A BLUEBERRY-ENRICHED DIET MAY AID IN THE AMELIORATION OF BONE LOSS IN THE OVARIECTOMIZED RAT MODEL

by

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To my mother, whose prayers were always with me throughout this challenging, yet fulfilling PhD journey…

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3Me-PAA	3-Methylphenylacetic acid
3OH-4MeBA	3-Hydroxy-4-methoxybenzoic acid
3OH-4-MePAA	3-Hydroxy-4-methoxyphenylacetic acid
3OH-4MePPA	3-Hydroxy-4-methoxyphenylpropionic acid
3OH-HA	3-Hydroxyhippuric acid
4OH-PAA	4-Hydroxyphenylacetic acid
ALP	Alkaline phosphatase
AUC	Area under the curve
BA-glcr	Benzoic acid-4-glucuronide
BA-O-S	Benzoic acid -4-O-sulfate
BB	Blueberry
BMC	Bone mineral content
BMP	Bone morphogenic protein
BSAP	Bone specific alkaline phosphatase
BV	Bone volume
BV/TV	Relative bone volume
C	Catechin
CA	Caffeic acid
CA-glcr	Caffeic acid 3-O-β-D-glucuronide
CA-O-S	Caffeic acid-3-O-sulfate
CBG	Cytosolic β -glycosidase
Chlr	Cholorogenic acid
Cmax	Maximum plasma concentration
Conn. D.	Connectivity density
Ct. Ar/Tt Ar	Cortical area to total area fraction
Ct. Th	Cortical Thickness
Cy	Cyanidin
Del	Delphinidin
EC	Epicatechin

Ec. Pm	Endocortical perimeter
ESI	Electrospray ionization
FeA	Ferulic acid
FeA-glcr	Ferulic acid 4-O-glucuronide
FeA-O-S	Ferulic acid-4-O-sulfate
glcr	Glucuronide
glcs	Glucosides
HA	Hippuric acid
HB	Highbush
IGF-1	Insulin-like growth factor
IL	Interleukin
L(i,j)	Transfer coefficient into compartment i from compartment j
LB	Lowbush
LPH	Lactase-phlorizin hydrolase
M(i)	Mass of compartment i.
Mal	Malvidin
Mapk	Macrophage colony-stimulating factor
Me	Methyl
Mont	Montgomery
MRP-2	Multidrug-resistance protein 2
Myr	Myricetin
Ntx	N-terminal telopeptide of type 1 collagen
Oc. N	Osteoclast number
OCN	Osteocalcin
OPG	Osteoprogerin
OVX	Ovariectomy
PCA	Principle component analysis
Peo	Peonidin
Pet	Petunidin
PPARγ	Peroxisome proliferator-activated receptor γ2
Ps. Pm	Periosteal perimeter

PTH	Parathyroid hormone
Q	Quercetin
RANKL	Receptor activator of nuclear factor kappa-B ligand
RE	Rabbiteye
Runx2	Runt-related transcription factor 2
SD	Sprague-Dawley
Tb. N	Trabecular number
Tb. Sp	Trabecular spacing
Tb. Th	Trabecular thickness
TMA	Total monomeric Anthocyanins
Tmax	Time of maximum plasma concentration
TNF-α	Tumor necrosis factor
TP	Total Phenolic
TV	Total volume
V_{0-}	Bone resorption
V_{0+}	Bone deposition
V_a	Calcium absorption
V_f	Endogenous excretion
V_F	Fecal excretion
V_u	Urinary excretion
WO	Washout
μCT	Micro computed tomography

ABSTRACT

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Osteoporosis is the most common bone disease in older adults and is characterized by low bone mass and increased fragility. Women are at a higher risk for osteoporosis because of the rapid loss of bone during menopause. The decline of estrogen is accompanied by an increased bone resorption and a decreased bone formation which results in negative bone balance. Due to adverse effects on the uterus, breast and cardiovascular system, hormone replacement therapy has been discouraged. Nutritional strategies for osteoporosis prevention are being sought. It has been suggested that (poly)phenol-rich fruits may have bone protective effects. Blueberries are one of the richest sources of (poly)phenols, thus the aim of this dissertation was to determine whether a blueberry-enriched diet could aid in bone loss prevention in the ovariectomized rat model.

There are hundreds of blueberry varieties which differ in (poly)phenol profiles and content. Five blueberry varieties (Ira, Montgomery, SHF2B1-21:3, Onslow and Wild Blueberry) were chosen to assess the bioavailability of its individual (poly)phenols. Bioavailability of individual phenolic metabolites was determined through a pharmacokinetic study in ovariectomized rats. The results showed that Montgomery blueberry had significantly higher bioavailability of malvidin, cyanidin and myricetin metabolites, while Ira had significantly higher bioavailability of quercetin metabolites, thus suggesting that the absorption of blueberry polyphenols and their potential to reach target tissues differed between blueberry varieties.

It is important to assess what is the most appropriate dose of blueberry necessary to exert beneficial effects on bone. To determine the most adequate dose of wild blueberry to prevent bone loss in ovariectomized rats, a randomized crossover study was carried out to assess the effects of four different blueberry doses on net bone calcium retention over a 10-day treatment period. The results showed that the only dose to significantly increase net bone calcium retention by 25.6% ($p = 0.0426$) was the 5% blueberry diet (% w/w), while the higher doses of 10% and 15% had no effect on net bone calcium retention. This informed the last study where Montgomery blueberry

and wild blueberry at a 5% dose (% w/w) were chosen to investigate the effects of an 8-week chronic feeding study on calcium metabolism, kinetics, bone microarchitecture and strength and polyphenol metabolism and distribution. A chronic consumption of the wild blueberry resulted in a trend towards minimal trabecular bone loss protection in comparison to the control diet ($p=0.08$). Kinetic modeling of calcium showed that the Montgomery blueberry had anabolic effects on bone through significantly increasing calcium absorption and bone deposition. The phenolic metabolism differed among blueberry varieties due to each berry's polyphenol content and profiles and a chronic consumption of blueberry resulted in significant changes in absorption and metabolism of polyphenols. The bone marrow was investigated to determine whether there was any accumulation of phenolic acids in the tissue. Hippuric acid accumulation was significantly higher with the Montgomery blueberry treatments in comparison to control diet. Interestingly, hippuric acid content in the bone marrow was significantly and positively correlated with bone deposition calculated from kinetic modeling. Although no differences were observed on bone mineral density, strength, and microarchitecture, previous studies with a duration of 12-14 weeks have shown significant protection of a blueberry-enriched diet on bone mineral density. Because our study showed a trend for increased trabecular bone ($p = 0.08$) with the blueberry treatments, we conclude that an 8-week treatment was insufficient time to detect significant differences between the control and blueberry treatments. Since previous researchers before us have reported significant attenuation to bone loss immediately after OVX, it is possible that blueberry that in our study, blueberry was unable to rescue bone once lost after ovariectomy.

A blueberry-enriched diet resulted in a minimal protection to bone after stabilized to OVX, but showed significant increases in calcium absorption and bone turnover in ovariectomized rats. Colonic metabolite profiles from the chronic consumption of blueberry significantly changed over time, thus providing an insight into the effects of blueberry consumption on polyphenol metabolism.

CHAPTER 1. INTRODUCTION

1.1 Osteoporosis as a public health concern and preventive strategies

Osteoporosis is the most common bone disease in older adults and is characterized by low bone mass and increased fragility that can result in fracture at the spine hip, or wrist (Imel, DiMeglio and Burr). It is caused by an increased bone resorption and decreased bone formation, resulting in a net bone loss. One out of three women will suffer from an osteoporotic fracture as will 1 out of 5 men (Foundation). It accounts for most hospital days than any other disease, including diabetes, heart attack and breast cancer (Sacco, Horcajada and Offord). It has also posed a great economic burden as health costs related to the disease are more than €31 billion per year in Europe and $20 billion in the United States. Prevalence of osteoporosis in the United States is estimated to increase by approximately 17.3 million (32%) from 2010 to 2030 (Wright, Looker, et al.) and costs are projected to continue increasing by almost 50% by the year 2025 (Burge et al.). Together, low bone mass and osteoporosis, affects 54% of older adults in the United States (Wright, Looker, et al.). Due to steady increases in life expectancy, osteoporosis prevention becomes of great concern for a better quality of life in aging populations.

It has been well established that nutrition plays an important role in the prevention of the onset of osteoporosis. An adequate intake of calcium in children and adolescents significantly increases total body and lumbar spine bone mineral content (BMC) (Huncharek, Muscat and Kupelnick) which may result in stronger bones less prone to fracture in the later stages of life. Throughout all life stages, an adequate intake of calcium and Vitamin D is essential for bone maintenance. Calcium intake recommendations vary depending life stage. During menopause an intake of 1200 mg Ca/day is recommended, but no additional protection is observed with an intake higher than the recommended, while an intake below the recommended results in significant bone

loss (Burckhardt). The increased calcium requirement for women is due the decreased Ca absorption that occurs after menopause.

During menopause, estrogen levels decline, causing a loss of its bone protective effects, leading to a period of rapid bone loss. As estrogen decreases, bone remodeling increases, leading to an increased release of Ca from bone which in turn suppresses parathyroid hormone (PTH), leading to a decrease in renal $1,25(OH)_2D$ production culminating in a decreased intestinal Ca absorption efficiency and an increased renal Ca excretion. The loss of estrogen, therefore leads to a net urinary calcium loss, causing a rapid bone loss (DiMeglio and Imel). Once the body has adapted to the loss of estrogen, the decreased intestinal Ca absorption efficiency and the enhanced urinary Ca excretion, there is an increase in the release of PTH, which helps restore the body's balanced state, slowing down the loss of bone. Although, an adequate intake of calcium and vitamin D is essential, they are insufficient to overcome the negative effects on bone from the loss of estrogen during menopause.

Since estrogen replacement therapy has been discouraged due to adverse effects on the uterus, breast and cardiovascular system (Rossouw et al.), other nutritional preventative strategies are being sought. Evidence from mechanistic and preclinical studies is accumulating showing that dietary polyphenols from plum, grape and blueberry may be capable of modulating bone turnover (Devareddy et al.; Hohman and Weaver; Pawlowski et al.; Li et al.). With an increased interest on blueberry's high phenolic content and health benefits, this dissertation project focused on determining three major issues. Since there are hundreds of blueberry varieties, we aimed to determine 1) whether there is a difference between the bioavailability of blueberry polyphenols in ovariectomized rats, 2) the most effective dose of blueberry consumption to increase net bone

calcium retention in ovariectomized rats and 3) the effects of a chronic blueberry consumption on bone loss prevention, calcium and polyphenol metabolism in ovariectomized rats.

1.2 Research Aims

1.2.1 Blueberry Polyphenol Bioavailability

Blueberries have a wide range of health promoting activities due to their high levels of (poly)phenols, including anthocyanins, flavonols, and phenolic acids. However, there are hundreds of varieties of blueberries and their phenolic content varies greatly (Yousef, Brown, et al.). Whether there is a difference in absorption and metabolism of polyphenols from different blueberry varieties is unknown. Thus, the goal of this study was to determine whether there is a difference in the bioavailability of individual flavonoids after an acute dose in ovariectomized rats. Five varieties of berries (Ira, Montgomery, Onslow, SHF2B1-21:3, Wild blueberry commercial mix) were selected based on principle component analysis (PCA) of their anthocyanin content and sourced from the North Carolina State University Blueberry Genome Repository.

1.2.2 Dose Response effects of blueberry on net bone calcium retention

A Blueberry-enriched diet has been shown to have bone health effects in animal models, not only in the adult postmenopausal model by attenuating bone loss, but also in young rats by increasing bone accrual during growth. The effect of blueberry on calcium metabolism has not yet been elucidated. Our objective was to determine the effective dose of blueberry consumption to increase net bone calcium retention in ovariectomized rats using ^{45}Ca technology. By determining the most effective dose, we were able to use that dose for a chronic feeding study to determine differences in bone mineral density and microarchitecture.

1.2.3 Effects of a chronic consumption of blueberry on bone health in ovariectomized rats

A chronic feeding with blueberry has shown to be effective in the attenuation of ovariectomy-induced bone loss in SD-rats. Previous studies have shown a significantly higher bone mineral density (BMD) in BB-fed rats in comparison to control rats (Devareddy et al.; Li et al.). The mechanism through which blueberries exert a beneficial effect on bone remains to be elucidated. No research has been conducted on the effects of a chronic consumption of blueberry on calcium metabolism. Thus, this study aimed to determine the effects of an 8-wk chronic consumption of two different varieties of blueberry on calcium balance, calcium kinetics, bone microarchitecture and polyphenol metabolism and distribution in ovariectomized rats.

CHAPTER 2. BACKGROUND

2.1 Osteoporosis

Osteoporosis is a major public health concern as it is the most common bone disease in older adults with an increasing incidence and a great economic burden. Osteoporosis is characterized by low bone mass and increased fragility that can result in fracture at the spine, hip, or wrist (Imel, DiMeglio and Burr) followed by an increased risk of mortality (Abrahamsen et al.) . Together, low bone mass and osteoporosis, affects 54% of older adults in the United States (Wright et al.). One out of three women will suffer from an osteoporotic fracture as will one out of five men (Foundation). In women, it is mainly attributed to the loss of estrogen's bone protective effects during menopause causing a rapid loss of bone (Hughes et al.). Osteoporosis accounts for most hospital days than any other disease, including diabetes, heart attack and breast cancer (Sacco, Horcajada and Offord). It also poses a great economic burden, as health costs related to the disease are more than $20 billion in the United States and €31 billion per year in Europe (Wright et al.). Since the world's population aged ≥ 65 years will double from 2010 to 2040 (Brauer et al.), prevention of osteoporosis becomes imperative for a better quality of life.

2.1.1 Pathogenesis of Osteoporosis

Bone is a dynamic tissue that is constantly undergoing remodeling. Old bone is replaced by new bone by the coupled activity between osteoblasts, the bone formation cells, and osteoclasts, the bone resorption cells (Nakamura et al.). The bone remodeling process starts with the activation phase in which osteoclasts are recruited to the surface of the bone to be resorbed. Once osteoclast maturation has occurred, the resorption phase starts by the generation of an acid microenvironment between the osteoclast and the surface of the bone which is being resorbed. Once osteoclasts have

concluded their activity, they undergo apoptosis and osteoblasts, are recruited to the bone surface to deposit new bone. The bone formation phase is initiated by the osteoblast deposition of collagen, which is then mineralized to form new bone (Gallagher and Sai). Thus, old bone is continually being replaced with new bone through a coupling activity between osteoclasts and osteoblasts.

During menopause, there is a loss of estrogen which leads to an increased bone resorption and a decrease in bone formation, resulting in net bone loss. Estrogen's protective effect on bone consists of the inhibition of osteoclast generation and induces osteoclast apoptosis, while also decreasing interleulkin-1 (IL-1), IL-6 and tumor necrosis factor (TNF-α), inhibiting osteoclast precursor proliferation and differentiation into mature osteoclasts. Estrogen also induces the apoptosis of mature osteoclasts by activating proapoptopic pathways. On osteoblasts and osteocytes, estrogen inhibits apoptosis by activating survival kinases ERKs and PI3-K, the inactivation of the proapoptopic protein Bad by phosphorylation (Bellido and Hill Gallant). With the loss of estrogen there is an overproduction of both osteoclasts and osteoblasts with a decrease in osteoclast apoptosis and an increased osteoblast and osteocyte apoptosis. All of this leads to a negative bone balance, leading to loss of bone during menopause (Bellido and Hill Gallant).

Nutrition plays a significant role in bone development and maintenance throughout the life stages. Most specifically, an adequate intake of calcium and vitamin D, is crucial for bone health (Holick et al.; Shin and Kim). Other than calcium and vitamin, recent studies have been focusing on other nutritional sources for bone health prevention after New *et al.* (New et al.), through a cross-sectional study in premenopausal women, reported a positive association between fruit consumption and bone mineral density in healthy adults. Although, it was first thought that the beneficial effect of fruits and vegetables on bone was due to an acid-base balance (New et al.), recent research suggests it's the phytochemical composition of fruits and vegetables that exerts the

beneficial effect on bone (Sacco, Horcajada and Offord). In a diet with adequate calcium and vitamin D intake, higher intake of fruits and vegetables can offer bone health benefits beyond those obtained by calcium and vitamin D intake alone.

2.1.2 Risk Factors

Age, ethnicity, race, genetics and nutrition are several of the factors that can influence one's development of osteoporosis (Imel, DiMeglio and Burr). Gender plays an important role in bone health as well. Women have smaller bodies, thus resulting in a smaller skeleton and smaller bone mass than men (Seeman). In comparison to men, they are also at higher risk for osteoporosis due to hormone deficiency during menopause (Cauley). The size of the skeleton is genetically programmed, and genetic predisposition for a smaller skeleton and greater bone loss during adulthood can lead to increased risk for osteoporosis (Smith et al.). Although genes encode for a specific phenotype, they also respond to environmental factors that can lead to epigenetic variation. Modifiable factors, such as an adequate nutrition, physical activity, smoke-free lifestyle and a low alcohol consumption throughout bone development can lead to an improved bone phenotype (Weaver et al.). Early interventions for a higher peak bone mass can significantly decrease one's risk of developing osteoporosis with age. The more bone mass one has, the more one can lose before developing the disease (Imel, DiMeglio and Burr). Thus, regardless of gender and genetics, living a healthy lifestyle may lead to an increased bone mass accrual and reduced risk for osteoporosis.

2.1.3 Current Therapeutic Strategies

More than one third of women reach menopause with low bone density, which is frequently worsened by years of inadequate calcium and/or vitamin D intake, resulting in a higher osteoporosis incidence (Andreopoulou and Bockman). Going through menopause with low bone

mass can result in a BMD of 2.5 standard deviations below the mean value for young sex-matched adults (Sacco, Horcajada and Offord). This reduced BMD at the femoral neck or spine is considered osteoporosis and patients are advised to undergo therapy to prevent a fracture. Although the rapid bone loss during menopause is due to estrogen deficiency, the use of estrogen replacement therapy is limited because of its adverse effects in the uterus, breast and cardiovascular system (Rossouw et al.). Current osteoporosis therapies substantially reduce the risk of vertebral and hip fractures. However, there are concerns of long-term usage safety (Reid).

Therapeutic treatment involves the use of agents such as bisphosphonates, denosumab, parathyroid hormone preparations, cathepsin K inhibitors and sclerostin antibodies (Reid). Bisphosphonates and denosumab inactivate and prevent osteoclast maturation, respectively. This reduces bone resorption, while preserving bone formation rates (Papapoulos et al.). Teriparatide is an anabolic agent that increases bone formation and has a greater effect if combined with antiresorptive agents. Cathepsin K inhibitors work by decreasing the cathepsin k released by the osteoclast, whose function is to digest type I collagen proteins from the bone matrix. Sclerostin inhibitors act through the increased activation of the Wnt/β-Catenin pathway that culminates in osteocyte and osteoblast survival and activation. Although these therapeutic agents can treat osteoporosis, they have adverse effects such as hypercalcemia, osteonecrosis of the jaw, fractures in the lateral cortex of the femoral shaft or gastrointestinal distress (Iñiguez-Ariza and Clarke; Papapoulos et al.; Reid; Andreopoulou and Bockman). These side effects have led to long-term usage concerns, suggesting a need for safe alternatives for bone formation and bone loss prevention.

Nutritional interventions, such as calcium and vitamin D play a significant role in bone health during aging as it has been shown that they are effective in reducing fracture risk among elderly individuals. A recent meta-analysis by Weaver *et al*. (Weaver et al.) reported that calcium

and Vitamin D reduce the risk of hip and vertebral fracture by 15% and a 30%, respectively. Unfortunately, a significant proportion of the world population does not meet the recommended intake levels for these nutrients (Cashman; Imel, DiMeglio and Burr). In the USA, 9 out of 10 and 4 out of 10 do not consume enough Vitamin D and Calcium, respectively (NHANES). This has led to an interest in alternative nutritional interventions such as (poly)phenol-rich fruits like blueberry, that, through molecular and cellular mechanisms, could potentially increase bone formation and prevent bone loss.

Evidence is accumulating that dietary polyphenols appear to be capable of modulating bone metabolism in mechanistic studies and relevant animal models. Associations between high intakes of polyphenol-rich foods and improved bone parameters have been reported and limited clinical data has also become recently available. To fully understand how these molecules are metabolized with signaling pathways involved in bone health, we will define polyphenols, their structure, and briefly describe their absorption and metabolism in the body.

2.2 Blueberry

2.2.1 The Fruit

Blueberries (*Vaccinium spp.*) are a commonly consumed fruit in the United States, whose demand has been increasing due to its reported health benefits. There are hundreds of varieties of blueberries, members of three major species, the low bush (wild blueberry) (*Vaccinium angustifolium Aiton.*), the highbush (*Vaccinium corymbosum L.*) and the rabbiteye (*Vaccinium ashes Read*) (Yousef et al.). Its production and consumption has been increasing over the years, with a per capita consumption increasing from 0.6 lb. to 1.5 lbs. between the years 2000-2010 (Yousef et al.). The increased demand for blueberries has risen mostly from its health benefits

towards the prevention of cardiovascular, neurodegenerative, and more recently, bone diseases (Devareddy et al.; A. Rodriguez-Mateos et al.; Feliciano et al.).

2.2.2 Bioactivity and health benefits

Blueberries' health benefits have been attributed to their high content of (poly)phenols, more specifically the flavonoids anthocyanins, flavonols, flavan-3-ols, proanthocyanidins, and phenolic acids (A. Rodriguez-Mateos et al.). Some of the many health benefits of blueberry consumption include the amelioration of oxidative stress in adults with metabolic syndrome (Nair et al.), increases in insulin sensitivity in obese men and women (Stull et al.) and improvement of cognitive function in the elderly with mild cognitive impairment (Krikorian et al.).The mechanism of action proposed for blueberry (poly)phenols include their activity as free radical scavengers and their possible influence in cell signaling, anti-inflammatory, and gene expression pathways (Yousef et al.; Lila et al.).

2.2.3 Flavonoids

Flavonoids are plant secondary metabolites that contribute blue and red coloring, synthesized as a response to environmental threats or microbial infections, and thus form part of the human and animal diet (Kumar and Pandey). They have shown to have protective effects against chronic diseases such as cancer, cardiovascular and neurodegenerative diseases (Trzeciakiewicz, Habauzit and Horcajada). Their chemical structure is based on a 15-carbon skeleton, consisting on two benzene rings, A and B, joined together by a heterocyclic pyrene ring, C, as shown in Figure 1.

Based on their oxidation level and pattern of substitution of the C ring, they are classified into six major groups, the anthocyanins, flavonols, flavanols, flavones, flavanones and

isoflavones (Ana Rodriguez-Mateos et al.; A. Rodriguez-Mateos et al.). Within the flavonoid group, blueberries are rich in anthocyanins (cyanidin, malvidin, peonidin, petunidin and delphinidin), flavonols (quercetin, kaempferol and myricetin), flavan-3-ols (e.g. epi/catechin), procyanidins and phenolic acids (chlorogenic acid) (A. Rodriguez-Mateos et al.).

2.2.4 Metabolism and Bioavailability

As polyphenols are ingested (Figure 2.2), they undergo extensive metabolism by phase II enzymes, resulting in polyphenolic conjugates that differ from the parent compound. The parent compound is rarely seen circulating in plasma; therefore, it is important to understand their metabolism and the major metabolites produced after ingestion that will eventually reach tissues of interest and exert their bioactive effect.

2.2.4.1 Anthocyanins

Anthocyanins occur as *O*-glycosides of their respective aglycone. The most common aglycones are malvidin, petunidin, cyanidin, pelargonidin and peonidin (Ronald). Once they are ingested, their absorption begins at the stomach and continues in the small intestine. Anthocyanins can be absorbed in their glycosidic form, unlike other polyphenols that need to be deglycosylated prior to absorption. In the intestine, their glycoside may be cleaved by lactate phlorizin hydrolase, resulting in anthocyanin aglycones which can passively enter the enterocytes (Kay).Once they are absorbed into circulation they are subject to phase II enzymatic metabolism, resulting in glucuronide, methyl and sulfate conjugates, which eventually are excreted through urine. Their bioavailability is quite low, reaching maximum plasma concentrations between 10-50 nM after a 50 mg dose, representing less than 0.1% absorption (Prior and Wu). The anthocyanins that are not absorbed in the small intestine, travel to the lower gut, where they are subject to further metabolism

by the gut microbiome, and may enter into circulation as low molecular weight phenolic acids, which are also subject to phase II metabolism and eventually excreted in the urine (Lila et al.).

2.2.4.2 Flavanols and flavones

In plant tissues, flavonols and flavones are linked to sugar moieties to form glycoside conjugates. Unlike anthocyanins, after ingestion, they must undergo enzymatic cleavage of the glycoside by cytosolic β-glycosidase (CBG) or lactase-phlorizin hydrolase (LPH). Once the aglycone is released, it is transported into epithelial cells either passively through simple diffusion or actively through multidrug-resistance protein 2 (MRP-2). In the epithelial cells, the aglycones are metabolized by phase-II enzymes and are conjugated with glucuronic acid, sulfate or methyl groups. If cleavage of the sugar unit does not occur, they cannot be absorbed and flavanols and flavanones travel to the lower digestive tract where they can be metabolized by the gut microbiome. Metabolites are then transported to the liver for further phase-II metabolism, transportation to other tissues to exert their bioactivity or excreted through the urine (MariuszKonrad, Kaeko and Junji).

2.2.4.3 Flavan-3-ols

Unlike other flavonoids, flavan-3-ols exist in plants mostly as aglycones instead of conjugates of a glycoside. Following their ingestion, absorption occurs by passive diffusion through the intestinal wall. Prior to being released from the epithelial cells into the bloodstream, they undergo phase-II enzymatic metabolism, yielding sulfate, glucuronide and/or methylated metabolites. These are then transported to other tissues or undergo further metabolism in the liver or enterohepatic recirculation.

After metabolism, polyphenol structures change and thus their bioactivity changes with them. Different chemical structures might be able to interact with other proteins and activate

distinct signaling pathways in bone remodeling. Knowledge of the metabolites that are present in plasma after consumption serves to choose a more appropriate compound for *in vitro* studies which will better mimic what actually takes place *in vivo*. An understanding of polyphenolic metabolite mechanism of action could potentially support associations between flavonoid intake and increased bone health that have been previously reported (Devareddy et al.; J. Zhang et al.).

2.3 Dietary polyphenols and Bone Health

A high intake of dietary flavonoids has been associated with a higher BMD in women. The flavonoid subgroups that were identified in these studies were flavonols, flavan-3-ols, flavanones, proanthocyanidins and anthocyanins. The foods that contributed the most to total flavonoid content were grapes, berries, wine, pear and fruit yogurts in the UK population (Welch et al.; Hardcastle et al.), while tea was the highest contributor in the Asian population (Z. Q. Zhang et al.). From the flavan-3-ols, catechin was the most abundant flavonoid which has been proven in mechanistic and pre-clinical studies to have bone health benefits (Choi and Hwang). Hardcastle *et al.* reported that all flavonoid subgroups, except flavones, had a significant positive correlation between intake and BMD at the femoral neck. This group was also the only one to analyze urinary bone resorption biomarkers, and reported that flavones had a mild, but significant negative correlation with bone resorption (Hardcastle et al.)

The type of flavonoid that is being consumed may predict for differences in BMD increases. Some flavonoid subgroups have a stronger correlation with an increased BMD than others. Welch *et al.* (Welch et al.) reported that the subclass of flavonoids that had the greatest effect on predicting BMD were the anthocyanins, which predicted a 3.5% and 3.1% increase at the spine and at the hip, respectively. Although, among the Asian population, this association was not found. Zhang *et al.*

did not find any correlation between anthocyanins and BMD. It is important to clarify that anthocyanin-rich berries were excluded from the food frequency questionnaires in the Asian population due to their low consumption. This could have had an effect in the absence of correlation between them and BMD. Interestingly, it was also reported that flavonoids have a different effect depending on sex steroid status. Postmenopausal women had a positive correlation between anthocyanin consumption and BMD while premenopausal women didn't (Hardcastle et al.).

2.3.1 In Vitro Studies

Molecular mechanisms through which polyphenols promote osteoblastogenesis and prevent osteoclastogenesis are still unclear. A reduction in the receptor activator of nuclear factor kappa-B ligand (RANKL) gene expression and bone resorption biomarkers after the consumption of a blueberry- , grape-, and dried plum-enriched diet in rats has led to recent *in vitro* studies. These have given insight into the mechanisms of action of polyphenols on bone health.

Zhang *et al.* investigated the effects of blueberry (BB) metabolites on bone health by obtaining serum from rats that had been fed a 1, 3, or 5% BB-diet. Cultures with the BB-metabolite enriched serum resulted in a decrease in RANKL expression in the bone marrow-derived stromal cell line ST2 (J. Zhang et al.). This shows that blueberry metabolites have the potential to decrease osteoclastogenesis by reducing the RANKL:OPG ratio, leading to a decrease in bone resorption.

Wnt/β-catenin signaling pathway increases bone mass through the upregulation of osteoblast promoting genes. To investigate whether polyphenols may be involved in this pathway, Chen *et al.* (Chen, Lazarenko, Wu, et al.) used the ST2 cells, a robust osteoblastogenesis cell line. The ST2 cells were treated with serum from rats that were fed a 10% BB-diet. The blueberry metabolites

promoted osteoblastogenesis through the activation of Wnt/β-Catenin pathway, as seen in an increase in β-Catenin accumulation in the cytoplasm and the nucleus. Polyphenols were also able to increase phosphorylation of p38, which together with MAPK, potentiated the downstream Wnt signaling cascade, resulting in an upregulation of expression of Runx2, ALP, OPG and OCN genes that lead to osteoblast differentiation. Thus, blueberry metabolites have the potential to increase bone formation through the activation of the Wnt/β-catenin signaling pathway.

Blueberry polyphenols have also shown to influence mesenchymal stem cell's commitment towards the osteoblast rather than adipocyte lineage. Seven phenolic acids can be identified in rat serum after being fed blueberries, from which hippuric acid (HA) is found in the highest concentration. At serum level concentrations, HA is able to stimulate C2C12 osteoblastic cell line maturation and ST2 cell line proliferation. In the 3T3-L1 pre-adipocyte cell line, differentiation was significantly inhibited by HA as seen by the down-regulation of gene expression of PPARγ. Even when rosiglitazone, an adipogenic agonist, was added to the culture, HA was still able to inhibit adipogenesis and the resulting cells had an osteoblast-like phenotype (Chen, Lazarenko, Zhang, et al.). These data suggest that commitment of mesenchymal stem cells can be influenced by polyphenols to favor bone formation instead of a fatty bone marrow that usually develops with age.

As polyphenols vary greatly in their chemical structures, it is important to identify which chemical structures can activate bone signaling pathways for future therapeutic treatments. Most research uses the whole matrix of polyphenols found in food, but only a few have investigated individual compounds. (+)Catechin, Syringetin, and HA can individually increase osteoblastogenesis, as seen in a significant increase in ALP activity, osteocalcin and BMP-2 after MC3T3 cell treatment (Choi and Hwang; Chen, Lazarenko, Zhang, et al.; Hsu et al.). (+)Catechin

also increases cell viability by decreasing TNF-α induced apoptosis of osteoblasts (Choi and Hwang). Although the whole matrix of polyphenols is more representative of a human diet, characterizing specific polyphenols can provide a better understanding of how their chemical structure might be interacting with signaling pathways to provide bone health benefits.

2.3.2 Blueberry-enriched diet and its effect on bone health in OVX rats

Two animal studies have elucidated the effects of a BB-enriched diet in the prevention of ovariectomy-induced bone loss in sprague-daily (SD) rats. Rats were fed either a 5% BB diet for 100 days (Devareddy et al.) or a 10% BB solution (w/w) administered daily by gavage for 12 weeks post-ovariectomy (Li et al.). Both studies showed that the BB-fed rats had a whole-body BMD no different from the Sham and significantly higher than the OVX control ($p < 0.05$). This increased BMD was due to higher bone volume per total volume (BV/TV%), trabecular thickness (Tb.Th.), trabecular number (Tb.N.), BFR/TV and decreased trabecular spacing (Tb.Sp.) and osteoclast number (Oc.N.) than OVX-control animals (Li et al.). Both studies reported a significant attenuation of bone loss in ovariectomized rats, showing how blueberry consumption during menopause can aid in prevention of hormone deficiency induced rapid bone loss.

