ± 438 ^b	1419 ± 175 ^{cd}	928 ± 363 ^c	1035 ± 303 ^{cd}	968 ± 124 ^c

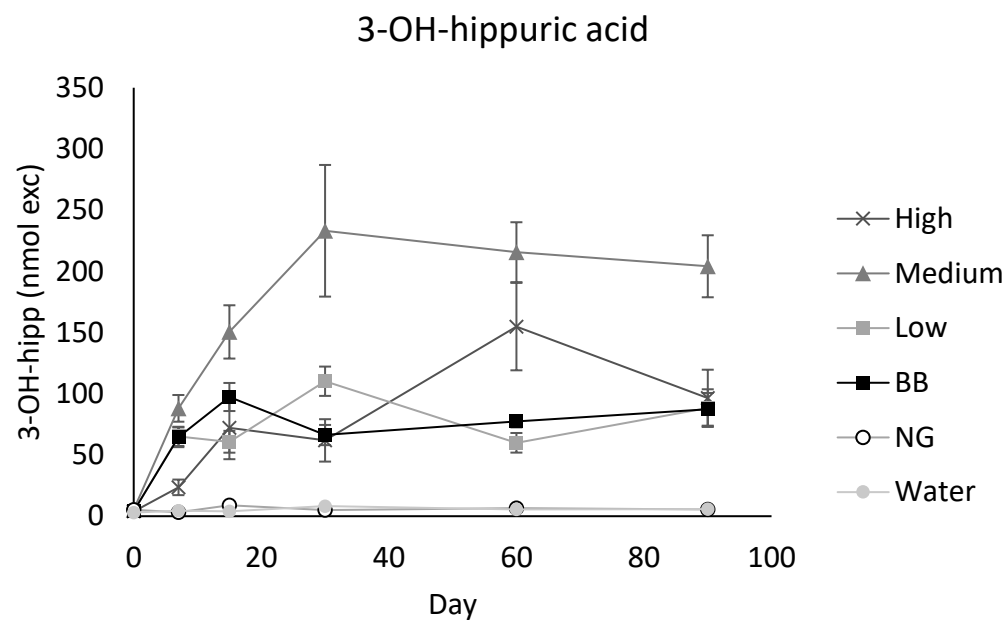


Figure C. 3 – Urinary excretion of 3-hydroxyhippuric acid over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	trace	trace	trace	trace	trace
Medium	nd	5.54 ± 2.60^b	4.76 ± 2.15^b	6.58 ± 3.71^b	6.30 ± 3.27^b	7.28 ± 2.75^b
High	nd	$7.31 \pm 3.59^{a,B}$	$13.4 \pm 5.75^{a,A}$	$10.6 \pm 4.28^{a,AB}$	$13.7 \pm 4.12^{a,A}$	$15.8 \pm 9.89^{a,A}$
BB	nd	trace	trace	trace	trace	trace
NG	nd	nd	nd	nd	nd	nd

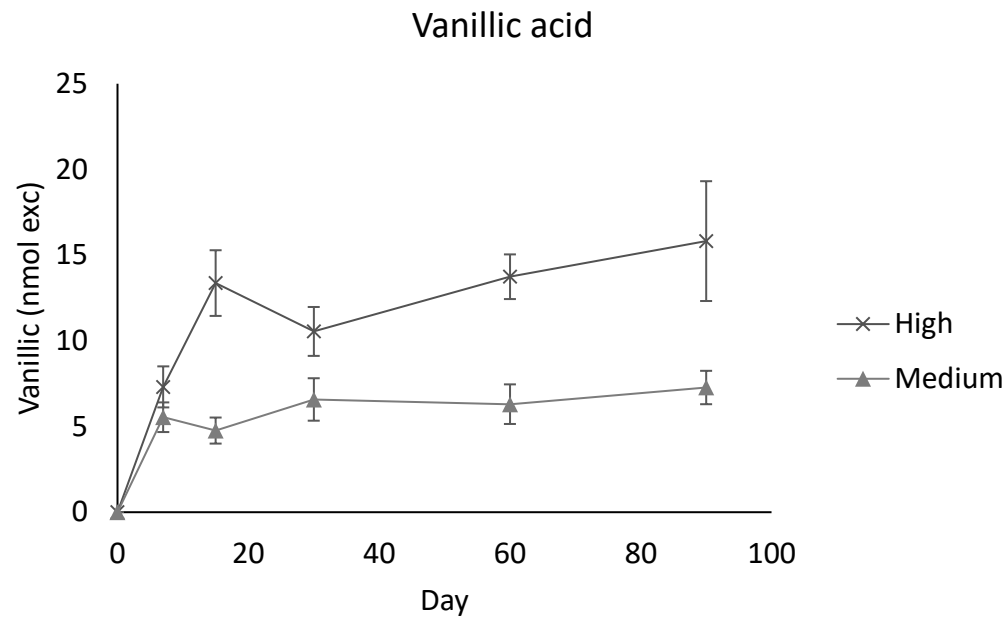


Figure C. 4 – Urinary excretion of vanillic acid over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	trace	trace	trace	trace	trace
Medium	nd	5.54 ± 2.60 ^b	4.76 ± 2.15 ^b	6.58 ± 3.71 ^b	6.30 ± 3.27 ^b	7.28 ± 2.75 ^b
High	nd	7.31 ± 3.59 ^{a,B}	13.4 ± 5.75 ^{a,A}	10.6 ± 4.28 ^{a,AB}	13.7 ± 4.12 ^{a,A}	15.8 ± 9.89 ^{a,A}
BB	nd	trace	trace	trace	trace	trace
NG	nd	nd	nd	nd	nd	nd

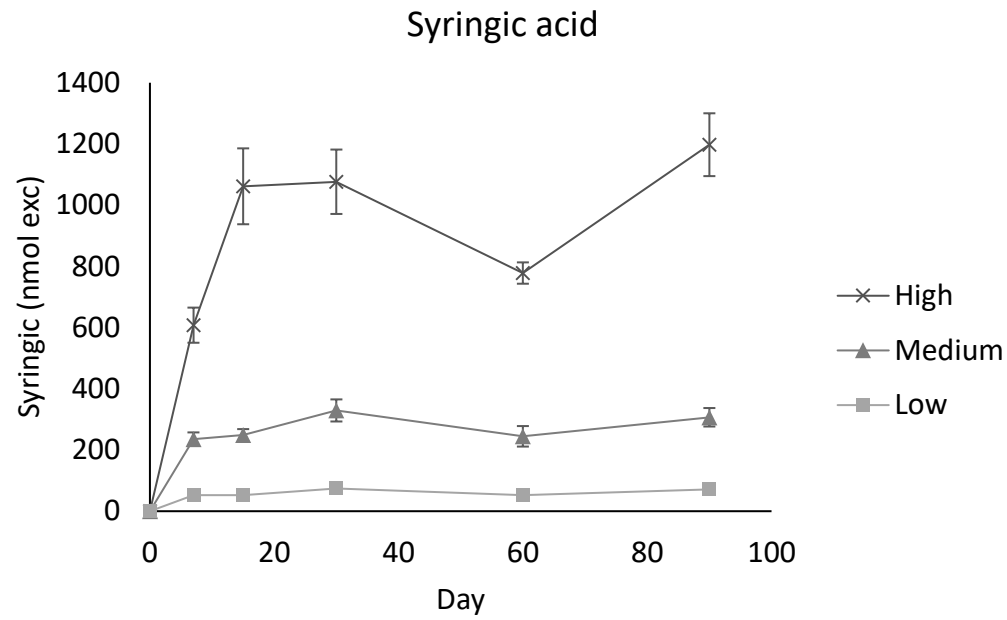


Figure C. 5 – Urinary excretion of syringic acid over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	52.8 ± 8.82 ^{c,AB}	52.1 ± 13.4 ^{c,AB}	74.3 ± 29.2 ^{c,A}	52.7 ± 19.9 ^{c,B}	71.6 ± 27.0 ^{c,AB}
Medium	nd	236 ± 64.6 ^b	249 ± 54.9 ^b	329 ± 108 ^b	245 ± 94.7 ^b	307 ± 85.3 ^b
High	nd	608 ± 172 ^{a,C}	1062 ± 372 ^{a,AB}	1076 ± 316 ^{a,AB}	778 ± 110 ^{a,B}	1197 ± 290 ^{a,A}
BB	nd	trace	trace	trace	trace	trace
NG	nd	nd	nd	nd	nd	nd

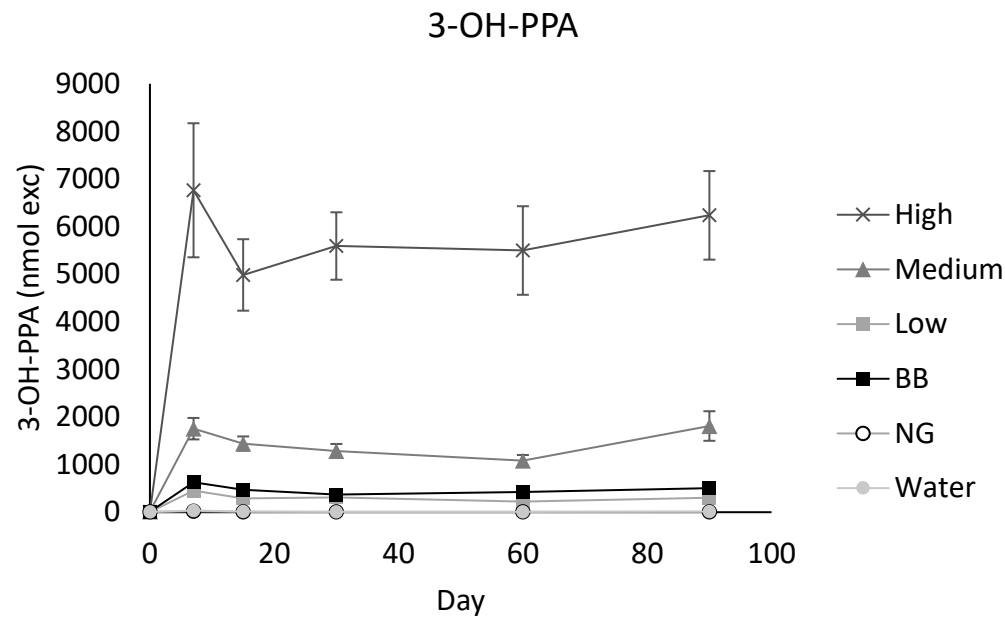


Figure C. 6 – Urinary excretion of 3-hydroxyphenylpropionic acid over 90 days.

	Day					
	0	7	15	30	60	90
Water	5.70 ± 3.18	35.3 ± 36.8 ^d	12.3 ± 5.07 ^d	10.4 ± 4.50 ^d	7.05 ± 1.67 ^d	12.5 ± 5.41 ^d
Low	5.98 ± 3.39 ^C	454 ± 132 ^{c,A}	290 ± 107 ^{c,AB}	311 ± 109 ^{c,AB}	221 ± 70.5 ^{c,B}	305 ± 60.8 ^{c,AB}
Medium	12.0 ± 7.35 ^B	1755 ± 677 ^{b,A}	1442 ± 422 ^{b,A}	1287 ± 441 ^{b,A}	1081 ± 341 ^{b,A}	1811 ± 879 ^{b,A}
High	7.46 ± 6.26 ^B	6762 ± 4224 ^{a,A}	4984 ± 2253 ^{a,A}	5591 ± 2124 ^{a,A}	5497 ± 2941 ^{a,A}	6235 ± 2631 ^{a,A}
BB	10.4 ± 9.14 ^B	629 ± 228 ^{c,A}	470 ± 99.0 ^{c,A}	370 ± 58.1 ^{c,A}	427 ± 169 ^{c,A}	507 ± 255 ^{c,A}
NG	7.82 ± 5.62	21.3 ± 19.4 ^d	10.6 ± 2.81 ^d	8.40 ± 4.89 ^d	10.5 ± 2.80 ^d	11.2 ± 4.18 ^d

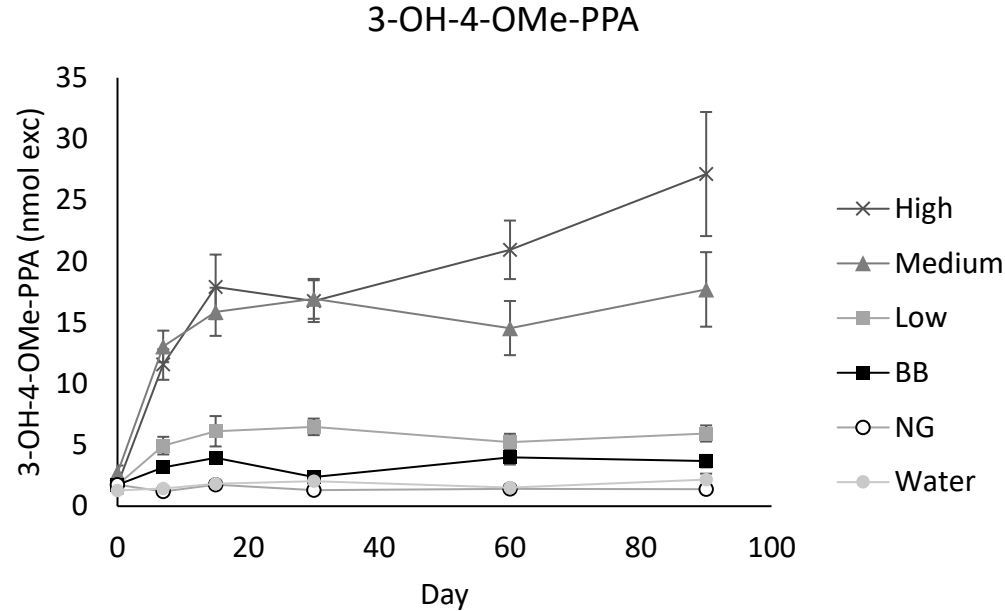


Figure C. 7 – Urinary excretion of 3-hydroxy-4-methoxyphenylpropionic acid over 90 days.

	Day					
	0	7	15	30	60	90
Water	1.29 ± 0.83	1.44 ± 0.59 ^c	1.84 ± 0.81 ^c	2.05 ± 0.72 ^c	1.51 ± 0.86 ^c	2.18 ± 1.45 ^c
Low	1.74 ± 1.2 ^B	4.95 ± 2.28 ^{b,A}	6.12 ± 3.92 ^{b,A}	6.48 ± 2.14 ^{b,A}	5.24 ± 2.14 ^{b,A}	5.94 ± 2.08 ^{b,A}
Medium	2.72 ± 1.82 ^B	13.05 ± 3.87 ^{a,A}	15.88 ± 5.56 ^{a,A}	16.94 ± 4.88 ^{a,A}	14.55 ± 6.25 ^{a,A}	17.71 ± 8.61 ^{a,A}
High	1.75 ± 0.5 ^C	11.57 ± 3.74 ^{a,B}	17.92 ± 7.92 ^{a,AB}	16.75 ± 5.12 ^{a,AB}	20.94 ± 7.56 ^{a,A}	27.13 ± 14.33 ^{a,A}
BB	1.74 ± 0.83 ^B	3.17 ± 0.9 ^{b,A}	3.96 ± 0.89 ^{b,A}	2.39 ± 0.59 ^{c,A}	3.99 ± 1.76 ^{b,A}	3.68 ± 1.25 ^{bc,A}
NG	1.75 ± 0.59	1.2 ± 0.4 ^c	1.76 ± 0.61 ^c	1.31 ± 0.4 ^c	1.42 ± 0.35 ^a	1.4 ± 0.4 ^c

Dihydrocaffeic acid sulfate

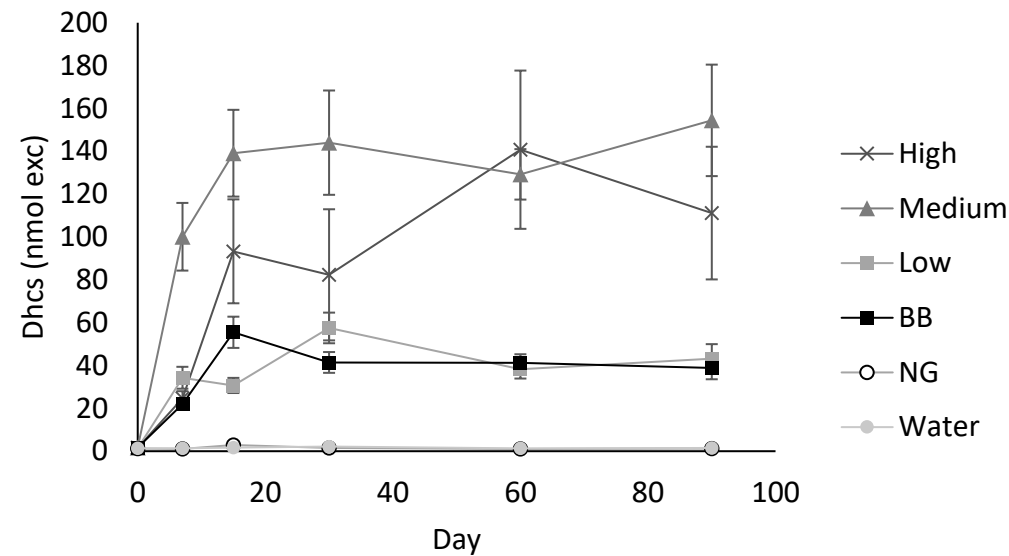


Figure C. 8 – Urinary excretion of dihydrocaffeic acid sulfate over 90 days.

	Day					
	0	7	15	30	60	90
Water	1.36 ± 1.05	1.4 ± 0.88 ^c	1.7 ± 0.66 ^d	2.18 ± 1.06 ^c	1.33 ± 0.54 ^c	1.49 ± 0.48 ^c
Low	1.36 ± 0.65 ^C	34.29 ± 16.15 ^{b,AB}	30.7 ± 11.17 ^{c,B}	57.54 ± 22.61 ^{b,A}	38.3 ± 13.83 ^{b,AB}	43.17 ± 21.54 ^{b,AB}
Medium	1.72 ± 1.03 ^B	100 ± 47.38 ^{a,A}	139 ± 57.28 ^{a,A}	144 ± 73.04 ^{a,A}	129 ± 33.3 ^{a,A}	154 ± 73.59 ^{a,A}
High	1.6 ± 0.75 ^D	24.72 ± 9.87 ^{b,C}	93.3 ± 72.75 ^{ab,AB}	82.33 ± 91.81 ^{b,B}	141 ± 117 ^{a,A}	111 ± 87.57 ^{ab,AB}
BB	1.72 ± 0.6 ^C	22.01 ± 7.51 ^{b,B}	55.51 ± 21.85 ^{bc,A}	41.44 ± 14.56 ^{b,AB}	41.26 ± 12.0 ^{b,AB}	38.79 ± 12.75 ^{b,AB}
NG	1.21 ± 1.05	1.13 ± 0.14 ^c	2.91 ± 0.26 ^d	1.51 ± 0.92 ^c	1.03 ± 0.36 ^c	1.15 ± 0.51 ^c

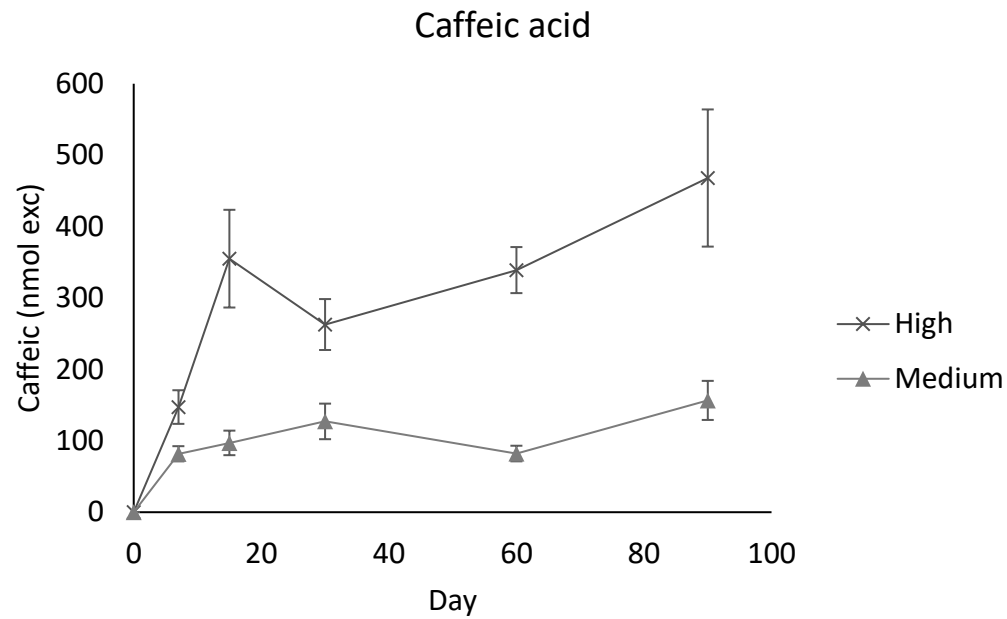


Figure C. 9 – Urinary excretion of caffeic acid over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	trace	trace	trace	trace	trace
Medium	nd	81.8 ± 31.9 ^b	97 ± 48.6 ^b	127.2 ± 74.7 ^b	82.2 ± 30.9 ^b	156.6 ± 77.4 ^b
High	nd	147.3 ± 70.6 ^{a,B}	355 ± 205.2 ^{a,A}	262.8 ± 106.9 ^{a,AB}	339 ± 102 ^{a,A}	468 ± 271.6 ^{a,A}
BB	nd	trace	trace	trace	trace	trace
NG	nd	nd	nd	nd	nd	nd

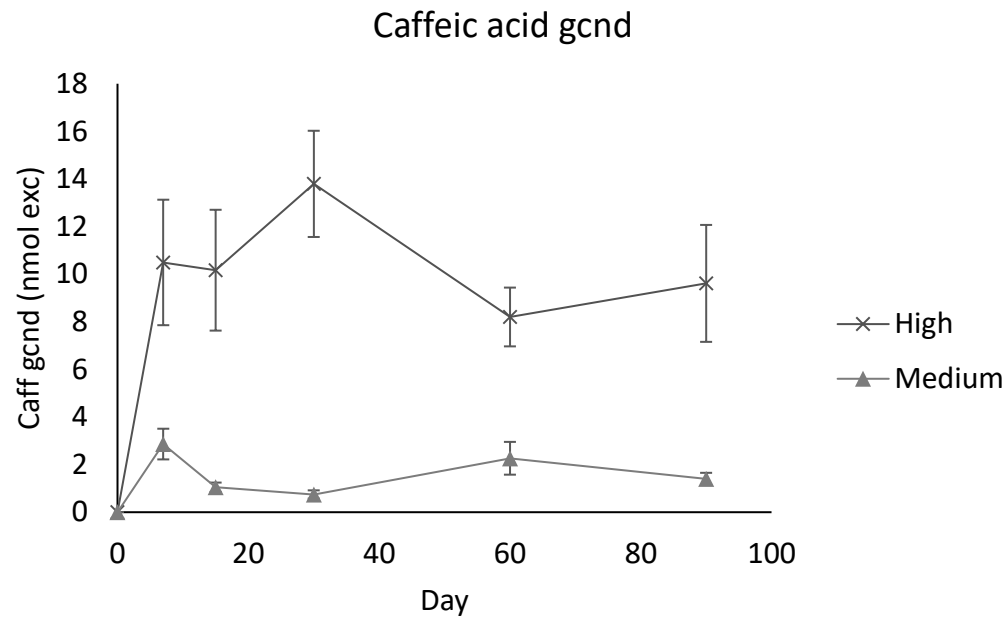


Figure C. 10 – Urinary excretion of caffeic acid glucuronide over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	nd	nd	nd	nd	nd
Medium	nd	2.86 ± 1.93 ^{b,A}	1.05 ± 0.56 ^{b,AB}	0.75 ± 0.52 ^{b,B}	2.27 ± 1.95 ^{b,AB}	1.4 ± 0.71 ^{b,AB}
High	nd	10.49 ± 7.91 ^a	10.17 ± 7.61 ^a	13.79 ± 6.69 ^a	8.2 ± 3.9 ^a	9.61 ± 6.95 ^a
BB	nd	nd	nd	nd	nd	nd
NG	nd	nd	nd	nd	nd	nd

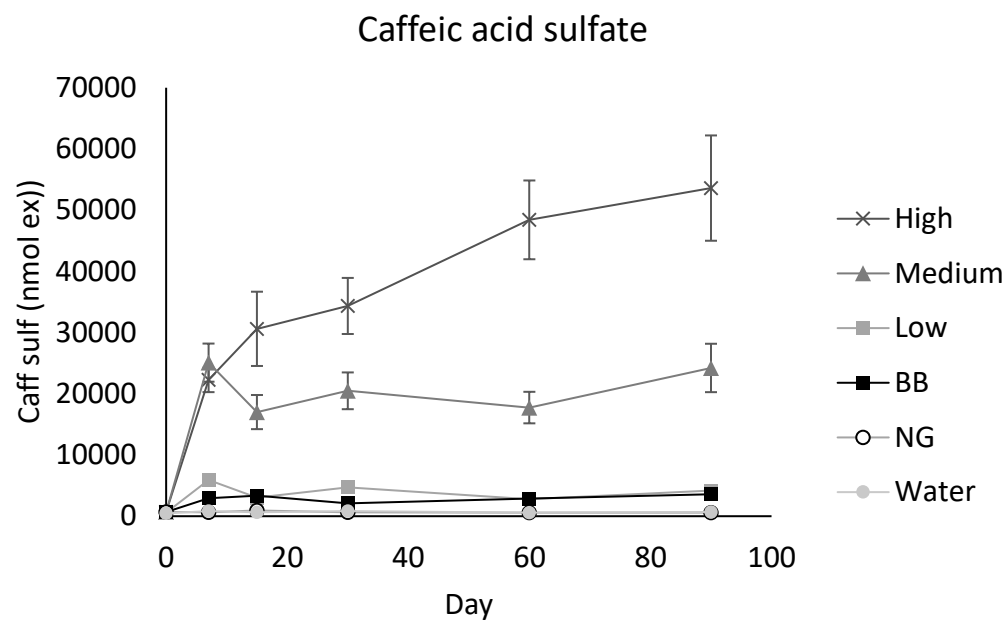


Figure C. 11 – Urinary excretion of caffeic acid sulfate over 90 days.

