ABSTRACT

BURTCH, HALEY LOUISE. Adaptation of an Existing *In Vitro* Digestion Model for High Throughput Phenolic Bioaccessibility Phenotyping within Cultivated (Highbush) Blueberry Varieties. (Under the Direction of Dr. Mario Ferruzzi).

The 2015 Dietary Guidelines for Americans (DGA) based recommendations for fruit and vegetable consumption on evidence supporting the role of nutrients and bioactives (phytochemicals) in meeting nutritional requirements. Intake of phytochemicals, defined as secondary plant metabolites (such as carotenoids and polyphenols), has widely been associated with a decreased risk of chronic and degenerative diseases. However, according to the USDA approximately 13% of US residents consume the recommended amount of vegetables, while 25% consume the recommended amount of fruit. Therefore, novel strategies are needed to address the gap in delivery of fruit and vegetable benefits to consumers. Given that the associated health impacts from fruits and vegetables are ultimately dependent on their phytochemical density and their absorption and metabolism (bioavailability), it has been suggested that advanced phenotyping combined with genetic techniques can be leveraged to breed fruits and vegetables with enhanced nutritive content and bioavailability.

Modern breeding strategies have advanced our knowledge of factors influencing polyphenol content in plant foods, such as polyphenol-rich highbush (HB) blueberries. However, potential traits related to phenolic bioavailability remain unknown due to limitations in phenotyping methods. While nutrient and bioactive bioavailability is most accurately measured by *in vivo* models, they are often costly, time-consuming, and constitute a bottleneck for high-throughput analysis. Alternatively, *in vitro* digestion models have proven useful in the estimation of factors impacting polyphenol digestive release and availability for absorption (bioaccessibility), a precursor of bioavailability. However, these models remain inefficient for screening large

germplasm collections. Therefore, high throughput (HT) models suitable for screening of larger HB blueberry populations are needed to advance our knowledge of genotypes and genetic factors influencing phenolic density and bioavailability.

An established static, relatively low throughput (LT), three stage *in vitro* gastrointestinal digestion model previously used for fruit phenolics was adapted to reduce tissue amounts, digestion volume and modified enzyme concentrations allowing for compatibility with a robotic fluid handling system (TECAN EVO 150) for HT. Bioaccessibility of phenolics from commercial HB blueberries was optimized for HT and validated against the LT model. The HT model was then used to screen phenolic bioaccessibility in a subset of 33 individual blueberry genotypes from a mapping population derived from a cross between two elite cultivars, Draper and Jewel (DxJ).

Bioaccessibility of anthocyanins (ANC), flavonols (FLAV), flavan-3-ols (F3L) and phenolic acids (PA) were well correlated (r=0.98) between HT and LT methods. Calculated CV ranged from 2.7-21.9%for HT and for LT 0.1-23.2%, respectively suggesting good reproducibility. The sum of total phenolic content within the DxJ subset ranged from 31.7– 81.1 mg/100g fresh weight (f.w.). Approximately 60% of the phenolics were ANC, followed by PA, predominately chlorogenic acid, representing 28%, while FLAV (<1%) and F3L (12%) accounted for the rest. Variation in relative bioaccessibility (%) was observed among all phenolic classes with ANC (0.3-11.7; $P<0.0001$), Acylated ANC (7.9-27.9; $P<0.0001$), FLAV (10.7-54.1; $P<0.0001$), FL3(1.0-49.1; $P<0.0001$) and PA (0.3-2.1; $P<0.0001$). Similarly, significant variation in absolute bioaccessibility (mg/100g f.w.) was noted among all phenolic classes with ANC (0.1-2.6 ; $P<0.0001$), acylated ANC (1.2-4.3; $P<0.0001$), PA (0.03-0.9; $P<0.0001$), FLAV (0.8-5.5; $P<0.0001$), and F3L (0.001-0.05; $P<0.0001$).

Therefore, the adapted HT *in vitro* digestion model provides a novel tool for phenotyping phenolic bioaccessibility in blueberry fruit. Application to a subset of DxJ genotypes, further establishes the need for continued screening of diverse blueberry germplasms and other plant materials in combination with genomic approaches. This will allow for greater understanding of specific genetic factors that influence polyphenolic density and bioavailability.

Adaptation of an Existing *In Vitro* Digestion Model for High Throughput Phenolic Bioaccessibility Phenotyping within Cultivated (Highbush) Blueberry Varieties.

by
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DEDICATION

To my family for their encouragement.