2.3.3 Polyphenol-rich foods and its effects on calcium absorption and retention

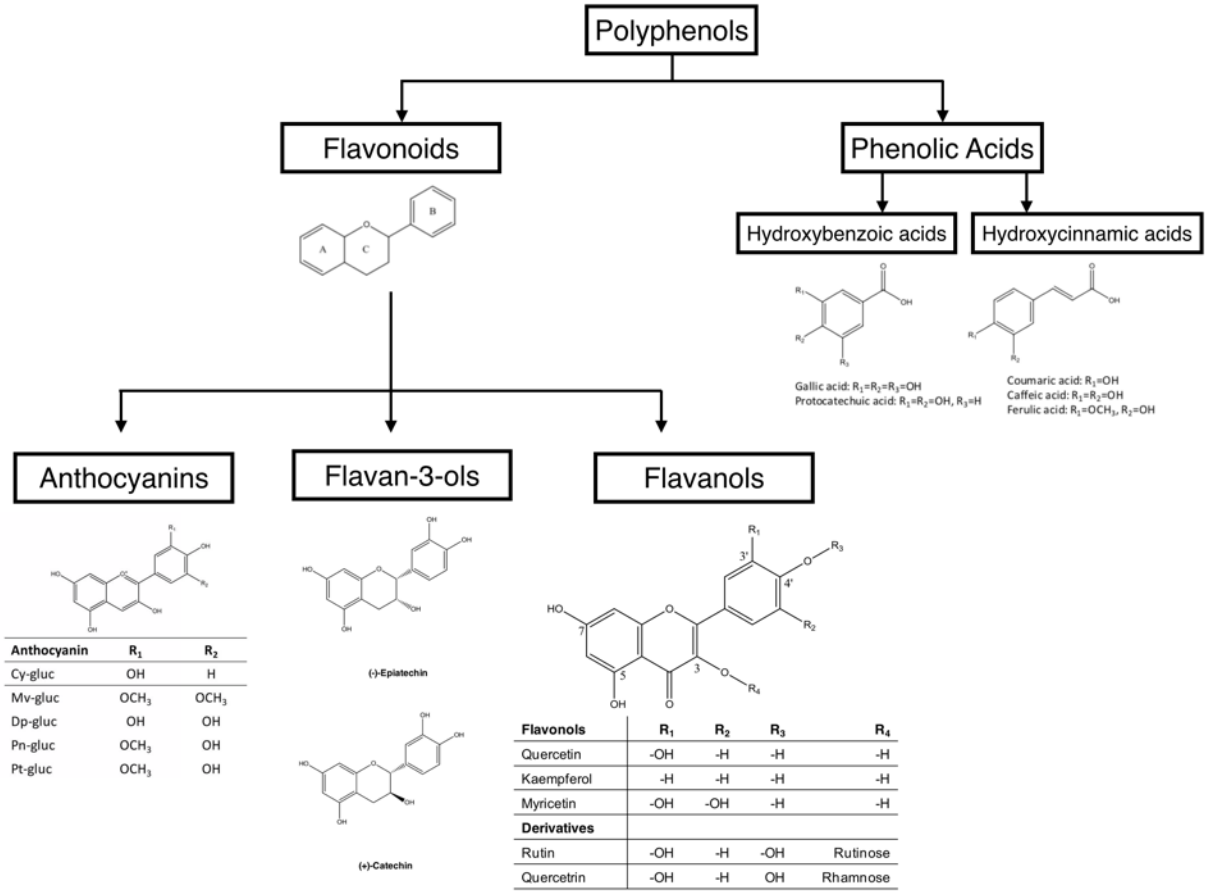
There have not been any studies which have assessed the effects of blueberry consumption on calcium absorption and retention. Other polyphenol-rich fruits, such as grape and dried plum have been investigated. Polyphenols from a grape- and dried plum-enriched diet increase calcium absorption and retention in OVX SD rats. Using ^{45}Ca, Pawlowski *et al*. (Pawlowski et al.) reported that a diet high in plum polyphenol extract (0.45% w/w) and resveratrol (0.2%w/w) during a 10-day treatment significantly increased calcium retention by 20% and 14% respectively. Grape seed extract- and grape-enriched diet, did not show any significant effects on calcium retention, but

they significantly decreased bone resorption as measured in a decrease in urine excretion of Ntx (Pawlowski et al.). A 25% grape-enriched diet feeding treatment of 8 weeks resulted in an increased calcium retention and absorption in comparison to baseline. These changes in calcium metabolism resulted in an increased cortical fraction and thickness and lower cortical porosity and endocortical perimeter, yielding significantly stronger bones ($p < 0.05$) (Hohman and Weaver). Polyphenols could potentially attenuate age-related decrease in calcium absorption, providing yet another mechanism for its bone health benefits.

2.3.4 Polyphenols in clinical studies

There are limited data on the effects of polyphenol-rich fruits on bone health. There have not been any blueberry and bone health clinical studies published, and the only polyphenol-rich fruit that has been studied in humans is plum, and data are limiting. Two studies were carried out by Arjmandi *et al.* (Hooshmand, Brisco and Arjmandi; Hooshmand et al.) where they reported increased increased levels of bone specific alkaline phosphatase (BSAP) and IGF-1 after a 90-day treatment and a significantly higher BMD in postemonopausal women supplemented with 100g of dried plum in comparison to women supplemented with 100g of dried apple. These studies have limitations, the first one being only a 90-day study without any BMD measurements and the second one not having an accurate control group. Therefore, clinical evidence of the efficacy of polyphenol-rich fruits on bone health are of great importance for the validation of preclinical and in vitro results.

2.4 Figures and Tables



Anthocyanin	R_1	R_2
Cy-gluc	OH	H
Mv-gluc	OCH_3	OCH_3
Dp-gluc	OH	OH
Pn-gluc	OCH_3	OH
Pt-gluc	OCH_3	OH

Flavonols	R_1	R_2	R_3	R_4
Quercetin	-OH	-H	-H	-H
Kaempferol	-H	-H	-H	-H
Myricetin	-OH	-OH	-H	-H
Derivatives				
Rutin	-OH	-H	-OH	Rutinose
Quercetrin	-OH	-H	OH	Rhamnose

Figure 2.1. Classification of Polyphenols present in blueberry

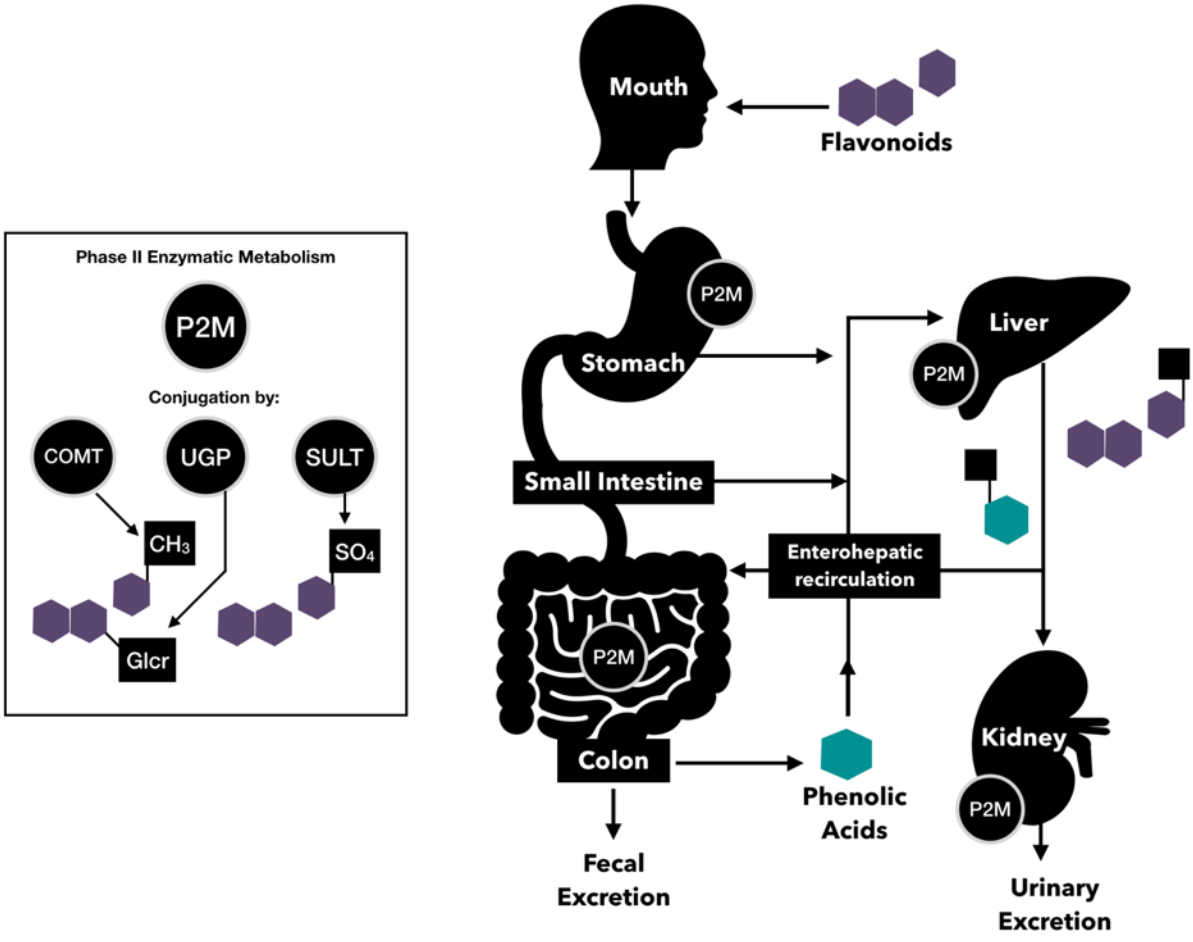


Figure 2.2. Flavonoid metabolism.

CHAPTER 3. ACUTE BIOAVAILABILITY OF (POLY)PHENOLIC CONTENT OF DIFFERENT VARIETIES OF VACCINIUM SPP. IN OVARIECTOMIZED RATS

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3.1 Abstract

Blueberries have a wide range of health promoting activities due to their high levels of (poly)phenols, including anthocyanins, flavonols, and phenolic acids. However, there are hundreds of varieties of blueberries and their phenolic content varies greatly. Thus, the goal of this investigation was to characterize the total phenolic content in 10 varieties of *Vaccinium spp.* berries as well as determine whether there is a difference in their bioavailability after an acute dose in ovariectomized rats. Ten varieties of berries (Ira, Legacy, Montgomery, Onslow, SHF2B1-21:3, Sampson, Cranberry, Bilberry, Wild blueberry and a Lowbush Wild Blueberry composite) were selected based on PCA analysis of their anthocyanin content and sourced from the North Carolina State University Blueberry Genome Repository. Berries were freeze-dried and phenolics were extracted with 2% formic acid 80:20 MeOH:H2O solvent and purified using solid phase extraction (SPE). Total phenolic content was determined via the Folin-Ciocalteu assay and individual phenolics where identified and quantified by HPLC-MS. For the bioavailability study, twenty Sprague-Dawley rats were given an acute dose of 25 mg of total phenolics/kg of body weight by gavage as a freeze-dried blueberry powder solution (aq.) and serial blood draws were collected over 8 hours. Plasma samples were extracted by SPE and analyzed through HPLC-MS/MS for

anthocyanin glycosides and flavonoid metabolites. Pharmacokinetic parameters (AUC, Cmax, and Tmax) were determined. Bilberry and LB composite have a significantly higher total phenolic content than the other varieties, while Cranberry and Sampson were the least concentrated. Montgomery blueberry had significantly higher bioavailability of malvidin and cyanidin glycosides. Montgomery and Ira varieties had the highest bioavailability for Me-Quercetin-glucuronide (glc) and Myricetin-glc metabolites, respectively ($p < 0.05$). The flavan-3-ols, catechin- (glc), epicatechin-glc and their respective methylated metabolites, were not significantly different between varieties of blueberries. Total (poly)phenolic content and bioavailability of individual phenolics varied widely among blueberry varieties. This study served as information for future investigations in cells, animals, and clinical trials.

3.2 Introduction

Blueberries (*Vaccinium spp.)* are a frequently consumed fruit in North America whose popularity has been on the rise in recent years due its potential role in the prevention of cancer, osteoporosis, cardiovascular and neurodegenerative diseases (Kang et al.; Devareddy et al.; A. Rodriguez-Mateos, T. Cifuentes-Gomez, T. W. George, et al.; Feliciano et al.). These beneficial health effects have been attributed to their high content of anthocyanins and phenolic acids (Yousef, Brown, et al.). Although all blueberry varieties are rich in these compounds, there are hundreds of varieties with varying anthocyanin and phenolic acid profiles, demonstrating potential for differences in polyphenol absorption, biotransformation and utilization.

Blueberries belong to three major species, the lowbush `wild blueberry´ (*Vaccinium angustifolium Aiton.*), the highbush `commercial blueberry´ (*Vaccinium corymbosum L.*) and the rabbiteye (*Vaccinium ashes Read*) (Yousef, Brown, et al.). Differences between these species

include bush height, machine harvestability, color, size and sweetness (Lila). Lowbush blueberries grow wild, vary in height, and are usually small in size. Due to their increased polyphenol content, they have a more astringent flavor and possess less attractive market characteristics, therefore, they are mainly frozen after harvesting and used as food ingredients. (Kalt, Ryan, et al.; Lila). In contrast, rabbiteye and highbush blueberries are selectively bred for commercialization because of their attractive fruit characteristics (MAKUS and MORRIS). Regardless of phenotype differences, all varieties of blueberries are rich in polyphenols, ranging in concentration of total phenolic (TP) content from 1.63 to 2.87 gallic acid equivalents (GAE) /mg of fresh weight (FW) in highbush and lowbush blueberries, respectively (Kalt, Ryan, et al.). The differences observed in polyphenol content are due to different genetic background, environmental factors, and harvest conditions during growth and storage (Kalt, Ryan, et al.; Lila; A. Rodriguez-Mateos, T. Cifuentes-Gomez, S. Tabatabaee, et al.).

Polyphenols are plant secondary metabolites produced as a mechanism to provide protection against environmental threats, as attractants to pollinators, as UV protection and to protect against microbial infection(A. Rodriguez-Mateos, D. Vauzour, et al.) (Lila). Although their main function is to assist in the plant's survival, they have proven to aid in human health as anticarcinogenic (Matchett et al.), antioxidant (Tang et al.) and anti-inflammatory (Huang et al.) compounds. Polyphenols are classified into three major groups as flavonoids, terpenoids, alkaloids and suphur-containing compounds (A. Rodriguez-Mateos, D. Vauzour, et al.).

From the flavonoid group, blueberries are rich in the subgroups anthocyanins, flavan-3-ols, flavonols, procyanidins and phenolic acids (A. Rodriguez-Mateos, T. Cifuentes-Gomez, S. Tabatabaee, et al.). These polyphenols have a common chemical structure comprised of a tricyclic C_6-C_3-C_6 flavonoid backbone (Taruscio, Barney and Exon) , with varying hydroxyl groups. The

anthocyanins in blueberries are cyanidin, malvidin, delphinidin, peonidin and petunidin in their glycosylated (-3-O-glucoside/arabinoside/galactoside) and acylated (-3-O-(6’’-acetyl)glycoside) forms (Yousef, Brown, et al.). The flavan-3-ols, (+)-catechin/epicatechin are found as monomers or polymers as procynidins B1 and B2. The flavonols quercetin, kaempferol and myricetin are present as aglycones or glycosylated forms and the major phenolic acids are chlorogenic acid and gallic acid. Anthocyanins and phenolic acids are the most prevalent of these compounds, thus past studies have focused predominantly on their characterization and bioavailability, usually disregarding the flavanols and flavan-3-ols (Yousef, Brown, et al.; Ayaz et al.; Cardenosa et al.).

Although blueberries are rich sources of polyphenols, their absorption is poor and difficult to assess due to degradation, colonic microbial transformation into phenolic metabolites, and rapid excretion through the urine (Thilakarathna and Rupasinghe). The absorption of polyphenols into circulation occurs mainly in the small intestine, through either passive or active absorption in the enterocytes in which they may experience a first-pass of phase II enzymatic metabolism, resulting in *O*-glucuronides, sulfate esters and *O*-methyl ester metabolites of the parent compound (Landete). Following absorption in the small intestine, they undergo further phase II enzymatic metabolism in the liver, resulting in methyl, glucuronide and sulfate derivatives, which facilitate their excretion through urine or undergo enterohepatic recirculation through bile, undergoing further phase II enzymatic metabolism until excreted (Lila et al.). The polyphenols that are not absorbed through the small intestine, travel to the lower gut and are hydrolyzed into small molecular weight colonic metabolites that can be readily absorbed into circulation (Del Rio et al.). The bioavailability of polyphenols is poor, clinical and animal studies have shown that the absorption of polyphenols in the upper gastrointestinal tract ranges between 1-2% absorption (Kay, Mazza and Holub).

However, recent studies suggest that anthocyanins and possibly other flavonoids have a longer residence time than expected due to bile enterohepatic recycling (Lila et al.; Kalt, Liu, et al.).

For this study, we compared flavonoid bioavailability, but since there exists ambiguity about the term bioavailability, we define it according to U.S. Food and Drug Administration (FDA) as "the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of drug action" (Administration). The site of action is unknown for flavonoids, but because we suppose that if they are absorbed into circulation they will eventually reach their target tissue, we chose the site of action to be plasma. Abiding by this definition of bioavailability, our study aimed at first characterizing the (poly)phenolic content of diverse highbush, lowbush and rabbiteye blueberry varieties and then determining whether the bioavailability of flavonoids varies among blueberry varieties in ovariectomized rats.

3.3 Methods

Chemicals. Commercial standards of cyaninidin-3-O-glucoside chloride (Cy-glc), (+)-catechin (C), (-)-epicatechin (EC) and quercetin-3-glucoside chloride (Q-3-glc), gallic acid, chlorogenic acid, kaempferol-3-rutinoside, quercetin-3-rutinoside, p-coumaric acid, ferulic acid, ellagic acid, resveratrol, procyanidin B1, procyanidin B2, epigallocatechin (EGC), epicatechin gallate (ECG), salicylic acid, caffeic acid, and quercetin-3-glucuronide as well as Folin-Ciocalteu's phenol reagent (2 N), hydrochloric acid, sodium carbonate, potassium chloride, formic acid, and L-ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC-MS grade solvents, including methanol, acetonitrile, and water, were purchased from VWR (Radnor, PA, USA). Ethyl gallate from Sigma-Aldrich (St. Louis, MO, USA) was used as internal standard to determine accuracy of the LC-MS/MS.

Blueberry cultivars for (poly)phenolic analysis. Recently, we screened anthocyanin content in blueberries from commercial cultivars and breeding selections from the North Carolina State University Piedmont Research Station (NCSU-PRS, Salisbury, NC, USA).(Yousef, Lila, et al.) Based on the characterization of 17 anthocyanins in 267 blueberry genotypes, we performed a principal component analysis (PCA) to select genotypes with maximal diversity in their anthocyanin – and, by extension, their (poly)phenolic – profiles.(McCabe et al.) Based on the PCA, we selected and sourced six cultivars from the NCSU-PRS collection: three rabbiteye varieties (*Vaccinium virgatum*), including Ira, Montgomery, and Onslow, and three southern highbush varieties (*V. corybosum*), including Legacy, Sampson, and SHF2B1-21:3. To maximize the diversity of (poly)phenolic profiles, we analyzed four additional members of the *Vaccinium* genus, including bilberries (*V. myrtillus*, Oregon Wild Harvest, Sandy, OR, USA), cranberries (*V. macrocarpon*, Cranberry Network, Wisconsin Rapids, WI, USA), and lowbush blueberries (*V. angustifolium*) from both wild blueberries and a commercially prepared wild blueberry composite (Wild Blueberry Association of North America, Old Town, MN, USA).

Blueberry extraction and purification of (poly)phenolics. Freeze dried berries were ground to a coarse powder using a KitchenAid coffee grinder (Benton Harbor, MI, USA). For each variety, three replicates were extracted for phenolic analysis. Each replicate (50 mg) was extracted with 5 mL of an extraction solution (80% methanol, 18% water, and 2% formic acid), vortexed, sonicated 30 minutes, and centrifuged at 6 °C and 3000*g* for 4 minutes. The supernatant was removed and the extraction repeated. The resulting supernatants were combined, and dried completely with nitrogen using a Meyer N-Evap (Organomation Association Inc., Berlin, MA, USA) with the water bath set to 37 °C.

Extracts were resolubilized in 5 mL of 2% formic acid in water and purified via solid phase extraction (Oasis HLB 1cc (30 mg) extraction cartridges, Waters Corp., Milford, MA, USA). Samples were purified using the following steps: 1) cartridge activation with methanol followed by distilled water, 2) loading the extracted blueberry sample, 3) sample clean up with 2% formic acid followed by a 95:5 water:methanol solution, and 4) elution with 2% formic acid in methanol. Eluates were dried completely under nitrogen, then resolubilized with 2% formic acid in water for analysis.

Blueberry quantification of total phenolics and anthocyanins. Total phenolics (TP) were measured via the Folin-Ciocalteu method, as described elsewhere.(Folin and Ciocalteu; Singleton, Orthofer and Lamuela-Raventos; Kulen, Stushnoff and Holm; Waterhouse) Briefly, 10 µL aliquots of purified, resolubilized blueberry extracts, 790 µL ddH2O, and 50 µL Folin-Ciocalteu reagent were vortexed and incubated at room temperature for 8 min. Then, 150 µL of 1N sodium carbonate was added, the mixture vortexed, and incubated in the dark at room temperature for 2 hr. Finally, 300 µL was transferred to a 96 well plate and the absorbance measured at 765 nm (Molecular Devices Spectra Max 100, Sunnyvale, CA, USA). Total phenolics in blueberry extracts were quantified in gallic acid equivalents (GAE).

Total monomeric anthocyanins (TMA) were measured using the pH differential method as previously described.(Song et al.) Briefly, 20 µL aliquots of each purified, resolubilized blueberry extract were transferred to two separate wells in a 96 well plate containing 280 µL of either 0.025 M potassium chloride buffer (pH 1.0) or 0.4 M sodium acetate buffer (pH 4.5). After incubating at room temperature for 15 min, the absorbance was measured at 520 and 700 nm. TMA were quantified in cyanidin-3-glucoside equivalents.

Blueberry quantification of individual phenolics. Purified blueberry extracts were filtered with 0.45 µm PTFE filters and 10 µL injected into a Waters 2695 HPLC-MS (Milford, MA, USA) with a Waters X-Bridge BEH Shield RP18 column (2.5 µm, 2.1 x 100 mm) heated to 40°C as previously described.(Song et al.; Furrer et al.) Anthocyanins were analyzed using a biphasic linear gradient at a flow rate of 0.25 mL/min, with mobile phases A (2% formic acid in water) and B (0.1% formic acid in acetonitrile) in the following ratios at 0, 15, 18, and 19 min, respectively: 90:10, 75:30, 70:30, and 90:10. Phenolic acids, stilbenoids, flavan-3-ols, and flavonols were analyzed using a biphasic linear gradient at a flow rate of 0.25 mL/min, with mobile phases A (0.4% formic acid in water) and B (0.1% formic acid in acetonitrile) in the following ratios at 0, 15, 17, and 18 min, respectively: 95:5, 65:35, 50:50, and 95:5.

Column eluate was split, with half going to a Waters 2996 photodiode array detector (PAD) and the other half going to a Waters Micromass ZQ mass spectrometer. Positive mode electrospray ionization (ESI+) was used to detect anthocyanins, with single ion responses (SIRs) set to *m/z* 287, 301, 303, 317, and 331 for cyanidin, peonidin, delphinidin, petunidin, and malvidin aglycone fragments, respectively. Settings for the mass spectrometer operating in ESI+ mode were: source temperature 150 °C, desolvation temperature 250 °C, nitrogen desolvation gas flow rate 250 L/hr, nitrogen cone gas flow rate 25 L/hr, capillary voltage 2500 V, cone voltage 50 V, and extractor voltage 3 V.

Negative mode ESI (ESI-) was used to detect phenolic acids, stilbenoids, flavan-3-ols, and flavonols. Settings for the mass spectrometer operating in ESI- mode were: source temperature 150 °C, desolvation temperature 250 °C, nitrogen desolvation gas flow rate 400 L/hr, nitrogen cone gas flow rate 60 L/hr, capillary voltage 3000 V, cone voltage 40 V, and extractor voltage 5 V. For phenolic acids, SIRs were set to *m/z* 137, 153, 163, 169, 179, 193,

223, 301, and 353 for salicylic, protocatechuic, p-coumaric, gallic, caffeic, ferulic, sinapic, ellagic, and chlorogenic acids, respectively. For stilbenoids, SIRs were set to *m/z* 227, 243, and 255 for resveratrol, piceatannol, and pterostilbene, respectively. For flavan-3-ols, SIRs were set to *m/z* 289, 305, 441, and 577 for catechin/epicatechin, EGC, ECG, and procyanidins B1/B2, respectively. For flavonols, SIRs were set to *m/z* 433, 447, 463, 477, 479, 593, and 609 for quercetin-3-arabinoside, kaempferol-3-glycosides[a] quercetin-3-rhamnoside, quercetin-3-glycosides, quercetin-3-glucuronide/isorhamnetin-3-glycosides, myricetin-3-glycosides, kaempferol-3-rutinoside, and quercetin-3-rutinoside, respectively. Due to low and inconsistent responses, results for salicylic acid, protocatechuic acid, p-coumaric acid, caffeic acid, ferulic acid, sinapic acid, ellagic acid, resveratrol, piceatannol, pterostilbene, EGC, ECG, and isorhamnetin-3-glycosides are not presented here.

Anthocyanins were quantified in cyanidin-3-glucoside equivalents; quercetin-3-arabinoside, quercetin-3-rhamnoside, quercetin-3-glucuronide, and myricetin-3-glycosides were quantified in quercetin-3-glucoside equivalents; catechin, procyanidin B1, and procyanidin B2 were quantified in epicatechin equivalents; kaempferol-3-glycosides were quantified in kaempferol-3-rutinoside equivalents; all other compounds (i.e., gallic acid, chlorogenic acid, epicatechin, quercetin-3-glycosides, kaempferol-3-rutinoside, and quercetin-3-rutinoside) were quantified based on their standards.

Bioavailability Study Design. All animal studies were conducted under the guidance and with protocols approved by the Purdue University Animal Care and Use Committee (Protocol 1405001076). Twenty 3-mo old rats were ovariectomized and, upon arrival, individually housed in stainless steel wire-bottom cages with a 12h on:off light cycle and fed chow diet and deionized water ad libitum. Rats were acclimated to their new housing and stabilized after their surgery for

2 months. Nine days prior to pharmacokinetic study, rats were put on a polyphenol-free diet (modified AIN93M diet where soybean oil was replaced with corn oil) and randomized to one of the five blueberry variety treatment groups: Montgomery, Ira, Onslow, SHF2B1-21:3, and wild blueberry composite mix (LB composite).

Blueberry doses for pharmacokinetic study. Freeze-dried blueberries were milled to a 0.5mm particle size. TP content was used to determine the individual dose of blueberry for each rat. Each dose was normalized to provide 25mg TP/kg of body weight (BW). Individual doses were dissolved in 2 ml of ultrapure water, vortexed, and administered to the rats on the day of the pharmacokinetic study via oral gavage.

Pharmacokinetics. Two days prior to the study, rats were anesthetized with isofluorane (3-5%) in an anesthesia chamber and maintained under anesthesia with a mask (1.5-3% isofluorane). A silastic catheter was implanted into the jugular vein and they were given burenorphine (0.1 mg/kg BW) to alleviate pain prior to regaining consciousness. Rats were allowed to rest for 48 hours after surgery and catheters were kept patent by flushing with heparinized saline (20 U/mL) every 12 h. Rats were fasted 8 hours prior to the study and ~ 400 µl of whole blood was collected at baseline (prior to gavage) and 0.25, 0.5, 1, 2, 4, 6 and 8 hrs post-gavage from the jugular catheter into heparinized tubes. Whole blood was processed to plasma by centrifugation at 4 ºC and 6000 rpm for 10 min. Two aliquots of 80 µl of plasma each were combined with 20 µl of acidified saline (1% ascorbic acid (AA), w/v%), flushed with N_2 gas and stored at -80 ºC until analysis. Rats were euthanized at the end of the 8 hr pharmacokinetic study by carbon dioxide overdose. Uterine horns were excised and weighed to confirm efficacy of ovariectomy.

Plasma metabolite solid phase extraction. Anthocyanin, flavonol and flavan-3-ol metabolites were extracted from plasma by solid-phase extraction. Samples were thawed at room temperature, diluted with 900 µl of 0.9% Saline, 1% AA (%w/v) and vortexed for 10 s. For anthocyanin metabolites, a 1-cc Oasis HLB cartridge (Waters, Milford, MA) was activated with 3 ml of methanol, followed by 3 ml of water. The sample was loaded into the cartridge, then washed with 2 ml of 2% FA (aq.) followed by 1 ml of 95:5 H20:MeOH. Samples were eluted with 2 ml of 2% FA MeOH (%v/v) and dried down with N_2 gas. Samples were resuspended in 100 µl of 2% FA (aq.) solution. For plasma flavan-3-ol and flavanol extraction, a 10 mg HLB cartridge was activated with 1 ml of methanol, followed by 1 ml of water. 1 ml of sample was loaded and washed with 1 ml of 1.5 M FA (aq.) followed by 1 ml of 5% methanol (aq.). Samples were eluted with 2 ml of 0.1% FA MeOH (%v/v) and dried down with N_2 gas. Dried extracts were resolubilized in acidified 20% acetonitrile (aq.) (0.1% FA %w/w) for LC-MS/MS analysis.

Analysis of plasma anthocyanin, flavanols and flavan-3-ol metabolites were performed using an Agilent 6400 triple quadrupole (QQQ) mass spectrometer equipped with an ESI source under multiple reaction monitoring modes (MRM). The method was previously described by Ho et al. (Ho et al.). Briefly, separation and characterization of metabolites was achieved using a Waters RP-C18 column (XBridge BEH Shield RP-C18, 2.5 µm, 2.1 x 100 mm). For (+)catechin/epicatechin, quercetin and myricetin metabolites, binary mobile phases were A: 0.1% FA (aq.) (v/v%) and B: 0.1% FA in acetonitrile (v/v%). The column was heated to 30 ºC and the system flow rate was 0.3 mL/min. For elution of the polyphenol metabolites, the binary gradient was: 10% B at 0 min, 40% B at 10 min, 95% B at 11 min and back to 10% B at 12-18 min. Fragmentor voltage was set at 135 V and collision energy was 17 eV in negative polarity for all compounds.

For anthocyanin metabolites, binary mobile phases were A: 2% FA (aq.) (v/v%) and B: 0.1% FA in acetonitrile (v/v%). The column was heated to 40 ºC and the system flow rate was 0.3 mL/min. For elution of the polyphenol metabolites, the binary gradient was: 5% B at 0 min, 25% B at 15 min, 30% B at 19 min and back to 5% B at 19–22.5 min. Fragmentor voltage was set at 135V and collision energy was 17eV in positive polarity for all compounds.

Calculations and Statistical Analysis. (Poly)phenolic characterization from blueberry raw material data are presented as mg (poly)phenol/100 g of berries (dry weight). Data from the bioavailability study were expressed as mean ± standard error of the mean (SEM). Pharmacokinetic parameter area under the curve from 0 to 8 h ($AUC_{0\text{-}8h}$) was calculated by the linear trapezoidal method using Microsoft Excel 2013 v.15. Maximum plasma concentration (C_{max}) and the time of the maximum plasma concentration (T_{max}) were obtained from the pharmacokinetic curve. Statistical analyses were performed using SAS 9.3 (SAS Institute, Cary, NC, USA). Data were analyzed via two-way ANOVA and one-way ANOVA for phenolic characterization and bioavailability, respectively. Significant differences were evaluated via the Tukey-Kramer HSD *post hoc* pairwise comparison test. Significance was accepted at $\alpha < 0.05$.

3.4 Results

Total phenolics and anthocyanins. Overall measures of phenolic content – including total phenolics (TP) and total monomeric anthocyanins (TMA) – are shown in Figure 3.1. TP ranged from 1951-4627 mg/100 g berry (dw), while TMA ranged from 369-1722 mg/100 g berry (dw), with bilberry and the low bush composite having higher levels of phenolics and TMA than their highbush counterparts. Interestingly, with the exception of the Sampson berries, the cultivars selected from the NCSU-PRS repository had similar amounts of both total phenolics and TMA.

Anthocyanin content. Anthocyanins are the most abundant phenolics present in *Vaccinium* species, accounting for approximately half of all phenolics in blueberries. Our results follow that trend, with each variety having a significant level of anthocyanins present.

For each variety of *Vaccinium*, we quantified cyanidin, delphinidin, malvidin, peonidin and petunidin in their glycosylated form (-3-O-galactoside/glucoside/arabinoside) and acylated forms. These results are illustrated in three different ways: 1) anthocyanins sharing the same aglycone (i.e., cyanidin, delphinidin, malvidin, peonidin, or petunidin) (Figure 3.2a), 2) anthocyanins with the same glycosylation (arabinoside, galactoside, or glucoside) (Figure 3.2b), and 3) quantification of individual anthocyanins (Table 3.1).

Considerable variation in the amounts and proportions of different anthocyanins was present across the 10 varieties we examined (Figure 3.2a; Table 3.1). Consistent with the higher levels of total phenolics and TMA observed in bilberry and low bush composite, these same varieties had the highest levels of anthocyanins when analyzed via HPLC-MS. Bilberry had the highest levels of delphinidin and cyanidin species and the low bush composite had the highest levels of malvidin and acylated anthocyanins. Cranberry had a dramatically different anthocyanin profile than all other berries, with the highest levels of peonidin but the lowest levels of delphinidin, malvidin, and petunidin species.

Glycosylation also varied among the varieties tested, with most exhibiting substantial amounts of arabinoside and galactoside derivates, but significant differences in the amount of glucoside derivatives (Figure 3.2b; Table 3.1). Some varieties (e.g., Ira, Legacy, and Sampson) had very low levels of glucosidic derivatives, while other varieties (e.g., Onslow, SHF2B1-21:3, wild blueberry, bilberry, and low bush composite) displayed levels of glucosidic derivatives equal to or greater than the arabinosidic and galactosidic derivatives.

Phenolic acids, flavan-3-ols, stilbenoids, and flavonols. Although anthocyanins make up a large proportion of the total phenolics present in *Vaccinium* berries, there are other key phenolics in these berries that may contribute to their bioactivity. We analyzed a number of these compounds, including phenolic acids (salicylic acid, protocatechuic acid, p-coumaric acid, gallic acid, caffeic acid, ferulic acid, sinapic acid, ellagic acid, and chlorogenic acid), stilbenoids (resveratrol, piceatannol, and pterostilbene), flavan-3-ols (catechin, epicatechin, EGC, ECG, procyanidin B1, and procyanidin B2), and flavonols (quercetin-3-arabinoside, kaempferol-3-galactoside, kaempferol-3-glucoside, quercetin-3-rhamnoside, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-glucuronide, isorhamnetin-3-galactoside, isorhamnetin-3-glucoside, myricetin-3-galactoside, myricetin-3-glucoside, kaempferol-3-rutinoside, and quercetin-3-rutinoside). The most prominent of these phenolics are presented in Tables 3.2 and 3.3, with chlorogenic acid (20-755 mg/100 g berry (dw)) and quercetin (93-418 mg/100 g berry (dw)) exhibiting the highest concentrations among these flavonoids. Flavan-3-ols present in comparatively low amounts for all varieties. As with anthocyanins, cranberry had a very different phenolic profile than other *Vaccinium* species evaluated here, with the lowest level of chlorogenic acid and the highest levels of epicatechin, quercetin-3-*O*-arabinoside, and myricetin-3-*O*-glycosides.

Overall, the six varieties selected from the NCSU-PRS collection showed considerable diversity in both their qualitative and quantitative phenolic profiles, confirming the selection of these varieties for use in this study. Additionally, by adding cranberry, bilberry, and low bush blueberries, we further increased the diversity in phenolic profiles for further study.