	Day					
	0	7	15	30	60	90
Water	457 ± 211	822 ± 332 ^d	620 ± 163 ^c	836 ± 64 ^d	595 ± 97 ^d	713 ± 102 ^d
Low	581 ± 252 ^D	5919 ± 2138 ^{b,A}	3064 ± 904 ^{b,BC}	4721 ± 1736 ^{b,AB}	2768 ± 1014 ^{c,C}	4203 ± 1745 ^{c,ABC}
Medium	665 ± 251 ^B	25089 ± 9344 ^{a,A}	17014 ± 7887 ^{a,A}	20487 ± 9008 ^{a,A}	17749 ± 7281 ^{b,A}	24222 ± 11182 ^{b,A}
High	707 ± 157 ^C	22268 ± 5987 ^{a,B}	30611 ± 18198 ^{a,B}	34349 ± 13737 ^{a,AB}	48414 ± 20353 ^{a,A}	53605 ± 24325 ^{a,A}
BB	685 ± 291 ^B	2914 ± 818 ^{c,A}	3346 ± 959 ^{b,A}	2123 ± 698 ^{c,A}	2870 ± 301 ^{c,A}	3585 ± 1554 ^{c,A}
NG	670 ± 380	615 ± 236 ^d	941 ± 208 ^c	642 ± 107 ^d	579 ± 176 ^d	593 ± 151 ^d

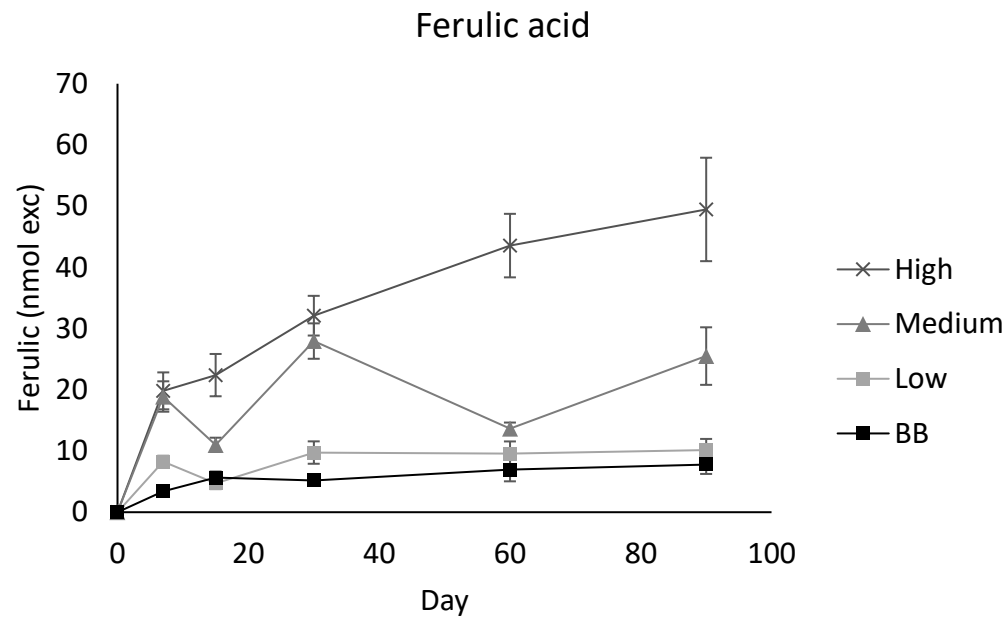


Figure C. 12 – Urinary excretion of ferulic acid over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	8.3 ± 2.92 ^{b,A}	4.71 ± 2.95 ^{b,B}	9.75 ± 5.78 ^{b,A}	9.59 ± 6.24 ^{bc,A}	10.15 ± 5.76 ^{b,A}
Medium	nd	18.9 ± 7.45 ^{a,ABC}	11.01 ± 3.3 ^{a,C}	27.96 ± 8.68 ^{a,A}	13.65 ± 2.82 ^{b,BC}	25.51 ± 13.3 ^{a,AB}
High	nd	19.83 ± 9.05 ^{a,B}	22.4 ± 10.37 ^{a,B}	32.11 ± 9.73 ^{a,AB}	43.56 ± 16.41 ^{a,A}	49.46 ± 23.9 ^{a,A}
BB	nd	3.42 ± 1.16 ^{c,B}	5.65 ± 3.03 ^{b,AB}	5.18 ± 2.07 ^{b,AB}	6.94 ± 5.69 ^{c,AB}	7.78 ± 3.74 ^{b,A}
NG	nd	nd	nd	nd	nd	nd

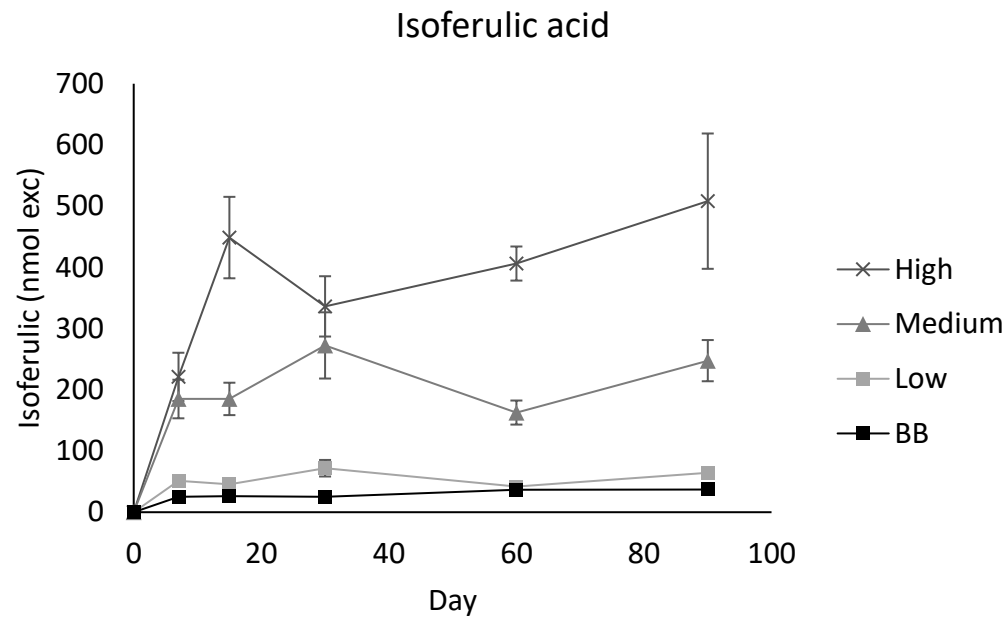


Figure C. 13 – Urinary excretion of isoferulic acid over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	51.4 ± 27.7 ^b	45.8 ± 24 ^c	71.9 ± 42.8 ^b	42 ± 19.5 ^c	64.3 ± 11.7 ^b
Medium	nd	184.9 ± 94.6 ^a	185.1 ± 74.9 ^b	272.5 ± 162.2 ^a	162.8 ± 55.6 ^b	247.6 ± 95.2 ^a
High	nd	221.1 ± 118.4 ^{a,B}	448.7 ± 199.5 ^{a,A}	336.3 ± 147.7 ^{a,AB}	406.3 ± 88 ^{a,A}	508.2 ± 312.6 ^{a,A}
BB	nd	25.3 ± 12.8 ^c	26.4 ± 14 ^c	25 ± 7.8 ^c	36.5 ± 18.8 ^c	37.1 ± 19.2 ^b
NG	nd	nd	nd	nd	nd	nd

Ferulic acid gcnd pk 1

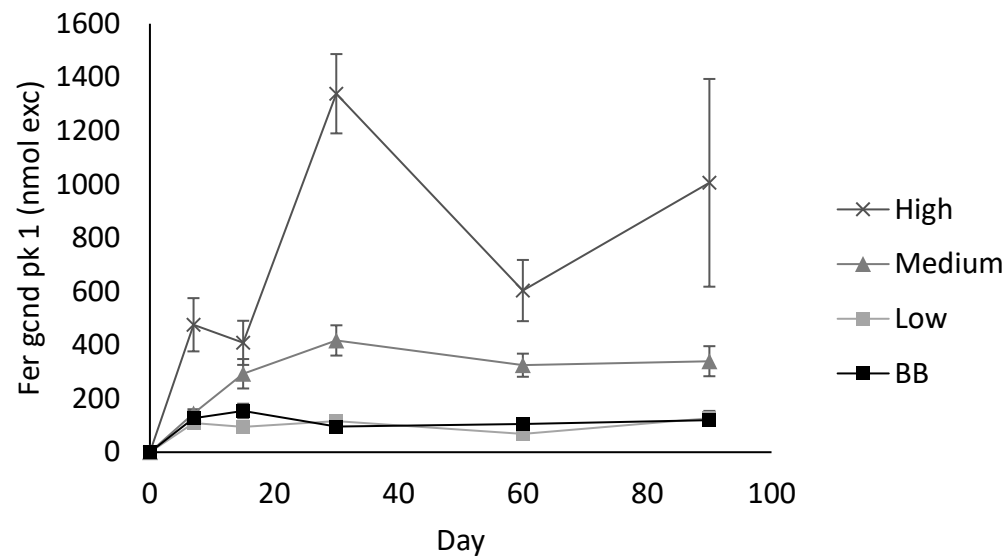


Figure C. 14 – Urinary excretion of ferulic acid glucuronide peak 1 over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	108.5 ± 47.1 ^b	94.3 ± 39.2 ^c	115.7 ± 56.4 ^a	68 ± 27.8 ^b	127 ± 86.2 ^b
Medium	nd	144.6 ± 47.6 ^{b,B}	293 ± 155.6 ^{ab,AB}	417.4 ± 169.6 ^{b,A}	324.9 ± 122.1 ^{a,A}	339.8 ± 158.7 ^{a,A}
High	nd	475.9 ± 298.3 ^{a,B}	408.5 ± 247.2 ^{a,B}	1338.6 ± 444.3 ^{a,A}	603.6 ± 361.4 ^{a,B}	1006 ± 1097 ^{a,B}
BB	nd	126.9 ± 80.3 ^b	154.2 ± 80.7 ^{bc}	95.4 ± 34.2 ^a	105.5 ± 30.7 ^b	120.3 ± 42.4 ^b
NG	nd	nd	nd	nd	nd	nd

Ferulic acid gcnd pk 2

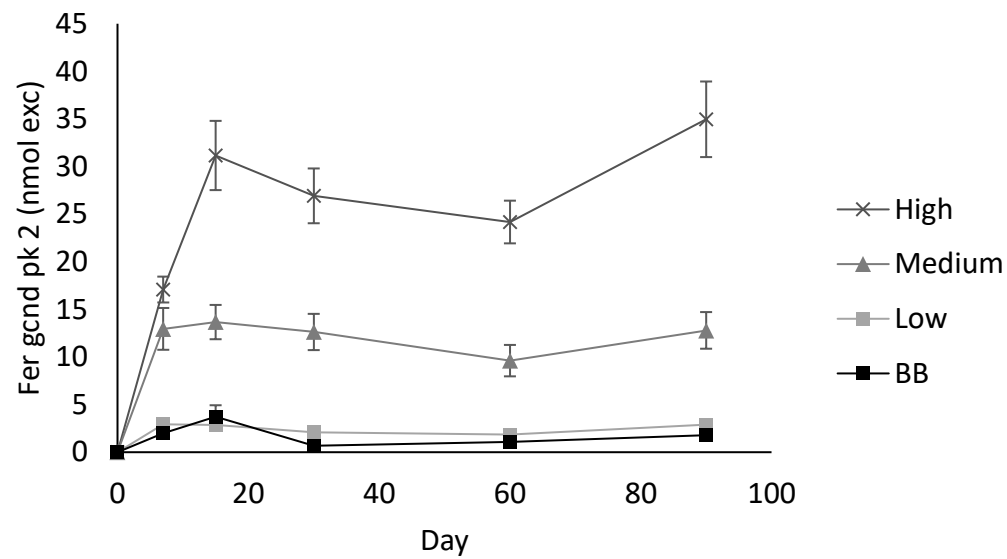


Figure C. 15 – Urinary excretion of ferulic acid glucuronide peak 2 over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	2.93 ± 1.17 ^b	2.87 ± 1.31 ^c	2.11 ± 1.46 ^c	1.85 ± 0.9 ^c	2.89 ± 1.28 ^c
Medium	nd	12.95 ± 6.59 ^a	13.67 ± 5.09 ^b	12.63 ± 5.71 ^b	9.62 ± 4.67 ^b	12.79 ± 5.44 ^b
High	nd	17.08 ± 4.07 ^a	31.16 ± 10.91 ^a	26.92 ± 8.63 ^a	24.18 ± 7.09 ^a	34.97 ± 11.23 ^a
BB	nd	2.0 ± 1.31 ^{b,AB}	3.73 ± 3.61 ^{c,A}	0.68 ± 0.45 ^{d,C}	1.08 ± 0.52 ^{c,BC}	1.78 ± 1.02 ^{c,AB}
NG	nd	nd	nd	nd	nd	nd

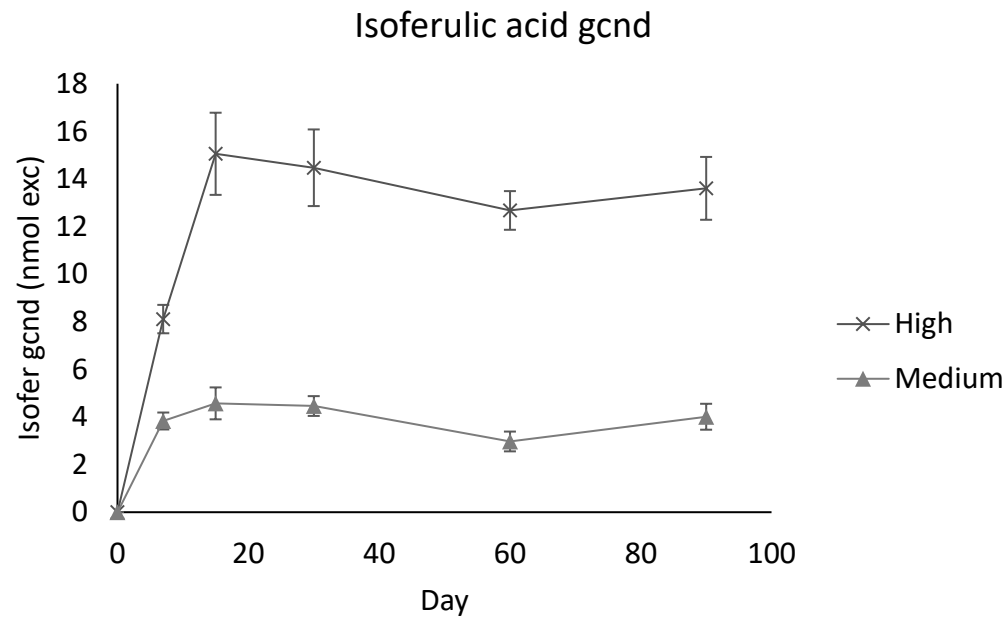


Figure C. 16 – Urinary excretion of isoferulic acid glucuronide over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	trace	trace	trace	trace	trace
Medium	nd	3.83 ± 1.07 ^{b,AB}	4.57 ± 1.89 ^{b,A}	4.46 ± 1.25 ^{b,A}	2.97 ± 1.18 ^{b,B}	4.01 ± 1.54 ^{b,AB}
High	nd	8.11 ± 1.79 ^{a,B}	15.06 ± 5.18 ^{a,A}	14.47 ± 4.83 ^{a,A}	12.68 ± 2.57 ^{a,A}	13.61 ± 3.73 ^{a,A}
BB	nd	trace	trace	trace	trace	trace
NG	nd	nd	nd	nd	nd	nd

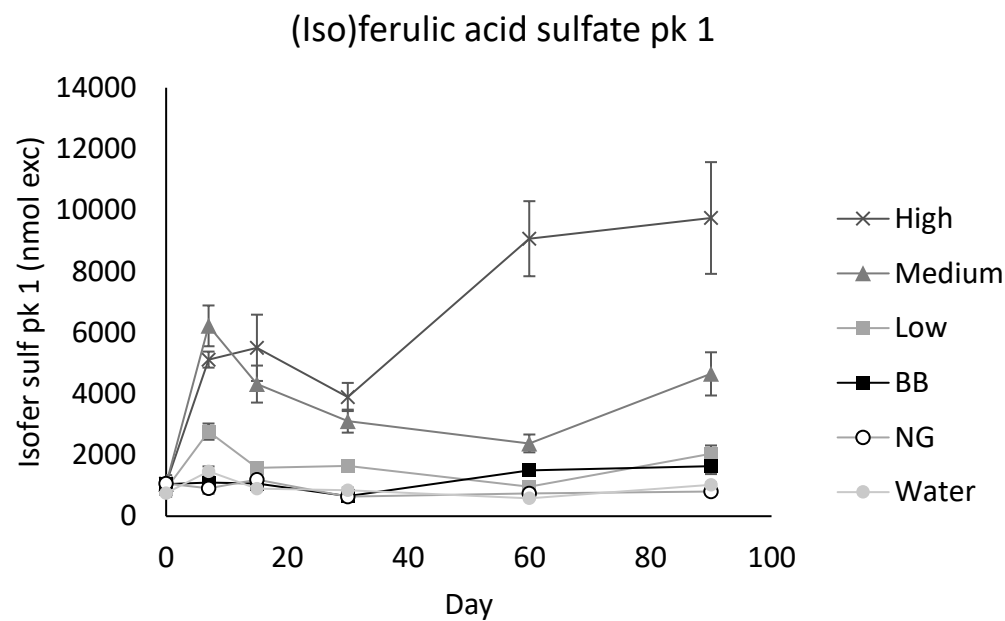


Figure C. 17 – Urinary excretion of (iso)ferulic acid sulfate peak 1 over 90 days.

	Day					
	0	7	15	30	60	90
Water	753 ± 355	1467 ± 505 ^c	902 ± 217 ^c	845 ± 265 ^c	584 ± 108 ^d	1025 ± 212 ^d
Low	893 ± 355 ^C	2764 ± 839 ^{b,A}	1579 ± 428 ^{b,B}	1641 ± 452 ^{b,B}	960 ± 305 ^{cd,C}	2038 ± 869 ^{c,AB}
Medium	1055 ± 387 ^D	6220 ± 2000 ^{a,A}	4318 ± 1709 ^{a,AB}	3106 ± 1126 ^{a,BC}	2378 ± 824 ^{b,C}	4650 ± 1997 ^{b,AB}
High	1066 ± 260 ^C	5117 ± 785 ^{a,B}	5503 ± 3248 ^{a,B}	3896 ± 1380 ^{a,B}	9067 ± 3878 ^{a,A}	9744 ± 5162 ^{a,A}
BB	1050 ± 459 ^{AB}	1101 ± 204 ^{c,A}	1064 ± 243 ^{bc,A}	656 ± 238 ^{c,B}	1502 ± 124 ^{bc,A}	1635 ± 633 ^{cd,A}
NG	1067 ± 549	910 ± 347 ^c	1196 ± 171 ^{bc}	641 ± 162 ^d	740 ± 289 ^d	806 ± 151 ^d

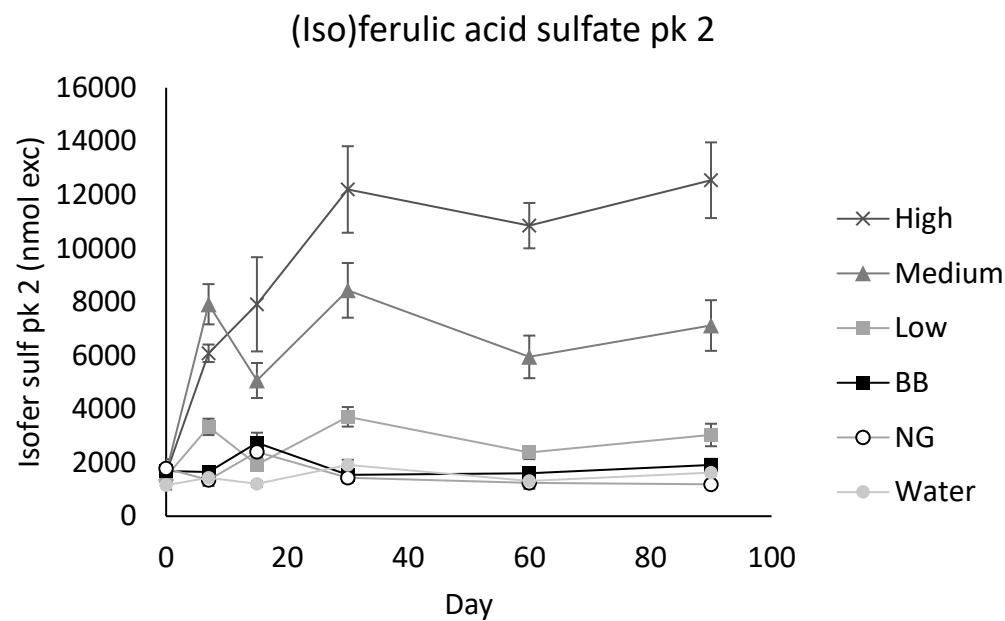


Figure C. 18 – Urinary excretion of (iso)ferulic acid sulfate peak 2 over 90 days.