BIOGRAPHY

Haley Louise Burtch was born on November 27th, 1993 in Rockford, Ohio. Upon completion of her Bachelor of Science degree in Nutrition, Fitness and Health and Dietetics from Purdue University in the Spring of 2016, she chose to further her education by pursuing a Master's degree from North Carolina State University in the field of Food Science. In June of 2017, Haley began her research, under the direction of Dr. Mario Ferruzzi, on polyphenol bioaccessibility in blueberries at the Plants for Human Health Institute in Kannapolis, North Carolina. Following completion of her graduate studies, Haley plans to apply her degree within the food industry.

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CHAPTER 1. REVIEW OF RELEVANT LITERATURE

1.1. Introduction

Fruits and vegetables have held a central place in dietary guidance by virtue of their density of vitamins, minerals, dietary fiber and, of more recent interest, their phytochemical content (Slavin and Lloyd 2012). Phytochemicals are broadly defined as secondary plant metabolites with putative human health benefits (R. H. Liu 2003). Intake of phytochemical rich fruits and vegetables has been associated with a decreased risk of chronic and degenerative diseases in humans including cardiovascular disease (Bazzano et al. 2002; Arai et al. 2000; Appel et al. 1997; Hertog et al. 1993), a multitude of cancers (lung, colon, breast, cervix, esophagus, oral cavity, stomach, bladder, pancreas, and ovary) (Bunea et al. 2013; Bagchi et al. 2004; Damianaki et al. 2000; Yi et al. 2005; Agudelo et al. 2018), diabetes (Marles and Farnsworth 1995; van Dam and Feskens 2002; Grace et al. 2009; Fan et al. 2013), obesity (Stull et al. 2010; Prior et al. 2010; Vuong et al. 2009), and neurodegenerative disorders (Joseph et al. 1999; Tavares et al. 2012; Papandreou et al. 2009; Krikorian et al. 2010).

Given the significant health benefits associated with fruit and vegetable nutrients and phytochemicals, the 2015 Dietary Guidelines for Americans (DGA) recommends consuming 2 cups of fruit and 2.5 cups of vegetables daily (USDA Dietary Guidelines for Americans 2015-2020) to meet daily nutrient needs and provide associated health benefits. While modest in levels, consumption of fruits and vegetables by the average American remains significantly below the recommended amounts. Specifically, in the 2015 DGA it was reported that only approximately 13% of the United States population was consuming fruits and vegetables at the recommended serving levels (USDA Dietary Guidelines for Americans 2015-2020). With such poor adherence to existing guidelines, novel strategies are required to improve the delivery of fruit and vegetable benefits.

Several factors have been linked to the low intake of fruits and vegetables including difficulty of modifying long-term consumer behavior, cost and lack of consistent access to fresh fruits and vegetables for low income consumers and the lack of alignment between current fruit and vegetable products and consumer lifestyles (Rekhy and McConchie 2014). Addressing both the production and consumption side of this equation has required collaborations amongst various disciplines with a focus on agriculture, food science and production, nutrition scientist and policy and consumer scientist.

In 2011, the Centers for Disease Control and Prevention published a guide titled, *"Strategies to Prevent Obesity and Other Chronic Diseases: The CDC Guide to Strategies to Increase the Consumption of Fruits and Vegetables"* to address 10 strategies (Table 1.1) designed to increase access to and improve the availability of fruits and vegetables, with the expectations that these changes will lead to increased consumption (CDC 2011). The strategies were focused on both policy and environmental changes and were selected on the best available evidence, as well as the knowledge and expertise of the authors and CDC partners. In short, the CDC encouraged all state and local levels to improve access and incorporate fruits and vegetables into more public settings, such as schools and places of employment. Similarly, the USDA suggests attainable options such as increasing the vegetable content of mixed dishes while decreasing the amounts of other food components that are often overconsumed, such as refined grains or meats high in saturated fat and/or sodium and including a salad or vegetable side dish along with incorporating more vegetables in general into meals and snacks. Additionally, the USDA also recommended incorporating more whole fruits into snacks, salads, side dishes and desserts (USDA Dietary Guidelines for Americans 2015-2020), thereby enhancing consumption opportunities.

While such strategies seek to improve consumer consumption of fruits and vegetables are in place, there has generally been no significant change in consumer behavior and fruit and vegetable intake over time (Casagrande et al. 2007; Lee-Kwan et al. 2017). Given the difficulty in changing consumer behavior and the challenges in addressing this challenge solely by increasing production of fruits and vegetables new strategies are needed to address the gap in delivery of fruit and vegetable benefits to American consumers.