Bioavailability of Anthocyanin metabolites. The anthocyanin metabolites detected circulating in plasma were cyanidin-3-glycosides (C-3-glcs), malvidin-3-glycosides (M-3-glcs),

peonidin-3-glycosides (Pn-3-glcs), and petunidin-3-glycosides (Pt-3-glcs). The pharmacokinetic curves for all berry varieties are shown in Figure 3.3. The pharmacokinetic parameters $AUC_{0\text{-}8h,}$ Cmax and Tmax are shown in Table 3.4. To determine bioavailability, the $AUC_{0\text{-}8h}$ and Cmax were normalized by dose of parent anthocyanin. Of the anthocyanin metabolites, only C-3-glcs and M-3-glcs from the Montgomery blueberry had significantly higher bioavailability in comparison to the other varieties of berries ($p < 0.05$) (Figure 3.4). The Cmax of anthocyanins did not differ among varieties, but when these values are normalized by dose, Cmax for C-3-glcs was significantly higher in Montgomery than in Ira, reaching a maximum concentration in plasma of 24.96 ± 3.72 nM/mg of C-3-glcs dosed. Montgomery also showed a higher Cmax for M-3-glcs of 14.65 ± 2.16 nM/mg M-3-glcs dosed than the other varieties ($p < 0.05$). All anthocyanins showed a similar Tmax between 0.25-1 h, regardless of blueberry variety.

Bioavailability of flavan-3-ol metabolites. For the flavan-3-ols, (+)-catechin and (-)-epicatechin, the metabolites identified and quantified in plasma were epicatechin-5-glucuronide (EC-glcr), catechin-5-glucuronide (C-glcr), 3'-O-methylepicatechin-5-glcr (MeEC-glcr) and 3'-O-methylcatechin-5-glcr (MeC-glcr). Pharmacokinetic curves for all varieties of blueberries are shown in Figure 3.5. $AUC_{0\text{-}8h}$ was normalized by total dose of the parent compound and results of the $AUC_{0\text{-}8h}$ comparisons of the different berries are shown in Figure 3.6. Ira variety reached a significantly higher plasma concentration of 40.22 ± 6.34 nM of C-glcr in comparison to LB composite which only reached a Cmax of 15.48 nM ($p < 0.05$). Ira, Onslow and Montgomery showed a significantly higher $AUC_{0\text{-}8h}$ than SHF2B1-21:3 and LB composite, but when the $AUC_{0\text{-}8h}$ was normalized by dose, there were no significant differences between the $AUC_{0\text{-}8h}$ /dose of flavan-3-ols, regardless of blueberry variety (Table 3.5).

Bioavailability of flavonol metabolites. The flavonol metabolites identified and quantified in plasma were quercetin-3-glucuronide (Q-glcr) and its methylated conjugate, methylquercetin-3-glucuronide (MeQ-gclr) and myricetin glucuronide (Myr-glcr) (Table 3.6). The pharmacokinetic curves for flavonols are shown in Figure 3.7. For Q-glcr, Montgomery had a greater $AUC_{0\text{-}8h}$ /mg Quercetin (Q) than SHF2B1-21:3 and LB Composite, but was no different than Onslow and Ira (Figure 3.8). Although the Cmax for Quercetin did not vary among blueberry varieties, when normalized by dose, Montgomery reached a significantly greater Cmax of 0.66 ±0.16 µM/mg Q than Ira, SHF2B1-21:3 and Wild BB at 0.05 ± 0.02, 0.21 ± 0.03, and 0.14 ± 0.02 µM/mg Q, respectively. Tmax did not differ between blueberry varieties. Montgomery also showed a higher bioavailability of MeQ-glcr than Ira. Interestingly, MeQ-glcr was only detected at trace levels in Ira blueberry, therefore showing a significantly lower $AUC_{0\text{-}8h}$ of 0.093 µM/mg Q in comparison to Montgomery's $AUC_{0\text{-}8h}$ of 0.705 ± 0.218 µM/mg Q. Tmax did not differ between blueberry variety, ranging between 30 min-120 min.

For Myr-glcr, significant differences were observed in the $AUC_{0\text{-}8h}$ and Cmax between the different varieties of blueberries (Table 3.6). Ira had a significantly higher bioavailability with $AUC_{0\text{-}8h}$/mg Myr of 0.315 ± 0.038 µM*h/mg Myr than all other blueberry varieties. Montgomery and Onslow followed Ira with an $AUC_{0\text{-}8h}$/mg Myr of 0.168 ± 0.028 and 0.115 ± 0.005 µM/mg Myr, respectively. Both SHF2B1-21:3 and LB composite, had low $AUC_{0\text{-}8h}$ of 0.001 ± 0.0003 and 0.014 ± 0.002 µM/mg Myr, respectively. Tmax was not significantly different among blueberry varieties. Regardless of the higher content of myricetin, glycosides in SHF2B1-21:3 and Wild BB, when normalized by dose, exhibit a bioavailability that is significantly lower than the other blueberry varieties whose low content of myricetin glycosides ranged between 2.2 - 4.86 mg/100g DW Berry.

3.5 Discussion

This study shows that different genetic varieties of blueberries contain differences in phenolic profiles and some varieties of blueberries have a higher bioavailability of individual (poly)phenols. Previous studies have determined differences between phenolic content of lowbush and highbush blueberries, reporting that lowbush blueberries contain a 2-fold increase in total phenolic content in comparison to highbush blueberries (Kalt, Ryan, et al.). This pattern was also observed in our study, with a 2.3-fold increased TP content of 4445 ± 95 mg GAE/100 g DW BB in the LB composite in comparison to 1951 ± 160 mg GAE/100 g DW BB in the highbush Sampson. This may be due to the harsh environmental conditions that lowbush wild blueberries are exposed to, leading to an increased production of flavonoids to aid in the plant's survival.

The anthocyanin metabolites for cyanidin and malvidin were significantly more bioavailable from the Montgomery variety. All anthocyanin metabolites from the different blueberry varieties had similar $AUC_{0\text{-}8h}$, but when $AUC_{0\text{-}8h}$ was normalized by dose, Montgomery blueberry was more bioavailable. Consistent with anthocyanin bioavailability studies in humans (Zhong et al.) and rats (T.-Y. Chen et al.; Nohara et al.), all anthocyanins reached a maximal plasma concentration 0.3-1 h post-dose. Anthocyanin absorption starts early in the stomach, as evidenced in this study by the high concentration of circulating anthocyanin metabolites as early as the first blood draw at 15 min post gavage. Most of the absorption occurs through the small intestine and the rate and extent at which they are absorbed may be affected by the aglycone, sugar moiety or acylated groups (Kurilich et al.). Acylation of anthocyanins highly influences their bioavailability. Charron et al. showed that nonacylated anthocyanins were 4-fold and 4.7-fold more bioavailable than the acylated anthocyainins in red cabbage and purple carrot

juice, respectively (Charron, Kurilich, et al.; Charron, Clevidence, et al.). Of all blueberry varieties, Montgomery showed significantly higher bioavailability of cyanidin and malvidin, which could be explained in part by Montgomery's almost non-existent content of acylated cyanidin (0.3 ± 0.6 mg / 100 g berry (dw)) and low content of acylated malvidin forms (11.1 ± 2.2 mg /100 g berry (dw)) while the other blueberries's anthocyanin profiles are comprised of ~5-20% acylated anthocyanins.

From the flavan-3-ols, (+)-catechin content varied widely between blueberry varieties, ranging from 3.9 ± 0.16 to 15.9 ± 1.28 mg C / 100 g DW of Sampson and Ira, respectively. Ira, Onslow and Montgomery have significantly higher content of (+)-catechin, 15.9 ± 1.28, 15.2 ± 0.57 and 12.8 ± 0.29 mg / 100g DW berry than SHF2B1-21:3 and LB composite. This higher (+)-catechin content in Ira variety is consistent with significantly higher plasma maximal concentration (Cmax) of 40.22 ± 6.34 nM for C-5-glcr in comparison to 15.48 ± 4.66 nM for LB Composite ($p < 0.05$). Although Cmax was significantly higher in Ira, when assessing the bioavailability by normalizing the $AUC_{0\text{-}8h}$ by dose, there were no differences between blueberry varieties. (-)-Epicatechin content was similar between all varieties, except for Onslow having significantly higher content than Ira variety with 3.24 ± 0.09 and 1.07 ± 0.25 mg EC / 100 mg DW berry, respectively ($p < 0.05$). But no significant difference was observed for $AUC_{0\text{-}8h}$ of epicatechin metabolites between blueberry varieties. Thus, the higher plasma response for (+)-catechin was only due to higher content, and not its bioavailability. All flavan-3-ol absorption occurred mostly in the small intestine, as evidenced by their maximal plasma concentration at 1-2 h post-gavage.

The flavanols quercetin and myricetin vary widely among blueberry varieties. Total quercetin was significantly higher in the LB composite than the rabbiteye varieties Ira, Montgomery and Onslow with a 4.5-fold higher concentration ($p < 0.05$) in the LB composite in comparison to rabbiteye Montgomery (418 ± 20.6 vs 92.5 ± 5.48 mg Total Quercetin / 100g DW berry, respectively.) Interestingly, although Montgomery had the lowest total quercetin content, its bioavailability was significantly higher than that of other berries for quercetin glucuronide ($p < 0.05$), and had the second highest bioavailability for its methylated conjugate ($p < 0.05$), with Ira, another rabbiteye, having significantly higher bioavailability for the MeQ-glcr than all other berries. This may be due to differences in the glycosylation of quercetin from the different blueberry varieties. The sugar moiety highly influences quercetin bioavailability as it needs be cleaved for absorption by lactase phlorizin hydrolase (LPH) or cytosolic β-glucosidase (CBG) at the enterocyte level. Once the aglycone is released, quercetin is then subject to phase II enzymatic conjugation with glucuronide/sulfates/methyl groups (Terao). Except for glucose, all other glycosides are poor substrates for enzymatic cleavage, limiting their absorption through the small intestine. Quercetin glycosides then travel to the lower gut, where they can be hydrolyzed by lower gut bacteria and absorbed (Erlund et al.), this early and late absorption can be observed in Figure 8, where quercetin exhibits a pharmacokinetic curve with double peaks for its MeQ-gclr metabolite, which is not uncommon and is consistent with previous literature. Yang et al. reported this same phenomenon with Q-glcr displaying peaks at 2 h and 8 h post dose, which in their study, was possibly caused by enterohepatic recirculation (L. L. Yang et al.). The peaks MeQ-glcr occurred at 0.5 h and 1-2 h post dose, which could have been caused by the more rapid absorption of the quercetin aglycone which can be passively absorbed by diffusion in comparison to the slower active absorption of quercetin glucoside.

The rabbiteye varieties, Montgomery, Ira and Onslow have a significantly lower concentration of myricetin in comparison to the highbush and lowbush varieties. There was as much as a 23-fold difference ($p < 0.05$) in concentration between Ira blueberry and SHF2B1-21:3, with 2.20 ± 0.12 and 52.1 ± 15.7 mg myricetin / 100 g DW berry, respectively. Although Ira and Montgomery had significantly lower content of myricetin, they reached significantly higher plasma concentrations 7- and 11-fold higher than SHF2B1-21:3 ($p < 0.05$), respectively. The bioavailability from Ira was significantly higher than all varieties, followed by Montgomery and Onslow, and a very low bioavailability was observed for SHF2B1-21:3 and LB composite. As with quercetin, myricetin has a low absorption rate because of its glycosides and although not quantified by our LC method, it is possible that the differences in myricetin bioavailability could have been due to differences in myricetin aglycone and glycoside content.

It is thought that the phenolic metabolites from blueberry are the ones that possess the bioactivity shown from its consumption. Since we rarely see the parent compounds circulating in plasma, it is more likely that the bioactive compounds in blueberry are the multiple metabolites produced after first-pass metabolism that will eventually reach target tissues and exert their health benefits. Because there are so many different metabolites produced after the consumption of berries, developing methods to asses tissue distribution of these compounds is of high importance. Due to limitations in plasma sampling volumes, this study focused mainly on small intestine absorption of flavonoid metabolites and thus were not able to investigate the large intestine absorption of colonic metabolites.

Assessing bioavailability of colonic metabolites is of high importance because only around 10% of flavonoids will enter into circulation through the small intestine, leaving 90% of flavonoids to be metabolized by the gut microbiome into small molecular weight phenolic acids

that are readily absorbed to exert a beneficial effect on health. Identifying these colonic metabolites, their distribution into tissues and their physiological concentrations will provide great insight into the bioactivity and adequate doses for cell culture studies to determine their mechanism of action.

Limitations in this study include the low sample size for the bioavailability study. Because we opted to give the same dose of total phenolic content (25 mg/kg BW), the amount of freeze-dried blueberry powder dosed varied. Thus, the rats fed with high TP blueberries were gavaged an average of 189 mg of blueberry powder dissolved in 2 ml of water, in comparison to low TP blueberries, like Montgomery or SHF2B1-21:3 which were dosed with an average of 285 mg of freeze dried BB. Dietary fiber constitutes 3%-3.5% of blueberry fruit weight (Michalska and Lysiak), since the lower TP blueberries had a higher weight to account for the 25mg TP, the ratio of TP to dietary fiber differed between the blueberries and might have had an effect on the absorption and metabolism of polyphenols. Other fruit contents that could have influenced their bioavailability are the percent fat, protein and sugar contents. Since these weren't considered in our study, we are unable to determine whether some of these differences in bioavailability might have been influenced by the food matrix effects.

This study suggests that blueberries with different genetic background contain different phenolic profiles with varying bioavailability of their polyphenols. This study served to inform preclinical and clinical studies on blueberry selection for the effects of chronic consumption on health outcomes.

3.6 Figures and Tables.

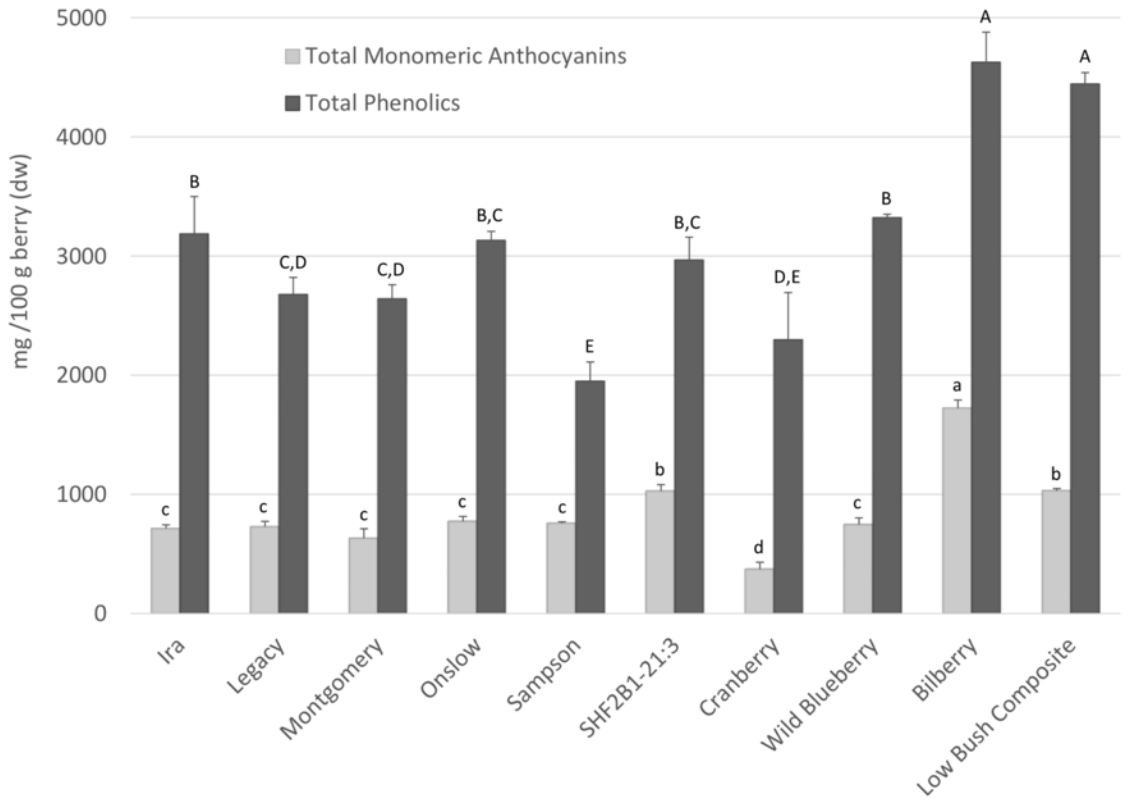

Figure 3.1. Total phenolics (in gallic acid equivalents) and total monomeric anthocyanins (TMA) in 10 varieties of *Vaccinium spp.* berries. Total phenolics were quantified via the Folin-Ciocalteu method and significant differences between varieties ($p < 0.05$) in total phenolic content are denoted by capital letters. TMA were quantified via the pH differential method and significant differences between varieties ($p < 0.05$) in TMA content are denoted by lower case letters. Data are shown as means and SDs.

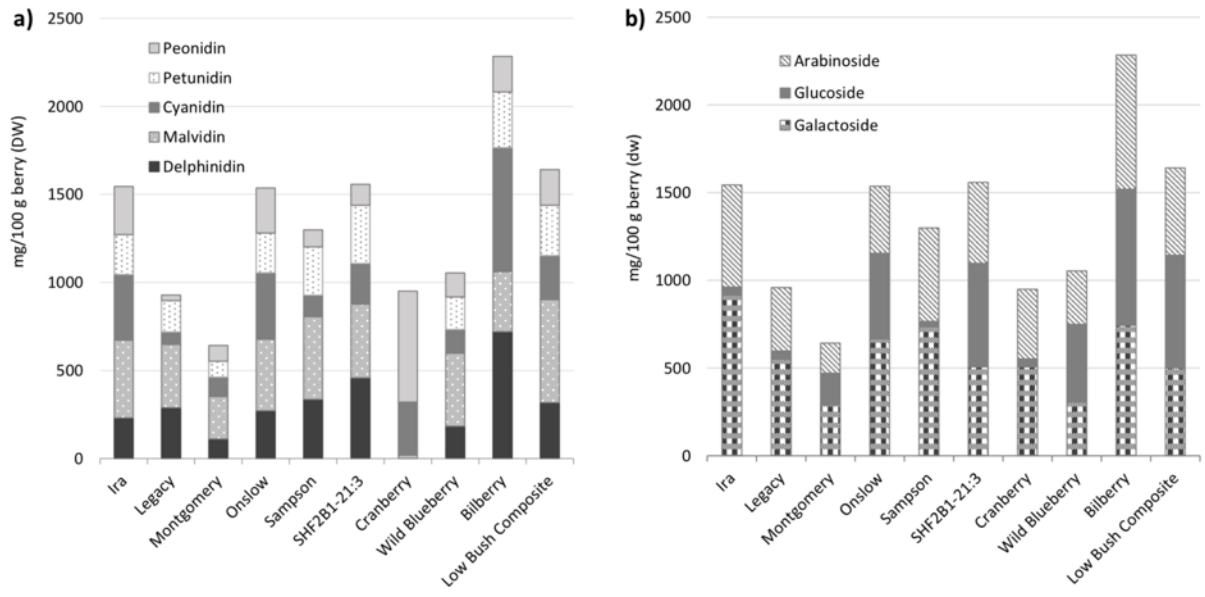

Figure 3.2 Anthocyanin content of 10 varieties of *Vaccinium spp.* berries. a) Sum of anthocyanins sharing the same aglycone, but having different glycosylation patterns. b) Sum of anthocyanins sharing the same glycosylation pattern, but having different aglycones.

Data are shown as means.

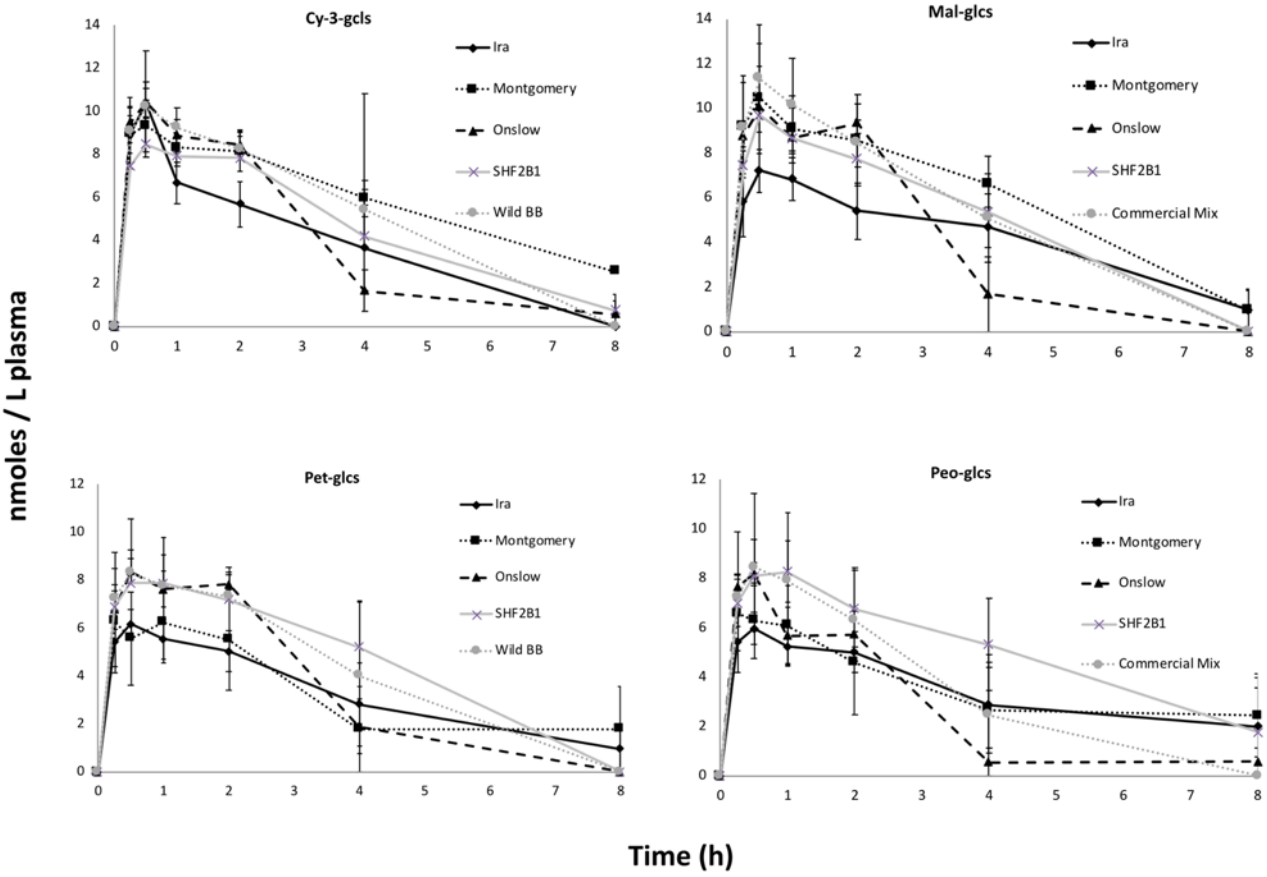

Figure 3.3 Plasma pharmacokinetic response of anthocyanin metabolites from different blueberry varieties. Data represented as mean ± SEM (n=4 rats / group). C-3-Glcs, cyanidin-3-glucosides; Mal-3-Glcs, malvidin-3-glucosides; Peo-3-Glcs, peonidin-3-glucosides; Pet-3-Glcs, petunidin-3-glucosides.

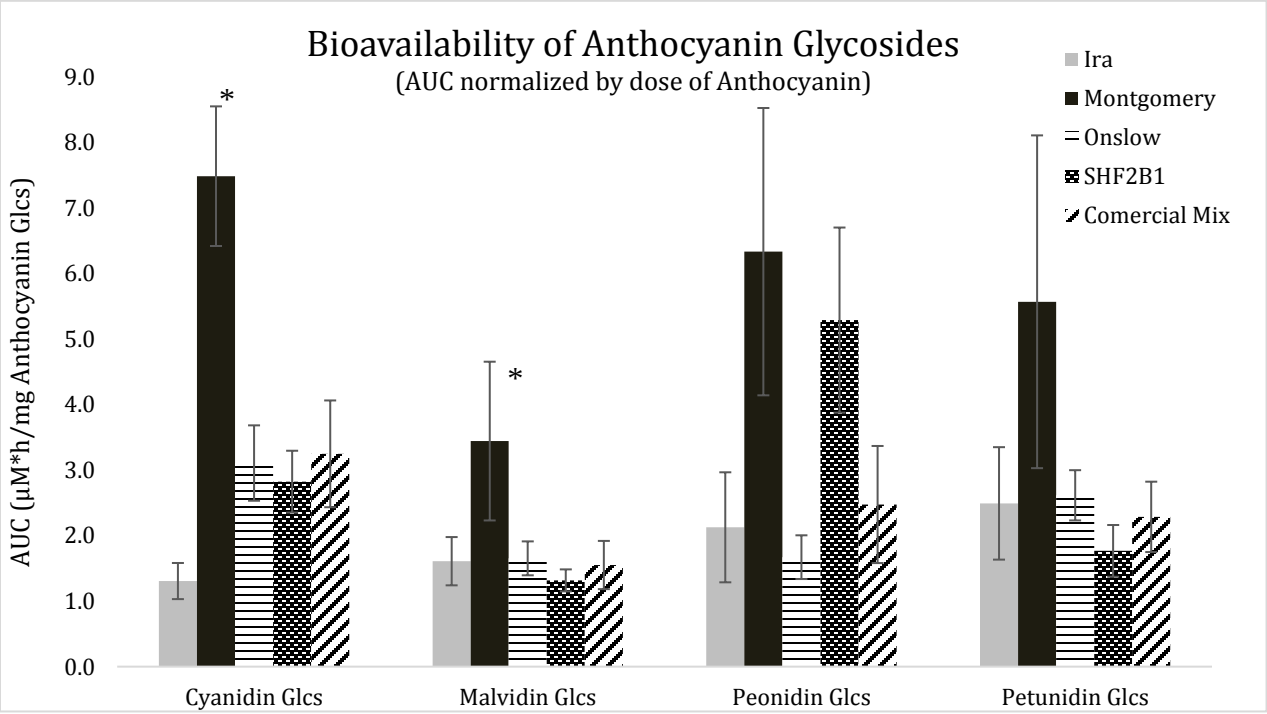

Figure 3.4 Area under the curve normalized by blueberry dose for anthocyanin metabolites. Malvidin, peonidin, cyanidin and petunidin glycosides were quantified circulating in plasma. $AUC_{0\text{-}8h}$ was normalized by blueberry dose. "*" represents significant differences between blueberry varieties ($p<0.05$). Data represented as mean ± SEM (n=4 rats / group). Glcs, glucosides.

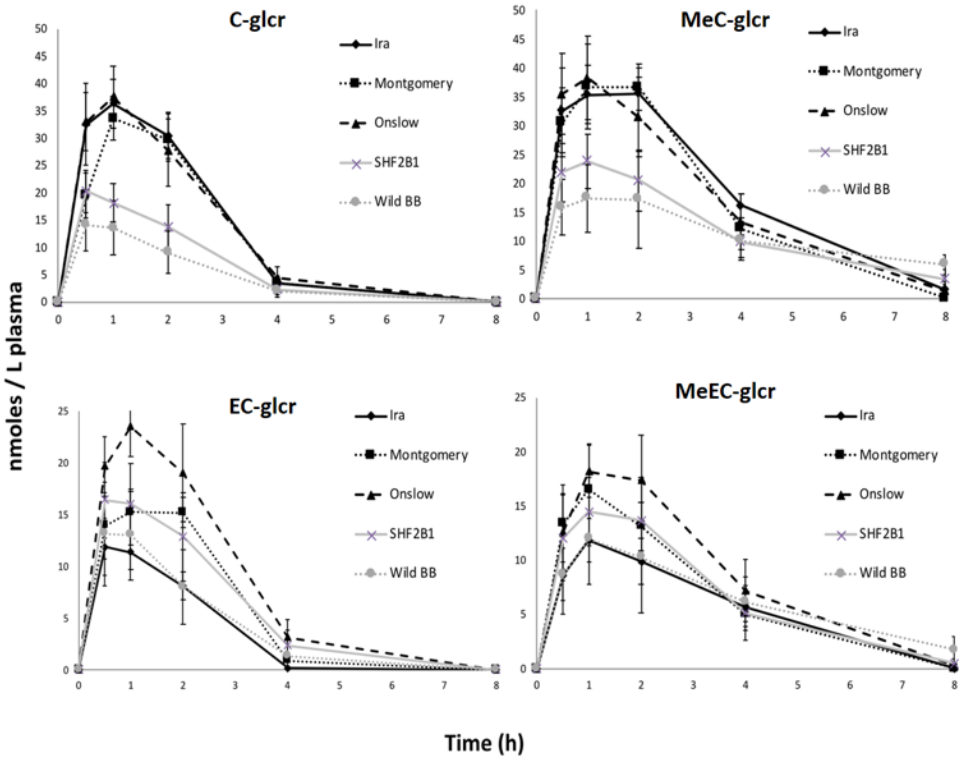

Figure 3.5 Plasma pharmacokinetic response of flavan-3-ols metabolites from different blueberry varieties. Data represented as mean ± SEM (n=4 rats / group). EC-glcr, epicatechin-5-glucuronide; C-glcr, catechin-5-glucuronide; EC-gclr, 3'-O-methylepicatechin-5-glcr; MeC-glcr, 3'-O-methylcatechin-5-glcr.

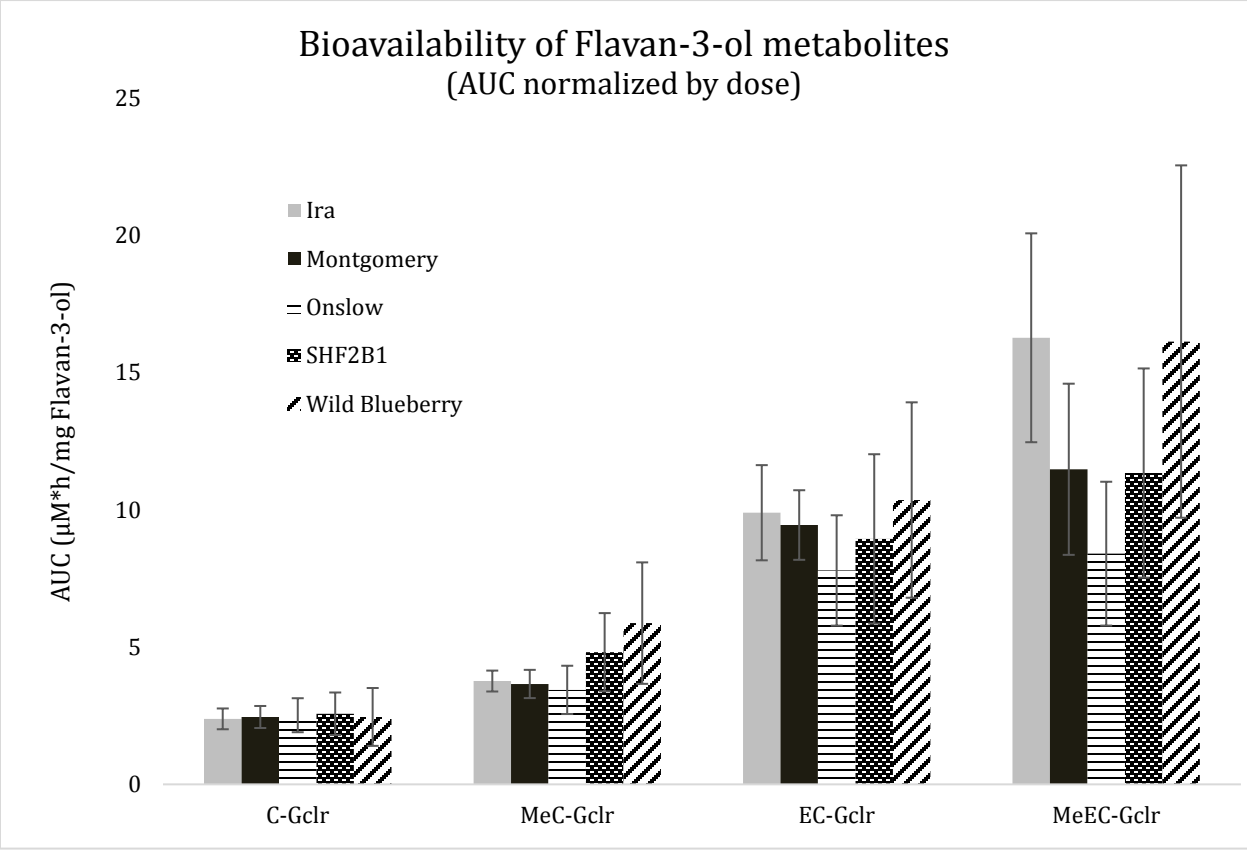

Figure 3.6 Area under the curve normalized by blueberry dose for flavan-3-ol metabolites. Flavan-3-ol AUC was determined via the trapezoidal method and normalized by blueberry dose. Data represented as mean ± SEM (n=4 rats / group). EC-glcr, epicatechin-5-glucuronide; C-glcr, catechin-5-glucuronide; EC-gclr, 3'-O-methylepicatechin-5-glcr; MeC-glcr, 3'-O-methylcatechin-5-glcr.