	Day					
	0	7	15	30	60	90
Water	1160 ± 512	1437 ± 445 ^c	1204 ± 342 ^c	1916 ± 572 ^c	1315 ± 274 ^d	1629 ± 306 ^d
Low	1479 ± 477 ^c	3335 ± 968 ^{b,AB}	1923 ± 479 ^{b,C}	3711 ± 1154 ^{b,A}	2374 ± 766 ^{c,BC}	3033 ± 1327 ^{c,ABC}
Medium	1628 ± 415 ^c	7915 ± 2254 ^{a,A}	5068 ± 1850 ^{a,B}	8435 ± 3064 ^{a,A}	5949 ± 2253 ^{b,AB}	7120 ± 2674 ^{b,AB}
High	1603 ± 388 ^c	6084 ± 981 ^{a,B}	7913 ± 5277 ^{a,B}	12201 ± 4846 ^{a,A}	10851 ± 2678 ^{a,A}	12546 ± 3997 ^{a,A}
BB	1686 ± 632 ^B	1650 ± 303 ^{c,B}	2743 ± 1129 ^{b,A}	1544 ± 546 ^{c,B}	1607 ± 172 ^{cd,B}	1920 ± 551 ^{cd,AB}
NG	1783 ± 704	1354 ± 449 ^c	2402 ± 406 ^b	1440 ± 381 ^c	1244 ± 448 ^d	1191 ± 165 ^d

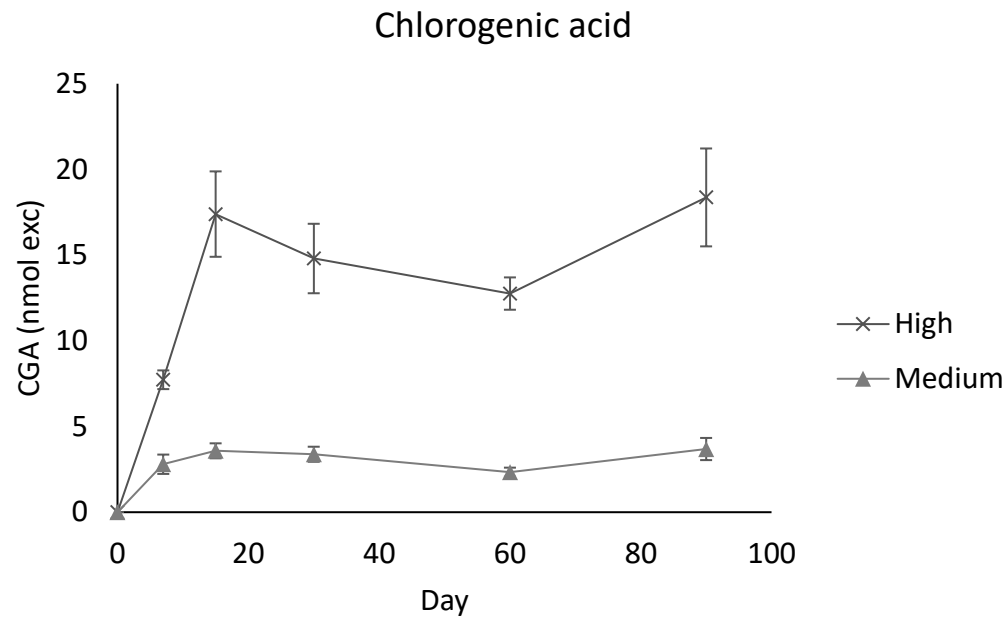


Figure C. 19 – Urinary excretion of chlorogenic acid over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	trace	trace	trace	trace	trace
Medium	nd	2.8 ± 1.69 ^b	3.58 ± 1.24 ^b	3.38 ± 1.32 ^b	2.34 ± 0.75 ^b	3.69 ± 1.83 ^b
High	nd	7.73 ± 1.64 ^{a,B}	17.39 ± 7.48 ^{a,A}	14.8 ± 6.08 ^{a,A}	12.76 ± 2.98 ^{a,A}	18.37 ± 8.08 ^{a,A}
BB	nd	trace	trace	trace	trace	trace
NG	nd	nd	nd	nd	nd	nd

Cyanidin-3-galactoside

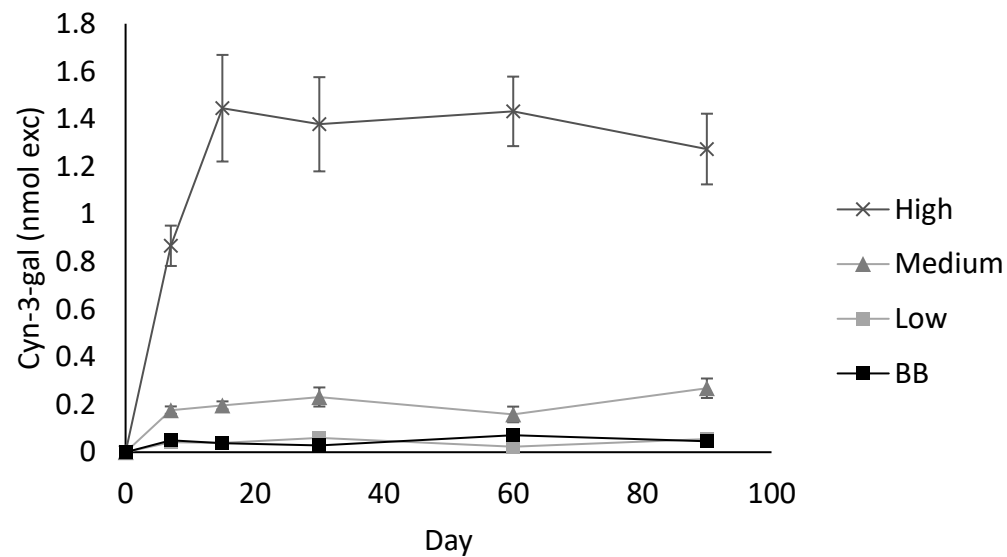


Figure C. 20 – Urinary excretion of cyanidin-3-galactoside over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	0.041 ± 0.023 ^{c,A}	0.039 ± 0.017 ^{c,A}	0.06 ± 0.032 ^{c,A}	0.023 ± 0.014 ^{c,B}	0.056 ± 0.022 ^{c,A}
Medium	nd	0.177 ± 0.047 ^b	0.197 ± 0.048 ^b	0.232 ± 0.12 ^b	0.159 ± 0.093 ^b	0.269 ± 0.116 ^b
High	nd	0.867 ± 0.254 ^a	1.445 ± 0.672 ^a	1.378 ± 0.594 ^a	1.432 ± 0.462 ^a	1.274 ± 0.42 ^a
BB	nd	0.05 ± 0.02 ^{c,A}	0.038 ± 0.017 ^{c,AB}	0.028 ± 0.017 ^{d,B}	0.071 ± 0.035 ^{b,A}	0.045 ± 0.035 ^{c,A}
NG	nd	nd	nd	nd	nd	nd

Cyanidin-3-glucoside

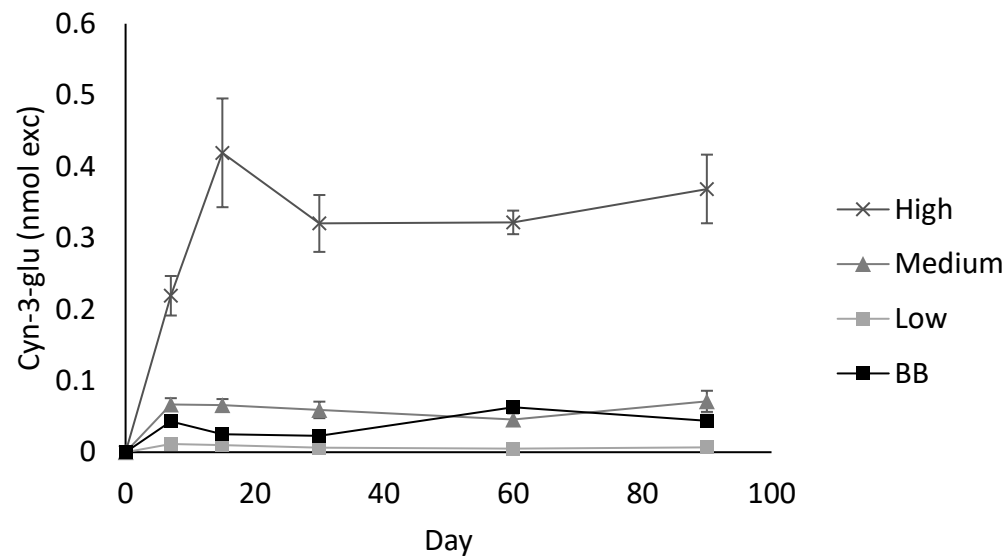


Figure C. 21 – Urinary excretion of cyanidin-3-glucoside over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	0.012 ± 0.006 ^{c,A}	0.01 ± 0.005 ^{d,AB}	0.007 ± 0.001 ^{d,AB}	0.005 ± 0.003 ^{c,B}	0.007 ± 0.005 ^{c,AB}
Medium	nd	0.067 ± 0.026 ^b	0.066 ± 0.024 ^b	0.059 ± 0.035 ^b	0.046 ± 0.024 ^b	0.071 ± 0.042 ^b
High	nd	0.219 ± 0.083 ^a	0.419 ± 0.228 ^a	0.32 ± 0.119 ^a	0.322 ± 0.052 ^a	0.369 ± 0.136 ^a
BB	nd	0.043 ± 0.019 ^{b,A}	0.025 ± 0.011 ^{c,AB}	0.023 ± 0.014 ^{c,B}	0.063 ± 0.012 ^{b,A}	0.044 ± 0.021 ^{b,A}
NG	nd	nd	nd	nd	nd	nd

Delphinidin-3-glycosides

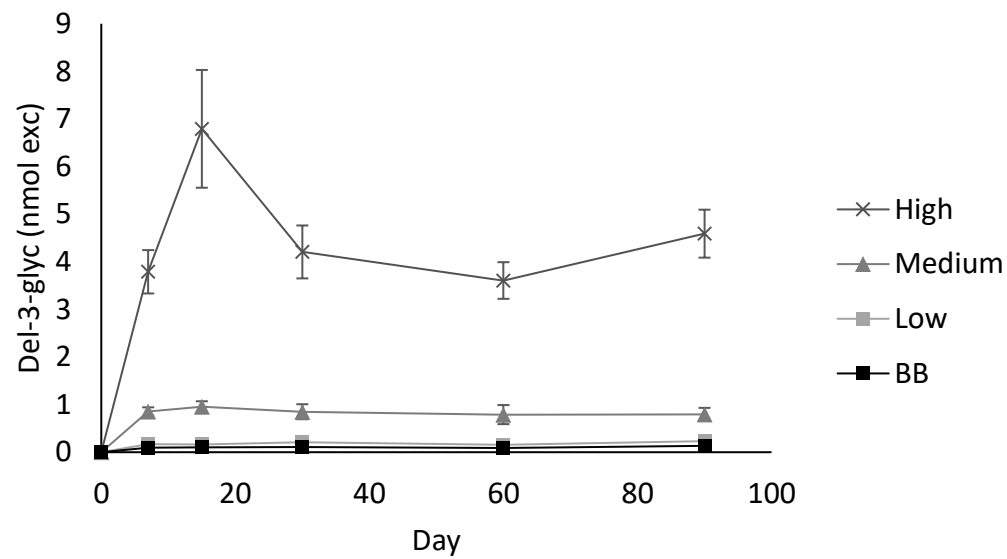


Figure C. 22 – Urinary excretion of delphinidin-3-glycosides over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	0.17 ± 0.09 ^c	0.16 ± 0.08 ^c	0.21 ± 0.12 ^c	0.16 ± 0.06 ^c	0.23 ± 0.1 ^c
Medium	nd	0.86 ± 0.26 ^b	0.96 ± 0.32 ^b	0.85 ± 0.47 ^b	0.79 ± 0.57 ^b	0.8 ± 0.38 ^b
High	nd	3.79 ± 1.37 ^a	6.79 ± 3.71 ^a	4.21 ± 1.67 ^a	3.61 ± 1.22 ^a	4.59 ± 1.43 ^a
BB	nd	0.1 ± 0.05 ^c	0.1 ± 0.08 ^c	0.11 ± 0.04 ^c	0.09 ± 0.04 ^c	0.13 ± 0.08 ^c
NG	nd	nd	nd	nd	nd	nd

Delphinidin-glucuronide

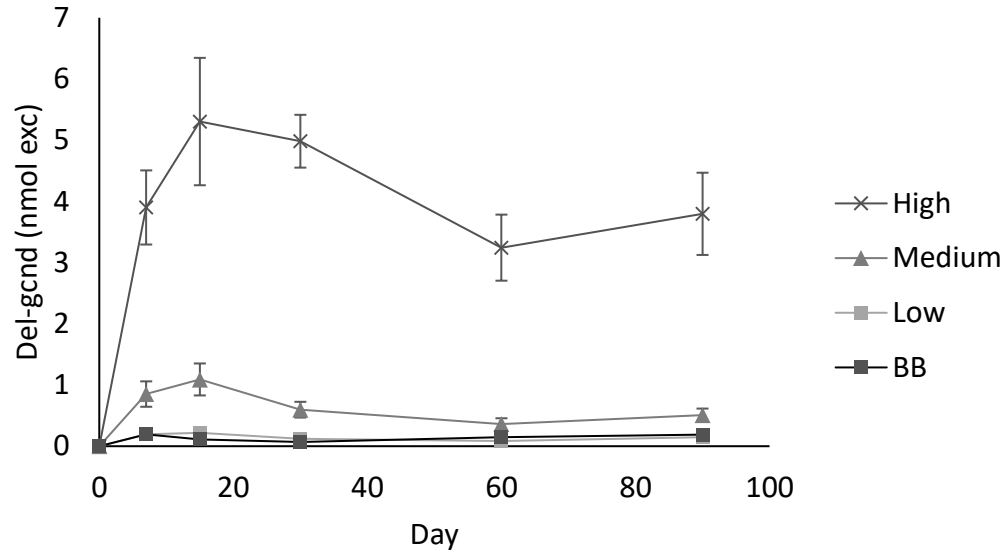


Figure C. 23 – Urinary excretion of delphinidin-3-glucuronide over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	0.19 ± 0.14 ^c	0.22 ± 0.13 ^c	0.12 ± 0.09 ^c	0.08 ± 0.06 ^c	0.15 ± 0.1 ^c
Medium	nd	0.85 ± 0.62 ^{b,AB}	1.09 ± 0.74 ^{b,A}	0.6 ± 0.39 ^{b,AB}	0.36 ± 0.27 ^{b,B}	0.51 ± 0.3 ^{b,AB}
High	nd	3.9 ± 1.82 ^a	5.3 ± 3.12 ^a	4.98 ± 1.29 ^a	3.24 ± 1.71 ^a	3.8 ± 1.9 ^a
BB	nd	0.19 ± 0.06 ^c	0.11 ± 0.09 ^c	0.07 ± 0.04 ^c	0.15 ± 0.1 ^{bc}	0.19 ± 0.12 ^{bc}
NG	nd	nd	nd	nd	nd	nd

Malvidin-3-galactoside

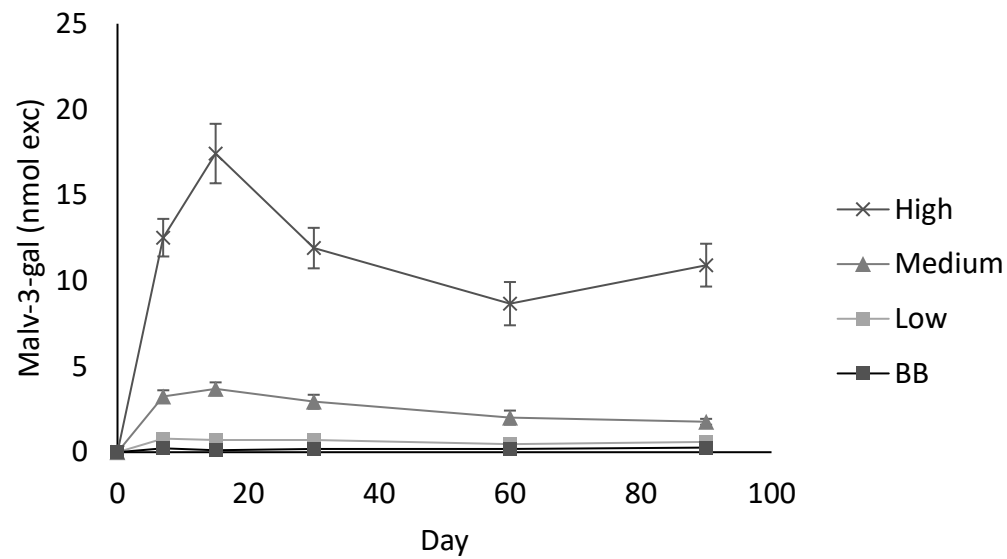


Figure C. 24 – Urinary excretion of malvidin-3-galactoside over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	0.79 ± 0.36 ^{c,A}	0.7 ± 0.3 ^{c,AB}	0.72 ± 0.52 ^{c,AB}	0.47 ± 0.25 ^{c,B}	0.61 ± 0.34 ^{c,AB}
Medium	nd	3.25 ± 1.08 ^{b,AB}	3.7 ± 1.07 ^{b,A}	2.95 ± 1.22 ^{b,AB}	2.02 ± 1.15 ^{b,B}	1.78 ± 0.48 ^{b,B}
High	nd	12.52 ± 3.29 ^{a,AB}	17.43 ± 5.2 ^{a,A}	11.91 ± 3.55 ^{a,AB}	8.67 ± 3.99 ^{a,B}	10.91 ± 3.53 ^{a,AB}
BB	nd	0.23 ± 0.1 ^{d,A}	0.12 ± 0.05 ^{d,B}	0.19 ± 0.09 ^{d,AB}	0.2 ± 0.09 ^{d,AB}	0.27 ± 0.12 ^{d,A}
NG	nd	nd	nd	nd	nd	nd

Malvidin-3-glucoside

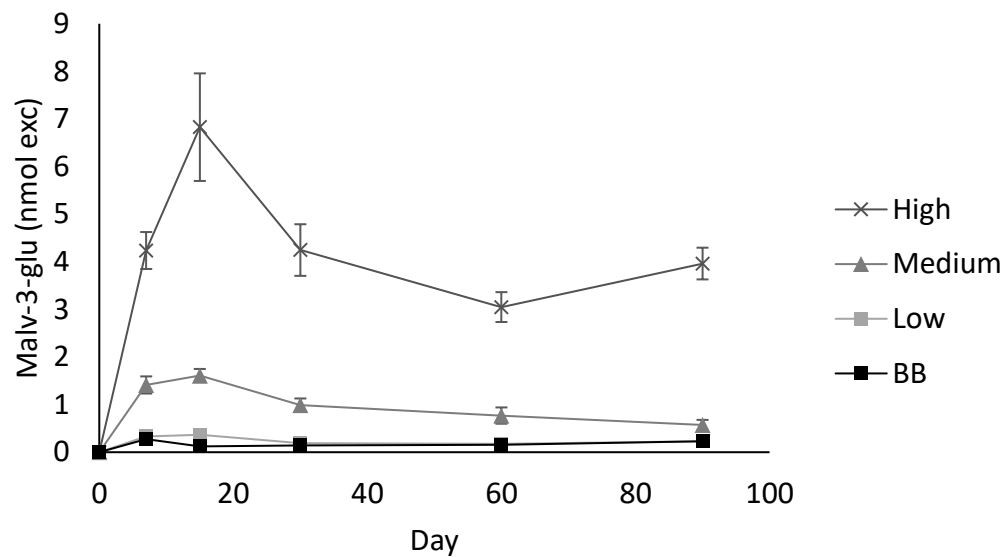


Figure C. 25 – Urinary excretion of malvidin-3-glucoside over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	0.33 ± 0.15 ^{c,AB}	0.37 ± 0.2 ^{c,A}	0.19 ± 0.14 ^{c,C}	0.18 ± 0.1 ^{c,C}	0.23 ± 0.15 ^{c,BC}
Medium	nd	1.41 ± 0.54 ^{b,AB}	1.61 ± 0.39 ^{b,A}	0.99 ± 0.41 ^{b,BC}	0.77 ± 0.48 ^{b,C}	0.57 ± 0.31 ^{b,C}
High	nd	4.24 ± 1.16 ^{a,AB}	6.83 ± 3.39 ^{a,A}	4.25 ± 1.63 ^{a,AB}	3.05 ± 0.99 ^{a,B}	3.96 ± 0.94 ^{a,AB}
BB	nd	0.27 ± 0.13 ^{c,A}	0.12 ± 0.04 ^{d,B}	0.14 ± 0.07 ^{c,B}	0.16 ± 0.06 ^{c,AB}	0.23 ± 0.1 ^{c,A}
NG	nd	nd	nd	nd	nd	nd