Table 1. 1. Strategies to increase the consumption of fruits and vegetables adapted from "Strategies to Prevent Obesity and Other Chronic Diseases: The CDC Guide to Strategies to Increase the Consumption of Fruits and Vegetables" (2011).

10 Strategies to Increase the Consumption of Fruits and Vegetables	
1.	**Promote food policy councils as a way to improve the food environment at state and local levels.** Rationale: Food policy council is created to develop policy related to healthy food access, including fruit and vegetable production, availability, and distribution. They develop strategies to provide high quality and affordable healthy food through policy and environmental changes.
2.	**Improve access to retail stores that sell high-quality fruits and vegetables or increase the availability of high-quality fruits and vegetables at retail stores in underserved communities.** Rationale: Research supports that greater access results in consumers having healthier diets, including higher intakes of fruits and vegetables (Larson, Story, and Nelson 2009),
3.	**Start or expand farm-to-institution programs in schools, hospitals, workplaces and other institutions.** Rationale: These programs allow individuals who take part in public and private institutions, high-quality, and regionally grown fruits and vegetables, which may encourage local and regional farmers to produce a variety of fruits and vegetables.
4.	**Start or expand farmers' markets in all settings.** Rationale: Farmers markets are relatively easy to implement given they are less costly than supermarkets and can be set up in a variety of locations (Austin and Elias 1996). They also provide a direct connection between consumers and the people who grow their food.
5.	**Start or expand community supported agriculture (CSA) programs in all settings.** Rationale: These programs Support regional fruit and vegetable production and distribution as a way to provide consumers with high-quality, affordable fresh produce, while encouraging farmers to produce these foods.
6.	**Ensure access to fruits and vegetables in workplace cafeterias and other food service venues.** Rationale: Given that many people spend the majority of their time at a workplace where they tend to at least one meal and several snack. Enhanced access encourages workers to eat healthier.
7.	**Ensure access to fruits and vegetables at workplace meetings and events.** Rationale: See rationale provided for *Strategy 6*
8.	**Support and promote community and home gardens.** Rationale: Such programs increase access to fresh produce and the ability to yield high-quality produce at low cost. It has been proven that higher exposure to fruits and vegetables may result in greater consumption (Blair, Giesecke, and Sherman 1991).
9.	**Establish policies to incorporate fruit and vegetable activities into schools as a way to increase consumption.** Rationale: Given the fact that such a low percentage of youth currently meet national recommendations for fruit and vegetable consumption, schools are in a unique position to influence and promote fruit and vegetable intake.
10.	**Include fruits and vegetables in emergency food programs.** Rationale, in 2007, approximately 11% of U.S. households were food insecure, which is defined as not having access to enough safe and nutritious food, at least sometime during the year (USDA ERS, Food Security in the United States, 2019). While many depend on food assistance, emergency food programs can have inadequate supplies of fruits and vegetables (Akobundu et al. 2004).

In developing strategies that could serve to improve delivery of fruit and vegetable benefits, one must consider that the health impacts derived from consumption of fruit and vegetables are dependent on the content of nutrients (macro and micronutrients) and phytochemicals (e.g.

carotenoids and phenolics), their absorption and metabolism (bioavailability), and ultimately their activities in the human body. Micronutrients and bioactive metabolites circulating in humans are implicated in biological effects and are considered markers of "delivery" of fruit- and vegetable-rich foods or diets (Pujos-Guillot et al. 2013; May et al. 2013; Andersen et al. 2014).With this in mind, a strategy focused on enhancing micronutrient and bioactive density as well as bioavailability from the most commonly consumed fruits and vegetables and their derived products can offer a promising path for consumers to achieve DGA guidelines within their current consumption patterns. This is especially true if the new products can be developed leveraging these raw materials that fit into consumer eating patterns. As a first step to deliver on this promise one must consider breeding and agronomic strategies that hold the potential to increase nutrients/phytochemical density in existing fruits and vegetables and/or enhance their bioavailability thereby delivering more from existing servings. This could serve not only to enhance the quality of fresh and minimally processed produce but also as the starting point for generation of novel ingredients suitable for production of new and improved fruit and vegetable products.