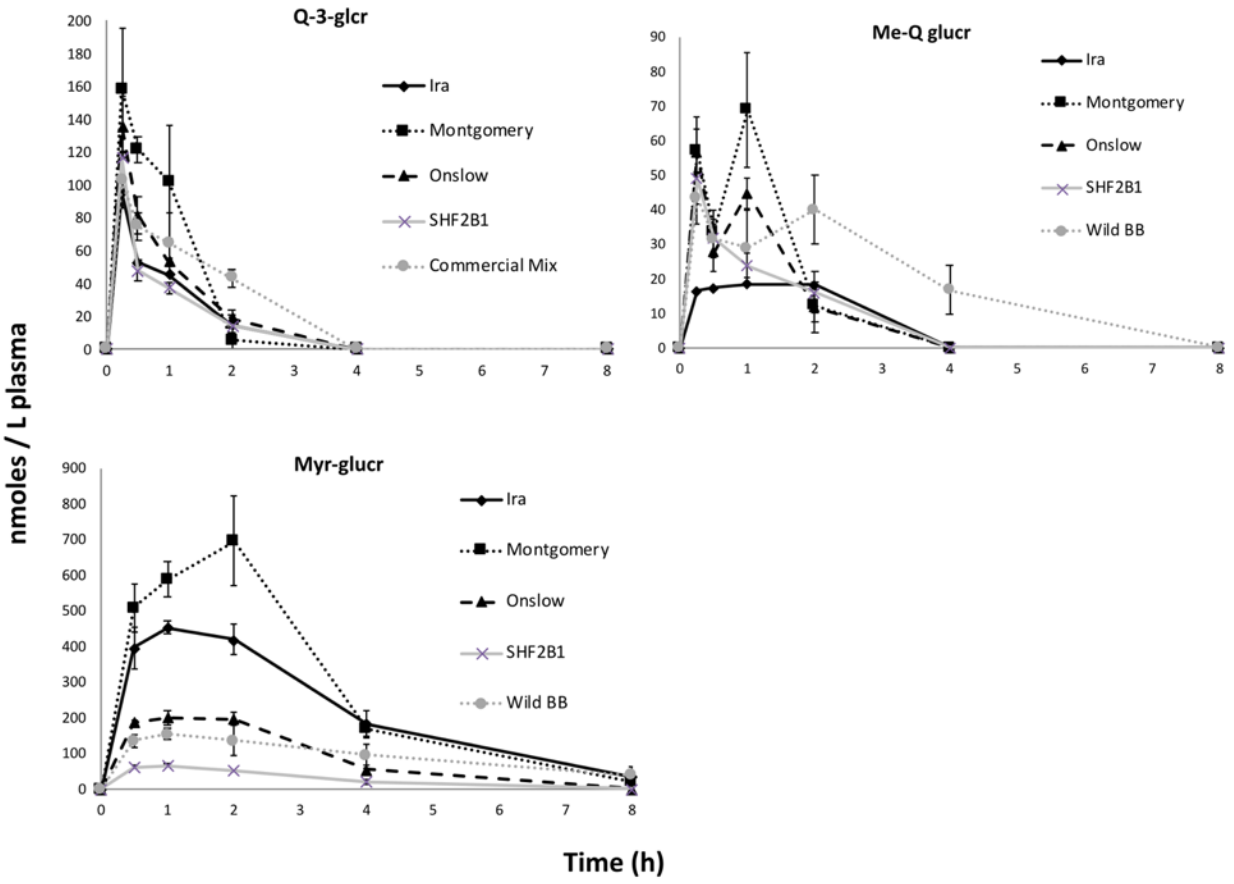

Figure 3.7 Plasma pharmacokinetic response of flavonol metabolites from different blueberry varieties. Data represented as mean ± SEM (n=4 rats / group). Q-3-glcr, quercetin-3-glucuronide; Me-Q-glucr, methylquercetin-3-glucuronide; Myr-glucr, myricetin glucuronide.

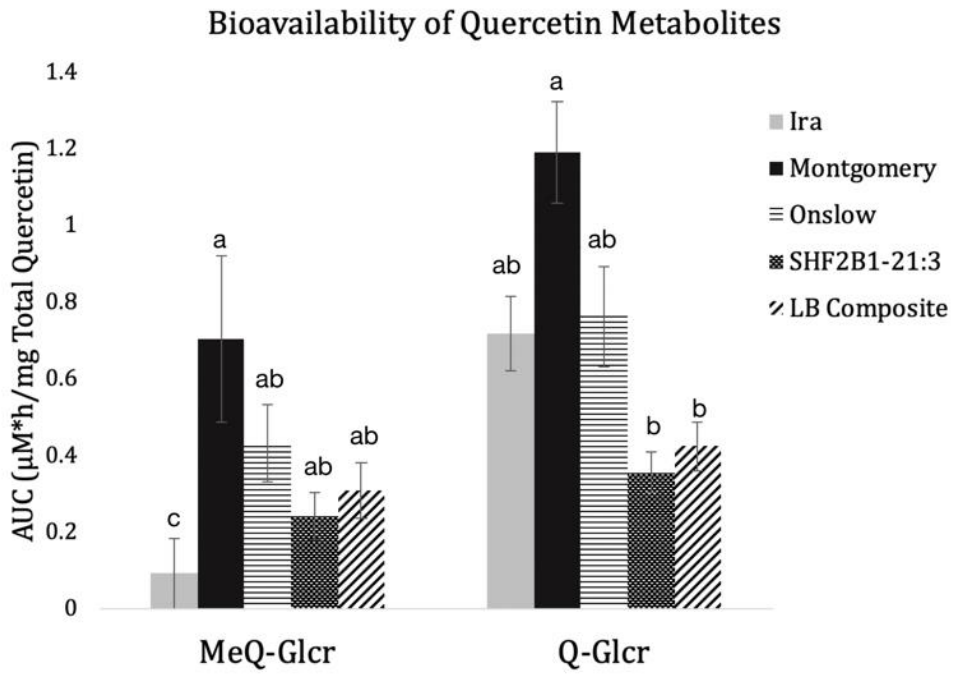

Figure 3.8 Bioavailability of Quercetin Metabolites. Letters represent significant differences ($p<0.05$) between the bioavailability of metabolites from different varieties. Data represented as mean and SEM. MeQ-Glcr, methylquercetin-3-glucuronide; Q-glcr, quercetin-3-glucuronide.

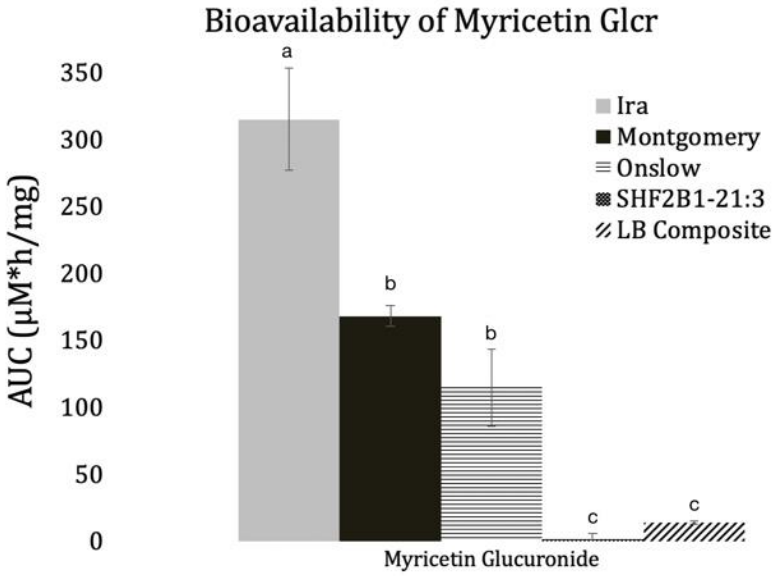

Figure 3.9 Bioavailability of Myricetin Metabolites**.** Letters represent differences ($p<0.05$) between the bioavailability of metabolites from different varieties. Data represented as mean and SEM.

Table 3.1 Anthocyanin content of 10 varieties of *Vaccinium spp.* (mg phenolic/100 g berry (dw)), from HPLC-MS analysis.[†]

		Delphinidin	Malvidin	Cyanidin	Petunidin	Peonidin	Total
Ira	3-galactoside	150 ± 3.78^{d,e}	237 ± 13.2^{a}	230 ± 6.0^{b}	142 ± 5.5^{b}	159 ± 9.3^{b}	916 ± 38^{A}
	3-glucoside	3.1 ± 0.2^{f}	14.5 ± 0.5^{e}	8.5 ± 0.1^{f}	3.7 ± 0.5^{e}	15.0 ± 0.6^{g}	44.8 ± 2.0^{E}
	3-arabinoside	78.5 ± 4.3^{d,e}	190 ± 15.4^{a}	134 ± 5.9^{b}	79.7 ± 5.5^{c,d}	99.7 ± 3.9^{b}	582 ± 35^{B}
	other forms	42.5 ± 5.9^{e}	42.4 ± 10.1^{d,e}	40.0 ± 8.6^{b,c,d}	16.7 ± 4.2^{e,f}	30.4 ± 6.4^{c,d}	172 ± 35^{E}
	Total	274 ± 14$^{\zeta}$	483 ± 39$^{\gamma,\delta}$	412 ± 21$^{\beta}$	242 ± 16$^{\varepsilon}$	304 ± 20$^{\beta}$	1715
Legacy	3-galactoside	184 ± 15.9^{b,c}	195 ± 12.8^{b}	41.5 ± 4.0^{i}	112 ± 9.9^{c}	17.7 ± 1.4^{f}	551 ± 44^{C}
	3-glucoside	3.5 ± 0.3^{f}	7.3 ± 1.4^{e}	0.2 ± 0.2^{g}	4.5 ± 1.3^{e}	0.2 ± 0.2^{h}	15.6 ± 3.3^{E}
	3-arabinoside	101 ± 7.7^{c}	156 ± 7.9^{b}	28.6 ± 2.6^{f}	62.0 ± 3.9^{e}	14.0 ± 1.2^{f}	362 ± 23^{E,F}
	other forms	62.4 ± 3.9^{d}	13.0 ± 5.1^{f}	2.5 ± 0.9^{f}	5.3 ± 1.6^{g}	0.5 ± 0.5^{e}	83.8 ± 12^{F}
	Total	351 ± 26$^{\delta,\varepsilon}$	372 ± 17$^{\varepsilon}$	72.8 ± 5.9$^{\eta}$	184 ± 14$^{\zeta}$	32.4 ± 2.6$^{\zeta}$	1013
Montgomery	3-galactoside	58.8 ± 12.8^{g}	94.9 ± 15.9^{d,e}	62.9 ± 14.9^{g,h}	40.5 ± 8.3^{f}	33.6 ± 5.4^{e,f}	291 ± 57^{D}
	3-glucoside	24.3 ± 5.7^{e}	74.1 ± 11.3^{d}	20.1 ± 4.2^{e}	28.0 ± 5.6^{d}	33.9 ± 5.6^{f}	180 ± 32^{D}
	3-arabinoside	28.5 ± 6.0^{g}	73.1 ± 8.3^{e}	26.0 ± 4.7^{f}	22.3 ± 3.9^{g}	21.5 ± 2.4^{e,f}	171 ± 25^{G}
	other forms	16.2 ± 1.3^{g}	11.1 ± 2.2^{f}	0.3 ± 0.6^{f}	4.3 ± 0.5^{g}	5.1 ± 1.2^{e}	37.1 ± 5.8^{F}
	Total	128 ± 26$^{\eta}$	253 ± 34$^{\zeta}$	109 ± 23$^{\zeta,\eta}$	95 ± 18$^{\eta}$	94 ± 13$^{\varepsilon}$	679
Onslow	3-galactoside	139 ± 2.6^{e}	142 ± 1.3^{c}	191 ± 2.7^{c}	97.4 ± 1.7^{c,d,e}	98.3 ± 3.0^{c}	668 ± 11^{B}
	3-glucoside	69.7 ± 1.0^{d}	156 ± 1.8^{c}	85.2 ± 1.1^{b}	78.5 ± 0.3^{c}	97.4 ± 0.4^{b}	486 ± 4.6^{C}
	3-arabinoside	64.1 ± 0.9^{e,f}	107 ± 0.9^{d}	100 ± 1.3^{c}	50.7 ± 0.3^{f}	60.7 ± 1.6^{c}	382 ± 5.1^{E}
	other forms	30.8 ± 2.2^{f}	37.3 ± 5.4^{e}	23.9 ± 5.3^{e}	21.1 ± 2.9^{e}	33.9 ± 5.2^{c}	147 ± 21^{E}
	Total	304 ± 3.2$^{\varepsilon,\zeta}$	442 ± 3.4$^{\delta}$	400 ± 1.8$^{\beta}$	248 ± 2.6$^{\varepsilon}$	290 ± 5.6$^{\beta}$	1684

Table 3.1 continued

Sampson	3-galactoside	203 ± 6.9^{b}	245 ± 2.9^{a}	67.1 ± 6.5^{f,g}	165 ± 3.1^{a}	57.0 ± 19.7^{d,e}	737 ± 39^{B}
	3-glucoside	5.0 ± 0.4^{f}	15.3 ± 1.2^{e}	1.22 ± 0.2^{f,g}	5.3 ± 0.3^{e}	2.1 ± 1.2^{h}	28.9 ± 3.4^{E}
	3-arabinoside	129 ± 4.2^{b}	209 ± 1.7^{a}	53.3 ± 5.7^{e}	104 ± 0.5^{a}	36.9 ± 14.5^{d}	532 ± 27^{B,C}
	other forms	62.1 ± 3.0^{d}	61.4 ± 2.0^{d}	2.0 ± 0.6^{f}	16.3 ± 0.8^{e,f}	4.4 ± 0.6^{e}	146 ± 7.0^{E}
	Total	399 ± 14$^{\delta}$	531 ± 3.7$^{\gamma}$	124 ± 13$^{\varepsilon,\zeta}$	291 ± 2.8$^{\delta}$	100 ± 36$^{\varepsilon}$	1444
SHF2B1-21:3	3-galactoside	166 ± 9.1^{c,d}	108 ± 3.8^{d}	93.0 ± 3.9^{e}	102 ± 6.1^{c,d}	39.0 ± 1.5^{d,e,f}	508 ± 24^{C}
	3-glucoside	159 ± 11.0^{b}	173 ± 10.4^{b}	65.8 ± 2.7^{c}	143 ± 8.2^{a}	48.5 ± 2.3^{d,e}	589 ± 35^{B}
	3-arabinoside	134 ± 6.4^{b}	136 ± 4.3^{c}	72.5 ± 2.2^{d}	86.7 ± 3.0^{b,c}	30.6 ± 1.2^{d,e}	461 ± 17^{D}
	other forms	130 ± 5.7^{b}	223 ± 4.3^{b}	43.5 ± 3.1^{b}	111 ± 2.2^{a}	43.3 ± 2.2^{b,c}	551 ± 17^{B}
	Total	589 ± 26$^{\beta}$	641 ± 22$^{\beta}$	275 ± 8.2$^{\delta}$	442 ± 18$^{\alpha}$	162 ± 6.0$^{\delta}$	2108
Cranberry	3-galactoside	nd	9.0 ± 0.4^{f}	157 ± 6.9^{d}	trace	342 ± 13.3^{a}	507 ± 21^{C}
	3-glucoside	nd	trace	4.6 ± 0.2^{f,g}	nd	41.7 ± 3.5^{e}	46.3 ± 3.7^{E}
	3-arabinoside	nd	11.0 ± 0.3^{f}	139 ± 1.2^{b}	trace	247 ± 4.7^{a}	396 ± 6.3^{E}
	other forms	90.4 ± 2.8^{c}	0.5 ± 0.5^{f}	41.7 ± 8.7^{b,c}	32.6 ± 0.2^{d}	94.8 ± 21.1^{a}	260 ± 33^{D}
	Total	90.4 ± 2.8$^{\eta}$	20.5 ± 0.4$^{\eta}$	342 ± 2.6$^{\gamma}$	32.6 ± 0.2$^{\theta}$	725 ± 0.2$^{\alpha}$	1210
Wild Blueberry	3-galactoside	58.8 ± 3.5^{g}	113 ± 5.4^{d}	42.7 ± 1.6^{h,i}	52.4 ± 2.8^{f}	40.7 ± 1.1^{d,e,f}	307 ± 14^{D}
	3-glucoside	78.7 ± 3.7^{d}	170 ± 6.5^{b,c}	50.4 ± 3.2^{d}	86.6 ± 4.8^{c}	55.9 ± 2.4^{d}	442 ± 21^{C}
	3-arabinoside	46.5 ± 2.5^{f,g}	132 ± 6.4^{c}	41.3 ± 2.5^{e}	46.1 ± 2.1^{f}	39.7 ± 1.5^{d}	305 ± 15^{F}
	other forms	93.2 ± 5.3^{c}	187 ± 14.0^{c}	27.2 ± 2.1^{d,e}	47.6 ± 3.8^{c}	58.6 ± 4.3^{b}	414 ± 29^{C}
	Total	277 ± 14$^{\zeta}$	602 ± 31$^{\beta}$	162 ± 9.2$^{\varepsilon}$	233 ± 13$^{\varepsilon}$	195 ± 8.8$^{\gamma,\delta}$	1468
Bilberry	3-galactoside	258 ± 9.4^{a}	79.0 ± 3.0^{e}	269 ± 11.5^{a}	92.1 ± 4.3^{d,e}	45.1 ± 1.2^{d,e}	744 ± 29^{B}
	3-glucoside	186 ± 9.1^{a}	162 ± 3.0^{b,c}	182 ± 6.3^{a}	131 ± 6.1^{a,b}	114 ± 2.9^{a}	775 ± 27^{A}
	3-arabinoside	277 ± 13.4^{a}	98.9 ± 1.5^{d}	253 ± 8.4^{a}	93.3 ± 3.6^{b}	43.1 ± 1.0^{d}	765 ± 28^{A}
	other forms	20.1 ± 1.1^{f,g}	13.9 ± 3.2^{f}	28.8 ± 5.9^{c,d,e}	13.7 ± 2.6^{f}	9.0 ± 2.1^{d,e}	85.6 ± 15^{F}
	Total	741 ± 33$^{\alpha}$	354 ± 7.8$^{\varepsilon}$	733 ± 28$^{\alpha}$	330 ± 15$^{\gamma}$	211 ± 5.5$^{\gamma}$	2369

Table 3.1 continued

Lowbush Composite	3-galactoside	107 ± 1.0^{f}	163 ± 1.7^{c}	85.4 ± 1.2^{e,f}	84.1 ± 0.6^{e}	62.9 ± 0.8^{d}	502 ± 5.2^{C}
	3-glucoside	125 ± 1.2^{c}	228 ± 2.4^{a}	85.2 ± 0.9^{b}	126 ± 1.0^{b}	77.7 ± 0.6^{c}	641 ± 6.1^{B}
	3-arabinoside	86.6 ± 2.1^{c,d}	194 ± 4.6^{a}	79.0 ± 1.0^{d}	76.5 ± 2.9^{d}	60.7 ± 0.8^{c}	497 ± 11^{C,D}
	other forms	168 ± 5.0^{a}	334 ± 7.3^{a}	65.4 ± 2.1^{a}	96.5 ± 1.9^{b}	96.0 ± 3.1^{a}	760 ± 19^{A}
	Total	487 ± 6.3$^{\gamma}$	918 ± 9.2$^{\alpha}$	315 ± 3.9$^{\gamma,\delta}$	383 ± 3.5$^{\beta}$	297 ± 3.7$^{\beta}$	2400

† Lowercase letters represent significant differences ($p < 0.05$) in individual anthocyanins between different *Vaccinium spp.* varieties; capital letters represent significant differences ($p < 0.05$) in anthocyanins with the same glycosylation/acylation pattern between varieties; Greek letters represent significant differences ($p < 0.05$) in anthocyanins sharing the same aglycone between varieties. “Other forms” represents the sum of anthocyanins with acylated side chains. “Total” represents the sum of all anthocyanins with the same parent anthocyanidin. “Trace” indicates compounds that were detected but below the LOQ, while “nd” indicates phenolics that were not detected.

Table 3.2 Phenolic acids and flavan-3-ols in 10 varieties of *Vaccinium spp.* (mg phenolic/100 g berry (dw)). †

	Phenolic Acids		Flavan-3-ols			
	Gallic Acid	Chlorogenic Acid	Catechin	Epicatechin	Procyanidin B1	Procyanidin B2
Ira	$0.70 \pm 0.09^{b,c,d}$	755 ± 46.7^{a}	15.9 ± 1.28^{a}	$1.07 \pm 0.25^{d,e}$	8.27 ± 0.96^{a}	0.75 ± 0.31^{e}
Legacy	$0.52 \pm 0.04^{c,d}$	520 ± 58.5^{b}	8.11 ± 0.67^{c}	0.71 ± 0.27^{e}	4.02 ± 0.23^{c}	$2.51 \pm 0.44^{d,e}$
Montgomery	$0.47 \pm 0.03^{c,d}$	493 ± 48.9^{b}	12.8 ± 0.29^{b}	$1.63 \pm 0.07^{c,d,e}$	6.56 ± 0.49^{b}	2.03 ± 0.23^{e}
Onslow	$0.57 \pm 0.19^{c,d}$	496 ± 30.5^{b}	15.2 ± 0.57^{a}	3.24 ± 0.09^{c}	$7.17 \pm 0.29^{a,b}$	$4.23 \pm 0.24^{c,d}$
Sampson	$0.92 \pm 0.09^{b,c}$	269 ± 9.03^{c}	3.90 ± 0.16^{d}	0.41 ± 0.28^{e}	0.91 ± 0.19^{d}	0.89 ± 0.44^{e}
SHF2B1-21:3	$0.66 \pm 0.04^{b,c,d}$	492 ± 26.4^{b}	6.97 ± 0.10^{c}	$1.89 \pm 0.88^{c,d,e}$	3.23 ± 0.26^{c}	$2.67 \pm 0.62^{d,e}$
Cranberry	0.38 ± 0.08^{d}	20.3 ± 0.64^{d}	1.93 ± 0.07^{e}	19.3 ± 0.82^{a}	trace	13.8 ± 1.49^{b}
Wild BB	$0.52 \pm 0.02^{c,d}$	566 ± 31.6^{b}	7.64 ± 0.78^{c}	$3.04 \pm 0.32^{c,d}$	6.21 ± 0.31^{b}	$4.62 \pm 0.48^{c,d}$
Bilberry	2.04 ± 0.37^{a}	$104 \pm 3.58^{c,d}$	0.06 ± 0.06^{f}	10.9 ± 1.75^{b}	trace	19.3 ± 1.33^{a}
LB Composite	1.11 ± 0.32^{b}	740 ± 25.1^{a}	8.02 ± 0.43^{c}	$1.67 \pm 0.64^{c,d,e}$	$7.02 \pm 0.53^{a,b}$	5.44 ± 0.62^{c}

† Letters represent significant differences ($p < 0.05$) in phenolics between varieties. "Trace" indicates compounds that were detected but below the LOQ.

Table 3.3 Flavonols in 10 varieties of *Vaccinium spp.* (mg phenolic/100 g berry (dw)).[†]

	Quer 3-arb	Quer 3-rham	Quer 3-glcs	Quer 3-gcnd	Quer 3-rut	Total Quer	Kaemp 3-glcs	Kaemp 3-rut	Myr 3-glcs
Ira	0.34 ± 0.14^{f}	58.1 ± 9.21^{b}	26.1 ± 3.18^{d}	8.92 ± 0.34^{c,d}	14.3 ± 1.30^{c,d,e}	108 ± 11.7^{e}	0.09 ± 0.08^{d}	trace	2.20 ± 0.12^{e}
Legacy	28.1 ± 7.30^{d}	24.7 ± 6.50^{c}	249 ± 29.1^{a}	1.14 ± 0.48^{d}	9.18 ± 2.16^{d,e,f}	313 ± 43.7^{b,c}	3.35 ± 1.03^{a}	0.57 ± 0.31^{b,c}	19.0 ± 1.40^{c,d}
Montgomery	17.3 ± 1.40^{d,e,f}	27.7 ± 1.35^{c}	23.9 ± 2.83^{d}	6.04 ± 0.59^{d}	17.6 ± 1.15^{c,d}	92.5 ± 5.48^{e}	0.59 ± 0.06^{c,d}	0.71 ± 0.11^{b,c}	4.86 ± 0.10^{d,e}
Onslow	26.3 ± 4.12^{d}	47.8 ± 4.76^{b}	33.3 ± 4.14^{d}	15.5 ± 0.44^{c}	7.68 ± 1.85^{e,f}	131 ± 5.70^{e}	0.73 ± 0.12^{c,d}	0.34 ± 0.19^{c}	2.35 ± 0.54^{e}
Sampson	92.7 ± 8.20^{b}	113 ± 6.15^{a}	124 ± 13.0^{c}	trace	31.3 ± 3.35^{a}	360 ± 22.4^{a,b}	1.10 ± 0.12^{c,d}	1.77 ± 0.36^{a}	26.1 ± 4.18^{c}
SHF2B1-21:3	20.6 ± 2.35^{d,e}	13.9 ± 2.14^{c,d}	156 ± 16.6^{c}	trace	4.81 ± 0.74^{f}	194 ± 19.1^{d}	0.64 ± 0.07^{c,d}	trace	52.1 ± 15.7^{a,b}
Cranberry	136 ± 11.0^{a}	21.1 ± 3.05^{c}	206 ± 15.8^{b}	trace	trace	363 ± 22.6^{a,b}	0.72 ± 0.26^{c,d}	nd	58.7 ± 9.74^{a}
Wild BB	26.9 ± 2.12^{d}	20.7 ± 1.94^{c}	161 ± 21.9^{c}	40.3 ± 6.84^{b}	20.9 ± 3.06^{b,c}	269 ± 25.5^{c}	1.59 ± 0.30^{b,c}	1.32 ± 0.22^{a,b}	22.4 ± 3.80^{c,d}
Bilberry	7.37 ± 1.26^{e,f}	1.09 ± 0.21^{d}	47.5 ± 6.38^{d}	38.5 ± 1.40^{b}	trace	94.5 ± 8.75^{e}	trace	trace	35.3 ± 8.01^{b,c}
LB Composite	64.1 ± 12.4^{c}	54.6 ± 7.43^{b}	210 ± 9.01^{a,b}	60.5 ± 4.19^{a}	29.0 ± 7.50^{a,b}	418 ± 20.6^{a}	2.28 ± 0.30^{a,b}	2.00 ± 0.51^{a}	27.8 ± 1.52^{c}

[†] Letters represent significant differences ($p < 0.05$) in phenolics between varieties. "Trace" indicates compounds that were detected but below the LOQ, "nd" indicates phenolics that were not detected, and "total quer" represents the total of all glycosides containing quercetin as the parent flavonol. Abbreviations: Quer = quercetin; arb = arabinoside; rham = rhamnoside; glcs = glycosides (galactoside + glucoside); gcnd = glucuronide; rut = rutinoside; Kaemp = kaempferol; Myr = myricetin.

Table 3.4 Pharmacokinetic Parameters for Anthocyanin Metabolites

Metabolite	Blueberry Variety	Normalized by Dose		Tmax (h)
		AUC (μM*h/mg)	Cmax (nM/mg)	
C-3-Glcs	Ira	1.31 ± 0.48	7.85 ± 2.47	0.3 ± 0.1
	Montgomery	7.48 ± 1.84*	24.96 ± 6.43	0.4 ± 0.1
	Onslow	2.82 ± 1.15	19.63 ± 13.83	0.9 ± 0.8
	SHF2B1	3.25 ± 0.82	10.82 ± 2.09	0.7 ± 0.3
	Commercial Mix	1.61 ± 1.41	17.38 ± 3.73	0.5 ± 0.0
Mal-3-Glcs	Ira	1.61 ± 0.74	6.42 ± 1.62	0.6 ± 0.3
	Montgomery	4.59 ± 0.95*	14.65 ± 6.31	1.0 ± 0.7
	Onslow	1.65 ± 0.52	8.83 ± 0.79	0.5 ± 0.0
	SHF2B1	1.32 ± 0.34	5.53 ± 2.08	0.6 ± 0.3
	Commercial Mix	1.55 ± 0.74	6.71 ± 2.76	0.5 ± 0.0
Peo-3-Glcs	Ira	2.13 ± 1.68	8.14 ± 2.51	0.4 ± 0.1
	Montgomery	6.33 ± 4.38	28.99 ± 7.88	0.6 ± 0.3
	Onslow	1.67 ± 0.67	10.93 ± 0.98	0.5 ± 0.0
	SHF2B1	5.29 ± 2.82	19.11 ± 5.63	0.7 ± 0.4
	Commercial Mix	3.30 ± 0.84	20.39 ± 5.04	0.4 ± 0.3
Pet-3-Glcs	Ira	2.49 ± 1.72	10.34 ± 2.53	0.4 ± 0.1
	Montgomery	5.57 ± 5.08	25.98 ± 13.41	0.6 ± 0.3
	Onslow	2.61 ± 0.77	13.08 ± 1.42	0.5 ± 0.0
	SHF2B1	1.77 ± 0.79	6.60 ± 1.86	0.7 ± 0.4
	Commercial Mix	2.28 ± 1.07	12.42 ± 5.16	0.9 ± 0.8

Data represented as mean ± SEM (n=4 rats / group). "*" represents significant differences between blueberry varieties, within each metabolite ($p<0.05$). Data represented as mean ± SEM (n=4 rats / group). C-3-Glcs, cyanidin-3-glucosides; Mal-3-Glcs, malvidin-3-glucosides; Peo-3-Glcs, peonidin-3-glucosides; Pet-3-Glcs, petunidin-3-glucosides.

Table 3.5 Pharmacokinetic Parameters for Flavan-3-ol Metabolites

Metabolite	Variety	AUC (µmol/L*h)	Cmax (µM)	Tmax (h)	AUC (µM*h/mg) Normalized by Dose	Cmax (µM/mg) Normalized by Dose
C-Glcr	Ira	0.098 ± 0.016	0.040 ± 0.006[a]	1.125 ± 0.315	2.386 ± 0.378	0.984 ± 0.154
	Montgomery	0.089 ± 0.013	0.034 ± 0.005[ab]	1.375 ± 0.375	2.454 ± 0.401	0.932 ± 0.145
	Onslow	0.099 ± 0.025	0.037 ± 0.007[ab]	1.250 ± 0.250	2.519 ± 0.619	0.955 ± 0.163
	SHF2B1-21:3	0.051 ± 0.016	0.020 ± 0.004[ab]	0.500 ± 0.000	2.572 ± 0.775	1.036 ± 0.199
	LB Composite	0.037 ± 0.016	0.015 ± 0.005[b]	1.875 ± 0.774	2.456 ± 1.054	1.031 ± 0.362
MeC-Glcr	Ira	0.154 ± 0.016	0.039 ± 0.005	1.125 ± 0.315	3.764 ± 0.380	0.962 ± 0.120
	Montgomery	0.134 ± 0.017	0.042 ± 0.007	1.500 ± 0.289	3.658 ± 0.515	1.078 ± 0.157
	Onslow	0.136 ± 0.036	0.039 ± 0.008	1.125 ± 0.315	3.442 ± 0.880	1.076 ± 0.172
	SHF2B1-21:3	0.096 ± 0.030	0.024 ± 0.005	0.750 ± 0.144	4.814 ± 1.425	2.000 ± 0.365
	LB Composite	0.089 ± 0.033	0.021 ± 0.007	1.875 ± 0.774	5.874 ± 2.213	1.623 ± 0.359
EC-Glcr	Ira	0.027 ± 0.005	0.013 ± 0.002	1.000 ± 0.354	9.896 ± 1.734	4.631 ± 0.880
	Montgomery	0.044 ± 0.005	0.018 ± 0.003	1.375 ± 0.375	9.448 ± 1.268	3.799 ± 0.701
	Onslow	0.065 ± 0.017	0.024 ± 0.004	1.500 ± 0.289	7.793 ± 2.010	2.855 ± 0.429
	SHF2B1-21:3	0.048 ± 0.018	0.017 ± 0.004	0.750 ± 0.144	8.942 ± 3.085	3.079 ± 0.743
	LB Composite	0.033 ± 0.011	0.014 ± 0.003	1.375 ± 0.875	10.358 ± 3.559	4.533 ± 1.634
MeEC-Glcr	Ira	0.045 ± 0.011	0.013 ± 0.002	0.750 ± 0.144	0.016 ± 0.004	0.007 ± 0.003
	Montgomery	0.054 ± 0.014	0.018 ± 0.003	1.250 ± 0.250	0.011 ± 0.003	0.003 ± 0.001
	Onslow	0.071 ± 0.023	0.024 ± 0.004	1.250 ± 0.250	0.008 ± 0.003	0.002 ± 0.001
	SHF2B1-21:3	0.061 ± 0.022	0.017 ± 0.004	1.125 ± 0.315	0.011 ± 0.004	0.004 ± 0.001
	LB Composite	0.051 ± 0.020	0.014 ± 0.003	1.500 ± 0.289	0.016 ± 0.006	0.005 ± 0.001

Data represented as mean ± SEM (n=4 rats / group). Letters represent significant differences ($p < 0.05$) in metabolites between varieties. EC-glcr, epicatechin-5-glucuronide; C-glcr, catechin-5-glucuronide; EC-gclr, 3'-O-methylepicatechin-5-glcr; MeC-glcr, 3'-O-methylcatechin-5-glcr.

Table 3.6 Pharmacokinetic parameters for flavan-3-ol metabolites.

Metabolite	Variety	AUC (μmol/L*h)	Cmax (μM)	Tmax (h)	AUC (μM*h/mg) Normalized by Dose	Cmax (μM/mg) Normalized by Dose
Q-Gclr	Ira	0.200 ± 0.029	0.096 ± 0.015	0.500 ± 0.000	0.718 ± 0.098^{ab}	0.046 ± 0.018^{d}
	Montgomery	0.312 ± 0.082	0.173 ± 0.038	0.625 ± 0.125	1.191 ± 0.342^{a}	0.658 ± 0.160^{a}
	Onslow	0.258 ± 0.027	0.135 ± 0.022	0.500 ± 0.000	0.763 ± 0.071^{ab}	0.402 ± 0.066^{ab}
	SHF2B1-21:3	0.193 ± 0.026	0.116 ± 0.013	0.500 ± 0.000	0.355 ± 0.050^{b}	0.215 ± 0.026^{bc}
	LB Composite	0.334 ± 0.040	0.111 ± 0.038	1.000 ± 0.354	0.424 ± 0.051^{b}	0.141 ± 0.023^{c}
MeQ-Gclr	Ira	0.026 ± 0.026	0.005 ± 0.005	2.000 ± 0.000	0.093 ± 0.093^{c}	0.004 ± 0.004^{c}
	Montgomery	0.185 ± 0.053	0.074 ± 0.017	1.250 ± 0.433	0.705 ± 0.218^{a}	0.281 ± 0.070^{a}
	Onslow	0.146 ± 0.035	0.059 ± 0.007	0.875 ± 0.375	0.432 ± 0.100^{ab}	0.174 ± 0.021^{ab}
	SHF2B1-21:3	0.132 ± 0.034	0.049 ± 0.008	0.500 ± 0.000	0.242 ± 0.060^{ab}	0.090 ± 0.015^{b}
	LB Composite	0.242 ± 0.054	0.056 ± 0.008	1.875 ± 0.774	0.309 ± 0.073^{ab}	0.071 ± 0.011^{b}
Myr-Gclr	Ira	1.779 ± 0.201^{a}	0.492 ± 0.024^{a}	0.875 ± 0.375^{b}	0.315 ± 0.038^{a}	0.087 ± 0.005^{a}
	Montgomery	2.345 ± 0.344^{a}	0.768 ± 0.132^{a}	2.000 ± 0.000^{ab}	0.168 ± 0.028^{b}	0.055 ± 0.011^{b}
	Onslow	0.696 ± 0.028^{b}	0.226 ± 0.010^{b}	1.250 ± 0.250^{b}	0.115 ± 0.005^{b}	0.037 ± 0.002^{b}
	SHF2B1-21:3	0.219 ± 0.022^{c}	0.068 ± 0.004^{c}	0.875 ± 0.125^{b}	0.001 ± 0.000^{c}	0.000 ± 0.000^{c}
	LB Composite	0.746 ± 0.111^{b}	0.195 ± 0.020^{b}	3.250 ± 0.750^{a}	0.014 ± 0.002^{c}	0.004 ± 0.000^{c}

Letters represent significant differences ($p < 0.05$) in pharmacokinetic parameters between blueberry varieties for each metabolite. Q-Gclr, quercetin-3-glucuronide; MeQ-Gclr, methylquercetin-3-glucuronide; Myr-Gclr, myricetin glucuronide. Data represented as mean ± SEM (n=4 rats / group).