Peonidin-3-galactoside

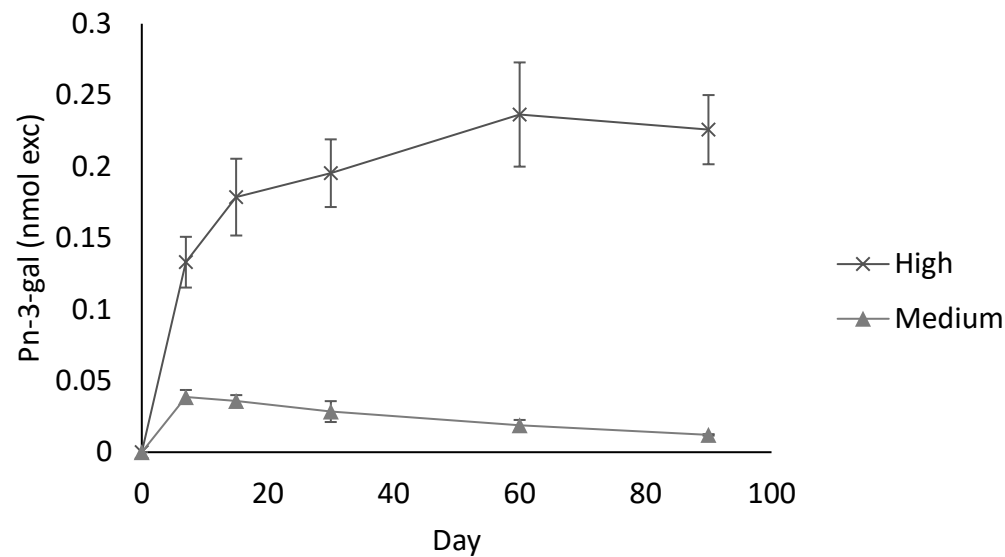


Figure C. 26 – Urinary excretion of peonidin-3-galactoside over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	trace	trace	trace	trace	trace
Medium	nd	0.039 ± 0.015 ^{b,A}	0.036 ± 0.011 ^{b,AB}	0.028 ± 0.022 ^{b,BC}	0.019 ± 0.011 ^{b,C}	0.012 ± 0.001 ^{b,C}
High	nd	0.133 ± 0.053 ^a	0.179 ± 0.081 ^a	0.195 ± 0.071 ^a	0.236 ± 0.115 ^a	0.226 ± 0.069 ^a
BB	nd	trace	trace	trace	trace	trace
NG	nd	nd	nd	nd	nd	nd

Peonidin-3-glucoside

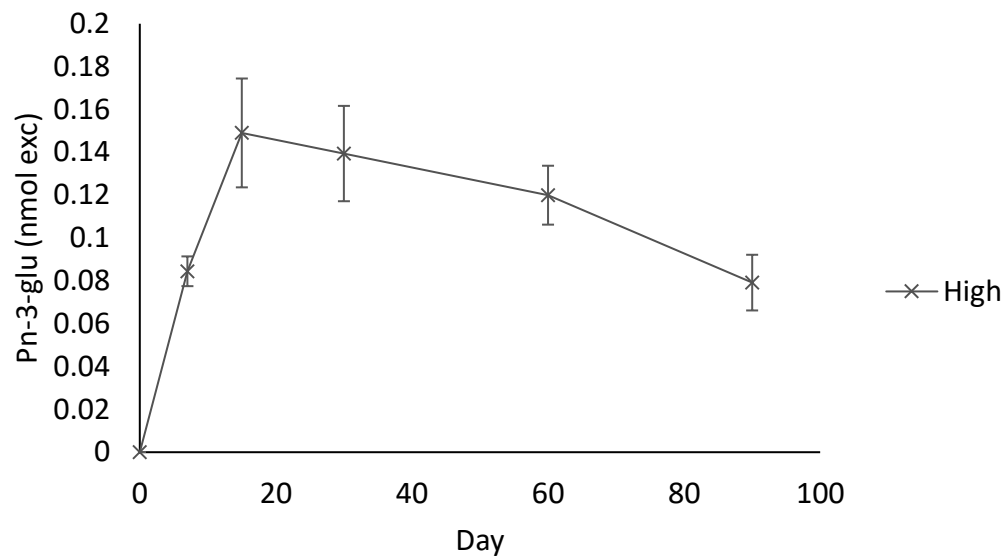


Figure C. 27 – Urinary excretion of peonidin-3-glucoside over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	trace	trace	trace	trace	trace
Medium	nd	trace	trace	trace	trace	trace
High	nd	0.084 ± 0.021 ^{BC}	0.149 ± 0.076 ^A	0.139 ± 0.067 ^{AB}	0.12 ± 0.043 ^{ABC}	0.079 ± 0.037 ^C
BB	nd	trace	trace	trace	trace	trace
NG	nd	nd	nd	nd	nd	nd

Petunidin-3-galactoside

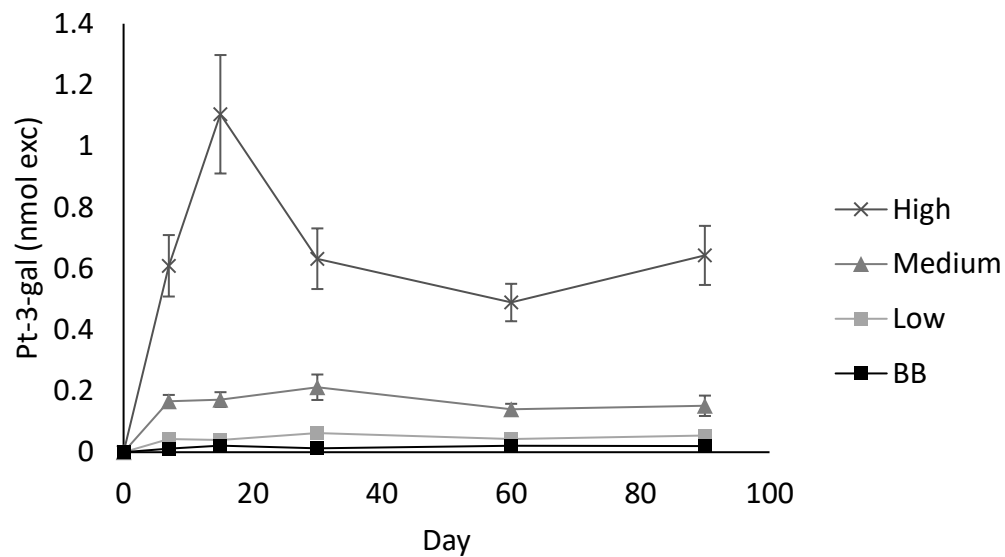


Figure C. 28 – Urinary excretion of petunidin-3-galactoside over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	0.043 ± 0.028 ^c	0.04 ± 0.02 ^c	0.063 ± 0.049 ^c	0.043 ± 0.024 ^c	0.054 ± 0.026 ^c
Medium	nd	0.167 ± 0.061 ^b	0.172 ± 0.068 ^b	0.212 ± 0.125 ^b	0.14 ± 0.051 ^b	0.152 ± 0.094 ^b
High	nd	0.609 ± 0.301 ^{a,AB}	1.104 ± 0.581 ^{a,A}	0.632 ± 0.297 ^{a,AB}	0.489 ± 0.193 ^{a,B}	0.643 ± 0.273 ^{a,AB}
BB	nd	0.012 ± 0.006 ^d	0.022 ± 0.01 ^c	0.013 ± 0.008 ^d	0.021 ± 0.012 ^c	0.02 ± 0.007 ^c
NG	nd	nd	nd	nd	nd	nd

Petunidin-3-glucoside

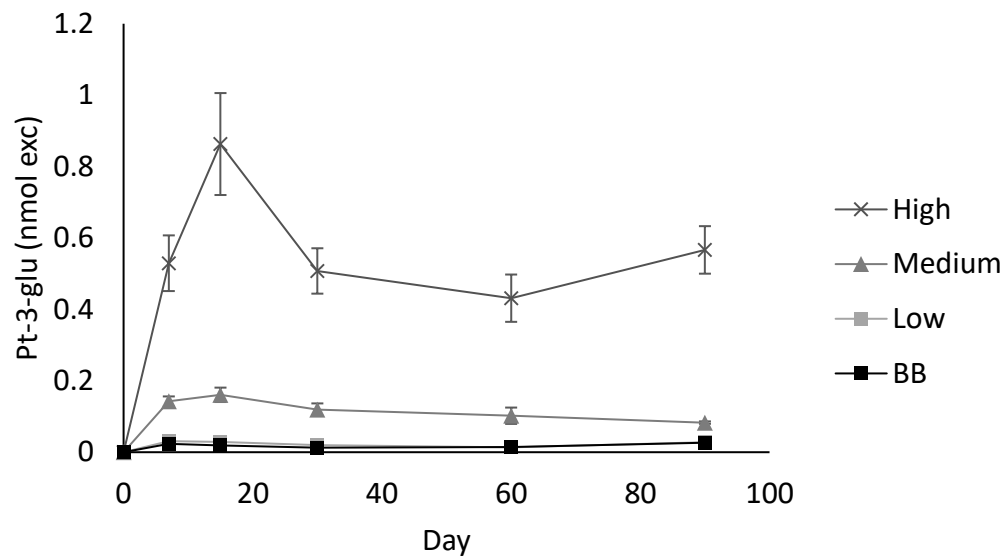


Figure C. 29 – Urinary excretion of petunidin-3-glucoside over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	0.031 ± 0.02 ^{c,A}	0.029 ± 0.015 ^{c,A}	0.019 ± 0.013 ^{c,AB}	0.014 ± 0.011 ^{c,B}	0.028 ± 0.016 ^{c,AB}
Medium	nd	0.142 ± 0.042 ^b	0.161 ± 0.057 ^b	0.12 ± 0.052 ^b	0.102 ± 0.065 ^b	0.083 ± 0.01 ^b
High	nd	0.529 ± 0.234 ^a	0.863 ± 0.428 ^a	0.508 ± 0.191 ^a	0.431 ± 0.209 ^a	0.567 ± 0.188 ^a
BB	nd	0.024 ± 0.011 ^{c,A}	0.019 ± 0.013 ^{c,AB}	0.012 ± 0.006 ^{c,B}	0.014 ± 0.005 ^{c,AB}	0.027 ± 0.019 ^{c,AB}
NG	nd	nd	nd	nd	nd	nd

Petunidin-glucuronide

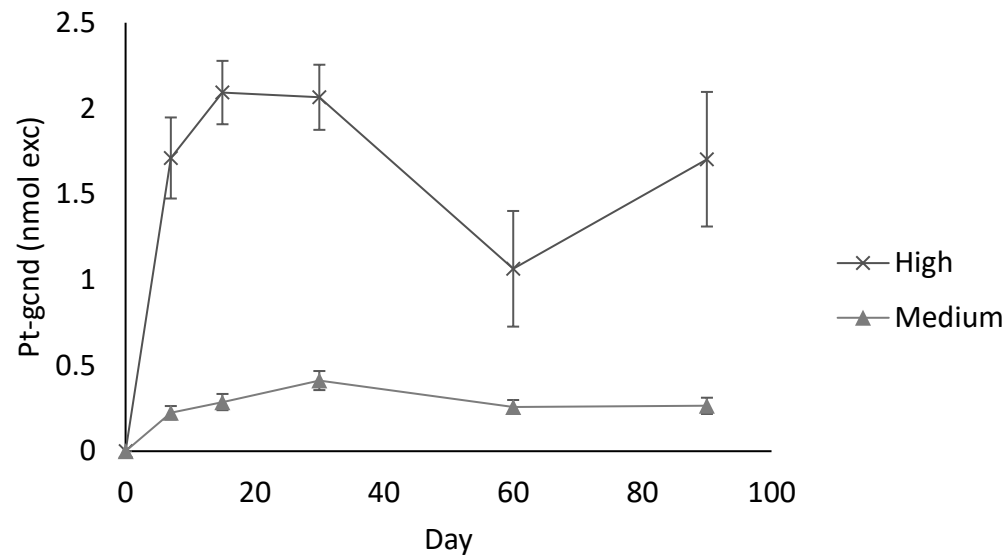


Figure C. 30 – Urinary excretion of petunidin-glucuronide over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	trace	trace	trace	trace	trace
Medium	nd	0.22 ± 0.12 ^b	0.29 ± 0.13 ^b	0.41 ± 0.17 ^b	0.26 ± 0.11 ^b	0.26 ± 0.13 ^b
High	nd	1.71 ± 0.71 ^{a,A}	2.09 ± 0.55 ^{a,A}	2.06 ± 0.57 ^{a,A}	1.06 ± 1.07 ^{a,B}	1.7 ± 1.11 ^{a,AB}
BB	nd	trace	trace	trace	trace	trace
NG	nd	nd	nd	nd	nd	nd

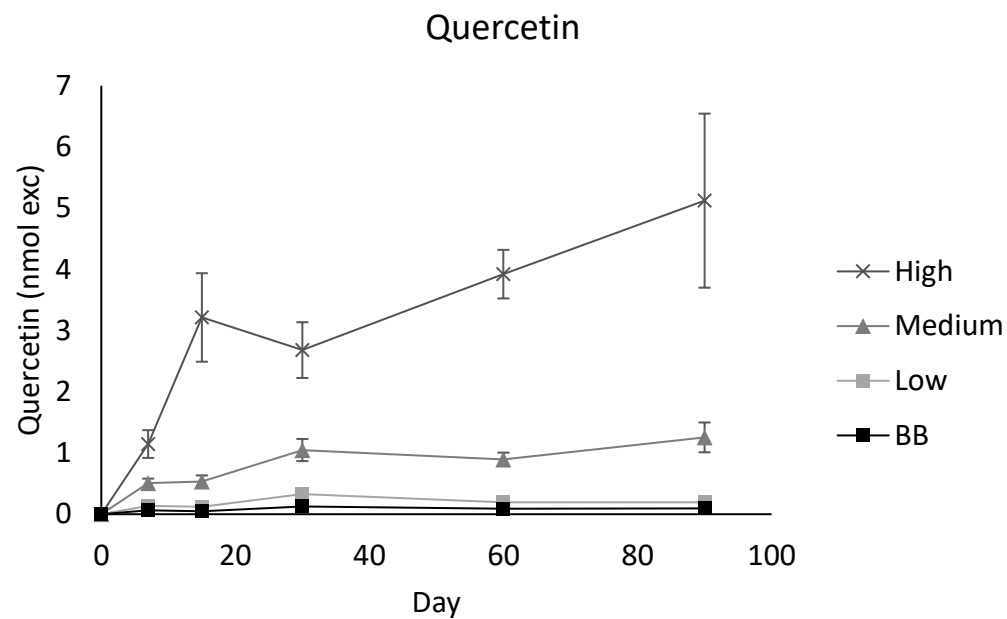


Figure C. 31 – Urinary excretion of quercetin over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	0.14 ± 0.09 ^{b,B}	0.12 ± 0.08 ^{c,B}	0.33 ± 0.19 ^{b,A}	0.20 ± 0.10 ^{c,AB}	0.20 ± 0.04 ^{b,AB}
Medium	nd	0.51 ± 0.22 ^a	0.53 ± 0.28 ^b	1.05 ± 0.54 ^a	0.9 ± 0.31 ^b	1.26 ± 0.69 ^a
High	nd	1.15 ± 0.68 ^{a,B}	3.21 ± 2.17 ^{a,A}	2.68 ± 1.37 ^{a,A}	3.92 ± 1.26 ^{a,A}	5.12 ± 4.02 ^{a,A}
BB	nd	0.06 ± 0.04 ^b	0.05 ± 0.05 ^c	0.13 ± 0.05 ^b	0.09 ± 0.11 ^c	0.1 ± 0.04 ^b
NG	nd	nd	nd	nd	nd	nd

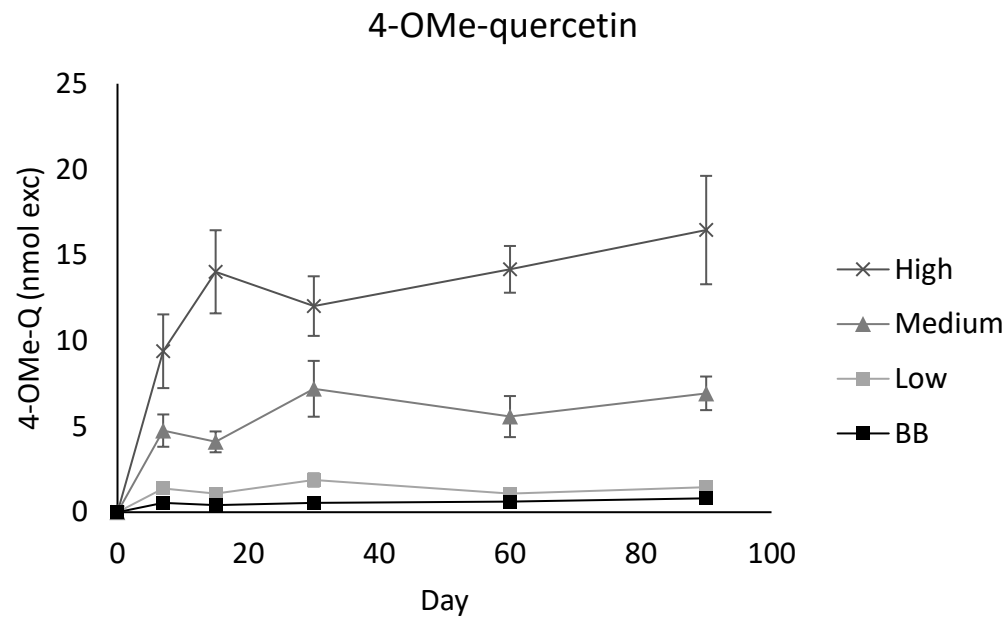


Figure C. 32 – Urinary excretion of 4-methoxyquercetin over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	1.39 ± 0.77 ^b	1.09 ± 0.63 ^c	1.88 ± 1.19 ^b	1.08 ± 0.65 ^b	1.46 ± 0.33 ^b
Medium	nd	4.76 ± 2.83 ^a	4.11 ± 1.72 ^b	7.2 ± 4.89 ^a	5.58 ± 3.39 ^a	6.94 ± 2.77 ^a
High	nd	9.39 ± 6.45 ^{a,B}	14.03 ± 7.27 ^{a,AB}	12.03 ± 5.22 ^{a,AB}	14.17 ± 4.31 ^{a,A}	16.46 ± 8.94 ^{a,AB}
BB	nd	0.54 ± 0.26 ^{c,AB}	0.42 ± 0.26 ^{d,B}	0.54 ± 0.14 ^{c,AB}	0.62 ± 0.26 ^{b,AB}	0.81 ± 0.62 ^{b,A}
NG	nd	nd	nd	nd	nd	nd

Quercetin gcnd pk 1

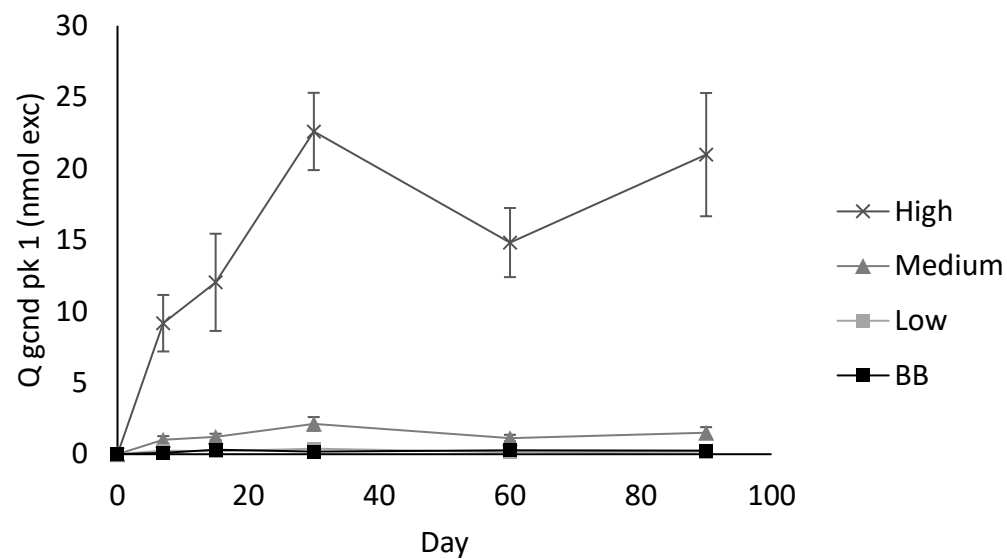


Figure C. 33 – Urinary excretion of quercetin-glucuronide peak 1 over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	0.26 ± 0.19 ^c	0.21 ± 0.17 ^c	0.39 ± 0.35 ^c	0.13 ± 0.07 ^c	0.19 ± 0.14 ^c
Medium	nd	1.01 ± 0.78 ^{b,B}	1.21 ± 0.65 ^{b,AB}	2.12 ± 1.43 ^{b,A}	1.13 ± 0.65 ^{b,AB}	1.52 ± 1.08 ^{b,AB}
High	nd	9.17 ± 5.93 ^{a,B}	12.03 ± 10.21 ^{a,AB}	22.6 ± 8.12 ^{a,A}	14.82 ± 7.65 ^{a,AB}	20.98 ± 12.2 ^{a,AB}
BB	nd	0.11 ± 0.08 ^{d,B}	0.31 ± 0.27 ^{c,A}	0.18 ± 0.12 ^{c,AB}	0.26 ± 0.22 ^{c,A}	0.26 ± 0.14 ^{c,AB}
NG	nd	nd	nd	nd	nd	nd

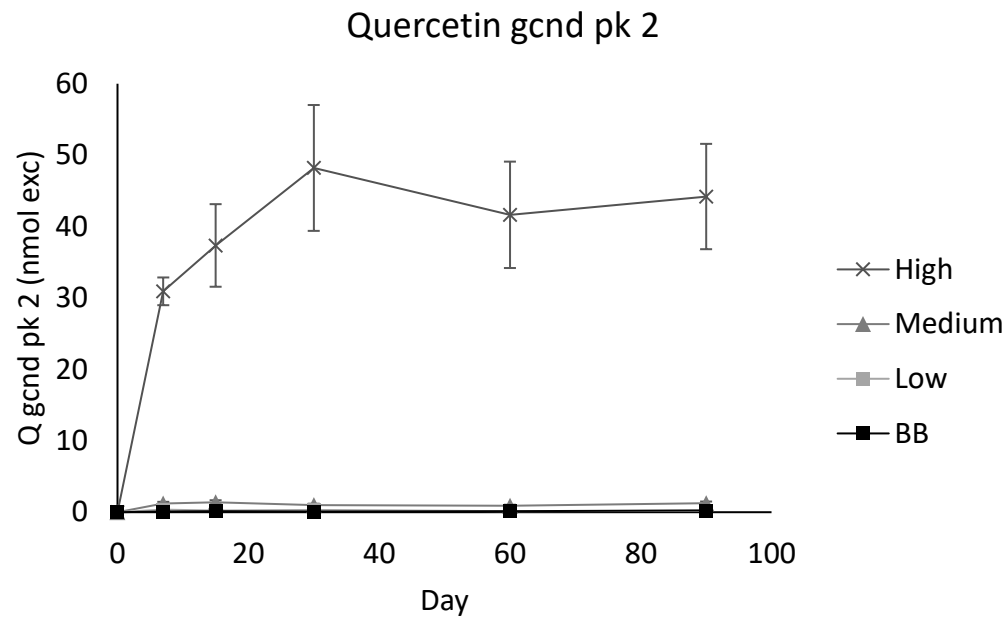


Figure C. 34 – Urinary excretion of quercetin-glucuronide peak 2 over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	0.36 ± 0.10 ^{c,A}	0.24 ± 0.15 ^{c,AB}	0.3 ± 0.17 ^{c,AB}	0.13 ± 0.09 ^{c,C}	0.21 ± 0.09 ^{c,B}
Medium	nd	1.23 ± 0.67 ^b	1.39 ± 0.81 ^b	1.0 ± 0.56 ^b	0.89 ± 0.45 ^b	1.24 ± 0.72 ^b
High	nd	30.93 ± 5.82 ^a	37.36 ± 17.35 ^a	48.21 ± 26.44 ^a	41.64 ± 23.56 ^a	44.21 ± 20.86 ^a
BB	nd	0.11 ± 0.09 ^{d,B}	0.15 ± 0.1 ^{c,AB}	0.11 ± 0.05 ^{d,B}	0.16 ± 0.07 ^{c,AB}	0.26 ± 0.12 ^{c,A}
NG	nd	nd	nd	nd	nd	nd