To date, there have been several advances in what is termed "nutrient-sensitive" breeding strategies that have advanced our understanding of factors and highlighted pathways for enhancing the content of nutrients and phytochemicals in fruits and vegetables (Davies and Espley 2013; Sanahuja et al. 2013; Raiola et al. 2015). Plant science and biotechnology programs utilizing both traditional breeding strategies as well as marker assisted breeding or genetic modification approaches (Davies and Espley 2013), have been extensively leveraged in the engineering of metabolic pathways crucial to phytochemical biosynthesis and accumulation. However, to date little is known regarding the variability or ability to modify traits relevant to bioavailability of

micronutrients and phytochemicals. This remains a critical piece of knowledge needed to enhance the potential impact of a single fruit or vegetable serving. This gap in the science is also connected to a general lack of a physiologically relevant model for "phenotyping" bioavailability across the large germplasm collections of individual fruits and vegetables required for such genetic and breeding programs. Such a phenotypic method is required to connect the nutritional impact of fruits and vegetables to the broader "omics" leveraged in advance breeding programs.

While nutrient and phytochemical bioavailability are best measured *in vivo* and in clinical settings, *in vitro* models of the gastrointestinal tract have proven useful in the estimation of factors impacting micronutrient and phytochemical digestive release and availability for absorption (bioaccessibility) (Peters et al. 2010; Reboul et al. 2006). Relying on predetermined physiological parameters that mimic the oral, gastric and small intestinal phases of digestion, such in vitro models have been effectively used to estimate food matrix and processing factors impacting micronutrient and phytochemical bioavailability (Neilson and Ferruzzi 2011; Neilson et al. 2009). In the case of screening relevant plant germplasm collections for nutritional delivery traits, iron bioaccessibility was proposed as a genetically variable trait in maize (Šimić et al. 2009). This, as well as vitamin A bioaccessibility, has been proposed as a target for breeding biofortified grains and other staple foods for at risk population (J. Aragón et al. 2018; Lipkie et al. 2013; Pixley, Palacios-Rojas, and Glahn 2011). However, the broader application of such methods has been challenged by logistical and practical considerations that limit overall throughput. Simply put, these labor-intensive models, while more efficient than clinical assessments, still have challenges to the broad application. This includes plant material sample size requirements, high level of manual processing, and overall analytical sensitivity. Overcoming these challenges and advancing throughput from dozens of samples per day to hundred would make it possible to leverage these

models for the full array of phytochemicals critical to fruit and vegetable benefits. The potential to develop a platform for this type of "functional phenotyping" of key fruit and vegetable germplasm collections will allow for us to leverage genomic approaches and facilitate identification of genetic factors that influence both density and bioavailability of micronutrients and/or phytochemicals from fruits and vegetables.

With this in mind, the focus of this thesis research was to generate a high throughput *in vitro* digestion model by adapting existing *in vitro* digestion conditions that have been traditionally applied to screen the impact of food matrix and formulation factors on polyphenol bioaccessibility from berries. This work will ultimately lead to a new functional high throughput method applicable to large blueberry germplasm collections designed to support future genomic studies. Utilizing said method will allow for efficient investigation of blueberry traits that influence nutrient density and bioavailability of target compounds. In support of this effort, the focus of this literature review is to provide an overview into blueberry polyphenols, with insight into their bioavailability and current models used to determine their bioaccessibility. This will include a comparison of the methods and models considered in the development of the current high throughput system.

1.2. Introduction to Polyphenols

Polyphenols are regarded as one of the most abundant and highly distributed classes of phytochemicals in nature with high concentrations found in plant foods and beverages including fruits and vegetables, tea, coffee and cocoa. They are defined as secondary metabolites generated through the shikimate pathway and the phenylpropanoid pathways in higher plants (Ben Haj Yahia et al. 2019; Harborne 1989).While not classified as essential nutrients, polyphenols are viewed as highly fundamental components of both human and animal diets (Bravo 1998). Interest in polyphenol is derived from their wide array of functions within plants, including their contribution

to natural pigmentation, flavor, reproduction and growth, and as natural defense against disease and pest (Bravo 1998). However, more recent interest stems from the association between polyphenol intake and bioactive properties and health-protective effects, including potential effects and antioxidant and free-radical scavenging abilities (Manach et al. 2004; Bravo 1998; R. H. Liu 2003).