CHAPTER 4. LOWER DOSES OF WILD BLUEBERRY ARE MORE EFFECTIVE AT INCREASING NET BONE CALCIUM RETENTION IN OVARIECTOMIZED RATS

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4.1 Abstract

Osteoporosis is the most common bone disease in postmenopausal women. Due to the loss of estrogen during menopause, there is an increase in oxidative stress, accompanied by inflammation that leads to an increase in bone resorption and a decrease in bone formation, resulting in rapid loss of bone. Because osteoporosis therapies such as hormone replacement therapy and antiresorptive treatments may result in negative side effects and a burden to the patient, it is of great interest to identify nutritional preventive strategies to attenuate the loss of bone during menopause. Because (poly)phenol-rich fruits, including grape, plum, and blueberry have been shown to have the potential to aid in bone loss prevention in ovariectomized (OVX) rats, this study aimed to determine the most effective dose of one of these fruits, i.e. blueberry, at increasing net bone calcium retention through urinary appearance of ^{45}Ca from pre-labeled skeleton that allows crossover between treatments and a rapid assessment of bone loss. Twenty 6-month old OVX rats went through a randomized-order crossover design study to determine the effect of four different blueberry (BB) diets at doses of 2.5%, 5%, 10% and 15% BB (w/w %) on net bone calcium retention. The only significantly effective dose at increasing net bone calcium retention by 25.6 % ($p = 0.0426$) was the 5% BB dose. This study suggests that

blueberry feeding has a hormesis effect. Further research needs to be conducted to elucidate whether the 10% and 15% are excessive doses that could be increasing oxidative stress negating the benefit of lower doses on bone health.

4.2 Introduction

Osteoporosis is the most common bone disease in older adults and is characterized by low bone mass and increased fragility that can result in fracture at the spine, hip, or wrist (Imel, DiMeglio and Burr). It is caused by an increased bone resorption and decreased bone formation, resulting in a net bone loss. Currently, NHANES data estimates that 16% of US men and 29.9% of US women have osteoporosis (Wright, Saag, et al.) and the prevalence of osteoporosis in this population is estimated to increase by approximately 17.3 million (32%) from 2010 to 2030 (Wright, Looker, et al.). Economically, it poses a great burden as it accounts for more hospital days than any other disease with health costs of more than $20 billion in the United States (Sacco, Horcajada and Offord). Costs which are predicted to increase by almost 50% by the year 2025 (Burge et al.). As we reach an era of longer life expectancy, osteoporosis prevention becomes of great concern for a better quality of life in aging populations.

Postmenopausal women have a greater risk of developing osteoporosis due to the loss of estrogen during menopause. During this period, the hormonal imbalance results in an increase in oxidative stress which leads to an increase in osteoclastogenesis, leading to an accelerated rate of bone resorption and a decrease in bone formation, resulting in rapid loss of bone (Clarke and Khosla; L. Kaume et al.). Adequate intake of calcium and vitamin D is essential for maintaining bone health, but does not prevent the loss of bone during this life stage (Holick et al.; Shin and Kim). Since hormonal replacement therapy was associated with an increased risk for

cardiovascular disease (D. Yang et al.), other nutritional preventive strategies are being sought, such as polyphenol-rich fruits, which may benefit bones by other mechanisms.

New *et al.*, through a cross-sectional study in premenopausal women, was the first to report a positive association between fruit consumption and bone mineral density in healthy adults (New et al.). Although, the authors attributed the beneficial effect of fruits and vegetables on bone to an acid-base balance, recent research suggests the phytochemical content of fruits and vegetables exert the beneficial effect on bone (Sacco, Horcajada and Offord), with evidence from plum, grape and blueberry (Hohman and Weaver; Pawlowski et al.; Hooshmand, Brisco and Arjmandi).

Blueberry (*Vaccinium* Spp) is a fruit rich in polyphenols including anthocyanins such as cyanidin, malvidin, delphinidin, peonidin and petunidin glycosides, phenolic acids such as chlorogenic, gallic, ferulic and ellagic acids, flavonoids and flavan-3-ols (Zhong et al.). These account for a total phenolic (TP) content ranging between 261.95 to 929.62 mg TP/100mg fresh weight (Sellappan, Akoh and Krewer), depending on the blueberry cultivar. *In vitro* experiments have shown that blueberry serum metabolites derived from blueberry-fed rats decrease the receptor activator of nuclear factor kappa-B ligand (RANKL) expression in bone marrow derived stromal cell line ST2, decreasing osteoclast resorptive activity (J. Zhang, O. P. Lazarenko, J. Kang, et al.). Hippuric acid (HA), a colonic metabolite from blueberry, increases osteoblast maturation and proliferation (J. R. Chen, O. P. Lazarenko, J. Zhang, et al.), which could potentially increase bone formation. In animal models, blueberry has been shown to increase bone mineral density (BMD) resulting from decreased bone resorption and increased bone formation, in diets containing 1-10% blueberry (Devareddy et al.; J. Zhang, O. P. Lazarenko, J. Kang, et al.; Li et al.). Thus, the aim of this study was to refine the effective dose of blueberry on net bone calcium retention in OVX rats using urinary appearance of ^{45}Ca from pre-labeled bone (Pawlowski et al.; Hohman and Weaver).

4.3 Methods

Rats. Twenty 4-month old female ovariectomized (OVX) Sprague Dawley rats (Harlan Laboratories) were individually housed in stainless steel wire-bottom cages with a 12-h on:off cycle and were fed chow diet and deionized water ad libitum. Rats were allowed to acclimate to their new environment and stabilize from ovariectomy for 75 days. On day 45 they were dosed via a tail-vein injection with 63 microcuries (μCi) of ^{45}Ca dissolved in 200μl of sterile. ^{45}Ca that was not deposited in bone was excreted from the soft tissues during the next 30 days prior to starting the study. On day 65, ten days prior to determination of baseline ^{45}Ca excretion, rats were changed to a AIN93-M polyphenol free diet (soybean oil was replaced with corn oil, Research Diets).

Diets. All diets were formulated to be isocaloric and to have equal amounts for macronutrients, vitamins and minerals (Table 4.1). The polyphenol free diet consisted of a modified version of AIN93-M diet in which soybean oil was replaced with corn oil to prevent confounding from residual soy isoflavones. The respective 2.5%, 5%, 10% and 15% blueberry diets were modified from the polyphenol free diet by modifying the quantities of sugars, maltodextrin and cellulose that the blueberry powder displaced. The freeze-dried blueberry powder was provided by the Wild Blueberry Association of North America (Old Town, ME), and freeze dried by Futureceuticals, Inc. (Momence, Il). The diets were prepared and pelleted under low temperature conditions to avoid polyphenol degradation (Research Diets, Inc. New Brunswick, NJ). Upon arrival, the diet was stored at -80 °C and weighed out portions for each rat were transferred to -20°C during treatment periods.

Study Design. This study was a randomized, crossover intervention to evaluate the dose response effect of a blueberry-enriched diet at a 2.5%, 5%, 10% and 15% freeze dried blueberry

diet on net bone calcium retention. Use of ^{45}Ca to determine net bone calcium etention implemented in this study was previously described by Pawlowksi et al. (Pawlowski et al.). Briefly, rat's bones are pre-labeled with ^{45}Ca isotope by administering 60 µCi of the isotope through a tail-vein injection. The rats are given a 30-day equilibration period for the ^{45}Ca that was not deposited in bone tissue to be excreted from the soft tissue through urine. During baseline, treatment and washout periods, daily urine is collected to determine the urinary ^{45}Ca and total calcium ratio in order to determine the treatment effect on bone calcium retention. As depicted in Figure 4.1, the study had a duration of a total of 91 days that consisted of an 11 day baseline period and four 10-day treatments followed by 10-day washout periods. Urine was collected as daily 24 h pools during baseline and treatment periods. During washout periods, rats were changed to a polyphenol-free diet and urine was collected daily except for day 5 to allow rats to rest on plexi-glass plates. For urine collection, rats were housed in metabolic cages that had a funnel attached to it to allow for urine collection and a screen that separated feces and debris from the urine. Each treatment is a different blueberry dose, and rats were randomly assigned to crossover through a combination of the four different diets.

^{45}Ca and Total Calcium Analysis. Urine was collected daily. Urine was decanted and diluted to the nearest 0.5 ml with ultra pure water; total urine volume was recorded. Scintillation vials were prepared with EcoLite(+) (MP Biomedicals LLC., Solon, OH) 1 ml of urine was used for baseline and washout periods. For urine from treatment periods, due to anthocyanin coloration from the diet, urine was diluted and 500 µL of 30% H_2O_2 (Macron Fine Chemicals, Center Valley, PA) were added to eliminate the pink coloring from the anthocyanins. ^{45}Ca counts per minute (CPM) were measured in a Beckman LS 6500 Scintillation counter (Beckman Instruments Inc., Fullerton). Total calcium was determined by vortexing samples, diluting and

analyzing them on the atomic absorption spectrometer, AAnalyst 300 (PerkinElmer Instruments, Waltham, MA). For each 24 h urine sample of each rat, the ratio of percent dose of ^{45}Ca to milligrams of total calcium was determined as the ^{45}Ca:Ca ratio. This ratio was used to compare calcium excretion during intervention and baseline/washout periods to determine the treatment effect percent bone calcium retention.

Statistics. Data were analyzed using SAS 9.3 (SAS Institute Cary, NC). To determine changes in bone calcium retention, the ratio of urinary ^{45}Ca to total Ca was transformed using the natural logarithm to correct for skewedness. For each rat, a simple linear regression model was fit through all of the nonintervention periods (baseline and washouts) creating a line that represents usual calcium excretion. During the intervention period, data points were used to generate a prediction equation for each rat which was subtracted from the experimental value measured during the blueberry treatment period. Differences between the predicted values and observed values reflect suppression of bone turnover. The means from each blueberry dose for each rat were averaged across all rats, and 95% confidence intervals were calculated. The values were exponentiated and reported in the original scale as percent improvement in calcium retention compared to baseline. Data are presented as the mean treatment effect and its 95% confidence interval. To determine differences between treatment diet effect on rat weight, weight gain, food intake, and feeding efficiency, a one way analysis of variances (ANOVA) was performed using JMP 12 (SAS Institute Cary, NC).

4.4 Results

Body Weight and Food Intake. Rats were weighed at the beginning and end of every treatment and their food intake was recorded to determine food intake (g/day) and feeding

efficiency. There were no significant differences in weight gain, food intake or food efficiency ratios among treatments, as shown in Table 4.2.

Bone Net Calcium Retention. The dose effect of the treatment on net bone calcium retention was determined by obtaining the log of the urinary ^{45}Ca:Ca ratio excreted during the treatment and washout periods. Baseline and washout periods were used to determine a regression line as control for each individual rat. The treatment effect was determined by comparing their ^{45}Ca:Ca ratio during treatment periods to their own control regression line. This allowed us to determine the treatment effect at each of the different blueberry doses. By averaging the mean distance of the ^{45}Ca:Ca ratio from treatment to the control line for each rat individually, we determined the effect of the different doses on bone net calcium retention. As shown in Figure 4.2, bone net calcium retention significantly increased by 25.6% ($p = 0.0426$) when the rats were on the 5% BB (w/w%), compared to a 24% increase at a 2.5%BB dose that failed to reach significance ($p = 0.0541$). At the higher doses of 10% and 15% BB, there was no significant effect on net bone calcium retention in ovariectomized rats.

4.5 Discussion

The most effective dose of freeze-dried wild blueberry at increasing bone net calcium retention in ovariectomized rats was identified from a range of 2.5% to 15% (% w/w). The crossover design of this study, in which rats served as their own control and were tested in all different treatments, allowed us to do a rapid assessment of the effects of blueberry on bone health with a relatively small sample size. We expected to see a dose-response increase in bone net calcium retention with increasing blueberry doses. However, lower doses resulted in higher

net bone calcium retention than higher doses, i.e. 25.6% for 5% BB dose and 24% for 2.5% BB dose ($p = 0.0541$) compared to no benefit at the higher doses of 10% BB and 15% BB.

Our findings with the higher doses are consistent with no benefit in bone Ca retention of a 9% and 25% BB diet (% w/w) in our previous study, using the same ^{45}Ca methodology (Pawlowski et al.). However, benefits with lower doses in our isotopic tracer study are supported by bone imaging outcomes. Using a 5% BB diet (w/w%), Devareddy et al. demonstrated through a 100-day study that a 5% dose protected ovariectomized rats from estrogen deficiency-induced bone loss, resulting in a significantly higher total BMD than OVX controls (Devareddy et al.).

Several studies have demonstrated that a diet rich in blueberries can attenuate bone loss in ovariectomized rats, at doses ranging from 5% BB to 10% blueberry for extended periods of time (Devareddy et al.; Li et al.)

Similar to our findings, a recent study using blackberry, a polyphenol-rich fruit similar to blueberry's phenolic content, evaluated the effects of a 5% and 10% (% w/w) blackberry diet in ovariectomized rats. They demonstrated that a 100-day treatment with 5% blackberry resulted in modest protection of tibial and femoral BMD, but no effect was seen with the higher dose of 10% (Lydia Kaume et al.).

The benefits of lower dose, but not higher doses, of blueberries may relate to redox status. After menopause, the body is depleted of estrogen and enters a stage of oxidative stress that causes an imbalance in osteoclast and osteoblast activity. Oxidative stress induces the differentiation and activation of osteoclasts, which leads to an accelerated rate of bone resorption that overcomes the rate of bone formation, leading to net bone loss (Huh et al.). This is consistent with Kaume et *al*.'s study which showed that doses higher than 10% of a polyphenol-rich fruit resulted in no beneficial effect on bone health (Lydia Kaume et al.).

The high levels of polyphenols in blueberries are thought to be the bioactive components that might act as antioxidants, reducing oxidative stress, and therefore, preventing bone loss. Even though toxic levels of polyphenols have not been established, our study suggests that consuming high levels of polyphenols might be counteracting its antioxidant capacity and voiding its beneficial effect on attenuating inflammation and oxidative stress that leads to bone loss. Blueberry polyphenols might be following the hormetic paradigm, in which smaller doses have opposite effects than higher doses (Hayes). Our data suggest future research should evaluate whether even higher levels that tested here are toxic to bone considering the availability of concentrated extracts on the market.

A strength of this study was the dose comparison of blueberries using a precise method to determine bone loss. A limitation of the study was that the only biological sample collected was urine, precluding measuring biomarkers to determine effects of blueberry on oxidative stress and inflammation. Blood collection was not practical in this cross over design of four short interventions. Nor did the design allow bone collection for analysis of mechanical and microarchitectural properties.

In summary, this study allowed determination of the most effective dose of blueberry (5%), which can now be tested for preventing menopausal bone loss, now in a chronic feeding to determine bone outcomes and potential mechanisms of action.

Our study demonstrated that a level of 5% blueberry in diets was more effective at preventing OVX-induced bone loss than higher doses.

4.6 Figures and Tables

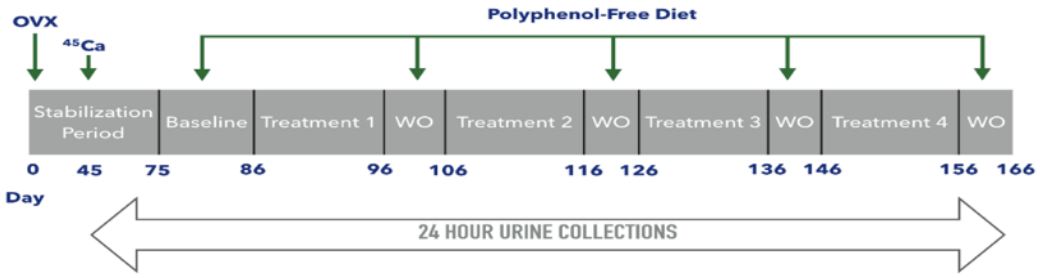

Figure 4.1. Study Design

Each rat was randomized to a specific sequence of blueberry doses. They all went through an 11 day baseline period, followed by four 10-day treatment periods, followed by a 10-day washout period (WO).

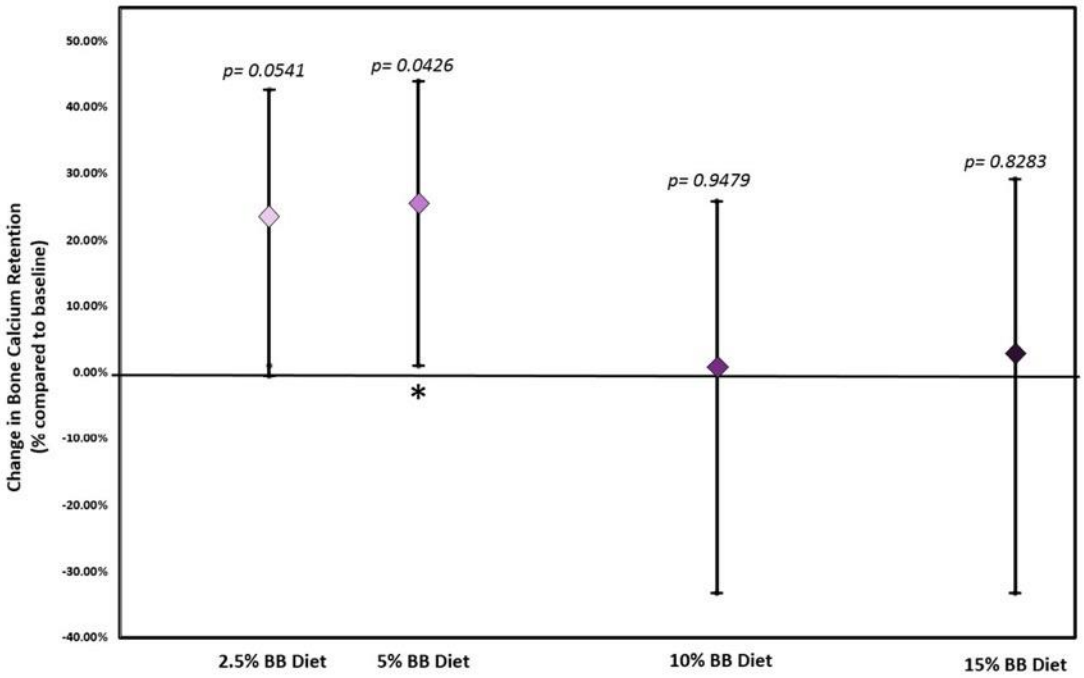

Figure 4.2 Change in Net Bone Calcium Retention.

The 5% BB dose significantly increased bone net calcium retention in ovariectomized rats. Four different doses were used to determine the effect of a diet rich in blueberry on bone net calcium retention in ovariectomized rats (n= 20 rats per diet). Results are reported as mean ± 95% confidence intervals. A value of zero represents no change from the regression line computed from baseline and washout periods. A confidence interval that does not cross the 0% change line indicates a significant change from the regression line at $\alpha = 0.05$.

Table 4.1 Modified AIN93-M Modified Diets per Treatment

	Polyphenol-Free	**2.5% BB**	**5% BB**	**10% BB**	**15% BB**
Ingredient			**g/kg of diet**		
Casein	140	140	140	140	140
L-Cysteine	1.8	1.8	1.8	1.8	1.8
Corn Starch[a]	495.7	468.2	470.7	474.7	479.7
Maltodextrin[a]	125	147	139	123	107
Sucrose[a]	100	84.5	69	38	7
Corn Oil[b]	40	40	40	40	40
Cellulose[a]	50	46	42	35	27
Mineral Mix[c]	35	35	35	35	35
Vitamin Mix	10	10	10	10	10
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5
TBHQ	0.008	0.008	0.008	0.008	0.008
Blueberry Powder	0	25	50	100	150
Total Phenolics	0	1.11	2.22	4.44	6.66

[a]Ingredients modified from original AIN93-M diet to match for the amount of fructose and fiber that the blueberry accounted for. [b]Corn oil was used to replace soybean oil from the original formulation to eliminate soy isoflavone residuals. [c]Mineral mix contains 0.5% Ca per kg of diet.

Table 4.2. Body Weight, Weight Gain, Food Intake and Feeding Efficiency Ratio[a] for the OVX rats during the study. There were no significant differences between any of the treatments. Results are expressed as Mean ± SEM

Treatment	Body Weight (g)	Weight Gain (g)	Food Intake (g/day)	Feeding Efficiency
2.5% BB Diet	320.4 ± 4.4	9.8 ± 1.1	15.4 ± 0.3	0.63 ± 0.07
5% BB Diet	319.9 ± 4.5	8.8 ± 1.3	15.5 ± 0.4	0.54 ± 0.07
10% BB Diet	319.6 ± 3.8	10.9 ± 1.2	15.6 ± 0.4	0.68 ± 0.06
15% BB Diet	321.3 ± 4.4	9.3 ± 1.1	14.5 ± 0.3	0.64 ± 0.07

[a]Feeding efficiency ratio as a change in body weight / food intake. NS differences determined by one-way ANOVA.

CHAPTER 5. CONSUMPTION OF A RABBITEYE BLUEBERRY-ENRICHED DIET RESULTS IN ACCUMULATION OF HIPPURIC ACID IN THE BONE MARROW AND INCREASED BONE DEPOSITION IN OVARIECTOMIZED RATS BUT FEW OTHER BONE BENEFITS

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5.1 Abstract

A chronic feeding with blueberry was shown to be effective in the attenuation of ovariectomy-induced bone loss in Sprague Dawley rats. Previous studies have shown a significantly higher BMD in BB-fed rats in comparison to control rats (Devareddy et al.; Li et al.). The mechanism through which blueberries exert a beneficial effect on bone remains to be elucidated. No research has been conducted on the effects of a chronic consumption of blueberry on calcium metabolism. Thus, this study aims to determine the effects of an 8-wk chronic consumption of two different varieties of blueberry on calcium balance, calcium kinetics, bone microarchitecture and polyphenol metabolism and distribution in ovariectomized rats.

Eighty 5-mo old OVX rats were sorted by weight and block-randomized to an 8 week chronic feeding treatment of a polyphenol-free (control), 5% wild lowbush blueberry (*V. angustifolium*), 5% `Montgomery´ rabbiteye blueberry (*V. Ashei*) or 2.5% `Montgomery´ rabbiteye blueberry diet (%w/w). During week 0 and week 8, rats were placed in metabolic cages

for 4 days and daily 24 h urine and feces collections were done to determine calcium absorption and retention. At the end of week 8, rats were dosed with ^{45}Ca and serial blood draws were done from baseline up to 48 h post-dose to map Ca kinetics. At sacrifice, the right femur was collected to determine differences in bone microarchitecture of trabecular and cortical bone through MicroCT. A 24 h urine collection was done at baseline and during week 8 of treatment to determine urinary phenolic acid excretion. Results show that a blueberry enriched diet had no effect on cortical bone microarchitecture, with a trend towards increased trabecular bone protection ($p = 0.08$). During baseline, a 2.5% Montgomery diet significantly increased Ca retention, but the effect was lost after the 8 weeks of treatment when there were no differences between treatments. Calcium kinetics results showed that Mont 5% significantly increase Ca absorption and bone turnover, although no differences in bone balance were detected between the treatments. Interestingly, an accumulation of hippuric acid in the bone marrow was detected with the blueberry treatments and it was significantly and positively correlated with bone deposition. The metabolism of phenolic acids was significantly affected by a chronic consumption of the treatment diets, resulting in phenolic acid profile and excretion shifts after the 8-week chronic study. In conclusion, a blueberry-enriched diet had minimal effects on bone after stabilized to ovariectomy and the effects differ depending on blueberry variety and dose.

5.2 Introduction

Osteoporosis is the major bone disease in older adults which can result in an increased mortality due to fractures at the spine, hip or femoral neck (Wright, Looker, et al.). It is a major public health concern, with an estimated two million fractures per year in the United States (US) and more hospital days than any other disease; with 432,000 hospital admissions and 2.5 million medical office visits per year (Michael Lewiecki et al.). Women are at a higher risk for

osteoporosis because of the loss of estrogen's protective effect on bone during menopause. When estrogen production declines, there is an increase in resorption rates and decreased formation rates, which results in a negative bone balance, leading to a rapid loss of bone. In the US, 29.9% of women have osteoporosis (Wright, Saag, et al.) and the lifetime risk of an osteoporotic fracture in women aged 50 and over is more than 40% (Kanis et al.). From the year 2002 to 2013, there was a significant decrease in fracture incidence at the hip in women, but this decline increased from the year 2013-2015, due to a decrease in DXA testing, an increased fear of osteoporosis treatment side effects (Michael Lewiecki et al.) or non-adherence to pharmacological treatment. A study conducted by Sattari et al. (2017) showed that only 21.6% of high-risk women diagnosed with osteoporosis in the Women's Health Initiative study underwent the recommended treatment to prevent future fractures(Sattari et al.). Due to the high incidence of osteoporosis and the low adherence to treatment, preventative nutritional alternatives are being sought.

Epidemiological (New et al.), preclinical (Devareddy et al.; Hohman and Weaver), and clinical studies (Hooshmand, Brisco and Arjmandi; Hooshmand, Chai, et al.; Hooshmand, Kern, et al.) suggest that polyphenol-rich fruits, such as blueberry, plum and grapes, may provide an alternative nutritional strategy to prevent bone loss in postmenopausal women. Polyphenols are secondary metabolites produced by plants to aid in their protection against stress or environmental threats (Lila). Human consumption of these compounds has shown to have beneficial effects to health, by acting as antioxidants (Tang et al.), anticarcinogenics (Matchett et al.), and anti-inflammatory (Huang et al.) compounds. One of the richest sources of polyphenols are blueberries, which contain anthocyanins, flavan-3-ols, flavonols and phenolic acids (Yousef, Brown, et al.). Previous preclinical studies have shown positive bone health effects in

ovariectomized (OVX) rats with both a 5% highbush blueberry diet (% w/w) or a 10% rabbiteye blueberry solution. Both studies showed significant attenuation of bone loss at the femur, assessed through bone mineral density (BMD) scans and bone resorption biomarker gene expression and protein levels (Devareddy et al.; Li et al.), but no study has investigated the effects of blueberry consumption on calcium metabolism. Since bone is a dynamic tissue that is in a constant state of remodeling, adequate absorption of calcium in the gut is essential to maintain serum calcium levels for normal bone mineralization (Weaver et al.). Menopause and aging is associated to a decrease in calcium absorption (Heaney et al.) which may lead to bone loss if calcium intake and vitamin D status are low (Park et al.). To prevent a significant loss of bone, adequate intake of Ca is essential. Increasing calcium intake above the recommended (1,200 mg Ca/d) shows no benefit on BMD (Burckhardt), an increased absorption of calcium might. Thus, the first aim of this study was to determine whether the bone protective effects of a blueberry-enriched diet during menopause are due to increased calcium utilization, resulting in increased bone mineral density, strength and microarchitecture.

Different genetic varieties of blueberry vary in (poly)phenol content and profiles. Lowbush wild blueberries have ~2-fold higher concentration of (poly)phenols in comparison to commercial rabbiteye blueberries (Kalt, Ryan, et al.). Preclinical evidence suggests that blueberry consumption has bone health benefits during growth (J. Zhang, O. P. Lazarenko, M. L. Blackburn, et al.; J. Zhang, O. P. Lazarenko, J. Kang, et al.) and menopause (Devareddy et al.; Li et al.) attributed to its phenolic content, but the specific (poly)phenols responsible for its bioactivity remain unclear. Blueberry contains more than 50 different (poly)phenols, including 5 anthocyanins (cyanidin, delphinidin, malvidin, peonidin and petunidin) and 3 flavonols (quercetin, myricetin and kaempferol), which all vary in their glycoside (3-

glucoside/arabonoside/galactoside/rhamnoside/rhutinoside), flavan-3-ol monomers (catechin and epicatechin) and polymers (procyanidins), and phenolic acids such as gallic acid, ellagic acid and chlorogenic acid (Ana Rodriguez-Mateos et al.). After ingestion, they undergo extensive intestinal and hepatic metabolism resulting in glucuronide, sulfate or methyl conjugates of the parent compound or are hydrolyzed by the gut microbiome into smaller colonic metabolites (Zhong et al.). Due to the phase II enzymatic metabolism of these compounds after ingestion, they have a very short half-life and are usually excreted in as short as half a day. In contrast, the smaller phenolic acids, produced by the gut microbiome are absorbed more slowly and remain in circulation for longer periods of time than conjugates of the parent compounds (Zhong et al.). Since their bioactivity will depend on their availability in plasma, we hypothesized that the smaller molecular weight colonic metabolites are the ones that provide the bone protective effects in ovariectomized rats. By using two different blueberry varieties with different phenolic profiles, our second aim was to 1) determine the absorption of blueberry polyphenols from two different blueberry varieties measured through a 24 h urinary excretion, 2) determine whether an 8-week chronic consumption of blueberry results in an increase/decrease in their absorption and metabolism, and 3) examine the presence of phenolic acids in the potential target tissue of the bone marrow.

5.3 Methods

Study Design. All animal procedures were approved by the Purdue Animal Care and Use Committee (Protocol 1612001508). Eighty 5-mo old Sprague Dawley rats were ovariectomized (Envigo) and upon arrival, were allowed to stabilize for a month after surgery. Rats were housed in shoebox cages in pairs with a 12-h on:off cycle. They were fed chow diet and deionized water ad libitum. Ten days prior to starting the treatment, rats were individually caged in wire-bottom

cages and changed to a polyphenol-free diet (modified AIN93M diet, Research Diets) to clear their systems of any polyphenols. Rats were randomized to an 8-week feeding treatment of a Control (polyphenol-free) diet, 2.5 % or 5 % `Montgomery´ highbush blueberry (Mont 2.5% or Mont 5%), or a 5% wild blueberry (Wild BB 5%) (% w/w). To avoid polyphenol degradation, diets were stored at -20 ºC, leftover food from the prior day was removed and rats were fed fresh pellets daily. At the beginning and end of the study, rats underwent bone mineral density scans, calcium balance study and 24 h urinary collection for phenolic excretion analysis. At the end of the study, rats underwent a calcium kinetic study, sacrificed through an overdose of carbon dioxide and bones were excised for further analyses.

Diets. All diet formulations were formulated to be isocaloric and to have equal amounts of macronutrients, vitamins and minerals. All diets were based on the purified AIN93M rodent diet (Research Diets, New Brunswick, NJ). Diet composition is shown in Table 5.1. The control diet was a polyphenol-free modified AIN93M diet in which soybean oil was replaced by corn oil to prevent confounding from residual isoflavones. Wild blueberry freeze dried powder was purchased from the Wild Blueberry Association of North America (Old Town, ME) and the `Montgomery´ highbush blueberry freeze-dried powder was provided by North Carolina State University's Plants for Human Health Institute (Kannapolis, NC). The three blueberry diets blueberry diets were modified from the polyphenol free diet by adjusting for the quantities of sugars, maltodextrin and cellulose that the blueberry powder displaced. The diets were prepared and pelleted under low temperature conditions to avoid polyphenol degradation (Research Diets, Inc. New Brunswick, NJ). Upon arrival, the diet was stored at -80 °C and weighed out portions for each rat were transferred to -20 °C during treatment.

Bone Mineral Density. Rats were anesthetized within a chamber with 3.5% Isofluorane, kept under anesthesia with a mask and placed on a Lunar PIXImus (GE Lunar Corp., Madison, WI) small animal bone densitometer. Scans were carried out during week 0 and week 8, one for the lumbar spine and another for the right femur. Scans were carried out at the beginning of the study, to determine baseline values and at the end of the 8-week treatment.

Bone imaging. Right femurs were excised and imaged with a micro-computed tomoghraphic (microCT) scanner (µCT 40; Scanco Medical) by using previously described methods (Hohman et al.). Scans were carried out at an isotropic resolution of 16 µm, and integration time of 190 ms, X-ray tube potential (peak) of 55 kVP. Distal femurs were scanned for 1 mm (62 slices) starting downwards after the disappearance of the growth plate. Segmentation values were sigma = 0.8 and support = 1, binarization threshold was 445.5. All trabecular measurements were calculated by using a three-dimensional direct model. Trabecular bone variables analyzed were bone volume fraction, connectivity density ($1/mm^3$), trabecular thickness (mm), trabecular number (1/mm) and trabecular spacing (mm). Cortical variables included cortical area fraction, cortical thickness (mm), periosteal perimeter (mm) and endocortical perimeter (mm).

Calcium balance. During week 1 and week 8, calcium balance studies were conducted. Rats were individually housed in stainless steel wire bottom metabolic cages. Daily 24 h urine and fecal collections were carried out for 4 consecutive days. Food intake was recorded and rats were weighed at the beginning and end of the 4-day balance period. Urine was collected in 50 ml tubes and centrifuged at 3500 rpm, for 10 minutes at 4 ºC. Urine was then decanted into 15 ml tubes to determine total volume and brought up to the nearest 0.5 ml with ultra pure water for volume accuracy. Urinary calcium was determined by diluting 100 µl of sample in 10 ml of 0.5

N HCl/0.5% lanthanum (III) chloride solution. Duplicate samples were analyzed through atomic absorption spectroscopy (AAnalyst 300; Perkin Elmer, Waltham MA). Fecal samples were ashed in a muffle furnace at 600 ºC for 10 days. Ashes were then dissolved with nitric acid and diluted with ultrapure water. For analysis, duplicate fecal samples were diluted with 2% nitric acid, and calcium was measured through inductively coupled plasma-optical emission spectrometry (ICP-OES; Optima 4300DV, Perkin Elmer, Shelton, CT, USA)

Calcium balance variables were calculated using the following equations:

$$\text{Net Ca absorption} = \text{Ca intake} - \text{fecal Ca} \quad (1)$$

$$\text{Net Ca retention} = \text{Ca intake} - (\text{fecal Ca} + \text{urinary Ca}) \quad (2)$$

$$\text{Net Ca Absorption efficiency} = (\text{Net Ca absorption}/\text{Ca intake}) \times 100 \quad (3)$$

^{45}Ca kinetics. During week 8, two days prior to the start of both the 4-day balance study and kinetic study, rats were anesthetized with isofluorane (3-5 %) in an anesthesia chamber and maintained under anesthesia with a mask (1.5-3 % isofluorane). A silastic catheter was implanted into the jugular vein and burenorphine (0.1 mg/kg BW) was administered to alleviate pain prior to animals regaining consciousness. Rats were allowed to rest for 48 hours after surgery and catheters were kept patent by flushing with heparinized saline (20 U/mL) every 12 h. Rats were fasted 8 hours prior to the kinetic study and ~ 200 µl of whole blood was collected at baseline. Rats were gavaged with one fourth of their daily calcium and blueberry intake which amounted to 80 mg of calcium acetate and 200 mg of freeze-dried blueberry powder dissolved in 2 ml of water. Control rats were gavaged with ultra pure water instead of blueberry. Rats were dosed

with 10 µCi or 5 µ Ci of ^{45}Ca orally or intravenously, respectively. After gavage, ~ 200 µl serial blood draws were carried out at minutes 5, 30, 60, 90, 120, 240, 360, 720, 1380 and 2880 and placed into heparinized tubes. Whole blood was processed to plasma by centrifugation at 4ºC and 6000 rpm for 10 min. For ^{45}Ca from plasma, urine and fecal samples were placed in scintillation vials with 15 ml of Ecolite scintillation liquid (MP Biomedicals) and counted via liquid scintillation counter (Beckman LS 6500; Beckman Coulter). The compartmental model is shown in Figure 5.1. Kinetic modeling was performed by using WINSAAM (NIH) as previously described (Cai et al.).