Quercetin gcnd pk 3

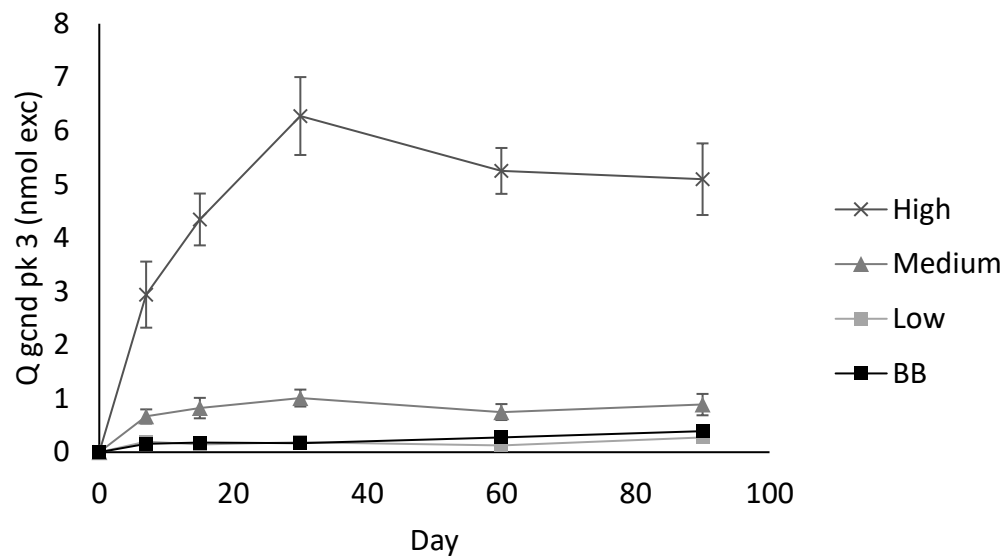


Figure C. 35 – Urinary excretion of quercetin-glucuronide peak 3 over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	0.2 ± 0.15 ^c	0.14 ± 0.13 ^c	0.19 ± 0.09 ^c	0.12 ± 0.09 ^c	0.28 ± 0.24 ^c
Medium	nd	0.67 ± 0.4 ^b	0.82 ± 0.54 ^b	1.01 ± 0.47 ^b	0.75 ± 0.42 ^b	0.89 ± 0.56 ^b
High	nd	2.94 ± 1.85 ^a	4.35 ± 1.45 ^a	6.28 ± 2.18 ^a	5.25 ± 1.35 ^a	5.1 ± 1.89 ^a
BB	nd	0.16 ± 0.09 ^c	0.18 ± 0.14 ^c	0.17 ± 0.11 ^c	0.28 ± 0.21 ^{bc}	0.39 ± 0.28 ^{bc}
NG	nd	nd	nd	nd	nd	nd

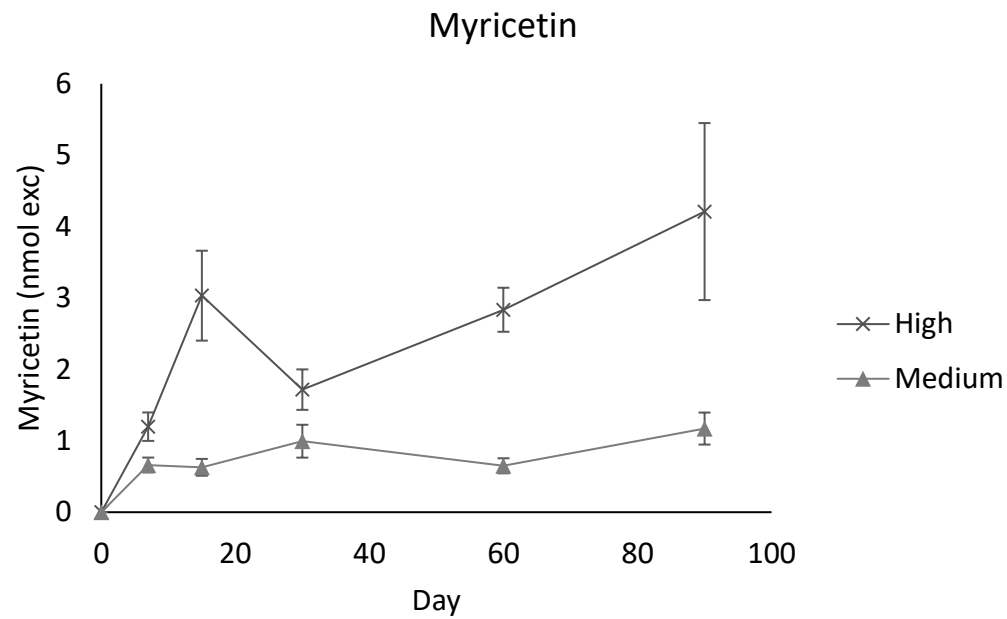


Figure C. 36 – Urinary excretion of myricetin over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	trace	trace	trace	trace	trace
Medium	nd	0.66 ± 0.31	0.63 ± 0.33 ^b	0.99 ± 0.69 ^b	0.65 ± 0.3 ^b	1.17 ± 0.63 ^b
High	nd	1.20 ± 0.60 ^B	3.03 ± 1.89 ^{a,A}	1.72 ± 0.85 ^{a,AB}	2.84 ± 0.98 ^{a,A}	4.21 ± 3.51 ^{a,A}
BB	nd	trace	trace	trace	trace	trace
NG	nd	nd	nd	nd	nd	nd

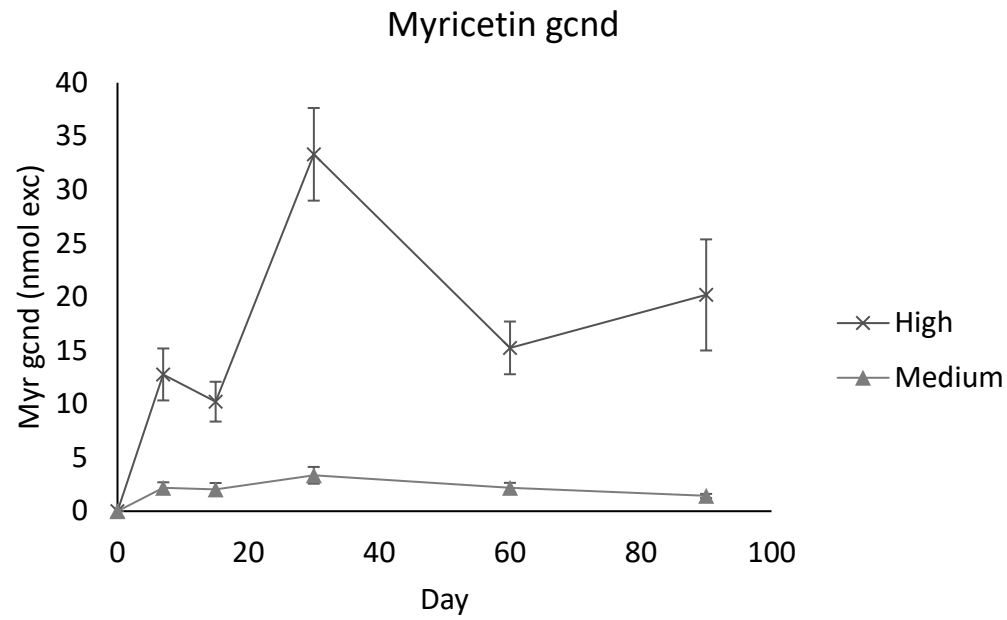


Figure C. 37 – Urinary excretion of myricetin-glucuronide over 90 days.

	Day					
	0	7	15	30	60	90
Water	nd	nd	nd	nd	nd	nd
Low	nd	nd	nd	nd	nd	nd
Medium	nd	2.20 ± 1.50 ^b	2.04 ± 1.68 ^b	3.35 ± 2.34 ^b	2.2 ± 1.27 ^b	1.43 ± 0.54 ^b
High	nd	12.77 ± 7.28 ^{a,B}	10.23 ± 5.58 ^{a,B}	33.32 ± 12.97 ^{a,A}	15.25 ± 7.78 ^{a,B}	20.19 ± 14.67 ^{a,AB}
BB	nd	nd	nd	nd	nd	nd
NG	nd	nd	nd	nd	nd	nd

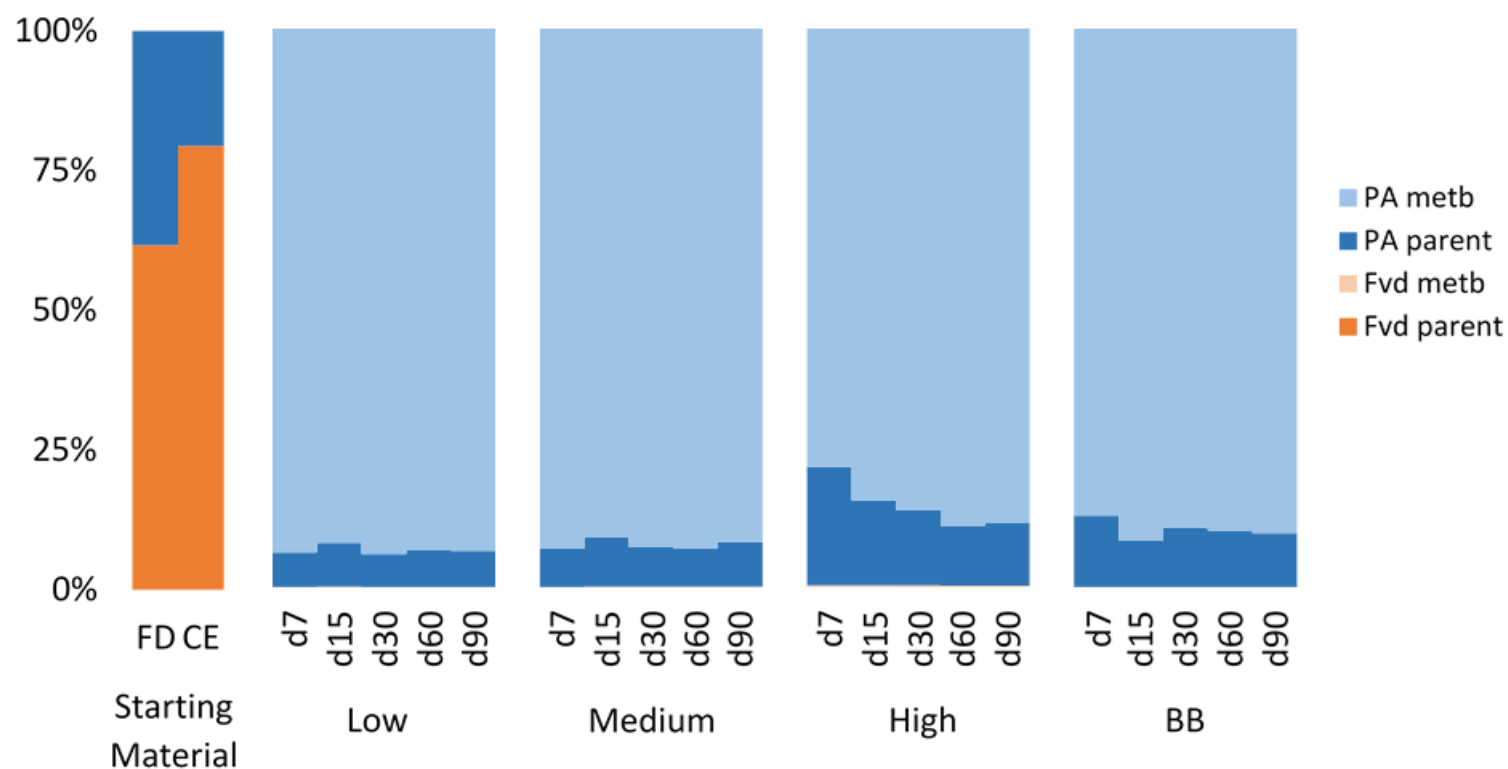


Figure C. 38 – Relative levels of phenolic acids and flavonoids in starting materials and urinary metabolites. Starting materials exhibited higher levels of flavonoids than phenolic acids, but after ingestion, urinary excretion of flavonoids is dwarfed by phenolic acids. Additionally, extensive phase II metabolism is observed, as a majority of excreted phenolic acids are excreted as phase-II metabolites, being conjugated to glucuronide or sulfate groups. (Note: hippuric acids NOT included in this figure.) FD = lyophilized whole blueberries; CE = purified blueberry polyphenol extract; PA = phenolic acids; Fvd = flavonoids; metb = metabolites.

APPENDIX D. STANDARD OPERATING PROCEDURES

D.1 – SOPs for raw materials

D.1.1 – Extraction of blueberry polyphenols.

1. Weigh out starting material in 15 mL centrifuge tube.
 - a. Freeze dried blueberries: use approx. 50 mg
 - b. Concentrated blueberry extract: use 10-20 mg
 - c. Rat diets: crush/grind first, then use approx. 1 g
 - d. Blueberry gavage preparations: amount varies based on dose and dilution
2. Add 5 mL extraction fluid (80:18:2 MeOH:H₂O:formic acid)
3. Vortex approx. 30s and ensure entire powder is wet and slurried.
4. Sonicate 30 min.
5. Centrifuge 5 min. at 3000 rpm and 4°C.
6. Transfer supernatant to clean glass test tube. Dry under nitrogen.
7. Re-extract pellet with another 5 mL extraction fluid; vortex 30s; sonicate 20 min.
8. Combine supernatants and dry completely under nitrogen.
9. Re-solubilize extract with 2% formic acid in H₂O. Cap test tube and vortex thoroughly.

D.1.2 – Resolubilization of crude extract for Folin procedure and SPE.

1. Re-solubilize extract with 2% formic acid in H₂O. Cap test tube and vortex thoroughly.
 - a. Use 5 mL for freeze dried BB and rat diets.
 - b. Use 10 mL for concentrated BB extract and blueberry gavage preparations

D.1.3 – Total Phenolic Analysis via Folin Ciocalteu Procedure

1. Prepare gallic acid standard curve.
 - i. Prepare 5 g/L gallic acid stock solution.
 - ii. Weigh out 250 mg gallic acid in 50 mL volumetric flask.
 - iii. Add 5 mL ethanol and dissolve gallic acid completely.
 - iv. Dilute to volume with distilled water.
- a. Prepare standard curve from stock solution in 50 mL volumetric flasks.
 - i. 500 mg/L = 5 mL stock solution, dilute to 50 mL with DI water
 - ii. 250 mg/L = 2.5 mL stock solution, dilute to 50 mL with DI water
 - iii. 100 mg/L = 10 mL 500 mg/L solution; dilute to 50 mL with DI water

- iv. 50 mg/L = 5 mL 500 mg/L solution; dilute to 50 mL with DI water
- v. 10 mg/L = 5 mL 100 mg/L solution; dilute to 50 mL with DI water
- b. Transfer gallic acid standards to 50 mL centrifuge tubes, wrap in foil, and store at 4°C for up to 1 month.
- 2. In 2 mL microfuge tube, mix 790 µL DI H₂O, 10 µL analyte, and 50 µL Folin reagent.
 - a. Do 3 replicates of each sample.
 - b. Run gallic acid standard curve at same time.
 - c. Include “blank” by running 3 replicates with H₂O.
 - d. Include vitamin C correction for each sample (see SPE section).
- 3. Vortex tubes, let sit 8 min.
- 4. Add 150 µL Na₂CO₃ solution, vortex, cap and let sit in dark for 2 hours.
- 5. Transfer 300 µL to 96 well plate and measure absorbance at 765 nm.
- 6. Calculate total polyphenol content in gallic acid equivalents (GAE), based on standard curve.

D.1.4 – Solid Phase Extraction (SPE) for polyphenols.

- 1. Condition SPE cartridge with 3x1 mL MeOH followed by 3x1 mL H₂O.
 - 2. *Load cartridge with sample.
 - a. 1 mL freeze dried BB and rat diets
 - b. 0.2-0.5 mL concentrated blueberry extract and gavage doses
 - 3. *Wash with 2x1 mL 2% formic acid in H₂O followed by 1 mL 95:5 H₂O:MeOH
 - 4. Elute with 2x1 mL 2% formic acid in MeOH
 - 5. Dry eluent completely under nitrogen.
 - 6. Resolubilize with 1 mL 1:1 methanol:water with 2% formic acid and vortex well. Filter with 0.45 µm filter into LC-vial, blanket with nitrogen, and store at -80°C until analysis.
- *Collect eluent from steps 2 and 3 for vitamin C correction with Folin assay.*

D.2 – Animal SOPs

D.2.1 – Body weight, food spill, and FER.

1. Animals should be weighed at least twice weekly.
2. Animals are housed in wire-bottom cages, with food weighed weekly before putting in hopper for animal.
3. Twice a week, when papers need to be changed, collect food spill from directly underneath cage, weigh, and discard.
4. Weigh food hopper once a week.
5. Food Efficiency Ratio (FER) is calculated by taking weekly body weight change and dividing by total food consumed during that week.

D.2.2 – Using metabolic cages for urine and fecal collection.

1. Place animals into metabolic cages immediately after receiving gavage dose.
2. Attach 50 mL centrifuge to bottom of cage to collect urine.
3. At the end of the collection interval, take down urine tubes and cap. Then, collect feces into 8 mL tube and freeze at -80°C for microbiome analysis.

D.2.3 – Blood collection for pharmacokinetics.

1. Perform jugular catheter surgery 2d prior to starting pharmacokinetics.
2. Flush implanted catheter every 12h with heparinized saline to keep patent.
3. When drawing blood for pharmacokinetics, clear catheter of saline, then use clean needle to draw blood, and refill catheter with heparinized saline.

D.2.4 – FITC administration for measuring intestinal permeability

1. Administer daily dose to animal via oral gavage.
2. Remove food and fast animal 4h.
3. Administer FITC via oral gavage at 50 mg/100 g bw.
4. Fast animal additional 4h.
5. Put animal under anesthesia with isoflurane and draw blood from jugular vein for FITC analysis (performed in Dr. Reddivari's lab).

D.2.5 – Necropsy procedures.

D.2.5.1 – Acute study necropsy.

1. Euthanize animal via CO₂ asphyxiation.

2. Lay animal on back and open abdominal cavity with scissors and cut through muscle to visually verify ovariectomy.
3. Excise both femurs and manually clean them of any excess tissue.
4. Wrap tissues in saline soaked gauze. Store in 8 mL tubes at -20°C until analysis.

D.2.5.2 – 90d necropsy procedures.

1. Two days prior to sacrifice, collect 24h urine sample (using metabolic cage) and send immediately to lab for urinalysis.
2. Fast animals 8h prior to sacrifice.
3. 24h after administering last oral gavage dose, sacrifice animals via CO₂ asphyxiation.
4. Open abdominal cavity with scissors, and extend the incision towards the chest, cutting through the sternum but being careful not to damage tissue.
5. Gently move organs to side and draw blood from abdominal aorta.
6. Sever jugular vein, then flush with 200 mL saline injected slowly into the left ventricle.
7. Collect tissues, pat dry, and weigh. Tissues collected for histology will be placed into 10% neutral buffered formalin (10:1 NBF:tissue), while tissues collected for polyphenol analysis are wrapped in foil and snap frozen in nitrogen and then stored at -80°C.
 - a. Liver – weigh whole liver, then separate large lobe for histology and keep remainder for polyphenol analysis
 - b. Kidney – collect both kidneys, weigh separately; use left kidney for histology and right kidney for polyphenol analysis
 - c. Spleen – weigh and save for histology
 - d. Pancreas – this is a relatively diffuse organ, so carefully separate from GI tract and abdominal fat, weigh, and save for histology
 - e. GI tract – collect entire GI tract, from stomach to rectum.
 - i. Stomach – flush contents several times with saline, save for histology
 - ii. Small intestine – flush contents with 3x10mL saline
 - a. Duodenum – 1 cm distal to the stomach, take a 1 cm long portion and save for histology in separate vial
 - b. Jejunum – find midpoint of small intestine and remove 1 cm long portion and save for histology in separate vial

- c. Ileum – 1 cm proximal to ileum, take 1 cm portion and save for histology in separate vial
 - d. Combine all remaining portions and save for polyphenol analysis
- iii. Cecum – flush contents several times with saline and save for histology
- iv. Colon – flush contents with 3x10mL saline; take 1 cm segment from midpoint of colon and save in separate vial for histology, use remainder for polyphenol analysis
- f. Heart – excise heart, weigh, and preserve for histology
- g. Lungs – excise all lobes of lungs, weigh, and preserve for histology
- h. Brain – sever upper portion of spinal cord and carefully remove skull to excise whole brain without damaging it; weigh and save for polyphenol analysis.
- 8. Collect bones for analysis; after collection, all bones should be wrapped in saline soaked gauze and stored in 8 mL tubes at 4°C until analysis.
 - a. Femur – excise right femur and manually clean excess tissue.
 - b. Tibia – remove right tibia and fibula and manually clean of excess tissue.
 - c. L1-L4 vertebrae – remove spine and collect T12-L4 vertebra; T12 is last rib with rib attached, and is useful marker for orienting spine at analysis stage.

D.3 – Extraction and analysis of polyphenols and ^{45}Ca from biological samples

D.3.1 – Processing biological samples and extracting polyphenols.

D.3.1.1 – Urine processing.

1. Immediately after collecting urine from animals in metabolic cages, centrifuge 10 minutes at 300 rpm and 4°C.
2. Decant supernatant to clean 15 mL centrifuge tube and record volume.
3. Transfer 0.5 mL urine to 2 mL vial and add 125 uL 0.5% formic acid in water to acidify to final concentration of 0.1% formic acid.
4. Flush with nitrogen, cap, and store at -80°C till analysis.

D.3.1.2 – Blood/plasma processing.

1. After collecting blood from animals, immediately transfer to lithium-heparinized tubes and gently invert tube several times to ensure anticoagulant mixes with blood.
2. Centrifuge blood at 4000 rpm for 10 minutes at 4°C.
3. Separate resulting plasma and acidify to final concentration of 0.1% formic acid.
4. Flush with nitrogen, cap, and store at -80°C till analysis.

D.3.1.3 – Tissue extraction.