Structurally, polyphenols are characterized by having at least one phenol unit (Gan et al. 2019) or an aromatic ring bearing one or more hydroxyl groups, including functional derivatives (Naczk and Shahidi 2006). The majority of polyphenols occur in nature as conjugates with sugars. Glycosidic forms include those where one or more sugar units are linked to hydroxyl groups. However, simpler forms exist consisting of direct linkage of the sugar residues to an aromatic carbon atom (Tsao 2010; Harborne 1989).

More than 8000 individual polyphenols exist(de la Rosa et al. 2019), and have been classified broadly according to their source of origin, biological function, and chemical structure (Tsao 2010). In the simplest terms, phenolic compounds can be classified into two main groups: flavonoids and nonflavonoids (Figure 1.1). Whereas flavonoids, which constitute the most important single group and most abundant form of polyphenols in fruits and vegetables, can be further subdivided into 6 classes, including flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (catechins and proanthocyanins). Meanwhile, nonflavonoids are primarily composed of phenolic acids, but additionally consist of stilbenes and lignans (Manach et al. 2004).

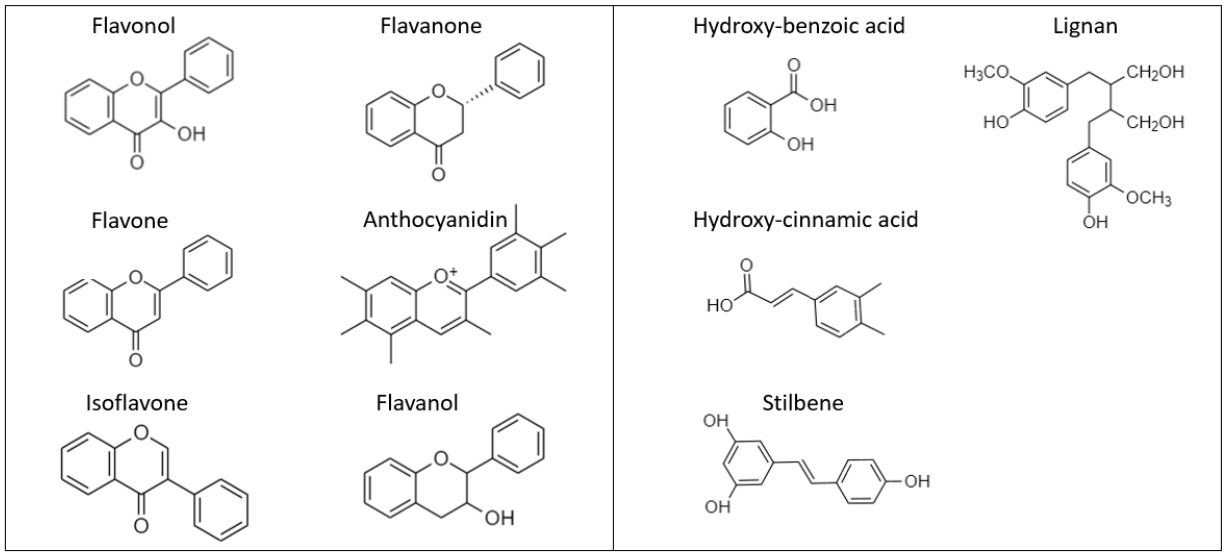


Figure 1. 1. Backbone structures of different flavonoid (left) and non-flavonoid (right) subclasses.

1.3. Flavonoids

Flavonoids are believed to comprise approximately two-thirds of all dietary polyphenolic compounds (de la Rosa et al. 2019; Bravo 1998). Their structural skeleton, known as a phenylbenzopryane skeleton, consists of a phenyl benzopyran which contain two phenyl rings joined through a heterocyclic pyran ring (Pereira et al. 2009). Classification into the acknowledged 6 subclasses is determined by differences in the pyran ring. Within each subclass, individual compounds differ in their pattern of hydroxylation and methylation of the two phenyl rings (de la Rosa et al. 2019).

1.3.1. Flavonols

Flavonols are abundant across many fruits and vegetables (Manach et al. 2004). Flavonols are considered to have significant antioxidant potential due to their pattern of hydroxylation, thus contributing to the potential prevention of diseases, such as cardiovascular disease and cancer

(Hollman and Katan 1999). The most common flavonol forms are kaempferol, quercetin, and myricetin and their associated derivatives. Major dietary sources of flavonols include apples (van der Sluis et al. 2001), onions (K. R. Price and Rhodes 1997), broccoli (Keith R. Price et al. 1998) and tomato (Crozier et al. 1997).