Blueberry phenolic analysis study design. The 24 h urinary phenolic acid excretion was assessed at baseline after the rat's first exposure to the blueberry and during week 8 of the treatment. All rats were fasted overnight and control rats were gavaged with 2 ml of deionized water, 185 mg or 370 mg of freeze-dried pulverized Montgomery blueberry dissolved in 2ml of water for Mont 2.5% and Mont 5% rats and 370 mg of freeze-dried pulverized wild blueberry dissolved in 2 ml of water for Wild BB 5% rats. Urine was collected for 24 h after the rats were gavaged and they were put on a polyphenol-free diet for the rest of the day to avoid contamination of urine from food spill. To remove food spill, urine samples were centrifuged at 3500 rpm for 10 min at 4 ºC. Two aliquots of urine were store with 1 % FA (aq.) in a 5:1 ratio, flushed with N_2 gas and stored at -80 ºC until analysis.

Urinary phenolic acid extraction. Phenolic acids from urine samples were extracted via SPE using the strataX, polymeric reversed phase microelution 96-well plate (Phenomenex Inc., Torrance, CA) using a Waters positive pressure-96 processor (Waters, Milford, MA). Solid-phase extraction was carried out as follows, the cartridge was preconditioned with 200 µl of 1% FA MeOH, followed by 200 µl 1% FA (aq.). The plate was equilibrated with 100 µl FA . 750 µl

of urine sample was loaded into each well and 10 µl of taxifolin was added into each well as internal standard. The plate was then washed with 200 µl of 1% FA (aq.), followed by 200 µl of 0.1% FA (aq.). The plate was kept under positive pressure for 30 min to dry out any remaining water. Samples were then eluted with 100 µl of 0.1% FA MeOH, twice. 10 µl of volume control (ethyl gallate) and IS (scopoletin) were added to each well before injection into UPLC-MS/MS.

*Bone marrow phenolic acid extraction***.** At sacrifice, the left femur was excised, chondyles were removed and the bone marrow was flushed out with 1ml of 0.1%FA saline solution, flushed with N_2 gas and stored at -80ºC until analysis. Bone marrow samples were sonified for 20 s, followed by a liquid-liquid extraction of fat by adding 5ml of hexane, vortexing the sample for 1min and then centrifuging at 3500rpm for 2min. Hexane was removed and liquid-liquid extraction was repeated twice. 3ml of 1% FA MeOH (%v/v) were added and sample was sonified for 10 s, centrifuged at 3500rpm for 2 min. The supernatant was decanted into new vial and extraction with 3ml of 1% FA MeOH was repeated twice. Samples were then filtered through a 0.45µm microfilter and dried down under N_2 gas. Samples were resolubilized in 0.1% FA (aq.) and then extracted through solid-phase extraction with a 1-cc Oasis HLB cartridge (Waters, Milford, MA). The cartridge was activated with 1ml of methanol followed by 1ml of wated. The sample was loaded into the cartridge and washed with 2ml of FA (aq.) followed by 1ml of 95:5 H_2O:MeOH. Samples were eluted with 2ml of 2% FA MeOh (% v/v) and dried down with N_2 gas. Dried samples were resuspended with 1ml of 0.2% FA H_2O for UPLC-MS/MS analysis.

*Urinary and Bone Marrow phenolic acid UPLC-MS/MS quantitation***.** LC-MS/MS analysis was conducted using Waters ACQUITY Xevo TQD instrument (Milford, MA). Inlet method was optimized for a run time of 6 minutes for an injection volume of 5 µl. The mobile

phase consisted of 0.1% formic acid (v/v) in water (mobile phase A) and 0.1% formic acid (% v/v) in acetonitrile (mobile phase B) at a flow rate of 5 mL/min. The gradient consisted of 0% B at 0 min, 6% B at 0.5 min, 9% B at 2 min, 13% B at 3 min, 35% B at 4.5 min, 0% B at 5.2 min, 0% B at 6 min. Mobile phase A was switched to 2% formic acid (% v/v) for the analysis of anthocyanins. ACQUITY UPLC BEH C18 column was used, and the column temperature was set at 40 °C. MS source parameters including cone voltage and collision energy were optimized by directly infusing individual standards of target compounds.

Randomization and Statistical analysis. For managing the workload, rats were divided into 3 cohorts that would start the study one week apart from each other. Each cohort contained equal numbers of rats per treatment group. To ensure that each treatment group had equal average body weights, rats were sorted by weight and block randomized. Each block consisted of 8 rats; 2 per treatment group. Data are presented as means ± SEMs. Differences between total phenolic content and individual phenolics in both blueberry varieties were determined through a Student's t-test. Differences between treatment groups for bone outcomes were assessed through a one-way ANOVA and for the urinary phenolic data, a two-way ANOVA was carried out to determine differences due to the treatment, time and treatment*time effects. For all parameters with significant p-values, intergroup differences were determined using Tukey's pairwise test. Due to the block randomization, all statistical analysis were analyzed using block nested within cohort as another variable. Correlations between the kinetic variables and hippuric acid content in the bone marrow, a bivariate fit was used and R^2 was determined by linear regression. A value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using JMP version 13 (SAS Institute).

5.4 Results

Bone imaging and microarchitecture. Bone mineral density was measured through a DXA Scan with Piximus at the right femur and the spine (L1-L4) at baseline and at 8 weeks after a chronic feeding of their treatment diet. All rats lost BMD at the lumbar spine, consistent with ovariectomy, but no differences were observed between treatments (Figure 5.2). At the left femur, the bone loss in Wild BB 5% was significantly lower than in the Control group ($p < 0.05$) (Figure 5.3).

Microarchitecture of the right femur was assessed through MicroCT, results are shown in Table 5.2. Wild BB 5% treatment significantly increased trabecular thickness in comparison to the control group ($p<0.05$), when using Dunnett's test, no significant differences were found through a one-way ANOVA ($p = 0.0843$). No differences were found in the midshaft femur for cortical bone or any other of the trabecular bone parameters measured.

Calcium Balance. Calcium balance was conducted during weeks 1 and 8 of treatment. As shown in Figure 5.4, during week 1, Ca absorption and Ca retention was significantly higher ($p < 0.05$) in rats that were fed the 2.5% Mont diet -with an average of 12.4 and 11.78 mg Ca / day, respectively, compared to rats on the control diet who had an average Ca absorption and Ca retention of 3.4 and 0.9 mg Ca / day, respectively. After 8 weeks of treatment diet, there was no difference between Ca absorption and retention due to different treatment diets. Calcium absorption efficiency (Figure 5.5) was significantly higher in the Mont 2.5% treatment group in comparison to the Control and Wild BB group ($p < 0.05$). Suggesting that different blueberry varieties and dosages have different effects on calcium balance. Wild blueberry had no effect on calcium metabolism, while Montgomery blueberry does. After 8 weeks of treatment, there were no significant differences between treatment groups, thus blueberry variety or dosage had no effect on calcium balance after a chronic blueberry consumption.

^{45}Ca Kinetics. Calcium kinetics and compartmental modeling were assessed by measuring the appearance of ^{45}Ca in plasma, urine and feces after a bolus dose of 5 µCi or 10 µCi for IV rats and oral rats, respectively. Ca tracee was also measured in feces and urine during the 4 consecutive balance days of week 8. After the kinetic modeling of the tracer and tracee, results (Table 5.3) show that the transfer coefficients for L(2,1) and L(1,2) were significantly higher in Wild BB 5% rats than in Mont 2.5% and Mont 5%, respectively. Wild BB 5% also had significantly lower transfer of Ca from compartment 9 to feces in compartment 5 (L(5,9)) than Mont 5% ($p < 0.05$). There were no differences between in the transfer coefficient for absorption from the upper GI tract (compartment 8) to plasma (compartment 1), nor in the urinary excretion transfer coefficient L(6,1).

Bone deposition (V_{0+}) and bone resorption (V_{0-}) were significantly higher in Mont 5% ($p<0.0001$ and $p < 0.05$, respectively), followed by Control and Mont 2.5%, with Wild BB 5% having the lowest values for V_{0+} and V_{0-}. Although there were differences in the calcium transport for bone deposition and bone resorption between the treatment groups, there were no differences in bone balance. Mont 5% had a significantly higher fractional ^{45}Ca absorption of 0.28 ± 0.01 in comparison to 0.23 - 0.24 in with the other treatments, suggesting that there are differences in the effects of different blueberry varieties on calcium absorption. Mont 5% had a significantly higher calcium absorption (V_a) of 18.2 ± 0.46 mg Ca/d than all the other treatments ($p = 0.0002$). Wild BB 5% had significantly lower total body Ca and lower calcium in compartments 2 and 3, in comparison to the other treatments ($p < 0.0001$).

Blueberry Phenolic Content. The phenolic content (TPC) of the blueberries was assessed via the Folin-ciocalteau assay. Wild blueberry has a significantly higher TPC of 3284.3 ± 236.7 mg GAE/100g DW in comparison to Montgomery Blueberry with a TPC of 2191.4 ± 67.1 mg

GAE/100g DW ($p < 0.05$) (Figure 5.6). Individual flavonoid content was also analyzed. Wild blueberry has a significantly higher content of ferulic acid, quercetin, quercetin-3-glucoside, and quercetin-3-rutinoside than the Montgomery blueberry (Table 5.4), from which quercetin-3-glucoside was ~16-fold higher ($p < 0.01$) in Wild Blueberry than in Montgomery blueberry (140.97 ± 10.05 and 8.37 ± 1.62 mg Q-3-glu /100 g DW blueberry, respectively). While wild blueberry had significantly higher content of flavanols, Montgomery had a ~2-fold significantly higher content of the flavan-3-ol epicatechin than in Wild Blueberry ($p < 0.01$) with 113.25 ± 1.28 in comparison to 56.92 ± 6.28 mg EC /100 g DW blueberry, respectively. There were no differences in ferulic acid, chlorogenic acid, myricetin, gallocatechin, epigallocatechin and feruloyquinic acids between the two blueberry varieties.

24 h Urinary phenolic excretion. At baseline and during week 8 of treatment, a 24h urinary phenolic excretion was collected and analyzed to determine whether there were differences in the absorption of blueberry polyphenols between the two different blueberries and whether a chronic feeding of the blueberries had an influence in urinary excretion. Figure 5.7 shows pie charts with the distribution of total urinary excretion of (poly)phenols by subclass derivatives at baseline and during week 8 of treatment, Table 5.5 shows the values corresponding to the pie charts and Table 5.6 shows the urinary excretion for each individual (poly)phenol. A graphic representation of the excretion of phenolic acid metabolites from blueberry treatments relative to control is shown in Figure 5.8 through a heat map. As expected, the control treatment had significantly lower excretion of all urinary phenolic acids when grouped by classes as benzoic acids, phenylacetic acids, phenylpropionic acids, hippuric acids, cinnamic acids and flavonols ($p < 0.0001$).

Benzoic acid derivatives. Urinary excretion of benzoic acid derivatives significantly increased in Mont 5% from 1.5 ± 0.13 µg of benzoic acids at baseline to 3.36 ± 0.36 µg after the 8-week chronic consumption. Mont 2.5% and Wild BB 5% increased as well, but failed to reach significance, while control remained constant over time and significantly lower than the blueberry treatments. The individual benzoic acids detected in urine were 3-hydroxy-4-methoxybenzoic acid (3OH-4MeBA), benzoic acid-4-glucuronide (BA-glcr) and benzoic acid -4-*O*-sulfate (BA-O-S). By week 8, BA-glcr urinary excretion significantly increased in both Mont 5% and Wild BB 5% while BA-O-S significantly decreased for all treatments, including control.

Phenylacetic acid derivatives. The Mont 5% blueberry treatment had a slight decrease in phenylacetic acid derivatives excreted in urine by week 8, from 1.5 ± 0.06 µg at baseline to 1.12 ± 0.08 µg by week 8 ($p < 0.0001$), while the other treatments remained constant over time. The individual phenylacetic acids detected and quantified in urine were 4-hydroxyphenylacetic acid (4OH-PAA), 3-methylphenylacetic acid (3Me-PAA) and 3-hydroxy-4-methoxyphenylacetic acid (3OH-4-MePAA). Of these, 4OH-PAA significantly decreased from 0.79 ± 0.04 µg at baseline to 0.53 ± 0.04 µg during week 8. The 3Me-PAA urinary excretion remained lower than the blueberry treatments and constant over time for the control rats, while all the blueberry treatments exhibited a significant decrease after a chronic consumption of the diet. Wild BB 5% treatment had a significantly higher urinary excretion of 3OH-4-MePAA at baseline and at week 8, than both Montgomery diets at both time points. While the urinary excretion for Montgomery berry remained the same over time, Wild BB 5% significantly increased the urinary excretion by week 8 from 0.4 ± 0.028 µg to 0.55 ± 0.027 µg. Regardless of dose, there were no differences in the urinary excretion of 3OH-4-MePAA between the Mont 2.5% and Mont 5% treatments.

Phenylpropionic acid derivatives. The phenylpropionic acid detected in urine was 3-hydroxy-4-methoxyphenylpropionic acid (3OH-4MePPA). The urinary excretion in all blueberry treatments was significantly higher than in the control and there was no effect on urinary excretion after a chronic consumption of the blueberries. Interestingly, Wild BB 5% and Mont 2.5% did not differ in urinary excretion of 3OH-4MePPA, while Mont 5% was significantly higher than the other blueberry treatments at baseline, but not significantly different from Wild BB 5% after the 8 weeks of treatment.

Hippuric acid derivatives. A chronic consumption of blueberry diet resulted in significant increases in urinary excretion of hippuric acids in all blueberry treatments except for the control treatment ($p < 0.05$). Although Mont 2.5% had significantly lower excretion of hippuric acids at baseline (3.83 ± 0.33 µg), by week 8 of treatment, the urinary excretion significantly increased to 5.9 ± 0.12 µg and was no different than the urinary excretion with Mont 5% (6.32 ± 0.172 µg). The individual hippuric acid derivatives detected in plasma were hippuric acid (HA) and 3-hydroxyhippuric acid (3OH-HA), of which urinary excretion of HA significantly increased in both Montgomery blueberry treatments by week 8, but remained the same in Wild BB 5%. 3OH-HA increased in all blueberry treatments, but was only significantly higher in Mont 2.5% and Wild BB 5%, while both compounds remained significantly lower than blueberry treatments and constant over time in the control rats.

Cinnamic acid derivatives. The 8-week chronic feeding had no effect on total cinnamic acid derivative excretion within each treatment. The urinary excretion of cinnamic acids was significantly lower in Mont 2.5% than in Mont 5% ($p < 0.01$), but by week 8 of treatment, regardless of the lower dose of blueberry, the urinary excretion of cinnamic acids in Mont 2.5% was not significantly different from Mont 5% or Wild BB 5% with 0.12 ± 0.001, 0.15 ± 0.0 and

0.15 ± 0.01 µg of cinnamic acids, respectively. The individual cinnamic acids detected in urine were caffeic acid (CA), caffeic acid 3-*O*-*β*-D-glucuronide (CA-glcr), caffeic acid-3-*O*-sulfate (CA-O-S), ferulic acid (FeA), ferulic acid 4-*O*-glucuronide (FeA-glcr), ferulic acid-4-*O*-sulfate (FeA-O-S) and cholorogenic acid (Chlr). Of these, CA-gclr significantly increased by week 8 in the Mont 5% diet ($p < 0.01$), but there was no change in the other treatments. The urinary excretion in both sulfate conjugates, CA-O-S and FA-O-S, decreased in all treatments, but only CA-O-S significantly decreased to a level of significance ($p< 0.0001$) after the 8-week treatment. For FA-glcr, Mont 2.5% increased significantly ($p < 0.0001$) after the 8 week treatement, resulting in no difference in urinary excretion compared to the higher dose blueberry treatments.

Flavonol derivatives. As expected, no flavonols were detected in urine from the control rats. Both Montgomery treatments had a significantly lower urinary excretion of flavonols than Wild BB 5%, the excretion or urinary flavonols in Wild BB 5% was 2-fold higher than in the Montgomery diets ($p < 0.0001$). Regardless of dose, by week 8, there were no significant differences between Mont 2.5% and Mont 5% urinary excretion of total flavonols with 0.2 ± 0.03 µg and 0.23 ± 0.02 µg, respectively. The individual flavonols detected in urine were quercetin (Q), quercetin-3-*β*-D-glucoside (Q-glu), 3-*O*-methylquercetin-3-*O*-*β*-D-glucuronide (MeQ-glcr), 4'-*O*-methylquercetin (MeQ), myricetin (Myr) and myricetin-3-glucuronide (Myr-glcr). Both Q and Q-glu urinary excretion remained constant over time, but were significantly lower in the Montgomery berries in comparison to Wild BB. MeQ-glcr remained constant and not different between the two Montgomery doses. Wild BB 5% showed significant increases in urinary excretion of MeQ-glcr. Regardless of dose, there were no significant differences in any of the quercetin metabolite excretion between Mont 2.5% and Mont 5% treatments. Both Myr and Myr-glcr urinary excretion did not differ between after a chronic consumption of the diet. Both

myricetin metabolite urinary excretion were significantly higher in the Montgomery treatment than in Wild BB 5% treatment ($p < 0.0001$).

Phenolics in bone marrow. For the first time, bone marrow was analyzed for phenolic content to determine whether there is accumulation of phenolic acids due to a chronic consumption of blueberry. At sacrifice, the left femur was excised and the bone marrow was flushed out. Phenolic compounds in the bone marrow were extracted and analyzed on the UPLC-MS/MS. Three different phenolic acids were detected in the bone marrow samples, 4-hydroxybenzaldehyde, 3-hydroxyphenylpropionic acid and hippuric acid, from which, only hippuric acid showed significant differences between the treatment groups and control. Hippuric acid content in the bone marrow was significantly higher in Mont 5% with 0.032 ± 0.003 µg HA, followed by Mont 2.5% with 0.019 ± 0.003 µg HA, in comparison to Control with a content of 0.006 ± 0.002 µg HA ($p < 0.0001$) (Figure 5.9). Interestingly, Wild BB 5%, even though having the highest phenolic content of the three treatment diets, had a hippuric acid content in the bone marrow no different from Control and Mont 2.5% diets with a content of 0.017 ± 0.002 µg HA. Hippuric acid content in the bone marrow was significantly correlated to bone deposition (V_{0+}). As shown in Figure 5.10a, with a correlation of 0.35, $R^2 = 0.122$ ($p = 0.0393$). HA content in the bone marrow was not significantly correlated to bone resorption (Figure 5.10b)

5.5 Discussion

In this 2-month study with different varieties and doses of blueberry, we found that the metabolism of calcium and (poly)phenols vary depending on blueberry variety and dose and that hippuric acid might play a role in the bone protective effects of blueberry consumption.

(Poly)phenol rich fruits such as blueberry, grape, and plum have been previously investigated for its potential to aid in osteoporosis prevention in preclinical studies (Hohman and

Weaver; Devareddy et al.; Li et al.; J. R. Chen, O. P. Lazarenko, J. Zhang, et al.), but the specific bioactive components of these fruits remains unclear. It is thought that the bioactive component of blueberries are their rich content of (poly)phenol but different blueberry varieties differ in (poly)phenol quantity and profiles, thus raising the question of whether there are differences in their bone health benefits. Our study is the first to relate urinary excretion of (poly)phenols and accumulation of a phenolic acid in the bone marrow of the femur, to bone health benefits. We further compared blueberry varieties.

To accurately assess the effects of (poly)phenols on bone health, it is important to understand their bioavailability, metabolism, distribution and possible site of action. Anthocyanin absorption is estimated to be between 2% and less than 1% of the dose (Lila et al.), and because phenolic acid metabolites derived from metabolism of (poly)phenols have been found in higher concentrations circulating in plasma(A. Rodriguez-Mateos, D. Vauzour, et al.; Nurmi et al.), we decided to focus on the phenolic acids and flavonols. The two berries that were chosen for this study were Montgomery blueberry, a rabbiteye blueberry which showed significant higher bioavailability of anthocyanins, myricetin and quercetin in a previous study in our lab, and the commercially available lowbush wild blueberry from the Wild Blueberry Association of North America. Consistent with the previously reported TPC of rabbiteye and lowbush blueberries(Kalt, Ryan, et al.), Montgomery blueberry had a significantly lower polyphenol content than the wild blueberry. Both blueberries also varied in flavonol and phenolic acid content which resulted in differences in the urinary excretion of phenolic acids. A total of 23 metabolites were found in urine, of which 3OH-4MePAA, HA, 3OH-HA, CA-gclr, FA-glcr, BA-glcr and MeQ-glcr significantly increased in at least one of the blueberry treatments. Interestingly, HA urinary excretion significantly increased in both Montgomery doses, but

remained constant in the Wild BB 5% treatment. HA is an end-stage metabolite that comes from the metabolism of many flavonoid parent compounds (Murota, Nakamura and Uehara), thus increases in HA may serve as an indication of overall rate of metabolism. This finding suggests that Montgomery blueberry induces an increase in metabolism after a chronic consumption, while the wild blueberry does not.

Polyphenols are identified in the body as xenobiotics and are exposed to phase II enzymatic metabolism for rapid excretion in the urine (Redan et al.) . They are conjugated with glucuronic acid, methyl or sulfate groups for their rapid excretion(A. Rodriguez-Mateos, D. Vauzour, et al.). Glucuronidation conjugates phenolic compounds with glucuronic acid by UDP-glucuronyl transferase (UGT), resulting in an increase in the hydrophilicity, facilitating their excretion. Glucuronide metabolites significantly increased after a chronic consumption in at least one of the blueberry treatments, which suggests that either a higher content of phenolics was absorbed, or activity of glucuronidases increased over time. In contrast, the sulfate metabolites significantly decreased in all treatments, including the control group, which suggests that decline in sulfate metabolites might be due to aging and menopause which may result in an alteration of the metabolizing capacity of sulfurotransferases.

Due to ovariectomy, all rats lost bone at the spine and at the left femur. Treatment with blueberry-enriched diet had no effect on bone mineral density at the spine (L1-L4) or at the left femur. There was no effect of a blueberry treatment on cortical bone, but there was a modest protection in trabecular bone loss in the Wild BB 5% group which had significantly higher tb. th. than the control rats, and was not significantly different to the other blueberry groups.

Our results are consistent with Devareddy et al.’s study, which conducted a 100-day 5% blueberry diet intervention in ovariectomized rats, which showed that a diet enriched with

blueberry significantly attenuated whole body BMD, but no significant differences were seen at the spine and at the femur (Devareddy et al.). Li et al. conducted a 12-week study where rats were gavaged daily with 10% rabbiteye blueberry solution (Li et al.), showing that blueberry treatment significantly attenuated ovariectomy induced bone loss at the left femur, with significant bone loss protection on trabecular bone measurements of BV/TV, Tb. Th, Tb. N and Tb. Sp. Contrary to Li et al., our study did not find significant differences between control group and blueberry group on trabecular bone microarchitecture. Differences between our study and theirs include the length of the study and the starting time of treatment. Our study had a duration of 8 weeks and treatment was started 1-mo post-ovariectomy while both Li et al. and Devareddy et al.'s studies began treatment immediately after ovariectomy and had a duration of 12-14 weeks (Li et al.; Devareddy et al.). Thus, they studied the ability of berries to attenuate OVX-induced bone loss, whereas, our study was measuring the ability of berries to rescue bone after stabilized to bone loss.

Although our study may not have been long enough to detect significant changes in BMD and bone microarchitecture, we were able to assess calcium balance at baseline and after the 8 week treatment, as well as a kinetic study of calcium during week 8. Through the calcium balance study, our results show that a lower dose of Mont 2.5% significantly increased calcium retention in comparison to control, but this effect was lost after the 8 week treatment, possibly due to an adaptation of the intestine (Wallace). The low Ca absorption, retention and absorption efficiency during week 1 is representative of rapid bone loss which is accompanied by a higher endogenous excretion, resulting in negative calcium balance, as was seen in one fourth of the rats at baseline in our study, while rats were still adjusting to the loss of estrogen. We can observe that by week 8, all rats have come to a more stable Ca homeostasis with no treatment effect.

Through calcium kinetics, we were able to determine differences between the blueberry varieties and doses on calcium metabolism. Montgomery blueberry showed significantly higher Calcium absorption (Va), bone deposition and bone resorption transfer coefficients than all the other treatments. This increased bone turnover and calcium absorption were not detected in the Wild BB 5%. On the contrary, rats on the 5% Wild BB diet had significantly lower bone deposition than the control and Mont 2.5%, but bone balance was not different than the other treatments, this finding might be due to Wild BB 5%'s lower total body calcium levels, reason for this finding remains unclear and needs further research.

Previous research has shown that phenolic acids derived from gut microbiota metabolism of polyphenols may have bone-formation promoting effects (J. R. Chen, O. P. Lazarenko, J. Zhang, et al.). Hippuric acid (HA), one of the most abundant phenolic acids circulating in rat plasma after blueberry consumption, significantly and dose-dependently increased alkaline phosphatase mRNA expression, an osteoblast differentiation biomarker (J. R. Chen, O. P. Lazarenko, J. Zhang, et al.) . HA was also able to induce osteoblast differentiation in ST2, CSC12, and primary mouse bone marrow stromal cells, while inhibiting the 3T3-L1 cell proliferation into adipocytes and favoring its differentiation into osteoblast-like cells. In the bone marrow environment, stromal cells and periosteal osteoblast precursors can be potential sources of new osteoblasts for bone formation (Ogita et al.), thus, the bone marrow was investigated to determine whether there is an accumulation of phenolic acids. This study shows for the first time, that HA accumulates in the bone marrow after a chronic feeding of blueberry, and the Mont 5% blueberry had significantly higher content of HA, regardless of its lower TPC. Because Mont 5% also exhibited a higher bone turnover rate than the other treatments, a correlation was carried out between hippuric acid content in the bone marrow and bone deposition and bone resorption

transfer coefficients from our kinetic modeling analysis. Bone deposition was significantly and positively correlated with HA content in the bone marrow, while there was no correlation with bone resorption. This finding suggests that HA explains some of the increased bone deposition in the Mont 5% rats, which may result from the increased osteoblast activity as previously reported by Chen et al.

One limitation of our study was that our study was only 8 weeks long and while sufficient to show loss in BMD over time due to OVX, it may not have been sufficient time to detect more subtle differences in bone mineral density and microarchitecture due to diet. Flavonoid intake has been shown to influence the quantity and quality of the gut microbiome, which can then have an effect on their own catabolism (Duda-Chodak et al.), thus influencing the absorption, excretion and profiles of phenolic acids. Our study has not yet assessed the effect of the blueberry chronic consumption on gut microbiome composition. This could help elucidate differences in the bacterial communities, which might be responsible for the shifts in urinary excretion of phenolic acids. Other that the effects of flavonoids on the microbiome composition, fiber content has also been shown to modulate the gut microbiome, thus it is worth investigating whether there are differences between the fiber content in different varieties of blueberries and how these play a role in flavonoid metabolism. Investigating the effects on microbiome modulation, as well as the fiber content in the berries will help elucidate the reason why the Montgomery blueberry seems to have higher metabolism of phenolic acids regardless of the fact that it has almost half the phenolic content than wild blueberry.

This study suggests that genetically different varieties of blueberries with different phenolic profiles and content have a different effect on phenolic and calcium metabolism, thus

resulting in differences on bone health benefits. HA might be one of the major phenolic acids responsible for blueberry's bone health benefits. Further research is needed to determine the exact molecular mechanism through which HA could induce bone formation.

5.6 Figures and Tables

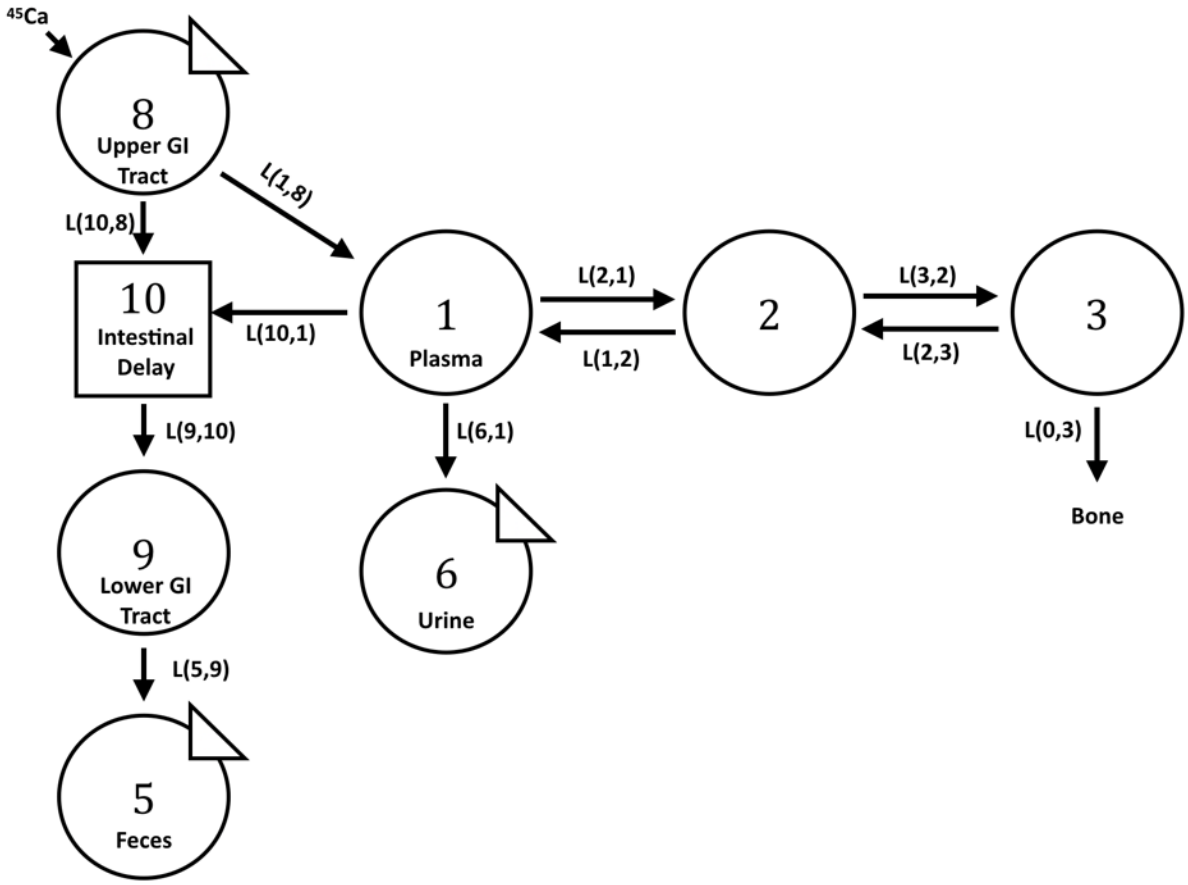

Figure 5.1 Compartmental model for oral calcium kinetics in ovariectomized rats. 10 µCi of ^{45}Ca was gavaged into each rat. Blood (compartment 1), feces (compartment 5) and urine (compartment 6) were sampled over 4 days after tracer administration. L(2,1), L(1,2), L(3,2), L(2,3) and L(10,1) were determined from rats administered 5 µCi of ^{45}Ca intravenously (n = 2-3/diet) and were then fixed for all rats within a diet group for oral kinetics. GI, gastrointestinal; L(*i*,*j*), fractional transfer rate (fraction/hour) into compartment *i* from compartment *j*.