1. Thaw tissues on ice.
2. Collect 0.5-1 g tissue in a 15 mL centrifuge tube for each extraction.
 - a. Brain – separate the right and left hemispheres of the brain along the central longitudinal fissure; use the entire left hemisphere (including cortex and cerebellum) for analysis and save right hemisphere.
 - b. Liver – from the largest lobe of the liver, separate a portion approximately 0.5-1 g to use for analysis and save remainder.
 - c. Kidney – cut the kidney in half from the connection point of the renal vasculature; separate the anterior portion of the kidney and use for polyphenol analysis; save posterior portion.
 - d. Small intestine – gently remove any remaining water or digesta from intestines, then randomly sample small segments along entire length of small intestine and collect together for analysis; save remaining portions.
 - e. Colon – cut colon lengthwise, from cecal connection to rectal end, leaving two halves; use one for analysis and save remainder.

3. Defat tissues.
 - a. Add 5 mL hexanes and homogenize tissue completely.
 - b. Vortex 10 minutes, then centrifuge at 3000 g for 10 minutes.
 - c. Decant and discard hexanes.
4. Extract phenolics.
 - a. Add 5 mL 1% formic acid in methanol to tissue and vortex mix 10 minutes.
 - b. Centrifuge 10 minutes at 3000 g.
 - c. Transfer supernatant to clean test tube and dry under nitrogen.
 - d. Repeat extraction once, combine supernatants and dry completely.
5. Cap and store extracted phenolics at -80°C until analysis.

D.3.2 – ⁴⁵Ca analysis.

1. Thaw bones at 4°C for 2d prior to analysis.
2. Weigh empty crucible, add femur and re-weigh crucible.
3. Place lid on crucible and put in muffle furnace to ash sample; temperature gradient ramps to 300°C at 4°C/minute, then dwells at 300°C for 15h, before ramping to 600°C at 4°C/minute, then dwells at 600°C for 5d.
4. Open furnace and allow to cool at least 4h prior to removing crucibles.
5. Weigh crucibles with ashed bone (but no lid).
6. Add 1 mL concentrated nitric acid (trace metals grade), replace lid, and let samples sit in fume hood overnight.
7. Transfer acid and dissolved bone to 25 mL volumetric flask, rinse crucible multiple times with ultrapure water and combine all rinses in flask; dilute to volume with ultrapure water; mix well.
8. Transfer to 50 mL centrifuge tube for storage.
9. Transfer 1 mL to scintillation vial and add 15 mL Ecolite; mix well and measure radioactivity on scintillation counter.

D.3.3 – Extraction and purification of polyphenols.

1. Prepare sample for extraction.
 - a. Urine: thaw samples at room temperature; put on ice once thawed.
 - b. Tissues: resolubilize with 2 mL 1% formic acid in water, sonicate 5 min.
2. Mix sample 1:1 with 1% formic acid in water in loading plate.

- a. Urine: mix 100 μ L urine with 100 μ L 1% formic acid.
 - b. Tissues: mix 500 μ L resolubilized tissue with 500 μ L 1% formic acid.
3. Precondition 96 well SPE plate with 200 μ L 1% formic acid in methanol followed by 200 μ L 1% formic acid in water.
4. Load sample from loading plate to SPE plate and add 20 μ L 50 μ M ethyl gallate as extraction efficiency control; use gentle positive pressure nitrogen to push through SPE plate.
 - a. Urine: load 100 μ L from loading plate.
 - b. Tissues: load 200 μ L from loading plate.
5. Wash with 200 μ L 1% formic acid in water followed by 200 μ L 0.1% formic acid in water.
6. Dry plate 15 minutes with nitrogen.
7. Change to elution plate and add 100 μ L 0.1% formic acid in methanol; soak 5 minutes.
8. Elute with nitrogen, add 20 μ L 50 μ M taxifolin as volume control; cap immediately with cap mat, and analyze via LCMS.

APPENDIX E. EXAMPLE SAS CODE USED FOR STATISTICAL ANALYSES

Data shortened for clarity, with a portion of the code relevant to that section of data shown.

E.1 – SAS code for Ch 2: Increasing Doses of Blueberry Polyphenols Alters Colonic Metabolism and Calcium Absorption in Ovariectomized Rats.

E.1.1 – Total urinary excretion of individual phenolic metabolites over 48h (one-way ANOVA)

```
data totexc;
input rat dose $ c3gal c3glu pt3sulf;
logc3gal=log(c3gal); logc3glu=log(c3glu); logpt3sulf=log(pt3sulf);
cards;
100 Control 0 0 0
101 Control 0 0 0
...
431 High 0.715719022 0.385252396 0.335682818
;
proc mixed data=totexc plots=all;
class dose rat; model logc3gal=dose / ddfm=kr;
random rat; lsmeans dose / adjust=tukey;
run;

proc mixed data=totexc plots=all;
class dose rat; model logc3glu=dose / ddfm=kr;
random rat; lsmeans dose / adjust=tukey;
run;

proc mixed data=totexc plots=all;
class dose rat; model logpt3sulf=dose / ddfm=kr;
random rat; lsmeans dose / adjust=tukey;
run;
```

E.1.2 – Pharmacokinetics of urinary excretion of individual phenolic metabolites over 48h (two-way ANOVA)

```
data PKexcPA;
input rat dose $ time $ ohpaa ohppa hipp ...;
logohpaa=log(ohpaa); logohppa=log(ohppa); loghipp=log(hipp); ...;
cards;
100 Control bsln 0.552991503 0.517374713 52.59122228
101 Control bsln 0.644355206 0.399443036 78.42138127
...
431 High t4 0.095414543 12.9420662 242.7113165
;
```

```
proc glimmix data=PKexcPA plots=residualpanel;
class dose rat time;
model logohpaa=dose|time / ddfm=kr;
random intercept / subject=rat(dose) type=arh(1);
lsmeans dose|time / slicediff=time adjust=tukey;
lsmeans dose|time / slicediff=dose adjust=tukey;
run;
```

```
proc glimmix data=PKexcPA plots=residualpanel;
class dose rat time;
model logohppa=dose|time / ddfm=kr;
random intercept / subject=rat(dose) type=arh(1);
lsmeans dose|time / slicediff=time adjust=tukey;
lsmeans dose|time / slicediff=dose adjust=tukey;
run;
```

```
proc glimmix data=PKexcPA plots=residualpanel;
class dose rat time;
model loghipp=dose|time / ddfm=kr;
random intercept / subject=rat(dose) type=arh(1);
lsmeans dose|time / slicediff=time adjust=tukey;
lsmeans dose|time / slicediff=dose adjust=tukey;
run;
```

E.1.3 – Pharmacokinetics of plasma appearance of individual phenolic metabolites over 48h

(two-way ANOVA)

```
data PKplasmaPA;
input rat dose $ time $ fersu hipp;
loghipp=log(hipp); logfersu=log(fersu);
cards;
100 control t0 0.013920993 0.050016974
100 control t06 0.026817412 0.099224426
100 control t12 0.041554008 0.056621224
...
431 high t36 0.023375953 2.503476297
431 high t42 0.066898784 2.166511342
431 high t48 0.044726836 1.633466296
;
```

```
proc glimmix data=PKplasmaPA plots=residualpanel;
class dose rat time;
model logfersu=dose|time / ddfm=kr;
random intercept / subject=rat(dose) type=arh(1);
lsmeans dose|time / slicediff=time adjust=tukey;
lsmeans dose|time / slicediff=dose adjust=tukey;
run;
```

```
proc glimmix data=PKplasmaPA plots=residualpanel;
class dose rat time;
```

```

model loghipp=dose|time / ddfm=kr;
random intercept / subject=rat(dose) type=arh(1);
lsmeans dose|time / slicediff=time adjust=tukey;
lsmeans dose|time / slicediff=dose adjust=tukey;
run;

```

E.1.4 – Total 48h urinary excretion of individual anthocyanin metabolites (one-way ANOVA)

```

data anthoexc;
input group $ resp;
cards;
cgal 0.141241277
cgal 0.162657678
...
cglu 0.168876528
cglu 0.222921953
;
proc mixed data=anthoexc plots=residualpanel;
class group; model resp=group / ddfm=kr;
lsmeans group / adjust=tukey;
run;

```

E.1.5 – ⁴⁵Ca absorption and femoral deposition (one-way repeated measures ANOVA)

```

data femurlong;
input ID Group $ Femur $ Measure;
cards;
101 control Right 0.287486132
102 control Right 0.447929601
103 control Right 0.307315606
...
428 high Left 0.399002994
429 high Left 0.52208444
430 high Left 0.45017086
;

proc sort data=femurlong;
by id femur;
run;

proc mixed data=femurlong;
class id group femur;
model measure = group femur / ddfm = kr residual;
repeated femur / subject = ID(group) type=cs;
lsmeans group / pdiff=control('control') ADJDFE=ROW adjust=dunnett;
run;

```

E.2 – SAS code for Ch 3: A 90 Day Oral Toxicity Study of Blueberry Polyphenols in Ovariectomized Sprague-Dawley Rats

Note: all stats run with and without non-gavaged control group. Code is the same for all analyses; only showing one example here for clarity

E.2.1 – Weekly body weight, % change in body weight, and FER (one-way ANOVA)

Code is similar for all analyses, so only 1 example shown here

```
data bw;
input rat dose $ cohort $ d0 d7;
logd0=log(d0); logd7=log(d7);
cards;
10    water A      315.3 315.5
11    water A      311.8 302.3
12    water B      286.9 283.2
...
88    BB      C      276.7 269.3
89    BB      C      294.8 286.9
100   nongvg   A      321.9 322.3
400   nongvg   B      281    278.5
600   nongvg   C      275.6 280
800   nongvg   C      290.7 287.6
;

proc mixed data=bw plots=residualpanel;
class cohort dose rat;
model logd0=dose cohort / ddfm=kr;
random rat / type=ar(1);
lsmeans dose cohort / adjust=tukey;
lsmeans dose cohort / pdiff=control('water') adjust=dunnett;
run;

proc mixed data=bw plots=residualpanel;
class cohort dose rat;
model logd7=dose cohort / ddfm=kr;
random rat / type=ar(1);
lsmeans dose cohort / adjust=tukey;
lsmeans dose cohort / pdiff=control('water') adjust=dunnett;
run;
```

E.2.2 – Total change in body weight, % bw, and FER (one-way ANOVA)

Code is similar for all analyses, so only 1 example shown here

```
data fer;
input rat dose $ d7 d14 total;
cards;
```

```

10   water 0.001893939      -0.020715631      0.011551791
11   water -0.099164927     -0.011494253      0.005555556
12   water -0.04214123      -0.009868421      0.018056605
...
88   BB      -0.083333333     -0.028290283      0.011517741
89   BB      -0.088465845      0.014008621      0.004226096
100  nongvg      0.00349345  0.063636364      0.017902813
400  nongvg      -0.02543235      0.014719411      0.011274735
600  nongvg      0.042145594     -0.00952381      0.018679409
800  nongvg      -0.032873807     -0.033991228     -0.002176279
;

```

```

proc mixed data=fer plots=residualpanel;
class dose rat; model d7=dose / ddfm=kr;
random rat / type=ar(1);
lsmeans dose / adjust=tukey;
lsmeans dose / pdiff=control('water') adjust=dunnett;
run;

```

```

proc mixed data=fer plots=residualpanel;
class dose rat; model d14=dose / ddfm=kr;
random rat / type=ar(1);
lsmeans dose / adjust=tukey;
lsmeans dose / pdiff=control('water') adjust=dunnett;
run;

```

```

proc mixed data=fer plots=residualpanel;
class dose rat; model total=dose / ddfm=kr;
random rat;
lsmeans dose / adjust=tukey;
lsmeans dose / pdiff=control('water') adjust=dunnett;
run;

```

E.2.3 – Tissue weights; absolute, % bw, and % brain weight (one-way ANOVA)

```

data tissuewt;
input rat dose $ wt ba bb;
logba=log(ba);
cards;

```

```

10   WATER      330.3      1.75      0.529821375
11   WATER      318.6      1.686     0.529190207
12   WATER      308.4      1.644     0.53307393
...
88   BB          289.1      1.674     0.579038395
89   BB          299.6      1.56      0.520694259
100  nongvg      347.1      1.736     0.500144051
400  nongvg      295.7      1.43      0.483598241
600  nongvg      297.1      1.676     0.564119825
800  nongvg      288.3      1.751     0.607353451
;

```

```

proc sort data=tissuewt out=mydata;
    by dose;
run;

proc mixed data=tissuewt plots=residualpanel;
class dose rat; model wt=dose / ddfm=kr;
random rat; lsmeans dose / pdiff=control('WATER') adjust=dunnett;
run;

proc mixed data=tissuewt plots=residualpanel;
class dose rat; model logba=dose / ddfm=kr;
random rat; lsmeans dose / pdiff=control('WATER') adjust=dunnett;
run;

proc mixed data=tissuewt plots=residualpanel;
class dose rat; model bb=dose / ddfm=kr;
random rat; lsmeans dose / pdiff=control('WATER') adjust=dunnett;
run;

```

E.2.4 – Hematology, serum biochemistry, and urinalysis (one-way ANOVA)

*****Code is similar for all analyses, so only 1 example shown here*****

```

data hematology;
input rat dose $ rbc hct hgb;
cards;
10    WATER 7.9    47    15
11    WATER 8.09   47    14.6
12    WATER 7.69   47.1  14.3
...
88    BB     8.22   46    15.1
89    BB     7.93   40    13.1
;

proc mixed data=hematology;
class dose rat; model rbc=dose / ddfm=kr;
random rat; lsmeans dose / pdiff=control('WATER') adjust=dunnett;
run;

proc mixed data=hematology;
class dose rat; model hct=dose / ddfm=kr;
random rat; lsmeans dose / pdiff=control('WATER') adjust=dunnett;
run;

proc mixed data=hematology;
class dose rat; model hgb=dose / ddfm=kr;
random rat; lsmeans dose / pdiff=control('WATER') adjust=dunnett;
run;

```

E.2.5 – FITC (one-way ANOVA)

```

data fitcnoNG;
input dose $ response;
cards;
water 270.9750856
water 386.554916
water 361.3285747
...
BB 154.5549756
BB 379.9399145
BB 257.7171751
;

proc mixed data=fitcnoNG plots=residualpanel;
class dose; model response=dose / ddfm=kr;
lsmeans dose / adjust=tukey;
run;

```

E.2.6 – BMD (one-way ANOVA)

```

data piximusraw;
input rat dose $ fBMD tBMD vBMD;
cards;
10 control 0.2011 0.1617 0.193
11 control 0.2019 0.1696 0.1757
12 control 0.2044 0.1582 0.1723
...
88 BB 0.2043 0.1697 0.1795
89 BB 0.1879 0.1586 0.1695
100 nongvg 0.2038 0.1673 0.1836
400 nongvg 0.1763 . 0.1525
600 nongvg 0.181 0.1544 0.1656
800 nongvg 0.1953 0.1621 0.1791
;

proc mixed data=piximusraw;
class dose rat; model fbmd=dose / ddfm=kr;
random rat; lsmeans dose / pdiff=control('control') adjust=dunnett;
run;

proc mixed data=piximusraw;
class dose rat; model tbmd=dose / ddfm=kr;
random rat; lsmeans dose / pdiff=control('control') adjust=dunnett;
run;

proc mixed data=piximusraw;
class dose rat; model vbmd=dose / ddfm=kr;
random rat; lsmeans dose / pdiff=control('control') adjust=dunnett;
run;

```

E.3 – SAS code for Ch 4: Changes in Blueberry Polyphenol Metabolism are Dependent on Dose and Food Matrix over 90 Days in Ovariectomized Sprague-Dawley Rats

Note: all stats run with and without non-gavaged control group. Code is the same for all analyses; only showing one example here for clarity

E.3.1 – Urinary excretion of individual phenolic metabolites over 90d (two-way ANOVA)

```
data toxPAdt;
input rat dose $ time $ hipp ohhip;
loghipp=log(hipp); logohhip=log(ohhip);
cards;
10    water    0    1810.66    4.47
11    water    0    649.18    1.75
12    water    0    1430.24    5.01
...
88    BB      90    2863.89    116.01
89    BB      90    3323.09    105.93
100   NG      90    885.65    3.74
400   NG      90    1146.56    7.87
600   NG      90    960.1    6.23
800   NG      90    879.9    4.75
;
proc glimmix data=toxPAdt plots=residualpanel;
class dose rat time;
model loghipp=dose|time / ddfm=kr;
random intercept / subject=rat(dose) type=arh(1);
lsmeans dose|time / slicediff=time adjust=tukey;
lsmeans dose|time / slicediff=dose adjust=tukey;
run;

proc glimmix data=toxPAdt plots=residualpanel;
class dose rat time;
model logohhip=dose|time / ddfm=kr;
random intercept / subject=rat(dose) type=arh(1);
lsmeans dose|time / slicediff=time adjust=tukey;
lsmeans dose|time / slicediff=dose adjust=tukey;
run;
```

E.3.2 – Urinary excretion of total phenolic metabolites, total phenolic acid metabolites, total flavonoid metabolites, and total hippuric acids over 90d (two-way ANOVA)

Code is similar for all analyses, so only 1 example shown here

```
data toxtotPPEnoNGdt;
input rat dose $ time $ totPA totantho totsum;
logtotPA=log(totPA); logtotantho=log(totantho); logtotsum=log(totsum);
cards;
10    water    0    5.78105    .    5.78105
```

```

11   water 0      2.65413      .      2.65413
12   water 0      5.25604      .      5.25604
13   water 0      4.78314      .      4.78314
...
88   BB    90     12.86979     1.5332     12.8713232
89   BB    90     11.76341     0.1252     11.7635352
;

proc glimmix data=toxtotPPEnoNGdt plots=residualpanel;
class dose rat time;
model logtotPA=dose|time / ddfm=kr;
random intercept / subject=rat(dose) type=arh(1);
lsmeans dose|time / slicediff=time adjust=tukey;
lsmeans dose|time / slicediff=dose adjust=tukey;
run;

proc glimmix data=toxtotPPEnoNGdt plots=residualpanel;
class dose rat time;
model logtotantho=dose|time / ddfm=kr;
random intercept / subject=rat(dose) type=arh(1);
lsmeans dose|time / slicediff=time adjust=tukey;
lsmeans dose|time / slicediff=dose adjust=tukey;
run;

proc glimmix data=toxtotPPEnoNGdt plots=residualpanel;
class dose rat time;
model logtotsum=dose|time / ddfm=kr;
random intercept / subject=rat(dose) type=arh(1);
lsmeans dose|time / slicediff=time adjust=tukey;
lsmeans dose|time / slicediff=dose adjust=tukey;
run;

```

E.3.3 – Tissue levels of individual phenolic metabolites (one-way ANOVA)

```

data tissuePPE;
input rat dose $ fersfs ohhips dhcsb;
logfersfs=log(fersfs); logohhips=log(ohhips); logdhcsb=log(dhcsb);
cards;
10   water .      .      12.90439539
11   water .      .      43.3478594
12   water .      22.8933165 86.89777659
...
88   BB    .      9.961726848 44.18253122
89   BB    3.293906065 187.3554861 46.01363744
100  NG    7.033088623 41.74074189 128.840556
400  NG    3.585356023 26.3955387 61.68481069
600  NG    .      20.20506122 57.51077937
800  NG    .      32.31767478 36.85412428
;

proc mixed data=tissuePPE plots=residualpanel;

```

```
class dose rat; model logfersfs=dose / ddfm=kr;  
random rat; lsmeans dose / adjust=tukey;  
run;
```

```
proc mixed data=tissuePPE plots=residualpanel;  
class dose rat; model logohhips=dose / ddfm=kr;  
random rat; lsmeans dose / adjust=tukey;  
run;
```

```
proc mixed data=tissuePPE plots=residualpanel;  
class dose rat; model logdhcsb=dose / ddfm=kr;  
random rat; lsmeans dose / adjust=tukey;  
run;
```

**APPENDIX F. FUTURECEUTICALS PRODUCT SPECIFICATION FOR
VITABLU American BLUEBERRY EXTRACT N1077 AND WILD
BLUEBERRY ASSOCIATION OF NORTH AMERICA (WBANA)
BLUEBERRY POWDER WBA1.1**

F.1 – Product specifications for blueberry extract used with study in chapter 2



SAMPLE MEMORANDUM

PURDUE UNIVERSITY
700 W State St
West Lafayette, IN, 47907

Thursday, January 25, 2018
Sample ID: SVDF145326

Attention: Pam Lachcik
(765) 534-8737

Ships via: Fed Ex Ground
Date Needed: 1/31/2018

Per the Request of: Leah Aylesworth

Sample	Item #	Qty.	Lot #
NorthAmerican Blueberry Extract	N1077	50g	249711636N1077

Please call if we can be of further assistance.
Samples of other ingredients available upon request.

Yours truly,
Leah Aylesworth

2692 N. State Rt. 1-17 • Mokenca, IL 60954 U.S.A
Phone: 888-452-6853 • Fax: 815-472-3529



Certificate of Analysis
VitaBlue®
 American Blueberry Extract
 N1077

Customer: **Perdue University**

P.O. -

Lot: **24971636N1077**

Order: -

Code: **N1077**

Date: **1/25/2018**

Manufacture Date: **9/6/2017**

Best if used by: **9/6/2019**

PRODUCT CHARACTERISTICS			
Botanical Name	<i>Vaccinium corymbosum</i>	Plant Part	Fruit
Description	Powder	Flavor	Characteristic
Kosher	Pareve per Orthodox Union	Organic	No
Carrier	No Carrier	Solvent	Ethanol/Water

PRODUCT PROFILE	METHOD	SPECIFICATION	TEST RESULT
Particle Analysis	FCCM P.2.1	90% through U.S.A. #80 sieve	98.1%
Moisture	FCCM P.1.1	5.0% Maximum	1.5%
Identity	FTIR	Characteristic	Complies
Irradiation Detection	PSI	Negative	Negative
Color	Visual	Dark Blue/Purple	Complies
ACTIVE ASSAY	METHOD	SPECIFICATION	TEST RESULT
Total Anthocyanins (By UV)	FCCM C.13.1	12% Minimum	17.84%
ANALYSIS	METHOD	SPECIFICATION	TEST RESULT
Heavy Metals by ICP-MS	AOAC 993.14M	NMT 10 ppm	Complies
Arsenic	AOAC 993.14M	NMT 5 ppm	0.108 ppm
Cadmium	AOAC 993.14M	NMT 3 ppm	<0.020 ppm
Lead	AOAC 993.14M	NMT 1 ppm	0.052 ppm
Mercury	AOAC 993.14M	NMT 0.5 ppm	0.223 ppm
MICROBIOLOGICAL PROFILE	METHOD	SPECIFICATION	TEST RESULT
Total Aerobic Plate Count	AOAC 990.12	NMT 5,000 CFU/g	<1,000 CFU/g
Yeast and Mold	AOAC 997.02	NMT 200 CFU/g	<10 CFU/g
Coliform	AOAC 991.14	NMT 10 CFU/g	<10 CFU/g
E. Coli	AOAC 991.14	Less than 10 CFU/g	Less than 10 CFU/g
Salmonella	Modified FDA BAM	Negative/375g	Negative/375g
Coag Postive Staph.	Modified FDA BAM	Negative/g	Negative/g

Page 1 of 2

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 1-888-452-6853



Certificate of Analysis
VitaBlue.
American Blueberry Extract
N1077

Customer: Perdue University

P.O. -

Lot: 24971636N1077

Order: -

Code: N1077

Date: 1/25/2018

Manufacture Date: 9/6/2017

Best if used by: 9/6/2019

INGREDIENT STATEMENT

Blueberry

COUNTRY OF ORIGIN

Blueberry: USA

QA Approval

Justin Madsen, Quality Compliance Manager

Date: 1/25/2018

Contact Information

Phone: (815) 507-1425

Fax: (815) 472-3850

E-Mail: jmadsen@futureceuticals.com

Status:	Current Review Date	Previous Review Date	Supersedes	Quality Compliance Coord.: Jessica Schweizer	Quality Compliance Admin.: Riley Jackson
Active	10/17/2017	10/10/2016	05 of 01/20/2014	<i>J. Schweizer</i>	<i>Riley Jackson</i>



Product Specification



American Blueberry Extract N1077

INGREDIENT STATEMENT

Blueberry

COUNTRY OF ORIGIN

Blueberry: USA

PACKAGING AND STORAGE

Pack Size: 5 kg

Packaging: Polyethylene Liner, Heat Sealed, Corrugated Package

Storage: Cool, Dry

Best if Used by or Retest Date: 24 months from date of manufacture when stored in original unopened package.