1.3.2. Flavones

Apigenin and luteolin are two of the most prevalent flavones found in nature. Apigenin is primarily in herbs like sage, oregano (W. Zheng and Wang 2001), and dried marjoram (Proestos and Komaitis 2006), while luteolin is similarly observed in herbs, as well as in artichokes (Romani et al. 2006). Similar to other flavonoid subclasses, evidence has supported the anti-inflammatory, anti-microbial, and anti-cancer effects of flavones (Jiang, Doseff, and Grotewold 2016).

1.3.3. Isoflavones

Isoflavones are a class of flavonoids that act as phytoestrogens, that is they have structural similarities with estradiol and the ability to initiate estrogenic-like effects (Manach et al. 2004). Common isoflavones include daidzein, genistein and glycitein. In general, isoflavones are found in leguminous plants, mainly soy-based, including soy paste, tempe and tofu (Murphy et al. 1999; T. Song et al. 1998)(Murphy et al. 1999). Interest regarding isolflavones revolves around their estrogenic properties and their potential role in the prevention of breast cancer and osteoporosis (Adlercreutz and Mazur 1997).

1.3.4. Flavanones

Despite being a relatively small group of compounds, flavanones contribute significantly to overall phenolic, specifically flavonoid, intake due to their widespread consumption (Manach et al. 2004). Naringin, found in grapefruit juice (Ho et al. 2000) and hesperidin, found in lemon (Miyake et al. 1998) and orange juice (Pupin, Dennis, and Toledo 1998), are considered the most

common of the flavanones and are found in high concentrations within citrus fruits and juices (Brat et al. 2006). Given their strong antioxidant and radical scavenging activity, they appear to be associated with the prevention of several chronic diseases. In addition to this potential, flavanones display antiviral, antimicrobial, anti-inflammatory, and anti-allergenic properties (Maria Tomas-Navarro 2014).

1.3.5. Anthocyanidins

Anthocyanins, the glycosides of anthocyanidins, are the compounds in plants responsible for their red, blue, and purple pigmentation (Martinez, Mackert, and McIntosh 2017)(Martinez, Mackert, and McIntosh 2017). Aside from giving fruits their signature red and blue hues, anthocyanins are associated with inhibiting various forms of cancer, cardiovascular disease, neurodegenerative disease, and diabetes (Kong et al. 2003; Bagchi et al. 2004; Andres-Lacueva et al. 2005; Guo and Xia 2014). Almost all anthocyanins are glycosylated derivatives of six common anthocyanidins including: delphinidin, cyanidin, peonidin, petunidin, malvidin, and pelargonidin (de la Rosa et al. 2019). The differences between individual anthocyanins relate to the number of hydroxyl groups, the nature and number of sugars attached to the molecule, the position of this attachment, and the nature and number of aliphatic or aromatic acids attached to sugars in the molecule (Kong et al. 2003). Anthocyanins are commonly found in blueberries, blackberries , red onion, grapes, red wine (Manach et al. 2004; Mazza 2018). Within fruits, anthocyanin content is typically found within the skin, the flesh of certain types of red fruit (Kayesh et al. 2013). Anthocyanins have been widely studied due to their association to their potential effects on health, such as reducing risk of cardiovascular disease, cancer, hyperlipidemia and other insulin-resistance-related diseases, like type-2-diabetes (Guo and Xia 2014; Bagchi et al. 2004; Kong et al. 2003).

1.3.6. Flavan-3-ols

Flavan-3-ols exist in two forms: monomeric (catechins) and polymeric (proanthocyanidins) (Manach et al. 2004). Common catechins include catechin, epicatechin and epigallocatechin which are found in cocoa (K. B. Miller et al. 2006), green tea (Khokhar and Magnusdottir 2002), fruit (Arts, van de Putte, and Hollman 2000) and red wine (Burns et al. 2000). Catechins are associated with high antioxidant functions that contribute to the prevention of chronic disease such as cancer and cardiovascular disease (Higdon and Frei 2003). Proanthocyaninidins, known as condensed tannins, are dimers, oligomers, and polymers of catechins (Manach et al. 2004) that are responsible, when combined with salivary proteins, for the astringency in fruit, beverages, and chocolate (Santos-Buelga and Scalbert 2000). Additionally, their intake is associated with inhibition of LDL oxidation and platelet aggregation, therefore they are shown to contribute to cardiovascular disease prevention (Santos-Buelga and Scalbert 2000).