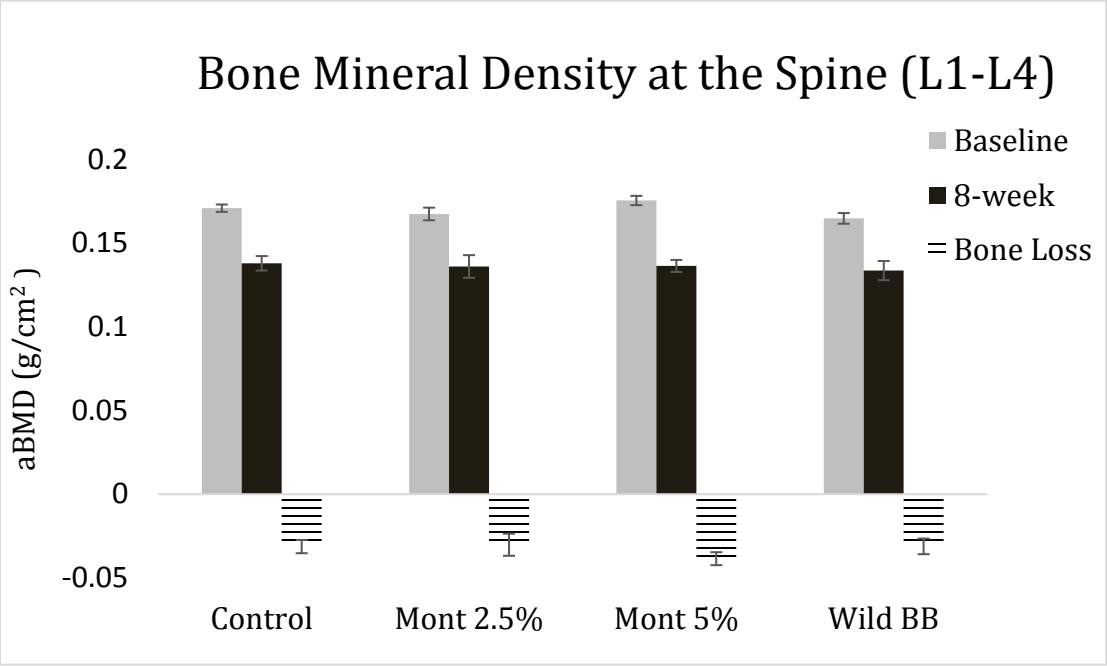

Figure 5.2 Bone Mineral Density at the Spine (L1-L4).). aBMD was measured at baseline, after 8-weeks of treatment diet. Bone loss refers to the difference between the 8-Week aBMD and the baseline aBMD. All rats lost bone, as expected after ovariectomy. No significant differences were observed between treatment groups ($p > 0.05$). (n=15-22 rats/group)

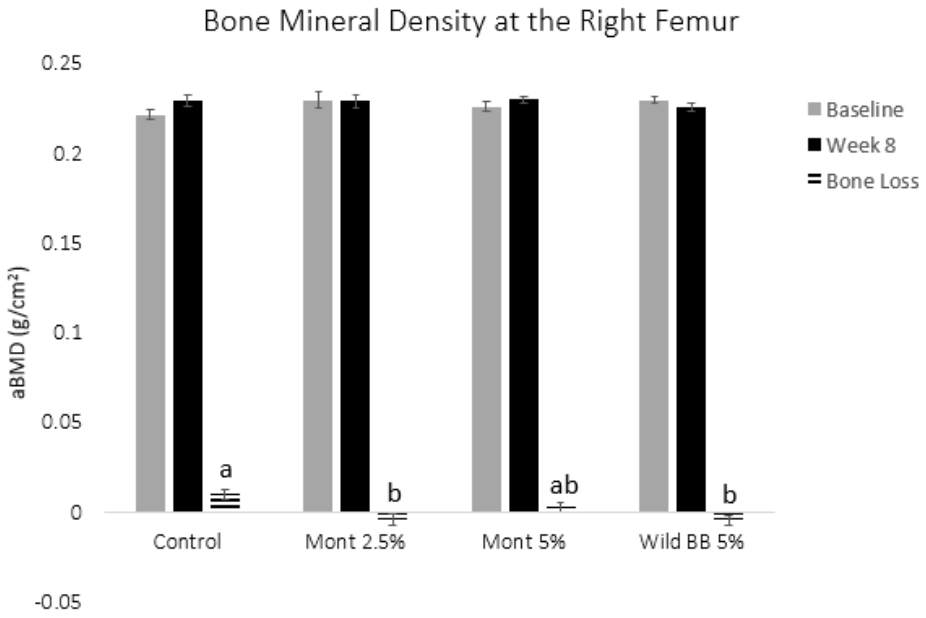

Figure 5.3 Bone Mineral Density at the Right Femur. aBMD was measured at baseline, after 8-weeks of treatment diet. Bone loss refers to the difference between the Week-8 aBMD and the baseline aBMD. All rats lost bone, as expected after ovariectomy. Different letters indicate significant differences between treatment groups ($p < 0.05$) (n=15-22 rats/group).

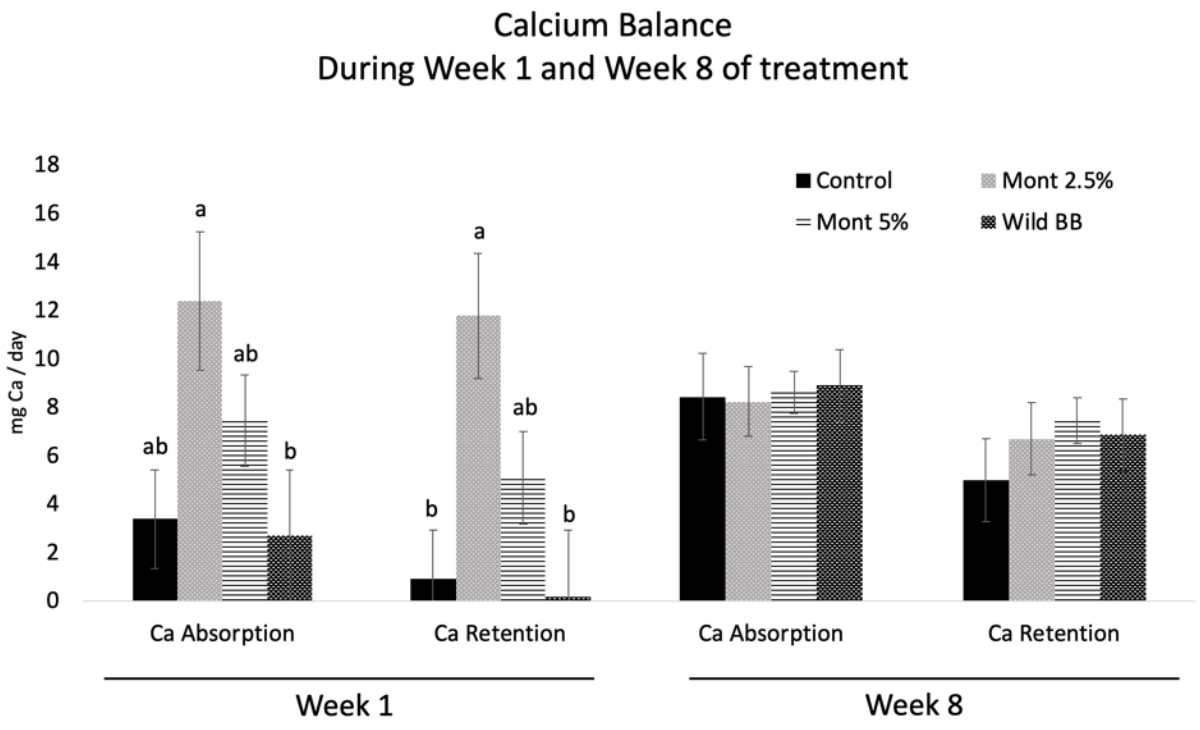

Figure 5.4 Calcium Balance during week 1 and week 8. Calcium balance in ovariectomized rats after 1 week and 8 weeks of treatment diet. Data are shown as mean ± SEM. Letters indicate significant differences between treatment groups for each outcome value, $p<0.05$. (n =15-22 rats)

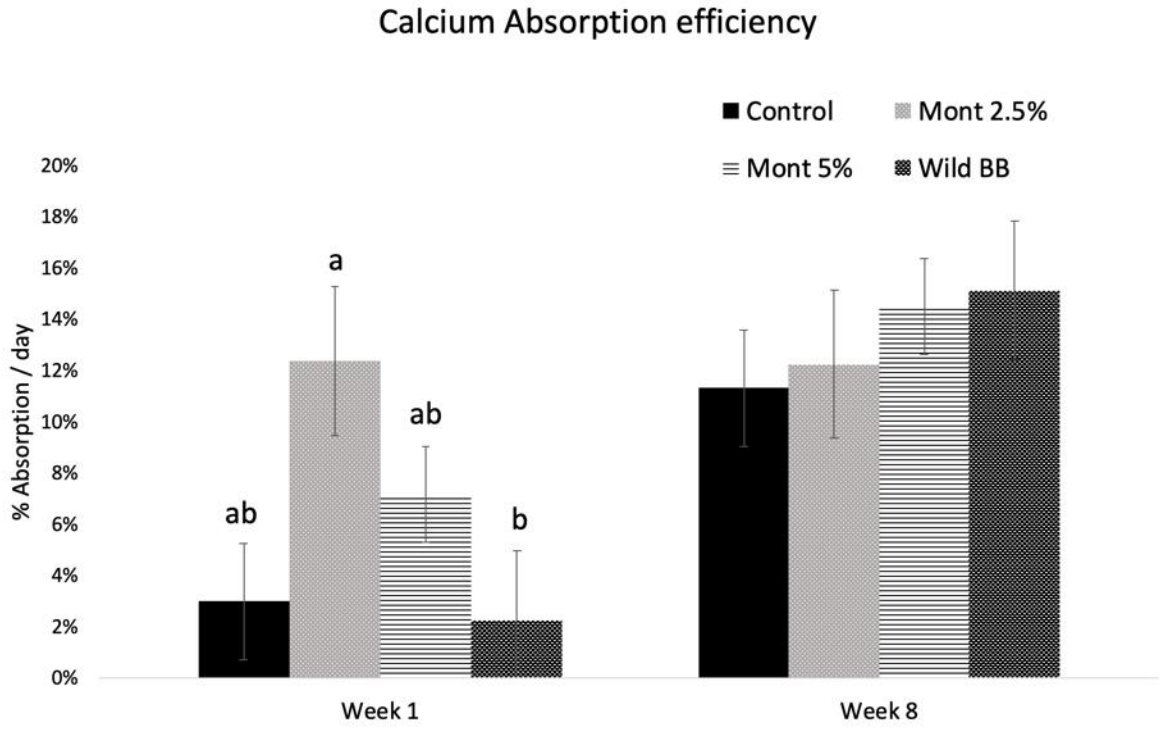

Figure 5.5 Calcium Absorption Efficiency. Differences between Ca absorption efficiency during week 1 are represented by different letters ($p<0.05$). There were no significant differences between treatment groups during week 8. Data are shown as mean ± SEM.

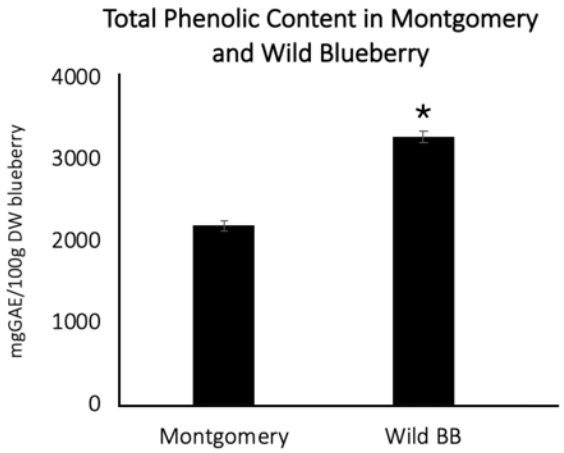

Figure 5.6 Total Phenolic Content in Montgomery blueberry and Wild Blueberry. Data are shown in mean and SEMs. "*" indicates significant differences between the two groups ($p < 0.05$)

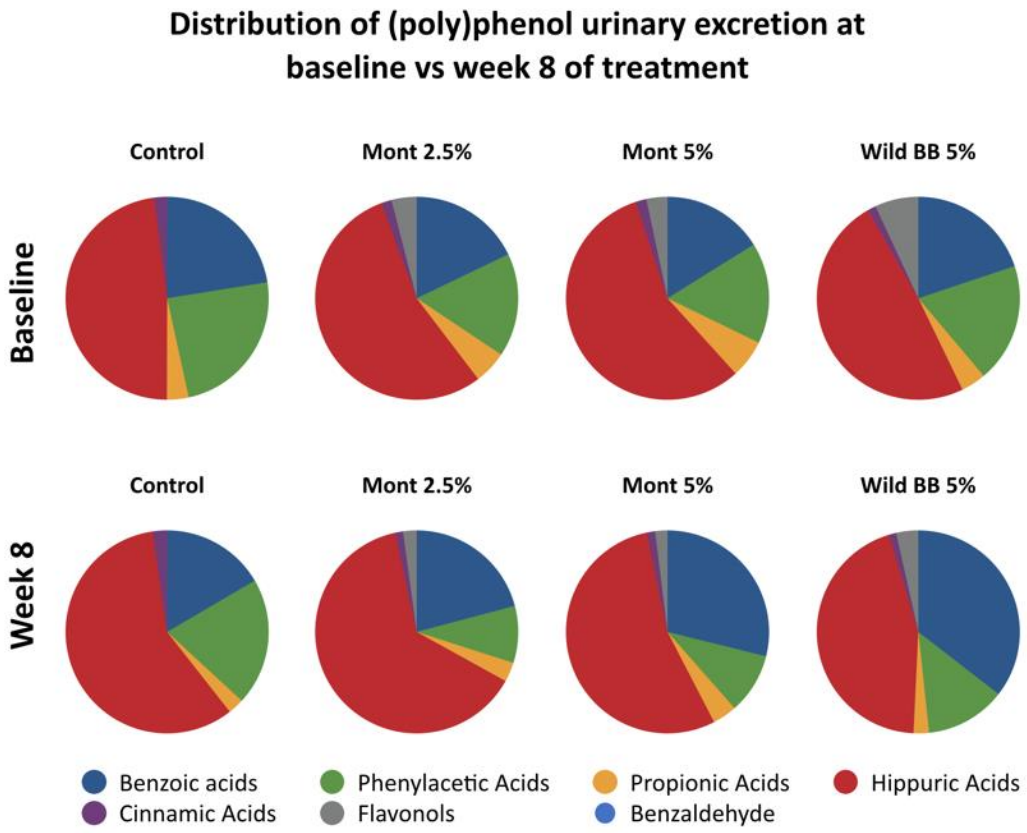

Figure 5.7. Distribution of (poly)phenol classes at baseline and week 8 of treatment. A 24 h urine excretion of phenolic acids was analyzed. Pie charts show the percent concentration of each class of (poly)phenols in each of the treatments groups at baseline and during week 8.

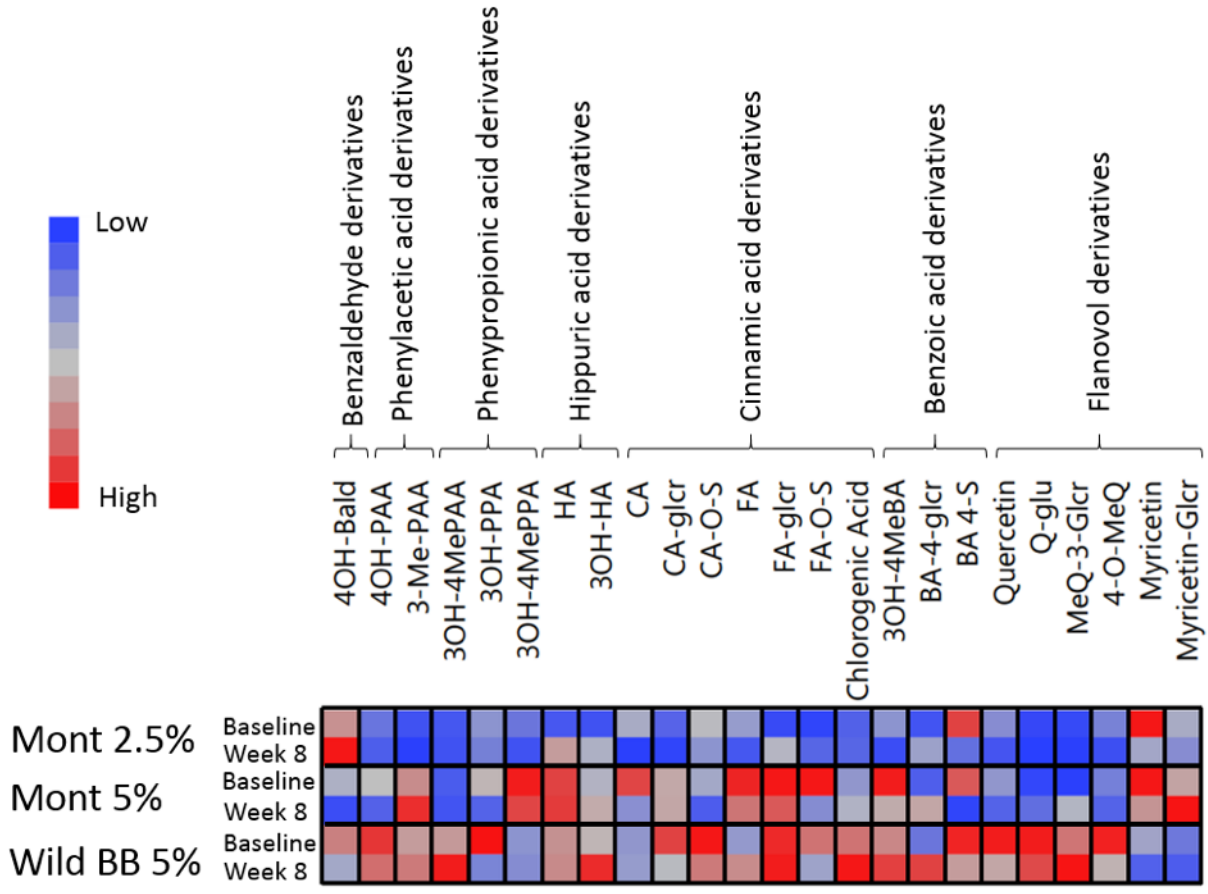

Figure 5.8. Heat Map of individual phenolic acid urinary excretion at baseline and week 8. Data is normalized to control, except for the flavonol derivatives which were not detected in control rats. Blue and red indicate low and high levels, respectively (n = 15-22 rats / treatment group)

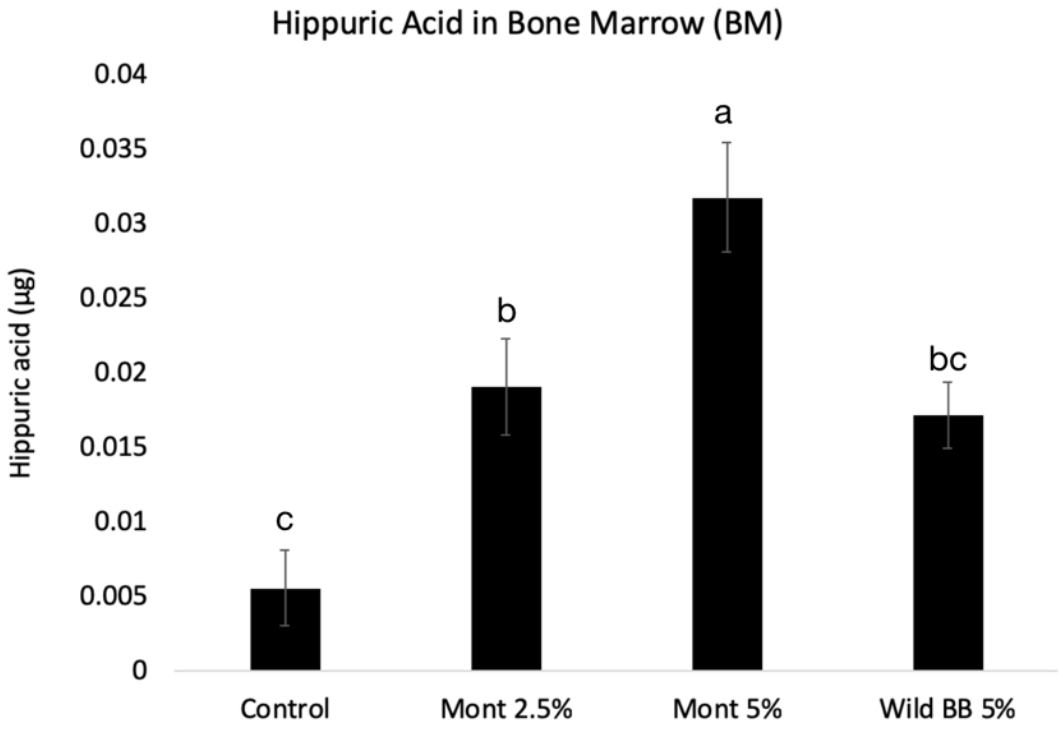

Figure 5.9 Hippuric Acid Content in Bone Marrow from the left femur. Values are means ± SEMs. Data were analyzed by one-way ANOVA followed by a Tukey's post hoc test. Different letters indicate significant differences ($p < 0.0001$). n = 8-10 rats/group.

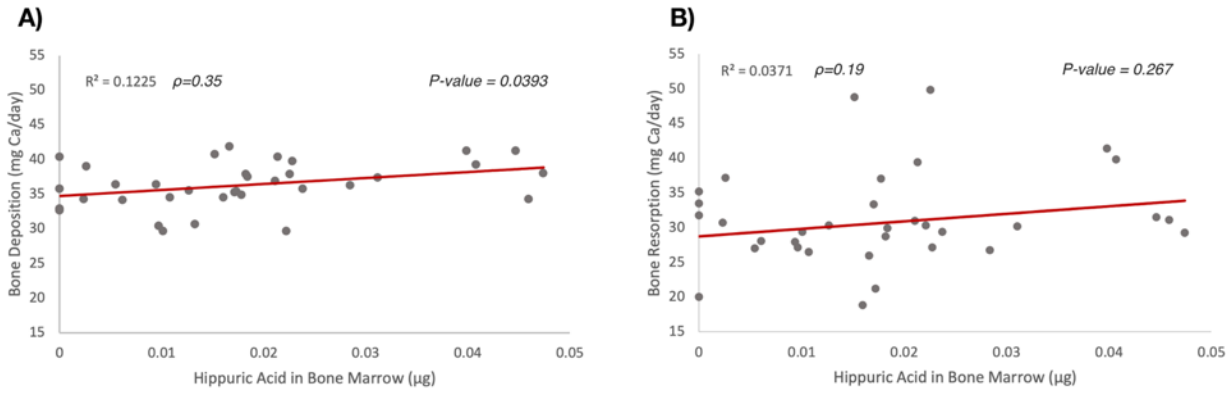

Figure 5.10. Correlation between Hippuric Acid content in Bone Marrow and A) Bone Deposition (V_{0+}) and B) Bone Resorption (V_{0-})

Table 5.1 Modified AIN93M diets per treatment

	Polyphenol-Free	**Mont 2.5%**	**Mont 5%**	**Wild BB 5%**
Ingredient		**g/kg of diet**		
Casein	140	140	140	140
L-Cysteine	1.8	1.8	1.8	1.8
Corn Starch[a]	495.7	468.2	470.7	470.7
Maltodextrin[a]	125	147	139	139
Sucrose[a]	100	84.5	69	69
Corn Oil[b]	40	40	40	40
Cellulose[a]	50	46	42	42
Mineral Mix[c]	35	35	35	35
Vitamin Mix	10	10	10	10
Choline Bitartrate	2.5	2.5	2.5	2.5
TBHQ	0.008	0.008	0.008	0.008
Montgomery Blueberry	0	25	50	0
Wild Blueberry	0	0	0	50

[a]Ingredients modified from original AIN93-M diet to match for the amount of fructose and fiber that the blueberry accounted for. [b]Corn oil was used to replace soybean oil from the original formulation to eliminate soy isoflavone residuals. [c]Mineral mix contains 0.5% Ca per kg of diet.

Table 5.2 Femur microarchitecture in ovariectomized rats after 8 weeks of a control or blueberry diet

	Diet			
Distal femur (trabecular bone)	**Control**	**Mont 2.5%**	**Mont 5%**	**Wild BB 5%**
BV/TV	0.068 ± 0.008	0.076 ± 0.01	0.074 ± 0.005	0.085 ± 0.006
Conn. D., 1/mm^3	20.22 ± 2.72	21.67 ± 2.97	21.11 ± 1.38	24.71 ± 2.23
Tb. N, 1/mm	2.38 ± 0.08	2.47 ± 0.07	2.4 ± 0.06	2.5 ± 0.06
Tb. Th, mm	0.069 ± 0.002	0.071 ± 0.002	0.072 ± 0.001	0.074 ± 0.002*
Tb. Sp, mm	0.423 ± 0.016	0.42 ± 0.013	0.429 ± 0.013	0.409 ± 0.01
Midshaft femur (cortical bone)				
Ct.Ar/Tt.Ar	0.602 ± 0.004	0.616 ± 0.007	0.602 ± 0.005	0.601 ± 0.005
Ct.Th, mm	0.615 ± 0.004	0.618 ± 0.005	0.608 ± 0.004	0.609 ± 0.004
Ps.Pm, mm	11.7 ± 0.08	11.73 ± 0.08	11.76 ± 0.08	11.76 ± 0.07
Ec.Pm, mm	7.16 ± 0.09	7.17 ± 0.07	7.25 ± 0.09	7.21 ± 0.09

Values are means ± SEMs, n=13-22 rats/group. *Different from control, as analyzed through Dunnett's test $p<0.05$. *By one-way ANOVA, there was a trend for significance with $p = 0.0843$. BV/TV, bone volume to total volume fraction; Conn. D., connectivity density; Tb. Th, trabecular thickness; Tb. Sp, trabecular spacing; Ct.Ar/Tt.Ar, cortical area to total area fraction; Ct. Th, cortical thickness; Ps.Pm, periosteal perimeter; Ec.Pm, endocortical perimeter; Ct.Po%, cortical porosity.

Table 5.3 Calcium kinetic variables in OVX rats fed with control or blueberry-enriched diets for 8 weeks

Transfer coefficient, fraction/h	Diet			
	Control	**Mont 2.5%**	**Mont 5%**	**Wild BB 5%**
L(2,1)	28.329 ± 0.279^{ab}	27.901 ± 0.587^{b}	30.086 ± 0.261^{ab}	30.573 ± 0.782^{a}
L(1,2)	2.564 ± 0.282^{ab}	2.557 ± 0.039^{ab}	2.407 ± 0.138^{b}	3.127 ± 0.176^{a}
L(3,2)	0.472 ± 0.026	0.472 ± 0.026	0.472 ± 0.026	0.472 ± 0.026
L(2,3)	0.114 ± 0.003	0.12 ± 0.005	0.111 ± 0.008	0.187 ± 0.033
L(0,3)	0.032 ± 0.002	0.033 ± 0.001	0.033 ± 0.001	0.035 ± 0.002
L(10,1)	0.311 ± 0.036	0.313 ± 0.005	0.327 ± 0.061	0.348 ± 0.066
L(1,8)	0.046 ± 0.007	0.02 ± 0.003	0.051 ± 0.005	0.045 ± 0.011
L(5,9)	0.722 ± 0.134^{ab}	0.621 ± 0.183^{ab}	0.833 ± 0.165^{a}	0.271 ± 0.061^{b}
L(6,1)	0.06 ± 0.003	0.07 ± 0.005	0.076 ± 0.005	0.069 ± 0.005
L(10,8)	0.486 ± 0.031	0.522 ± 0.038	0.394 ± 0.027	0.484 ± 0.033
Calcium Transport (mg Ca/d)				
V_f	10.02 ± 0.13^{b}	10.06 ± 0.11^{b}	10.5 ± 0.09^{b}	11.27 ± 0.19^{a}
V_F	56.56 ± 1.33	60.33 ± 1.86	54.76 ± 1.44	58.36 ± 1.63
V_u	1.97 ± 0.09	2.15 ± 0.18	2.36 ± 0.13	2.14 ± 0.17
V_{o+}	35.66 ± 0.39^{b}	35.54 ± 0.45^{b}	39.98 ± 0.33^{a}	32.97 ± 0.59^{c}
V_{o-}	28.94 ± 1.71^{ab}	32.18 ± 2.16^{ab}	34.43 ± 1.49^{a}	27.03 ± 1.78^{b}
V_a	15.61 ± 0.66^{b}	14.3 ± 0.84^{b}	18.2 ± 0.46^{a}	16.16 ± 0.42^{b}
Bone balance	6.72 ± 1.73	5.16 ± 1.87	6.59 ± 1.25	5.94 ± 1.74
Abs tracer (fract)	0.23 ± 0.01^{b}	0.24 ± 0.01^{b}	0.28 ± 0.01^{a}	0.24 ± 0^{b}
Abs Tracee (fract)	0.28 ± 0.02	0.24 ± 0.03	0.3 ± 0.02	0.29 ± 0.03
Compartment mass (mg Ca)				
M(1)	1.34 ± 0.01	1.34 ± 0.01	1.34 ± 0.01	1.35 ± 0.02
M(2)	14.26 ± 0.18^{b}	14.02 ± 0.18^{b}	15.95 ± 0.14^{a}	12.76 ± 0.22^{c}
M(3)	46.16 ± 0.56^{b}	45.02 ± 0.52^{b}	50.3 ± 0.43^{a}	39.14 ± 0.64^{c}
Total body Ca	61.76 ± 0.76^{b}	60.37 ± 0.69^{b}	67.6 ± 0.57^{a}	53.25 ± 0.87^{c}

Values are means ± SEMs. Different letters indicate significant differences between treatment groups, $P < 0.05$. V_f, endogenous excretion; V_F, Fecal excretion, V_u, urinary excretion; V_{0+}, bone deposition, V_{0-}, bone resorption; V_a, calcium absorption; M(*i*), mass of compartment *i*.

Table 5.4. Flavonoid content in Montgomery and Wild blueberry

Flavonoid (mg/100g DW blueberry)	**Montgomery**	**Wild BB**
Ferulic Acid	1.31 ± 0.01^{b}	3.49 ± 0.15^{a}
Chlorogenic Acid	331.41 ± 28.02	351.6 ± 30.68
Myricetin	1.26 ± 0.23	2.28 ± 0.29
Quercetin	0.25 ± 0.02^{b}	0.81 ± 0.09^{a}
Q-3-Glu	8.37 ± 1.62^{b}	140.97 ± 10.05^{a}
Q-3-Rut	4.88 ± 1.71^{b}	43.75 ± 3.2a
Epicatechin	113.25 ± 1.28^{a}	56.92 ± 6.28^{b}
Gallocatechin	51.49 ± 2.92	53.26 ± 7.71
Epigallocatechin	19.4 ± 1.63	25.73 ± 3.65
Feruloyquinic Acid	13.64 ± 1.07	7.69 ± 1.61

Values are means ± SEMs. Different letters indicate significant differences between blueberry varieties, $P < 0.05$. Data was analyzed through student's test with Tukey Kramer's post hoc test.

Table 5.5 Total urinary phenolic acids by subclass derivatives.

Total urinary phenolic acids by subclass derivatives (µg)								
	Control		**Mont 2.5%**		**Mont 5%**		**Wild BB 5%**	
	Baseline	**Week 8**	**Baseline**	**Week 8**	**Baseline**	**Week 8**	**Baseline**	**Week 8**
Benzoic Acids	0.79 ± 0.05^{cd}	0.59 ± 0.04^{d}	1.25 ± 0.12^{bc}	1.94 ± 0.21^{b}	1.5 ± 0.13^{b}	3.36 ± 0.36^{a}	1.92 ± 0.14^{a}	4.66 ± 0.58^{a}
Phenylacetic Acids	0.85 ± 0.08^{cd}	0.7 ± 0.07^{d}	1.2 ± 0.04^{cd}	0.84 ± 0.04^{bc}	1.5 ± 0.06^{ab}	1.12 ± 0.08^{c}	1.83 ± 0.12^{a}	1.68 ± 0.08^{a}
Phenylpropionic Acids	0.024 ± 0.003^{d}	0.018 ± 0.002^{d}	0.072 ± 0.009^{bc}	0.056 ± 0.007^{c}	0.111 ± 0.01^{a}	0.089 ± 0.009^{ab}	0.075 ± 0.006^{bc}	0.064 ± 0.006^{bc}
Hippuric Acids	1.69 ± 0.11^{e}	2.08 ± 0.18^{e}	3.83 ± 0.33^{d}	5.9 ± 0.12^{ab}	5.27 ± 0.23^{bc}	6.32 ± 0.172^{a}	4.72 ± 0.27^{cd}	5.85 ± 0.27^{ab}
Cinnamic Acids	0.07 ± 0.01^{d}	0.08 ± 0.01^{cd}	0.11 ± 0.01^{bc}	0.12 ± 0^{abc}	0.16 ± 0.01^{a}	0.15 ± 0.01^{ab}	0.13 ± 0.01^{ab}	0.15 ± 0.01^{ab}
Flavonols	N.D.	N.D.	0.28 ± 0.03^{bcd}	0.2 ± 0.03^{b}	0.31 ± 0.03^{cd}	0.23 ± 0.02^{bc}	0.67 ± 0.07^{e}	0.46 ± 0.05^{de}

Values are shown as means ± SEM. Data was analyzed through a two-way ANOVA to determine differences in total (poly)phenol subclass between treatment and time (baseline or week 8). Different letters within subclass derivatives indicate significant differences ($p < 0.05$).