Status:	Current Review Date	Previous Review Date	Supersedes	Quality Director Approval: Kim Rivard	Quality Material Compliance: Justin Madsen
Active	10/17/2017	10/10/2016	05 of 01/20/2014		

CUSTOMER APPROVAL

Company Name: _____
Signature: _____ Date: _____
Printed Name: _____ Title / Position: _____

Invalid without Approval Signatures



Discovering Tomorrow's Health Today

Product Specification



American Blueberry Extract

N1077

PRODUCT CHARACTERISTICS

Botanical Name	<i>Vaccinium corymbosum</i>	Plant Part	Fruit
Description	Powder	Flavor	Characteristic
Kosher	Pareve per Orthodox Union	Organic	No
Carrier	No Carrier	Solvent	Ethanol/Water

PRODUCT PROFILE

Particle Analysis
Moisture
Identity
Irradiation Detection
Color

METHOD

FCCM P.2.1
FCCM P.1.1
FTIR
PSL
Visual

SPECIFICATION

90% through U.S.A. #80 sieve
5.0% Maximum
Characteristic
Negative
Dark Blue/Purple

ACTIVE ASSAY

Total Anthocyanins (By UV)

METHOD

FCCM C.13.1

SPECIFICATION

12% Minimum

ANALYSIS

Heavy Metals by ICP-MS
Arsenic
Cadmium
Lead
Mercury

METHOD

AOAC 993.14M
AOAC 993.14M
AOAC 993.14M
AOAC 993.14M
AOAC 993.14M

SPECIFICATION

NMT 10 ppm
NMT 5 ppm
NMT 3 ppm
NMT 1 ppm
NMT 0.5 ppm

MICROBIOLOGICAL PROFILE

Total Aerobic Plate Count
Yeast and Mold
Coliform
E. Coli
Salmonella
Coag Postive Staph.

METHOD

AOAC 990.12
AOAC 997.02
AOAC 991.14
AOAC 991.14
Modified FDA BAM
Modified FDA BAM

SPECIFICATION

NMT 5,000 CFU/g
NMT 200 CFU/g
NMT 10 CFU/g
Less than 10 CFU/g
Negative/375g
Negative/g

Status:	Current Review Date	Previous Review Date	Supersedes	Quality Director Approval: Kim Rivard	Quality Material Compliance: Justin Madsen
Active	10/17/2017	10/10/2016	05 of 01/20/2014		

Customer Approval Initial: _____ Date: _____

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F.2 – Product specifications for blueberry extract used with study in chapters 3 & 4



Product Specification



American Blueberry Extract

N1077

PRODUCT CHARACTERISTICS

Botanical Name	<i>Vaccinium corymbosum</i>	Plant Part	Fruit
Description	Powder	Flavor	Characteristic
Kosher	Pareve per Orthodox Union	Organic	No
Carrier	No Carrier	Solvent	Ethanol/Water

PRODUCT PROFILE

Particle Analysis
Moisture
Identity
Irradiation Detection
Color

METHOD

FCCM P.2.1
FCCM P.1.1
FTIR
PSL
Visual

SPECIFICATION

90% through U.S.A. #80 sieve
5.0% Maximum
Characteristic
Negative
Dark Blue/Purple

ACTIVE ASSAY

Total Anthocyanins (By UV)

METHOD

FCCM C.13.1

SPECIFICATION

12% Minimum

ANALYSIS

Heavy Metals by ICP-MS
Arsenic
Cadmium
Lead
Mercury

METHOD

AOAC 993.14M
AOAC 993.14M
AOAC 993.14M
AOAC 993.14M
AOAC 993.14M

SPECIFICATION

NMT 10 ppm
NMT 5 ppm
NMT 3 ppm
NMT 1 ppm
NMT 0.5 ppm

MICROBIOLOGICAL PROFILE

Total Aerobic Plate Count
Yeast and Mold
Coliform
E. Coli
Salmonella
Coag Postive Staph.

METHOD

AOAC 990.12
AOAC 997.02
AOAC 991.14
AOAC 991.14
Modified FDA BAM
Modified FDA BAM

SPECIFICATION

NMT 5,000 CFU/g
NMT 200 CFU/g
NMT 10 CFU/g
Less than 10 CFU/g
Negative/375g
Negative/g

Status:	Current Review Date	Previous Review Date	Supersedes	Quality Director Approval: Kim Rivard	Quality Material Compliance: Justin Madsen
Active	10/17/2017	10/10/2016	05 of 01/20/2014		

Customer Approval Initial:

Date: 10-22-18

Page 1 of 2

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1-888-452-6853



Discovering Tomorrow's Health Today

Product Specification



American Blueberry Extract

N1077

INGREDIENT STATEMENT

Blueberry

COUNTRY OF ORIGIN

Blueberry: USA

PACKAGING AND STORAGE

Pack Size: 5 kg

Packaging: Polyethylene Liner, Heat Sealed, Corrugated Package

Storage: Cool, Dry

Best if Used by or Retest Date: 24 months from date of manufacture when stored in original unopened package.

Status:	Current Review Date	Previous Review Date	Supersedes	Quality Director Approval: Kim Rivard	Quality Material Compliance: Justin Madsen
Active	10/17/2017	10/10/2016	05 of 01/20/2014	<i>[Signature]</i>	<i>[Signature]</i>

CUSTOMER APPROVAL

Company Name: Purdue University
Signature: *Pamela Lachcik* Date: 10-22-18
Printed Name: Pamela Lachcik Title / Position: Lab Tech

Invalid without Approval Signatures



Certificate of Analysis
VitaBlue.
 American Blueberry Extract
 N1077

Customer: Purdue University

P.O. 10192018

Lot: 07181636N1077

Order: 69559

Code: N1077

Date: 10/23/2018

Manufacture Date: 3/12/2018

Best if used by: 3/12/2020

PRODUCT CHARACTERISTICS			
Botanical Name	<i>Vaccinium corymbosum</i>	Plant Part	Fruit
Description	Powder	Flavor	Characteristic
Kosher	Pareve per Orthodox Union	Organic	No
Carrier	No Carrier	Solvent	Ethanol/Water
PRODUCT PROFILE	METHOD	SPECIFICATION	TEST RESULT
Particle Analysis	FCCM P.2.1	90% through U.S.A. #80 sieve	92.7%
Moisture	FCCM P.1.1	5.0% Maximum	1.3%
Identity	FTIR	Characteristic	Complies
Irradiation Detection	PSL	Negative	Negative
Color	Visual	Dark Blue/Purple	Complies
ACTIVE ASSAY	METHOD	SPECIFICATION	TEST RESULT
Total Anthocyanins (By UV)	FCCM C.13.1	12% Minimum	16.63%
ANALYSIS	METHOD	SPECIFICATION	TEST RESULT
Heavy Metals by ICP-MS	AOAC 993.14M	NMT 10 ppm	Complies
Arsenic	AOAC 993.14M	NMT 5 ppm	0.048 ppm
Cadmium	AOAC 993.14M	NMT 3 ppm	<0.020 ppm
Lead	AOAC 993.14M	NMT 1 ppm	0.053 ppm
Mercury	AOAC 993.14M	NMT 0.5 ppm	0.024 ppm
MICROBIOLOGICAL PROFILE	METHOD	SPECIFICATION	TEST RESULT
Total Aerobic Plate Count	AOAC 990.12	NMT 5,000 CFU/g	<1,000 CFU/g
Yeast and Mold	AOAC 997.02	NMT 200 CFU/g	20 CFU/g
Coliform	AOAC 991.14	NMT 10 CFU/g	<10 CFU/g
E. Coli	AOAC 991.14	Less than 10 CFU/g	Less than 10 CFU/g
Salmonella	Modified FDA BAM	Negative/375g	Negative/375g
Coag Postive Staph.	Modified FDA BAM	Negative/g	Negative/g

Page 1 of 2

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 1-888-452-6833



Certificate of Analysis
VitaBlue.
American Blueberry Extract
N1077

Customer: Purdue University P.O. 10192018
Lot: 07181636N1077 Order: 69559 Code: N1077 Date: 10/23/2018
Manufacture Date: 3/12/2018 Best if used by: 3/12/2020

INGREDIENT STATEMENT
Blueberry

COUNTRY OF ORIGIN
Blueberry: USA

QA Approval
Justin Madsen, Quality Compliance Manager

Date: 10/23/2018

Contact Information

Phone: (815) 507-1425

Fax: (815) 472-3850

E-Mail: jmadsen@futureceuticals.com

Status:	Current Review Date	Previous Review Date	Supersedes	Quality Compliance Coord.: Jessica Schweizer	Quality Compliance Admin.: Riley Sutyak
Active	10/17/2017	10/10/2016	05 of 01/20/2014	<i>J. Schweizer</i>	<i>Riley Sutyak</i>

F.3 – Product specifications for lyophilized blueberries used in all studies



Product Specification WBANA Blueberry Powder WBA1.1

PRODUCT CHARACTERISTICS

Botanical Name	<i>Vaccinium angustifolium</i>	Plant Part	Fruit
Description	Powder	Flavor	Characteristic
Kosher	No	Organic	No

PRODUCT PROFILE

Particle Analysis
Moisture
Identification
Irradiation Detection
Color

METHOD

FCCM P.2.1
FCCM P.1.1
FTIR
PSL
Visual

SPECIFICATION

98% through U.S.A. #40 sieve
4.0% Maximum
Characteristic
Negative
Purple

ACTIVE ASSAY

Total Polyphenols
Total Anthocyanins
Total ORAC FN

METHOD

FCCM C.2.4
FCCM C.13.1
FCCM C.1.3

SPECIFICATION

Report Only
Report Only
Report Only

ANALYSIS

Heavy metals by ICP-MS
Arsenic
Cadmium
Lead
Mercury

METHOD

AOAC 993.14M
AOAC 993.14M
AOAC 993.14M
AOAC 993.14M
AOAC 993.14M

SPECIFICATION

NMT 10 ppm
NMT 5 ppm
NMT 3 ppm
NMT 1 ppm
NMT 0.5 ppm

MICROBIOLOGICAL PROFILE

Total Aerobic Plate Count
Yeast and Mold
Coliform
E. Coli
Salmonella
Coag. Pos. Staph.

METHOD

AOAC 990.12
AOAC 997.02
AOAC 991.14
AOAC 991.14
Modified FDA BAM
Modified FDA BAM

SPECIFICATION

NMT 100,000 CFU/g
NMT 10,000 CFU/g
NMT 50 CFU/g
Less than 10 CFU/g
Negative/375g
Negative/g

Status:	Current Review Date	Previous Review Date	Supersedes	Quality Director Approval: Dr. Boris Nemzer	Quality Operations Approval: Ed Lindquist
Active	01/29/2015	11/20/2014	04 of 11/09/2010		

Customer Approval Initial: _____ Date: _____

Page 1 of 2

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Product Specification
WBANA Blueberry Powder
WBA1.1

INGREDIENT STATEMENT

Blueberry, Silica Dioxide (1%)

PACKAGING AND STORAGE

Pack Size: Multiple Pack Sizes

Packaging:

Storage: Cool, Dry

Best if Used by or Retest Date: 24 months from date of manufacture when stored in original unopened package.

Status:	Current Review Date	Previous Review Date	Supersedes	Quality Director Approval: Dr. Boris Nemzer	Quality Operations Approval: Ed Lindquist
Active	01/29/2015	11/20/2014	04 of 11/09/2010	<i>Boris Nemzer</i>	<i>Ed Lindquist</i>

CUSTOMER APPROVAL

Company Name: _____

Signature: _____ Date: _____

Printed Name: _____ Title / Position: _____

Invalid without Approval Signatures



Certificate of Analysis

WBANA Blueberry Powder

WBA1.1

Customer: Wild Blueberry Association

P.O. Letter 4/16

Lot: 01254356WBA1.1

Order: 43524

Code: WBA1.1

Date: 6/17/2015

Manufacture Date: 1/12/2015

Best if used by: 1/12/2017

PRODUCT CHARACTERISTICS			
Botanical Name	<i>Vaccinium angustifolium</i>	Plant Part	Fruit
Description	Powder	Flavor	Characteristic
Kosher	No	Organic	No
PRODUCT PROFILE	METHOD	SPECIFICATION	TEST RESULT
Particle Analysis	FCCM P.2.1	98% through U.S.A. #40 sieve	100%
Moisture	FCCM P.1.1	4.0% Maximum	0.8%
Identification	FTIR	Characteristic	Complies
Irradiation Detection	PSL	Negative	Negative
Color	Visual	Purple	Complies
ACTIVE ASSAY	METHOD	SPECIFICATION	TEST RESULT
Total Polyphenols	FCCM C.2.4	Report Only	2.9%
Total Anthocyanins	FCCM C.13.1	Report Only	1.9%
Total ORAC FN	FCCM C.1.3	Report Only	2,125 μ mole TE/g
ANALYSIS	METHOD	SPECIFICATION	TEST RESULT
Heavy metals by ICP-MS	AOAC 993.14M	NMT 10 ppm	Complies
Arsenic	AOAC 993.14M	NMT 5 ppm	0.032 ppm
Cadmium	AOAC 993.14M	NMT 3 ppm	<0.020 ppm
Lead	AOAC 993.14M	NMT 1 ppm	<0.020 ppm
Mercury	AOAC 993.14M	NMT 0.5 ppm	0.030 ppm
MICROBIOLOGICAL PROFILE	METHOD	SPECIFICATION	TEST RESULT
Total Aerobic Plate Count	AOAC 990.12	NMT 100,000 CFU/g	1,000 CFU/g
Yeast and Mold	AOAC 997.02	NMT 10,000 CFU/g	90 CFU/g
Coliform	AOAC 991.14	NMT 50 CFU/g	< 10 CFU/g
E. Coli	AOAC 991.14	Less than 10 CFU/g	Less than 10 CFU/g
Salmonella	Modified FDA BAM	Negative/375g	Negative/375g
Coag. Pos. Staph.	Modified FDA BAM	Negative/g	Negative/g

Page 1 of 2

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Certificate of Analysis
WBANA Blueberry Powder
WBA1.1

Customer: **Wild Blueberry Association** P.O. Letter **4/16**
 Lot: **01254356WBA1.1** Order: **43524** Code: **WBA1.1** Date: **6/17/2015**
 Manufacture Date: **1/12/2015** Best if used by: **1/12/2017**

INGREDIENT STATEMENT

Blueberry, Silica Dioxide (1%)

QA Approval
 Signature: *[Signature]*

Date: 6/17/2015

Contact Information
 Phone: (815) 472-6853

Fax: (815) 472-3529

E-Mail: Bnemzer@vandrunen.com

Status:	Current Review Date	Previous Review Date	Supersedes	Quality Director Approval: Dr. Boris Nemzer	Quality Operations Approval: Ed Lindquist
Active	01/29/2015	11/20/2014	04 of 11/09/2010	<i>[Signature]</i>	<i>[Signature]</i>



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Date Issued: June 17, 2015

Product Name: WBANA Blueberry Powder

Product Item #: WBA1.1

VDF FutureCeuticals has an allergen program in place that effectively monitors and controls the "Big 8" allergens recognized by the FDA. Those items included in the VDF FutureCeuticals allergen program are listed below.

Component & Derivatives	Present in Product	Present in other products manufactured on the same line	Present in the same manufacturing plant or warehouse
Peanut	No	No	No
Tree Nut	No	No	No
Dairy (yogurt, cheese)	No	Yes	Yes
Eggs	No	Yes	Yes
Fin Fish (Anchovy)	No	No	Yes
Shell Fish	No	No	No
Soy (soynuts)	No	Yes	Yes
Wheat	No	Yes	Yes

*The items listed below are considered to be sensitivities but are not regulated by the FDA.

Component & Derivatives	Present in Product	Present in other products manufactured on the same line	Present in the same manufacturing plant or warehouse
Corn	No	Yes	Yes
Celery	No	Yes	Yes
Mustard	No	Yes	Yes
Sesame	No	Yes	Yes
Cereals/grains containing gluten (spelt, kamut, oat, barley)	No	Yes	Yes
Sulphites	No	No	Yes
Lupin	No	No	No
Monosodium Glutamate (MSG)	No	No	No
Seed	No	Yes	Yes



*Sales & Customer Service Office
2692 N. State Rt. 1-17 - Momence, IL 60954*



Certificate of Analysis

Lynne Bell
University Of Reading
School of Psychology
Whiteknights Campus
Reading
RG6 6AL

Report No: P16-00744
Purchase Order: (None Supplied)
Date Received: 29th January 2016
Date Started: 3rd February 2016

Page 1 of 1

Analysis of Freeze Dried Blueberry Powder

Sample Code: **P16-00744-1** Your Refs: **01254356WBA1.1**
Description: **Freeze dried blueberry powder**

<u>Method</u>	<u>Analysis</u>	<u>Result</u>	<u>Units</u>
TM350	Fructose	36.0	g/100g
TM350	Glucose	34.0	g/100g
TM162	Vitamin C	335	mg/100g

The following analyses were subcontracted

<u>Method</u>	<u>Analysis</u>	<u>Result</u>	<u>Units</u>
	Soluble and Insoluble Dietary Fibre	16.0	g/100g

⚡ These results relate only to the sample(s) tested and do not guarantee the bulk of the material to be of equal quality. This report shall not be reproduced, except in full, without the written approval of RSSI. RSSI staff were not responsible for sampling and cannot be held liable in respect of the use to which this information is put. All samples will be retained for a period of one month (or ten days, if perishable) from the date of this certificate.

Science
With
Service

Approved By:
Aurel Covadi
Senior Scientist 1
(Functional Ingredients)
19 February 2016

READING SCIENTIFIC SERVICES LTD
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Pepper Lane, Reading, RG6 6LA
Tel: +44 (0)118 918 4000
Fax: +44 (0)118 9868932
email: enquiries@rssl.com
web: www.rssl.com

VITA

Dennis P Cladis, MS²

*Graduate Research & Teaching Assistant
Department of Food Science
Purdue University*

Career Goal: Become an independent researcher in food and nutritional toxicology.

Education

- 2020 (exp)** **Ph.D. Food Science**, Purdue University (GPA: 3.8/4.0)
Thesis: *Consuming High Doses of Blueberry Polyphenols is Safe but Induces Dose-Dependent Shifts in Metabolism.*
Advisors: Drs. Mario Ferruzzi & Connie Weaver
- 2014** **M.S. Food Science**, Purdue University (GPA: 3.8/4.0)
Thesis: *Fatty Acids and Mercury in Seventy-Seven Species of Commercially Available Finfish in the United States.*
Advisor: Dr. Charles Santerre
- 2012** **M.S. Inorganic Chemistry**, Purdue University (GPA: 3.9/4.0)
Thesis: *Multielectron Chemistry with a Redox Active Uranium Complex.*
Advisor: Dr. Suzanne Bart
- 2009** **B.A. Mathematics & Chemistry** (Spanish Minor)
DePauw University, Magna Cum Laude (GPA: 3.7/4.0)

Professional Appointments

- 2012-present** **Graduate Research & Teaching Assistant**, Depts Nutrition Sci & Food Sci, Purdue Univ
- 2010-12** **Graduate Research & Teaching Assistant**, Dept Chemistry, Purdue Univ
- 2009-10** **Graduate Teaching Assistant**, Dept Chemistry, Univ Northern Colorado

Other Professional Activities

- 2004-present** High School Certified Baseball Umpire & Soccer Referee
Selected to referee 2018 & 2019 Soccer State Finals
- 2002-13** Mathematics and Chemistry Tutor
- 2005** Intern at Pinnacle Actuarial Resources, Inc.