1.4. Nonflavonoids

Compared to flavonoids, nonflavonoids are smaller and simpler in terms of structure (de la Rosa et al. 2019). Nonflavonoids can be divided into 3 separate subclasses including phenolic acids, stilbenes, and lignans.

1.4.1. Phenolic Acids

Phenolic acids account for approximately one-third of all dietary polyphenolics are divided into two classes: derivatives of cinnamic acid and benzoic acid (Manach et al. 2004; de la Rosa et al. 2019). They consist of a single phenyl group substituted by one carboxylic group and at least one hydroxyl group, however are differentiated by the varying length of the chain containing the carboxylic group (de la Rosa et al. 2019).

A. Hydroxycinnamic acids

Hydroxycinnamic acids, the more common of the two classes, is generally found in fresh fruits and vegetables such as blueberries, kiwi, apple, artichokes, herbs, potatoes, as well as coffee (Naczk and Shahidi 2006; Schuster and Herrmann 1985; Manach et al. 2004). The main representatives of these acids include p-coumaric acid, ferulic, caffeic, sinapic, and chlorogenic (combination of quinic acid) acids (Manach et al. 2004). While caffeic acid is the main constituent within fruits and vegetables (Haghi and Hatami 2010), chlorogenic composes a majority of the phenolic acid profile in coffee (Farah and Donangelo 2006). Research in cell and animal studies suggests that hydroxycinnamic acids may help modulate inflammation, obesity, and diabetes (Maurya and Devasagayam 2010).

B. Hydroxybenzoic acids

Hydroxybenzoic acids are rarely found in their free form. Instead, they are generally glycosylated, linked to small organic acids, or attached to physical components of the plant cells (Manach et al. 2004). Gallic, vanillic, and syringic acid are amongst the most common hydroxybenzoic acids (de la Rosa et al. 2019). While their content is generally low, hydroxybenzoic acid can be found within berries, tea, and herbs (Schuster and Herrmann 1985; Tomás-Barberán and Clifford 2000). Given their relatively low content and availability, limited nutritional studies have been conducted on hydroxybenzoic acid.

1.4.2. Stilbenes

Structurally, stilbenes consist of two benzene rings joined by a molecule of ethanol or ethylene (Moreno and Peinado 2012). Resveratrol, a popular derivative of stilbenes, is a naturally occurring antioxidant found in grapes, red wine, berries, and peanuts and is by far the most extensively studied stilbene given its associated beneficial health effects (Maru et al. 2014; Ren

and Lien 1997). The greatest dietary source of resveratrol is fresh grape skin and therefore its associated form of red wine (Bianchini and Vainio 2003; Y. Wang et al. 2006). Many studies have concluded anticarcinogenic effects by resveratrol intake (Damianaki et al. 2000; Chang, Lee, and Ko 2000; Y. Wang et al. 2006).

1.5. Blueberry polyphenols and intake of polyphenolic-rich blueberries

A prime example of a polyphenolic rich fruit is the blueberry. Blueberries can be divided into 3 predominate cultivated species within the genus *Vaccinium* section (or sub-genus) *Cyanococcus* (Ericaceae family), including highbush (Vaccinium corymbosum L.), rabbiteye (Vaccinium virgatum Ait.), and lowbush "wild" (Vaccinium angustifolium Ait.) (Retamales and Hancock 2018). Most commercial production, however, consists primarily of highbush and lowbush blueberries, while rabbiteye are of more importance in the southeast regions of North America (Retamales and Hancock 2018).

Blueberries are well known for their diverse array and relatively high concentration of phenolic compounds (Figure 1.2), including anthocyanins, phenolic acids, flavanols, and flavan-3-ols. Five major anthocyanins (delphinidin, cyanidin, petunidin, peonidin, and malvidin) and their associated glycosides have been commonly identified in blueberries. Regarding phenolic acids, chlorogenic acid, p-coumaric acid, caffeic acid, ferulic acid have been identified. Quercetin, syringetin, kaempferol, myricetin derivatives comprise the flavanol profile, epicatechin and catechin contribute to the flavan-3-ol content. Polyphenol content within blueberries has been seen to vary according to several factors including growing location, year, species and specific genotype (Connor et al. 2002), harvest stage (degree of maturation and ripeness) (Castrejón et al. 2008), and post-harvest storage conditions (Kalt et al. 1999).