Table 5.6 Total Urinary excretion of individual phenolics in all treatments at baseline and week 8

Total 24 h Urinary Excretion (µg)	Diet							
	Control		Mont 2.5%		Mont 5%		Wild BB 5%	
	Baseline	Week 8	Baseline	Week 8	Baseline	Week 8	Baseline	Week 8
Phenylacetic Acid Derivatives								
4-Hydroxyphenylacetic acid	0.75 ± 0.08^{abc}	0.58 ± 0.08^{bc}	0.72 ± 0.03^{abc}	0.5 ± 0.03^{c}	0.79 ± 0.04^{ab}	0.53 ± 0.04^{c}	0.87 ± 0.07^{a}	0.65 ± 0.04^{abc}
3-Methylphenylacetic acid	0.01 ± 0.001^{d}	0.013 ± 0.002^{d}	0.363 ± 0.029^{b}	0.24 ± 0.017^{c}	0.601 ± 0.02^{a}	0.464 ± 0.029^{b}	0.592 ± 0.032^{a}	0.433±0.017^{b}
3-Hydroxy-4-methoxyphenylacetic acid	0.082 ± 0.003^{a}	0.086 ± 0.004^{a}	0.083 ± 0.003^{a}	0.079 ± 0.003^{a}	0.091 ± 0.002^{a}	0.083 ± 0.003^{a}	0.4 ± 0.028^{b}	0.55 ± 0.027^{c}
Propionic Acid Derivatives								
3-Hydroxy-4-methoxyphenylpropionic acid	0.024 ± 0.003^{d}	0.018 ± 0.002^{d}	0.072 ± 0.009^{bc}	0.056 ± 0.007^{c}	0.111 ± 0.01^{a}	0.089 ± 0.009^{ab}	0.075 ± 0.006^{bc}	0.064 ± 0.006^{bc}
Benzaldehyde Derivatives								
4-Hydroxybenzaldehyde	0.003 ± 0.0003^{ab}	0.002 ± 0.0002^{bc}	0.003 ± 0.0003^{a}	0.002 ± 0.0002^{bc}	0.003 ± 0.0002^{a}	0.001 ± 0.0001^{c}	0.003 ± 0.0003^{a}	0.001 ± 0.0001^{c}
Hippuric Derivatives								
Hippuric Acid	1.68 ± 0.11^{e}	2.23 ± 0.11^{e}	3.8 ± 0.33^{d}	5.86 ± 0.12^{ab}	5.23 ± 0.23^{bc}	6.27 ± 0.17^{a}	4.68 ± 0.27^{bc}	5.79 ± 0.26^{ab}
3-Hydroxyhippuric acid	0.018 ± 0.001^{e}	0.021 ± 0.003^{e}	0.024 ± 0.002^{de}	0.042 ± 0.005^{bc}	0.037 ± 0.002^{bc}	0.048 ± 0.004^{b}	0.034 ± 0.002^{cd}	0.061 ± 0.004^{a}

Table 5.6 continued

Cinnamic Acid Derivatives								
Caffeic acid	0.009 ± 0.002^{d}	0.009 ± 0.002^{cd}	0.039 ± 0.006^{ab}	0.027 ± 0.004^{bcd}	0.05 ± 0.005^{a}	0.035 ± 0.004^{ab}	0.036 ± 0.004^{ab}	0.028 ± 0.004^{bc}
Caffeic acid 3-O-β-D-glucuronide	0.005 ± 0.001^{c}	0.007 ± 0.002^{bc}	0.007 ± 0.002^{bc}	0.01 ± 0.001^{bc}	0.011 ± 0.001^{bc}	0.018 ± 0.002^{a}	0.012±0.002^{ab}	0.013 ± 0.002^{ab}
Caffeic acid-3-O-sulfate	3.81 ± 0.26^{a}	2.35 ± 0.18^{b}	3.88 ± 0.26^{a}	2.29 ± 0.22^{b}	3.92 ± 0.12^{a}	2.12 ± 0.15^{b}	4.54 ± 0.32^{a}	2.65 ± 0.16^{b}
Ferulic Acid	0.031 ± 0.003^{c}	0.031 ± 0.003^{bc}	0.053 ± 0.007^{ab}	0.043 ± 0.004^{abc}	0.065 ± 0.005^{a}	0.052 ± 0.005^{ab}	0.053 ± 0.004^{a}	0.055 ± 0.006^{a}
Ferulic acid 4-O-glucuronide	0.024 ± 0.003^{bc}	0.031 ± 0.005^{ab}	0.016 ± 0.002^{c}	0.03 ± 0.003^{abd}	0.029 ± 0.003abc	0.038 ± 0.003^{a}	0.029 ± 0.003^{abc}	0.041 ± 0.004^{a}
Ferulic Acid-4-O-sulfate	5.81 ± 0.64^{bc}	5.9 ± 0.77^{bc}	4.58 ± 0.55^{c}	5.71 ± 0.37^{bc}	8.45 ± 0.45^{a}	6.2 ± 0.36^{abc}	7.35 ± 0.61^{ab}	6.78 ± 0.37^{abc}
Chlorogenic Acid	N.D.	N.D.	0.0009 ± 0.0001^{ab}	0.0007 ± 0.0001^{a}	0.0014 ± 0.0001^{c}	0.0016 ± 0.0003^{bc}	0.0022 ± 0.0002^{c}	0.0022 ± 0.0005^{c}

Table 5.6 Continuation

Benzoic Acid Derivatives								
3-hydroxy-4-methoxybenzoic acid	0.022 ± 0.004^{b}	0.031 ± 0.005^{ab}	0.024 ± 0.005^{ab}	0.015 ± 0.001^{b}	0.038 ± 0.003^{a}	0.024 ± 0.003^{ab}	0.032 ± 0.004^{ab}	0.02 ± 0.004^{b}
Benzoic Acid-4-glucuronide	0.78 ± 0.04^{cd}	0.58 ± 0.04^{d}	1.24 ± 0.12^{bc}	1.93 ± 0.21^{b}	1.47 ± 0.13^{b}	3.34 ± 0.36^{a}	1.9 ± 0.14^{b}	4.64 ± 0.57^{a}
Benzoic Acid-4-O-sulfate	7.44 ± 0.43^{a}	5.35 ± 0.4^{b}	7.85 ± 0.39^{a}	4.51 ± 0.33^{b}	8.75 ± 0.54^{a}	4.29 ± 0.29^{b}	8.04 ± 0.45^{ab}	5.19 ± 0.29^{b}

Table 5.6 continued

Flavonol Derivatives								
Quercetin	N.D.	N.D.	0.008 ± 0.001^{a}	0.005 ± 0^{abc}	0.008 ± 0^{bc}	0.006 ± 0.001^{ab}	0.016 ± 0.002^{d}	0.012 ± 0.002^{cd}
Quercetin 3-β-D-glucoside	N.D.	N.D.	0.0015 ± 0.0001^{a}	0.0015 ± 0.0001^{a}	0.0015 ± 0.0001^{a}	0.0021 ± 0.0002^{a}	0.0049 ± 0.0004^{b}	0.0045 ± 0.0003^{b}
3'-O-Methyl Quercetin 3-O-β-D-Glucuronide	N.D.	N.D.	0.0021 ± 0.0004^{b}	0.0031 ± 0.0006^{b}	0.0027 ± 0.0005^{b}	0.0057 ± 0.0011^{ab}	0.0045 ± 0.0008^{b}	0.0118 ± 0.0024^{a}
4'-O-methyl Quercetin	N.D.	N.D.	0.25 ± 0.03^{ab}	0.18 ± 0.03^{a}	0.26 ± 0.02^{ab}	0.2 ± 0.02^{ab}	0.67 ± 0.08^{c}	0.42 ± 0.05^{bc}
Myricetin	N.D.	N.D.	0.005 ± 0.001^{b}	0.002 ± 0^{b}	0.005 ± 0^{a}	0.004 ± 0.001^{ab}	0.002 ± 0^{b}	0.001 ± 0^{c}
Myricetin-3-glucuronide	N.D.	N.D.	0.0074 ± 0.0011^{b}	0.0077 ± 0.0011^{b}	0.0111 ± 0.0017^{ab}	0.0148 ± 0.0018^{a}	0.0061 ± 0.0006^{b}	0.0059 ± 0.0007^{b}

Values are shown as means ± SEM. Data was analyzed through a two-way ANOVA to determine differences in urinary phenolic excretion between treatment and time (baseline or week 8). Different letters within subclass derivatives indicate significant differences ($p < 0.05$).

CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

Different blueberry varieties vary widely in (poly)phenol content and profiles which in turn results in differences in bioavailability of individual phenolics. It is unknown whether the differences in bioavailability are due to the sugar moieties attached to the individual phenolics, the fiber content in the berries or other nutrients such as fat and protein from the food matrix that may be influencing their bioavailability. Results from our study showed that Montgomery and Ira varieties have the highest bioavailability for Me-Quercetin-glc and Me-Myricetin-glc metabolites, respectively ($p < 0.05$). From the anthocyanin metabolites, Montgomery had a higher bioavailability of cyanidin-glycosides in comparison to the other varieties. The flavan-3-ols, catechin-glucuronide (glc), epicatechin-glc and their respective methylated metabolites, were not significantly different between varieties of blueberries. Bioavailability of individual phenolics varied widely among blueberry varieties, but although Montgomery and Ira blueberries have a higher bioavailability, they are not commercial produced, thus their availability for consumption is limited.

Dose plays a very important role when assessing efficacy of treatment and when making dietary recommendations for health benefits. Thus, we conducted a dose response study with a commercially available wild blueberry composite to determine most effective dose for increased net bone calcium retention in ovariectomized rats. Of the four different doses investigated (2.5%, 5%, 10% and 15%), only the 5% BB diet significantly increased net bone calcium retention by 25.6% (p-value = 0.0426). The 2.5% BB diet increased net bone calcium retention by 24%, but failed to reach significance (p-value=0.054). Interestingly, the higher doses of 10% and 15% BB did not have an effect on net bone Ca retention, suggesting that blueberry exhibits the hormetic

paradigm where higher doses show opposite effects than lower doses, and that consuming high levels of polyphenols might be counteracting its antioxidant capacity and voiding its beneficial effects on attenuating inflammation and oxidative stress that leads to bone loss. Although surprising, this data enables nutrition scientists to make dietary suggestions for increased bone health that are attainable. A 5% BB diet, is equivalent to a daily consumption of 1 cup of fresh blueberries, while a 15% blueberry dose is equivalent to 3 cups of fresh blueberries and compliance to such a high dose may be difficult.

This 5% BB dose and the highly bioavailable Montgomery blueberry, led us to conduct a chronic feeding study to determine whether this increased net bone calcium retention in ovariectomized would translate into significant attenuation of bone loss, assessed through bone microarchitecture and bone mineral density. Results showed that a blueberry enriched diet had no effect on cortical bone microarchitecture, with a trend towards minimal increased trabecular protection ($p = 0.0843$). During baseline, a 2.5% Montgomery diet significantly increased Ca retention, but the effect was lost after the 8 weeks of treatment when there were no differences between treatments. Calcium kinetics results showed that Mont 5% significantly increased Ca absorption and bone turnover, although no differences in bone balance were detected between the treatments. Interestingly, an accumulation of hippuric acid in the bone marrow was detected with the blueberry treatments and it was significantly and positively correlated with bone deposition
It is possible that our study may not have been long enough to detect substantial differences between the blueberry treatments and control or that blueberry is not effective at rescuing bone after OVX-induced bone loss.

The design of this study allowed for the comparison of urinary profiles of phenolic acids after the consumption of different varieties and doses of blueberries. These data showed another

more complex picture of blueberry (poly)phenol metabolism, with differences in phenolic acid profiles between blueberry varieties at baseline and further changes after the 8 week chronic feeding in comparison to baseline values and blueberry treatment. The metabolites investigated were phenolic metabolites derived mostly from gut microbiome metabolism. Although it was not determined in our study, it is important to evaluate how the microbiome composition was affected by the different blueberries and thus which bacterial communities were responsible for the changes in metabolism. The Montgomery blueberry has nearly half as much phenolic content than the wild blueberry, but resulted in a significantly higher amount of urinary HA and HA accumulation in the bone marrow, thus suggesting a higher degree of polyphenol metabolism. A study to determine the food matrix differences between Montgomery blueberry and wild blueberry, as well as its absorption, would help us further understand what makes Montgomery polyphenol bioavailability and metabolism so different than wild blueberry.

Further research to elucidate the effects of blueberry on bone health should evaluate treatment starting at three different time periods, i.e. pre-ovariectomy, immediately after ovariectomy and 2 months post-ovariectomy. This would allow elucidation of the age and sex-steroid status that would benefit most from a nutritional intervention for bone health. Our study has shown the accumulation of hippuric acid in the bone marrow and has provided the concentration at which it is present in the marrow, thus, further in vitro studies could provide insight into the mechanism of action using the physiological concentration of HA as found in this study.

To conclude, a blueberry-enriched diet resulted in a minimal protection to bone after stabilized to OVX, with significant increases in calcium absorption and bone turnover in ovariectomized rats.

REFERENCES

Abrahamsen, B., et al. "Excess Mortality Following Hip Fracture: A Systematic Epidemiological Review." *Osteoporos Int* 20.10 (2009): 1633-50. Print.

Administration, Food and Drug. "Code of Federal Regulations, Title 21 - Food and Drugs, Chapter 1 Food and Drug Administration, Department of Health and Human Servives, Subchapter D Drugs for Human Health, Part 314 Applications for Fda Approval to Market a New Drug Definitions,." Print.

Andreopoulou, P., and R. S. Bockman. "Management of Postmenopausal Osteoporosis." *Annu Rev Med* 66 (2015): 329-42. Print.

Ayaz, F. A., et al. "Separation, Characterization, and Quantitation of Phenolic Acids in a Little-Known Blueberry (Vaccinium Arctostaphylos L.) Fruit by Hplc-Ms." *J Agric Food Chem* 53.21 (2005): 8116-22. Print.

Bellido, Teresita, and Kathleen M. Hill Gallant. "Chapter 15 - Hormonal Effects on Bone Cells." *Basic and Applied Bone Biology*. Ed. Allen, David B. BurrMatthew R. San Diego: Academic Press, 2014. 299-314. Print.

Brauer, C. A., et al. "Incidence and Mortality of Hip Fractures in the United States." *JAMA* 302.14 (2009): 1573-9. Print.

Burckhardt, P. "Calcium Revisited, Part Iii: Effect of Dietary Calcium on Bmd and Fracture Risk." *Bonekey Rep* 4 (2015): 708. Print.

Burge, R., et al. "Incidence and Economic Burden of Osteoporosis-Related Fractures in the United States, 2005-2025." *J Bone Miner Res* 22.3 (2007): 465-75. Print.

Cai, J., et al. "Calcium Bioavailability and Kinetics of Calcium Ascorbate and Calcium Acetate in Rats." *Exp Biol Med (Maywood)* 229.1 (2004): 40-5. Print.

Cardenosa, V., et al. "Influence of Genotype, Cultivation System and Irrigation Regime on Antioxidant Capacity and Selected Phenolics of Blueberries (Vaccinium Corymbosum L.)." *Food Chem* 202 (2016): 276-83. Print.

Cashman, K. D. "Calcium Intake, Calcium Bioavailability and Bone Health." *Br J Nutr* 87 Suppl 2 (2002): S169-77. Print.

Cauley, J. A. "Estrogen and Bone Health in Men and Women." *Steroids* 99.Pt A (2015): 11-5. Print.

Charron, Craig S., et al. "Effect of Dose Size on Bioavailability of Acylated and Nonacylated Anthocyanins from Red Cabbage (Brassica Oleracea L. Var. Capitata)." *Journal of Agricultural and Food Chemistry* 55.13 (2007): 5354-62. Print.

Charron, Craig S., et al. "Bioavailability of Anthocyanins from Purple Carrot Juice: Effects of Acylation and Plant Matrix." *Journal of Agricultural and Food Chemistry* 57.4 (2009): 1226-30. Print.

Chen, J. R., et al. "Dietary-Induced Serum Phenolic Acids Promote Bone Growth Via P38 Mapk/Beta-Catenin Canonical Wnt Signaling." *J Bone Miner Res* 25.11 (2010): 2399-411. Print.

Chen, J. R., et al. "Diet-Derived Phenolic Acids Regulate Osteoblast and Adipocyte Lineage Commitment and Differentiation in Young Mice." *J Bone Miner Res* 29.5 (2014): 1043-53. Print.

Chen, Tzu-Ying, et al. "Influence of Diabetes on Plasma Pharmacokinetics and Brain Bioavailability of Grape Polyphenols and Their Phase Ii Metabolites in the Zucker Diabetic Fatty Rat." *Molecular nutrition & food research* 61.10 (2017): 10.1002/mnfr.201700111. Print.

Choi, E. M., and J. K. Hwang. "Effects of (+)-Catechin on the Function of Osteoblastic Cells." *Biol Pharm Bull* 26.4 (2003): 523-6. Print.

Clarke, B. L., and S. Khosla. "Physiology of Bone Loss." *Radiol Clin North Am* 48.3 (2010): 483-95. Print.

Del Rio, D., et al. "Bioavailability of Catechins from Ready-to-Drink Tea." *Nutrition* 26.5 (2010): 528-33. Print.

Devareddy, L., et al. "Blueberry Prevents Bone Loss in Ovariectomized Rat Model of Postmenopausal Osteoporosis." *J Nutr Biochem* 19.10 (2008): 694-9. Print.

DiMeglio, Linda A., and Erik A. Imel. "Chapter 13 - Calcium and Phosphate: Hormonal Regulation and Metabolism." *Basic and Applied Bone Biology*. Ed. Allen, David B. BurrMatthew R. San Diego: Academic Press, 2014. 261-82. Print.

Duda-Chodak, A., et al. "Interaction of Dietary Compounds, Especially Polyphenols, with the Intestinal Microbiota: A Review." *Eur J Nutr* 54.3 (2015): 325-41. Print.

Erlund, I., et al. "Pharmacokinetics of Quercetin from Quercetin Aglycone and Rutin in Healthy Volunteers." *Eur J Clin Pharmacol* 56.8 (2000): 545-53. Print.

Feliciano, R. P., et al. "Plasma and Urinary Phenolic Profiles after Acute and Repetitive Intake of Wild Blueberry." *Molecules* 21.9 (2016). Print.

Folin, O., and V. Ciocalteu. "On Tyrosine and Tryptophane Determinations in Proteins." *Journal of Biological Chemistry* 73.2 (1927): 627-50. Print.

Foundation, International Osteoporosis. "Fracture Statistics." 2015. Web.

Furrer, A., et al. "Changes in Phenolic Content of Commercial Potato Varieties through Industrial Processing and Fresh Preparation." *Food Chemistry* 218 (2017): 47-55. Print.

Gallagher, J. C., and A. J. Sai. "Molecular Biology of Bone Remodeling: Implications for New Therapeutic Targets for Osteoporosis." *Maturitas* 65.4 (2010): 301-7. Print.

Hardcastle, A. C., et al. "Associations between Dietary Flavonoid Intakes and Bone Health in a Scottish Population." *J Bone Miner Res* 26.5 (2011): 941-7. Print.

Hayes, D. P. "Adverse Effects of Nutritional Inadequacy and Excess: A Hormetic Model." *Am J Clin Nutr* 88.2 (2008): 578S-81S. Print.

Heaney, R. P., et al. "Calcium Absorption in Women: Relationships to Calcium Intake, Estrogen Status, and Age." *J Bone Miner Res* 4.4 (1989): 469-75. Print.

Ho, Lap, et al. "Identification of Brain-Targeted Bioactive Dietary Quercetin-3-O-Glucuronide as a Novel Intervention for Alzheimer's Disease." *The FASEB Journal* 27.2 (2013): 769-81. Print.

Hohman, E. E., et al. "Bioavailability and Efficacy of Vitamin D2 from Uv-Irradiated Yeast in Growing, Vitamin D-Deficient Rats." *J Agric Food Chem* 59.6 (2011): 2341-6. Print.

Hohman, E. E., and C. M. Weaver. "A Grape-Enriched Diet Increases Bone Calcium Retention and Cortical Bone Properties in Ovariectomized Rats." *J Nutr* 145.2 (2015): 253-9. Print.

Holick, M. F., et al. "Guidelines for Preventing and Treating Vitamin D Deficiency and Insufficiency Revisited." *J Clin Endocrinol Metab* 97.4 (2012): 1153-8. Print.

Hooshmand, S., J. R. Brisco, and B. H. Arjmandi. "The Effect of Dried Plum on Serum Levels of Receptor Activator of Nf-Kappab Ligand, Osteoprotegerin and Sclerostin in Osteopenic Postmenopausal Women: A Randomised Controlled Trial." *Br J Nutr* 112.1 (2014): 55-60. Print.

Hooshmand, S., et al. "Comparative Effects of Dried Plum and Dried Apple on Bone in Postmenopausal Women." *Br J Nutr* 106.6 (2011): 923-30. Print.

Hooshmand, S., et al. "The Effect of Two Doses of Dried Plum on Bone Density and Bone Biomarkers in Osteopenic Postmenopausal Women: A Randomized, Controlled Trial." *Osteoporos Int* 27.7 (2016): 2271-9. Print.

Hsu, Y. L., et al. "Syringetin, a Flavonoid Derivative in Grape and Wine, Induces Human Osteoblast Differentiation through Bone Morphogenetic Protein-2/Extracellular Signal-Regulated Kinase 1/2 Pathway." *Mol Nutr Food Res* 53.11 (2009): 1452-61. Print.

Huang, W., et al. "Antioxidant and Anti-Inflammatory Effects of Blueberry Anthocyanins on High Glucose-Induced Human Retinal Capillary Endothelial Cells." *Oxid Med Cell Longev* 2018 (2018): 1862462. Print.

Hughes, D. E., et al. "Estrogen Promotes Apoptosis of Murine Osteoclasts Mediated by Tgf-Beta." *Nat Med* 2.10 (1996): 1132-6. Print.

Huh, Y. J., et al. "Regulation of Osteoclast Differentiation by the Redox-Dependent Modulation of Nuclear Import of Transcription Factors." *Cell Death Differ* 13.7 (2006): 1138-46. Print.

Huncharek, M., J. Muscat, and B. Kupelnick. "Impact of Dairy Products and Dietary Calcium on Bone-Mineral Content in Children: Results of a Meta-Analysis." *Bone* 43.2 (2008): 312-21. Print.

Imel, Erik A., Linda A. DiMeglio, and David B. Burr. "Chapter 16 - Metabolic Bone Diseases." *Basic and Applied Bone Biology*. Ed. Allen, David B. BurrMatthew R. San Diego: Academic Press, 2014. 317-44. Print.

Iñiguez-Ariza, Nicole M., and Bart L. Clarke. "Bone Biology, Signaling Pathways, and Therapeutic Targets for Osteoporosis." *Maturitas* 82.2 (2015): 245-55. Print.

Kalt, W., et al. "Anthocyanin Metabolites Are Abundant and Persistent in Human Urine." *J Agric Food Chem* 62.18 (2014): 3926-34. Print.

Kalt, W., et al. "Interspecific Variation in Anthocyanins, Phenolics, and Antioxidant Capacity among Genotypes of Highbush and Lowbush Blueberries (Vaccinium Section Cyanococcus Spp.)." *J Agric Food Chem* 49.10 (2001): 4761-7. Print.

Kang, J., et al. "Phenolic Acids of the Two Major Blueberry Species in the Us Market and Their Antioxidant and Anti-Inflammatory Activities." *Plant Foods Hum Nutr* 70.1 (2015): 56-62. Print.

Kanis, J. A., et al. "European Guidance for the Diagnosis and Management of Osteoporosis in Postmenopausal Women." *Osteoporos Int* 24.1 (2013): 23-57. Print.

Kaume, L., et al. "Cyanidin 3-O-Beta-D-Glucoside Improves Bone Indices." *J Med Food* 18.6 (2015): 690-7. Print.

Kaume, Lydia, et al. "Antioxidant-Rich Berries Exert Modest Bone Protective Effects in Postmenopausal Smokers without Improving Biomarkers of Bone Metabolism." *Journal of Functional Foods* 9 (2014): 202-10. Print.

Kay, C. D., G. J. Mazza, and B. J. Holub. "Anthocyanins Exist in the Circulation Primarily as Metabolites in Adult Men." *J Nutr* 135.11 (2005): 2582-8. Print.

Krikorian, R., et al. "Blueberry Supplementation Improves Memory in Older Adults." *J Agric Food Chem* 58.7 (2010): 3996-4000. Print.

Kulen, O., C. Stushnoff, and D. G. Holm. "Effect of Cold Storage on Total Phenolics Content, Antioxidant Activity and Vitamin C Level of Selected Potato Clones." *Journal of the Science of Food and Agriculture* 93.10 (2013): 2437-44. Print.

Kumar, S., and A. K. Pandey. "Chemistry and Biological Activities of Flavonoids: An Overview." *ScientificWorldJournal* 2013 (2013): 162750. Print.

Kurilich, A. C., et al. "Plasma and Urine Responses Are Lower for Acylated Vs Nonacylated Anthocyanins from Raw and Cooked Purple Carrots." *J Agric Food Chem* 53.16 (2005): 6537-42. Print.

Landete, J. M. "Updated Knowledge About Polyphenols: Functions, Bioavailability, Metabolism, and Health." *Crit Rev Food Sci Nutr* 52.10 (2012): 936-48. Print.

Li, T., et al. "Rabbiteye Blueberry Prevents Osteoporosis in Ovariectomized Rats." *J Orthop Surg Res* 9 (2014): 56. Print.

Lila, M. A., et al. "Unraveling Anthocyanin Bioavailability for Human Health." *Annu Rev Food Sci Technol* 7 (2016): 375-93. Print.

Lila, Mary Ann. "He Nature-Versus-Nurture Debate on Bioactive Phytochemicals: The Genome Versus Terroir." *J. Sci. Food Agric* 86 (2006): 2510-15. Print.

MAKUS, DONALD J., and JUSTIN R. MORRIS. "A Comparison of Fruit of Highbush and Rabbiteye Blueberry Cultivars1." *Journal of Food Quality* 16.6 (1993): 417-28. Print.

MariuszKonrad, Piskula, Murota Kaeko, and Terao Junji. "Bioavailability of Flavonols and Flavones." *Flavonoids and Related Compounds*. Oxidative Stress and Disease: CRC Press, 2012. Print.

Matchett, M. D., et al. "Inhibition of Matrix Metalloproteinase Activity in Du145 Human Prostate Cancer Cells by Flavonoids from Lowbush Blueberry (Vaccinium Angustifolium): Possible Roles for Protein Kinase C and Mitogen-Activated Protein-Kinase-Mediated Events." *J Nutr Biochem* 17.2 (2006): 117-25. Print.

McCabe, GP, et al. "Principal Components as a Tool in the Selection of Blueberry Germpasm for Biomedical Experiments." *Stat Med* submitted (2018). Print.

Michael Lewiecki, E., et al. "Hip Fracture Trends in the United States, 2002 to 2015." *Osteoporos Int* 29.3 (2018): 717-22. Print.

Michalska, A., and G. Lysiak. "Bioactive Compounds of Blueberries: Post-Harvest Factors Influencing the Nutritional Value of Products." *Int J Mol Sci* 16.8 (2015): 18642-63. Print.

Murota, K., Y. Nakamura, and M. Uehara. "Flavonoid Metabolism: The Interaction of Metabolites and Gut Microbiota." *Biosci Biotechnol Biochem* 82.4 (2018): 600-10. Print.

Nair, A. R., et al. "Blueberry Supplementation Attenuates Oxidative Stress within Monocytes and Modulates Immune Cell Levels in Adults with Metabolic Syndrome: A Randomized, Double-Blind, Placebo-Controlled Trial." *Food Funct* 8.11 (2017): 4118-28. Print.

Nakamura, T., et al. "Estrogen Prevents Bone Loss Via Estrogen Receptor Alpha and Induction of Fas Ligand in Osteoclasts." *Cell* 130.5 (2007): 811-23. Print.

New, S. A., et al. "Nutritional Influences on Bone Mineral Density: A Cross-Sectional Study in Premenopausal Women." *Am J Clin Nutr* 65.6 (1997): 1831-9. Print.

NHANES. "Percent Below Estimated Average Requirement for Vitamin D." *Day 1-2, Food Sources Only* (2009-2010). Print.

Nohara, Chiaki, et al. "Daily Consumption of Bilberry (Vaccinium Myrtillus L.) Extracts Increases the Absorption Rate of Anthocyanins in Rats." *Journal of Agricultural and Food Chemistry* 66.30 (2018): 7958-64. Print.

Nurmi, T., et al. "Metabolism of Berry Anthocyanins to Phenolic Acids in Humans." *J Agric Food Chem* 57.6 (2009): 2274-81. Print.

Ogita, M., et al. "Differentiation and Proliferation of Periosteal Osteoblast Progenitors Are Differentially Regulated by Estrogens and Intermittent Parathyroid Hormone Administration." *Endocrinology* 149.11 (2008): 5713-23. Print.

Papapoulos, S., et al. "Five Years of Denosumab Exposure in Women with Postmenopausal Osteoporosis: Results from the First Two Years of the Freedom Extension." *J Bone Miner Res* 27.3 (2012): 694-701. Print.

Park, C. Y., et al. "Calcium and Vitamin D Intake Maintained from Preovariectomy Independently Affect Calcium Metabolism and Bone Properties in Sprague Dawley Rats." *Osteoporos Int* 25.7 (2014): 1905-15. Print.

Pawlowski, J. W., et al. "Plum and Soy Aglycon Extracts Superior at Increasing Bone Calcium Retention in Ovariectomized Sprague Dawley Rats." *J Agric Food Chem* 62.26 (2014): 6108-17. Print.

Prior, R. L., and X. Wu. "Anthocyanins: Structural Characteristics That Result in Unique Metabolic Patterns and Biological Activities." *Free Radic Res* 40.10 (2006): 1014-28. Print.

Redan, B. W., et al. "Altered Transport and Metabolism of Phenolic Compounds in Obesity and Diabetes: Implications for Functional Food Development and Assessment." *Adv Nutr* 7.6 (2016): 1090-104. Print.

Reid, I. R. "Short-Term and Long-Term Effects of Osteoporosis Therapies." *Nat Rev Endocrinol* 11.7 (2015): 418-28. Print.

Rodriguez-Mateos, A., et al. "Impact of Cooking, Proving, and Baking on the (Poly)Phenol Content of Wild Blueberry." *J Agric Food Chem* 62.18 (2014): 3979-86. Print.

Rodriguez-Mateos, A., et al. "Procyanidin, Anthocyanin, and Chlorogenic Acid Contents of Highbush and Lowbush Blueberries." *J Agric Food Chem* 60.23 (2012): 5772-8. Print.

Rodriguez-Mateos, A., et al. "Bioavailability, Bioactivity and Impact on Health of Dietary Flavonoids and Related Compounds: An Update." *Arch Toxicol* 88.10 (2014): 1803-53. Print.

Rodriguez-Mateos, Ana, et al. "Procyanidin, Anthocyanin, and Chlorogenic Acid Contents of Highbush and Lowbush Blueberries." *Journal of Agricultural and Food Chemistry* 60.23 (2012): 5772-78. Print.

Ronald, L. Prior. "Anthocyanins." *Flavonoids and Related Compounds*. Oxidative Stress and Disease: CRC Press, 2012. Print.

Rossouw, J. E., et al. "Risks and Benefits of Estrogen Plus Progestin in Healthy Postmenopausal Women: Principal Results from the Women's Health Initiative Randomized Controlled Trial." *JAMA* 288.3 (2002): 321-33. Print.

Sacco, S. M., M. N. Horcajada, and E. Offord. "Phytonutrients for Bone Health During Ageing." *Br J Clin Pharmacol* 75.3 (2013): 697-707. Print.

Sattari, M., et al. "Osteoporosis in the Women's Health Initiative: Another Treatment Gap?" *Am J Med* 130.8 (2017): 937-48. Print.

Seeman, Ego. "Pathogenesis of Bone Fragility in Women and Men." *The Lancet* 359.9320 (2002): 1841-50. Print.

Sellappan, S., C. C. Akoh, and G. Krewer. "Phenolic Compounds and Antioxidant Capacity of Georgia-Grown Blueberries and Blackberries." *J Agric Food Chem* 50.8 (2002): 2432-8. Print.

Shin, C. S., and K. M. Kim. "Calcium, Is It Better to Have Less?--Global Health Perspectives." *J Cell Biochem* 116.8 (2015): 1513-21. Print.

Singleton, V. L., R. Orthofer, and R. M. Lamuela-Raventos. "Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent." *Oxidants and Antioxidants, Pt A* 299 (1999): 152-78. Print.

Smith, D. M., et al. "Genetic Factors in Determining Bone Mass." *J Clin Invest* 52.11 (1973): 2800-8. Print.

Song, Brian J., et al. "Photo- and Thermodegradation of Anthocyanins from Grape and Purple Sweet Potato in Model Beverage Systems." *Journal of Agricultural and Food Chemistry* 61.6 (2013): 1364-72. Print.

Stull, A. J., et al. "Bioactives in Blueberries Improve Insulin Sensitivity in Obese, Insulin-Resistant Men and Women." *J Nutr* 140.10 (2010): 1764-8. Print.

Tang, J. S., et al. "Bioavailable Blueberry-Derived Phenolic Acids at Physiological Concentrations Enhance Nrf2-Regulated Antioxidant Responses in Human Vascular Endothelial Cells." *Mol Nutr Food Res* 62.5 (2018). Print.

Taruscio, T. G., D. L. Barney, and J. Exon. "Content and Profile of Flavanoid and Phenolic Acid Compounds in Conjunction with the Antioxidant Capacity for a Variety of Northwest Vaccinium Berries." *J Agric Food Chem* 52.10 (2004): 3169-76. Print.

Terao, Junji. "Factors Modulating Bioavailability of Quercetin-Related Flavonoids and the Consequences of Their Vascular Function." *Biochemical Pharmacology* 139 (2017): 15-23. Print.

Thilakarathna, S. H., and H. P. Rupasinghe. "Flavonoid Bioavailability and Attempts for Bioavailability Enhancement." *Nutrients* 5.9 (2013): 3367-87. Print.

Trzeciakiewicz, A., V. Habauzit, and M. N. Horcajada. "When Nutrition Interacts with Osteoblast Function: Molecular Mechanisms of Polyphenols." *Nutr Res Rev* 22.1 (2009): 68-81. Print.

Wallace, Joseph M. "Chapter 6 - Skeletal Hard Tissue Biomechanics." *Basic and Applied Bone Biology*. Ed. Allen, David B. BurrMatthew R. San Diego: Academic Press, 2014. 115-30. Print.

Waterhouse, AL. "Determination of Total Phenolics." *Current Protocols in Food Analytical Chem* 6.1 (2002): I1.1.1-I1.1.8. Print.

Weaver, C. M., et al. "Calcium Plus Vitamin D Supplementation and Risk of Fractures: An Updated Meta-Analysis from the National Osteoporosis Foundation." *Osteoporos Int* (2015). Print.

Welch, A., et al. "Habitual Flavonoid Intakes Are Positively Associated with Bone Mineral Density in Women." *J Bone Miner Res* 27.9 (2012): 1872-8. Print.

Wright, N. C., et al. "The Recent Prevalence of Osteoporosis and Low Bone Mass in the United States Based on Bone Mineral Density at the Femoral Neck or Lumbar Spine." *J Bone Miner Res* 29.11 (2014): 2520-6. Print.

Wright, N. C., et al. "The Impact of the New National Bone Health Alliance (Nbha) Diagnostic Criteria on the Prevalence of Osteoporosis in the United States: Supplementary Presentation." *Osteoporosis International* 28.11 (2017): 3283-84. Print.

Yang, D., et al. "Effect of Hormone Replacement Therapy on Cardiovascular Outcomes: A Meta-Analysis of Randomized Controlled Trials." *PLoS One* 8.5 (2013): e62329. Print.

Yang, L. L., et al. "Pharmacokinetic Comparison between Quercetin and Quercetin 3-O-Beta-Glucuronide in Rats by Uhplc-Ms/Ms." *Sci Rep* 6 (2016): 35460. Print.

Yousef, G. G., et al. "Efficient Quantification of the Health-Relevant Anthocyanin and Phenolic Acid Profiles in Commercial Cultivars and Breeding Selections of Blueberries (Vaccinium Spp.)." *J Agric Food Chem* 61.20 (2013): 4806-15. Print.

Yousef, G. G., et al. "Impact of Interspecific Introgression on Anthocyanin Profiles of Southern Highbush Blueberry." *Journal of the American Society for Horticultural Science* 139.2 (2014): 99-112. Print.

Zhang, J., et al. "Feeding Blueberry Diets in Early Life Prevent Senescence of Osteoblasts and Bone Loss in Ovariectomized Adult Female Rats." *PLoS One* 6.9 (2011): e24486. Print.

Zhang, J., et al. "Feeding Blueberry Diets to Young Rats Dose-Dependently Inhibits Bone Resorption through Suppression of Rankl in Stromal Cells." *PLoS One* 8.8 (2013): e70438. Print.

Zhang, Z. Q., et al. "Association between Dietary Intake of Flavonoid and Bone Mineral Density in Middle Aged and Elderly Chinese Women and Men." *Osteoporos Int* 25.10 (2014): 2417-25. Print.

Zhong, S., et al. "Characterization of Wild Blueberry Polyphenols Bioavailability and Kinetic Profile in Plasma over 24-H Period in Human Subjects." *Mol Nutr Food Res* 61.12 (2017). Print.