Honors & Awards

2019	Runner-Up, ASN Emerging Leaders in Nutrition, Dietary Bioactive Components RIS
2019	Vitamin World Travel Award
2018	Catherine Peachy Oral Presentation Award
2016	Mary E. Fuqua Graduate Scholarship
2015	Certificate of Excellence in Research (Office of Interdisciplinary Grad. Pgm.)
2015	Teaching Academy Graduate Teaching Award (TA of the year, Nutrition Sci)
2014	Burdock Group Student Travel Award – Food Laws & Regulations Division
2014	IFT Research Paper Competition Finalist, Nutrition Division
2014	Phi Tau Sigma inductee, Hoosier chapter (Food Science Honor Society)
2011	Phi Lambda Upsilon inductee, Purdue chapter (Chemistry Honor Society)
2010-11	Ross Graduate Fellowship
2009	Sigma Delta Pi inductee (Spanish Language Honor Society)
2009	H.E.H. Greenleaf Award for excellence in mathematics
2009	Mathematical Association of America Award for excellence in mathematics
2007-09	Dow Chemical Company Scholar
2008	John D. Ricketts Prize for excellence in physical chemistry
2008	Phi Lambda Upsilon inductee, DePauw chapter (Chemistry Honor Society)
2008	Chi Alpha Sigma inductee (National College Athlete Honor Society)

Professional Development

2019	General Mills Leadership and Development Seminar Series, Purdue Univ
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Professional Affiliations

American Association for the Advancement of Science (AAAS, since 2017)
American Chemical Society (ACS, since 2011)
American Society for Nutrition (ASN, since 2017)
Institute of Food Technologists (IFT, since 2012)
Phi Tau Sigma (Hoosier chapter, since 2014)
Society of Toxicology (SOT, since 2019)

Service to the University/Department

2019	Judged Future Farmers of America (FFA) Food Sci. Career Dev. state competition
2013-15	4H Food Science & Nutrition Workshop Volunteer
2006-15	National Chemistry Week Volunteer
2011-12	Phi Lambda Upsilon social committee
2011	ChemTeens Conference Volunteer
2011	Science Fair Judge for Lafayette Central Catholic High School science fair

RESEARCH AND SCHOLARSHIP

Research Interests

- Food and nutritional toxicology
- Polyphenols & bioactives
- Micronutrients & dietary supplements
- Metabolomics

Publications

*Journal Articles (*corresponding author)*

1. Hodges, JK*; Cao, S; **Cladis, DP**; Weaver, CM. **2019**. Lactose Intolerance and Bone Health: The Challenge of Ensuring Adequate Calcium Intake. *Nutrients*, 11 (4): 718 (17p). DOI: 10.3390/nu11040718
2. Weaver, CM*; Stone, MS; Lobene, AJ; **Cladis, DP**; Hodges, JK. **2018**. What is the Evidence Base for a Potassium Requirement? *Nutr Today*, 53 (5): 184-195. DOI: 10.1097/NT.0000000000000298
3. Furrer, A; **Cladis, DP**; Kurilich, A; Monoharon, R; Ferruzzi, MG*. **2017**. Changes in Phenolic Content of Commercial Potato Varieties Through Industrial Processing and Fresh Preparation. *Food Chem.* 218:47-55. DOI: 10.1016/j.foodchem.2016.08.126
4. Kiernicki, JJ; **Cladis, DP**; Fanwick, PE; Zeller, M; Bart, SC*. **2015**. Synthesis, Characterization, and Stoichiometric U-O Bond Scission in Uranyl Species Supported by Pyridine(diimine) Ligand Radicals. *J. Am. Chem. Soc.*, 137: 11115-11125. DOI: 10.1021/jacs.5b06217
5. **Cladis, DP***; Zhang, R; Tan, X; Craig, B; Santerre, CR. **2015**. A Postharvest Correlation between Swordfish (*Xiphias gladius*) Size and Mercury Concentration in Edible Tissues. *J. Food Prot.*, 78 (2): 396-401. DOI: 10.4315/0362-028X.JFP-14-449
6. Kleiner, AC; **Cladis, DP**; Santerre, CR*. **2015**. A Comparison of Actual Versus Stated Label Amounts of EPA and DHA in Commercial Omega-3 Dietary Supplements in the United States. *J. Sci. Food Agric.*, 95 (6): 1260-1267. DOI: 10.1002/jsfa.6816
7. **Cladis, DP**; Kleiner, AC; Freiser, HH; Santerre, CR*. **2014**. Fatty Acid Profiles of Commercially-Available Finfish Fillets in the United States. *Lipids*, 49 (10): 1005-1018. DOI: 10.1007/x11745-014-3932-5 (Cited in the Scientific Report of the 2015 Dietary Guidelines Advisory Committee)
8. **Cladis, DP**; Kleiner, AC; Santerre, CR*. **2014**. Mercury Content in Commercially Available Finfish in the United States. *J. Food Prot.*, 77 (8): 1361-1366. DOI: 10.4315/0362-028X.JFP-14-097 (Cited in Advice About Eating Fish, From the EPA and FDA, 82 FR 6571)

9. **Cladis, DP**; Kiernicki, JJ; Fanwick, PE; Bart, SC*. **2013**. Multi-electron Reduction Facilitated by a Trianionic Pyridine(diimine) Ligand. *Chem. Commun.*, 49 (39): 4169-4171. DOI: 10.1039/c2cc37193f
10. Mohammad, A; **Cladis, DP**; Forrest, WE; Fanwick, PE; Bart, SC*. **2012**. Reductive Heterocoupling Mediated by Cp*₂U(2,2'-bpy). *Chem. Commun.*, 48 (11): 1671-1673. DOI: 10.1039/c2cc16200h

Submitted manuscripts

1. McCabe, GP*; Ferruzzi, MG; Brown, AF; Lila, MA; **Cladis, DP**; Weaver, CM. Principal Components as a Tool in the Selection of Blueberry Germplasm for Biomedical Experiments. *Stat. Med.*
2. Maiz, M[^]; **Cladis, DP**[^]; Lachcik, PJ; Janle, EM; Lila, MA; Ferruzzi, MG; Weaver, CM*. Acute Bioavailability of (Poly)phenolic Content of Different Varieties of *Vaccinium spp.* in Ovariectomized Rats. *J Funct Foods.* (^Authors contributed equally)
3. **Cladis, DP**; Li, S; Reddavari, L; Cox, A; Ferruzzi, MG; Weaver, CM*. A 90-day Oral Toxicity Study of Blueberry Polyphenols in Ovariectomized Sprague-Dawley Rats. *Food Chem Toxicol.*
4. Cao, S; **Cladis, DP**; Weaver, CM*. Calcium Absorption and Metabolism is Influenced by Age, Sex, Race, Bioactive Constituents and the Gut Microbiome. *J Ag Food Chem.*

Articles in preparation

1. **Cladis, DP**; Debelo, H; Lachcik, PJ; Ferruzzi, MG; Weaver, CM*. Increasing doses of blueberry polyphenols alters colonic metabolism and calcium absorption in ovariectomized rats.
2. Maiz, M; Henry, C; Lachcik, PJ; **Cladis, DP**; Lila, MA; Ferruzzi, MG; McCabe, G; Weaver, CM*. Lower Doses of Wild Blueberry and More Effective at Increasing Net Calcium Retention in Ovariectomized Rats.
3. **Cladis, DP**; Cooper, K; Ferruzzi, MG; Weaver, CM. Changes in blueberry polyphenol metabolism are dependent on dose and food matrix over 90 days in ovariectomized rats.

Planned Manuscripts

1. Esmaeilzadeh, H; **Cladis, DP**; dos Santos, A. Reactive Oxygen Species in Erythrocytes: A novel method for measuring changes in rodents over 90 days.
2. Li, S; **Cladis, DP**; Reddavari, L. Changes in colonic markers of inflammation over 90 days with increasing doses of blueberry polyphenols.

Departmental seminars

1. **Cladis, DP. 2019.** "Metabolic Fate of Bioactive Polyphenols". *Purdue Univ*, West Lafayette, IN, September 24.
2. **Cladis, DP. 2017.** "Health Effects of Increased Levels of Anthocyanin Consumption". *Purdue Univ*, West Lafayette, IN, November 16.
3. **Cladis, DP. 2013.** "Chemical Contaminants Produced during High Temperature Processing of Food", *Purdue Univ*, West Lafayette, IN, October 29.
4. **Cladis, DP. 2010.** A Review of $\text{ACu}_3\text{B}_4\text{O}_{12}$ Ordered Perovskites and 'Heavy-Mass Behavior of Ordered Perovskites $\text{ACu}_3\text{B}_4\text{O}_{12}$ ($A = \text{Na, Ca, La}$)'. *Univ Northern Colorado*, Greeley, CO, April 2.

Abstracts Presented (*presenter listed in italics*)

International Meetings

1. **Cladis, DP;** Martin, BR; Naja, F; Donángelo, CM; Shyur, L-F; Yang, FL; Wiafe-Addai, B; Welch, A; Weaver, CM. **2018.** Mineral and Phenolic Content in Representative Diets from Seven Countries for the International Breast Cancer and Nutrition (IBCN) Project. *8th International Breast Cancer Prevention Symposium*, West Lafayette, IN, Oct 9, oral. ([Catherine Peachy Oral Presentation Award winner](#))
2. **Cladis, DP;** Martin, BR; Naja, F; Donángelo, CM; Shyur, L-F; Yang, FL; Wiafe-Addai, B; Welch, A; Weaver, CM. **2018.** Mineral and Phenolic Content in Representative Diets from Eight Countries for the International Breast Cancer and Nutrition Project. *8th International Breast Cancer Prevention Symposium*, West Lafayette, IN, Oct 8, poster.

National Meetings

1. **Esmailzadeh, H; Cladis, DP;** Serpa, PBS; Elshafie, NO; Lachcik, PJ; Weaver, CM; Santos, AP. **2019.** Evaluation of Oxidative Damage in Erythrocytes of Rats Exposed to High Doses of Blueberry Polyphenols. *American Society for Veterinary Clinical Pathology & American College of Veterinary Pathologists Nat'l Mtg*, San Antonio, TX, Nov 11, poster.
2. **Cladis, DP;** Debelo, H; Ferruzzi, MG; Weaver, CM. **2019.** Colonic Metabolism of Blueberry Polyphenols in Ovariectomized Rats Increases in a Dose-Response Fashion. *American Society for Nutrition Nat'l Mtg*, Baltimore, MD, June 11, oral.
3. **Cladis, DP;** Debelo, H; Ferruzzi, MG; Weaver, CM. **2019.** Colonic Metabolism of Blueberry Polyphenols in Ovariectomized Rats Increases in a Dose-Response Fashion. *American Society for Nutrition Nat'l Mtg*, Baltimore, MD, June 8, poster #OR34-06-19. ([Runner-Up, ASN Emerging Leaders in Nutrition, Dietary Bioactive Components RIS](#))

4. **Cladis, DP**; Andolino, C; Calvert, R; Running, CR. **2019**. Demonstrating protein functionality in foods: an innovative teaching lab for students in any setting. *Institute of Food Technologists Nat'l Meeting*, New Orleans, LA, June 3, poster #P02-001.
5. **Cladis, DP**; Martin, BR; Naja, F; Donángelo, CM; Shyur, L-F; Yang, FL; Wiafe-Addai, B; Welch, A; Weaver, CM. **2018**. Mineral and Phenolic Content in Representative Diets from Eight Countries for the International Breast Cancer and Nutrition Project. *American Society for Nutrition Nat'l Mtg*, Boston, MA, June 10, poster.
6. *Maiz-Rodriguez, M*; **Cladis, DP**; Lachick, PJ; Lila, MA; Ferruzzi, MG; Weaver, CM. **2016**. Acute Bioavailability of (Poly)phenolic Content of Different Varieties of *Vaccinium spp.* in Ovariectomized Rats. *Experimental Biology Nat'l Meeting*, San Diego, CA, April 3, poster. ([Finalist, Emerging Leaders in Nutrition Science Competition](#))
7. *Furrer, A*; **Cladis, DP**; Kurilich, A; Hawkins, G; Manoharan, R; Ferruzzi, MG. **2015**. Effect of Fresh Preparation and Industrial Processing on Stability of Potato Phytochemicals. *Experimental Biology Nat'l Meeting*, Boston, MA, March 31, poster. ([2nd place, Emerging Leaders in Nutrition, Dietary Bioactive Research Interest Section](#))
8. Kleiner, AC; **Cladis, DP**; *Santerre, CR*. **2014**. Fatty Acid Composition of Commercial Fish Oil Supplements in the United States. *Environmental Protection Agency Fish Contaminant Forum*, Alexandria, VA, September 23, poster.
9. **Cladis, DP**; Kleiner, AC; *Santerre, CR*. **2014**. Mercury Content of Commercial Fish from Across the U.S. *Environmental Protection Agency Fish Contaminant Forum*, Alexandria, VA, September 23, poster.
10. **Cladis, DP**; Kleiner, AC; Freiser, HH; *Santerre, CR*. **2014**. Fatty Acid Profiles of Commercial Fish from Across the U.S. *Environmental Protection Agency Fish Contaminant Forum*, Alexandria, VA, September 23, poster.
11. **Cladis, DP**; Tan, X; Craig, BA; *Santerre, CR*. **2014**. Mercury in Commercially-Available U.S. Finfish & a Fish Size-Mercury Correlation for Swordfish. *International Assoc. for Food Protection Nat'l Meeting*, Indianapolis, IN, August 6, poster #P3-125.
12. **Cladis, DP**; Kleiner, AC; Freiser, HH; *Santerre, CR*. **2014**. Fatty Acid Profiles of Commercial Fish from Across the U.S. *Institute of Food Technologists Nat'l Meeting*, New Orleans, LA, June 23, poster #138-05. ([Finalist, IFT Graduate Research Paper Competition, Nutrition Division](#))
13. Kleiner, AC; **Cladis, DP**; *Santerre, CR*. **2014**. Commercial Fish Oil Supplements in the United States – Fatty Acid Composition. *Institute of Food Technologists Nat'l Meeting*, New Orleans, LA, June 23, poster #138-24.

14. **Cladis, DP**; Kleiner, AC; Santerre, CR. **2014**. Mercury Content of Commercial Fish from Across the U.S. *Institute of Food Technologists Nat'l Meeting*, New Orleans, LA, June 24, poster #254-10.
15. **Kiernicki, JJ**; **Cladis, DP**; Fanwick, PE, Bart, SC. **2013**. Reactivity of Highly Reduced Uranium Complexes Bearing Redox Active Pyridine(diimine) Ligands. *American Chemical Society Nat'l Meeting*, Indianapolis, IN, September 10, oral.
16. **Bart, SC**; **Cladis, DP**; Kraft, SJ; Schaefer, BA; Kiernicki, JJ; Fanwick, PE. **2012**. Multi-Electron Processes at Uranium Mediated by Redox-Active Ligands. *Gordon Research Conference*, Newport, RI, July 10, oral.
17. **Bart, SC**; **Cladis, DP**; Schaefer, BA; Kraft, SJ; Fanwick, PE. **2012**. Multi-electron Processes Involving Uranium Complexes Supported by Redox-active Ligands. *American Chemical Society Nat'l Meeting*, San Diego, CA, March 27, oral.
18. **Mohammad, A**; **Cladis, DP**; Fanwick, PE; Bart, SC. **2012**. Synthesis and Reactivity of Trivalent Uranium Complex (Containing a Reduced Monoanionic Bipyridine) with Carbonylated Small Molecules and O-transfer Agent." *American Chemical Society Nat'l Meeting*, San Diego, CA, March 25, oral.

Local Meetings

1. **Cladis, DP**; Debelo, H; Ferruzzi, MG; Weaver, CM. **2019**. Colonic Metabolism of Blueberry Polyphenols in Ovariectomized Rats Increases in a Dose-Response Fashion. *Office of Interdepartmental Graduate Programs Spring Poster Session*, West Lafayette, IN, May 1, poster.
2. **Esmailzadeh, H**; **Cladis, DP**; Silva Serpa, PB; Lachcik, PJ; Weaver, CM; dos Santos, AP. **2019**. Effects of blueberry toxicity on reactive oxygen species production and red blood cell morphology. *Purdue International Scholar Research Symposium*, West Lafayette, IN, April 17, poster.
3. **Westendorf, SG**; **Cladis, DP**. **2019**. Health and Safety of Blueberry Polyphenols Using a 90-Day Oral Toxicity Model in Rats. *Purdue Undergraduate Research Conference*, West Lafayette, IN, April 9, poster #202b.
4. **Cooper, K**; **Cladis, DP**. **2019**. Quantification and Analysis of Blueberry Polyphenols. *Purdue Undergraduate Research Conference*, West Lafayette, IN, April 9, poster #163b.
5. **Cladis, DP**; Martin, BR; Naja, F; Donángelo, CM; Shyur, L-F; Yang, FL; Wiafe-Addai, B; Welch, A; Weaver, CM. **2018**. Mineral and Phenolic Content in Representative Diets from Eight Countries for the International Breast Cancer and Nutrition Project. *Office of Interdepartmental Graduate Programs Spring Poster Session*, West Lafayette, IN, May 2, poster.

6. **Cladis, DP**; Martin, BR; Naja, F; Donángelo, CM; Shyur, L-F; Yang, FL; Wiafe-Addai, B; Welch, A; Weaver, CM. **2018**. Mineral and Phenolic Content in Representative Diets from Eight Countries for the International Breast Cancer and Nutrition Project. *Nutrition Science Corporate Affiliates Poster Session*, West Lafayette, IN, February 23, poster.
7. **Cladis, DP**; McCabe, GP; Lila, MA; Weaver, CM; Ferruzzi, MG. **2016**. (Poly)phenol Content and Stability of *Vaccinium spp.* Berries. *Whistler Center Annual Meeting*, West Lafayette, IN, May 10, poster.
8. **Maiz-Rodriguez, M**; **Cladis, DP**; Lachick, PJ; Lila, MA; Ferruzzi, MG; Weaver, CM. **2016**. Acute Bioavailability of (Poly)phenolic Content of Different Varieties of *Vaccinium spp.* in Ovariectomized Rats. *Health and Disease: Science, Technology, Culture and Policy Graduate Student Poster Competition*, West Lafayette, IN, March 28, poster. (1st place, Wellness and Prevention category)
9. **Maiz-Rodriguez, M**; **Cladis, DP**; Lachick, PJ; Lila, MA; Ferruzzi, MG; Weaver, CM. **2016**. Acute Bioavailability of (Poly)phenolic Content of Different Varieties of *Vaccinium spp.* in Ovariectomized Rats. *Nutrition Science Corporate Affiliates Poster Session*, West Lafayette, IN, February 26, poster.
10. **Cladis, DP**; Maiz-Rodriguez, M; Lila, MA; Weaver, CM; Ferruzzi, MG. **2015**. Characterization of Ten Varieties of *Vaccinium spp.*: (Poly)Phenol Content of Different Berries. *Bioactives and Health Symposium*, West Lafayette, IN, August 28, poster.
11. **Maiz-Rodriguez, M**; **Cladis, DP**; Lachick, PJ; Janle, E; Ferruzzi, MG; Weaver, CM. **2015**. Bioavailability of Flavonoids from Different Blueberry (*Vaccinium spp.*) Cultivars in Ovariectomized Rats. *Bioactives and Health Symposium*, West Lafayette, IN, August 28, poster.
12. **Furrer, A**; **Cladis, DP**; Kurilich, A; Hawkins, G; Manoharan, R; Ferruzzi, MG. **2015**. Effect of Fresh Preparation and Industrial Processing on Stability of Potato Phytochemicals. *Whistler Center Annual Meeting*, West Lafayette, IN, May 12, poster.
13. **Cladis, DP**; Zhang, R; Tan, X; Craig, BA; Santerre, CR. **2015**. Swordfish Size-Mercury Correlation: New Tools for Postharvest Safety Analysis. *Office of Interdepartmental Graduate Programs Spring Poster Session*, West Lafayette, IN, April 1, poster. (Winner, Certificate of Excellence in Research Award, Office of Interdisciplinary Graduate Programs)
14. **Cladis, DP**; Zhang, R; Tan, X; Craig, BA; Santerre, CR. **2015**. A Postharvest Size-Mercury Correlation for Swordfish. *Nutrition Science Corporate Affiliates Poster Session*, West Lafayette, IN, February 27, poster.
15. **Kleiner, AC**; **Cladis, DP**; Santerre, CR. **2014**. Fatty Acid Composition of Commercial Fish Oil Dietary Supplements in the U.S. *Office of Interdepartmental Graduate Programs Spring Poster Session*, West Lafayette, IN, April 2, poster.

16. **Cladis, DP**; Kleiner, AC; Freiser, HH; Santerre, CR. **2014**. To Eat or Not to Eat: Omega-3's and Mercury in Seafood from Across the United States. *Office of Interdepartmental Graduate Programs Spring Poster Session*, West Lafayette, IN, April 2, poster.
17. **Cladis, D. P.**; Kleiner, A. C.; Santerre, C. R. **2014**. Mercury in Commercially-Available U.S. Finfish. *Nutrition Science Corporate Affiliates Poster Session*, West Lafayette, IN, February 28, poster.
18. **Kleiner, AC; Cladis, DP**; Santerre, CR. **2014**. Fatty Acid Composition of Commercial Omega-3 Dietary Supplements in the U.S. *Nutrition Science Corporate Affiliates Poster Session*, West Lafayette, IN, February 28, poster.
19. **Cladis, DP**; Kleiner, AC; Freiser, HH; Santerre, CR. **2014**. EPA and DHA in Commercially-Available U.S. Finfish. *Nutrition Science Corporate Affiliates Poster Session*, West Lafayette, IN, February 28, poster.
20. **Cladis, DP**; Kleiner, AC; Freiser, HH; Santerre, CR. **2013**. Omega-3 Fatty Acids and Mercury in Seventy-Seven Species of Commercial Fish. *Next Generation Scholars Fair*, West Lafayette, IN, November 3, poster.
21. **Kiernicki, JJ; Cladis, DP**; Fanwick, PE; Bart, SC. **2012**. Reactivity of Highly Reduced Uranium Complexes bearing Redox Active Pyridine(diimine) and Bulky Ancillary Ligands. *Purdue Indiana Notre Dame Universities Inorganic Chemistry Conference*, South Bend, IN, December, poster.
22. **Cladis, DP**; Mohammad, A; Fanwick, PE; Bart, SC. **2011**. C-O Bond Activation in Redox-Active Uranium Complexes. *Purdue Indiana Notre Dame Universities Inorganic Chemistry Conference*, Bloomington, IN, November 5, poster.

TEACHING AND ADVISING

Teaching

Teaching Assistant (7 unique courses; 18 total sections taught)

- 2017-18** *Food Chemistry* (NUTR 453, taught twice); Lab TA & delivered one lecture, Dept Nutrition Sci, Purdue Univ
- 2018** *Nutrition throughout the Life Cycle* (NUTR 365); Grader & delivered one lecture, Dept Nutrition Sci, Purdue Univ
- 2013-14** *Food Science I* (NUTR 205, taught five times); Head TA (twice) & lab TA (three times), Dept Nutrition Sci, Purdue Univ
- 2012** *Transition Metal and Organometallic Chemistry* (CHM 647); Grader & delivered two lectures, Dept Chemistry, Purdue Univ
- 2010-11** *General Chemistry* (CHM 115, taught three sections); Lab & recitation TA, Dept Chemistry, Purdue Univ
- 2010** *Chemistry for Citizens* (CHEM 102); Lab TA, Dept Chemistry & Biochemistry, Univ Northern Colorado
- 2009-10** *General Chemistry I* (CHEM 111, taught five sections); Lab TA, Dept Chemistry & Biochemistry, Univ Northern Colorado

Teaching Certificates

- 2018** Certificate of Foundations in College Teaching
- 2018** Graduate Teaching Certificate (GTC)

Research Advising

Graduate Students

- 2019-present** Kendal Schmitz (PhD student in Hill-Gallant lab)
Trained & mentored in animal work; assisted in planning & completing two animal studies

Undergraduate Students (13 students)

- 2018-19** Kaitlyn Cooper (Fr, Honors Biology major, class of 2022)
Poster abstract: Quantification and Analysis of Blueberry Polyphenols
- 2018-19** Sam Westendorf (Fr, Honors Biology major, class of 2022)
Poster abstract: Health and Safety of Blueberry Polyphenols Using a 90-Day Oral Toxicity Model in Rats
- 2018-19** *Berries & Bone project, mentored through all aspects of performing an animal study and developing a research paper:*
Josie Austin (Fr, Nutrition Sci major, class of 2022)
Kaylee Brunsting (Soph, Honors Nutrition Sci major, class of 2022)
Lindsey Bullerman (Fr, Food Sci major, class of 2022)

Abby Emigh (Jr, Nutrition Sci major, class of 2020)
Samuel Loebig (Jr, Public Health major, class of 2021)
Samantha Meima (Jr, Nutrition Sci major, class of 2020)
Emilee Pfeifer (Jr, Nutrition Sci major, class of 2020)
Erin Roy (Jr, Nutrition Sci major, class of 2020)
Xinyue Xie (Soph, Food Sci major, class of 2021)

2012-13

Fatty Acids & Mercury in Finfish, supervised lab work and data entry:

Sara Foresman, RD, CDSN (Clinical dietician at Arizona Burn Center;
Purdue BS in Dietetics & Nutrition 2015)

Alayne Meyer, MS (Genetic Counselor at Nationwide Children's Hospital;
Purdue BS in Agricultural & Biological Engineering 2015)