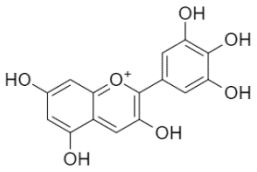
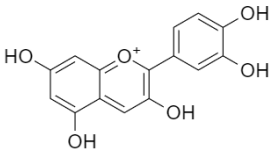
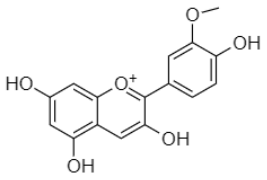
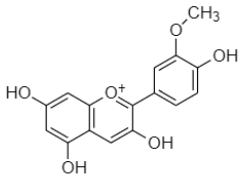
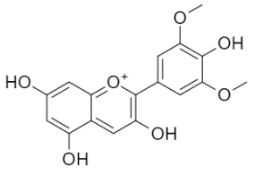
In recent years, blueberries have been deemed a "superfood" by the emergence of supportive scientific research concluding associated health benefits (Kalt, Joseph, and Shukitt-Hale 2007). Specifically, the abundance of polyphenols found in blueberries have been highly regarded for their antioxidant and anti-inflammatory potential (Wei Zheng and Wang 2003; He and Liu 2006; Cirico and Omaye 2006; Duthie, Gardner, and Kyle 2003), as a result of these compounds interacting either independently or synergistically (Seeram et al. 2004). Such effects have been determined to contribute to the prevention and improvement of several chronic diseases, including type 2 diabetes (Martineau et al. 2006; Grace et al. 2009), neurodegenerative diseases (ie. Alzheimers) (Brewer et al. 2010; Joseph et al. 1999; Papandreou et al. 2009), cancer (Bunea et al. 2013; Kraft et al. 2005), and heart disease (Basu et al. 2010; Curtis et al. 2019).

While blueberry consumption specifically is associated with a reduced risk of such diseases, less is known regarding the individual differences in potential health benefits between unique blueberry genotypes. With interest in understanding underlying mechanism by which blueberries deliver on these benefits has grown, phenotyping including phenolic and micronutrient phenotyping have been conducted on select germplasm collections (Howard, Clark, and Brownmiller 2003; Kalt et al. 2001; Connor et al. 2002; Bunea et al. 2013; Grace et al. 2014). Such insights have proven useful to breeding programs interested in enhancing phenolic content and potential health benefits.

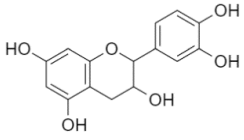
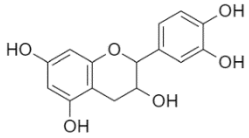
Rising interest in fruits such as the blueberry has resulted in growth of global blueberry production by 40% between the years 2012 and 2016 (North American Blueberry Council (NABC 2017), as did blueberry consumption per capita within the United States. According to the USDA Economic Research Service (ERS), consumption of blueberries increased by 599% from 1994-2014, which was the greatest growth of any other fruit or vegetable.

Figure 1. 2. Representative polyphenols found in blueberries.

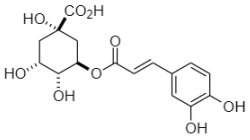
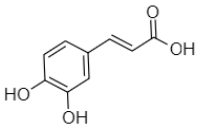
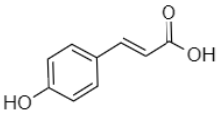
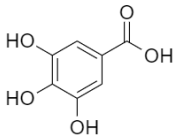
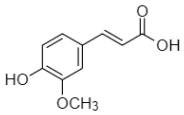
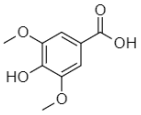
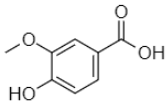
A. Anthocyanin compounds in blueberries.

Polyphenolic Compounds	Structure	References
Anthocyanins		(Correa-Betanzo et al. 2014) (Y. Liu et al. 2014) (Gao and Mazza 1994) (Barnes et al. 2009) (Grace et al. 2014) (Kader et al. 1996)
Delphinidin 3-galactoside 3-galactoside acetylated 3-glucoside 3-glucoside acetylated 3-arabinoside 6-acetyl 3-glucoside		
Cyanidin 3-galactoside 3-galactoside acetylated 3-glucoside 3-glucoside acetylated 3-arabinoside 3-arabinoside acetylated		
Peonidin 3-galactoside 3-galactoside acetylated 3-glucoside 3-glucoside acetylated 3-arabinoside		
Petunidin 3-galactoside 3-galactoside acetylated 3-glucoside 3-glucoside acetylated 3-arabinoside		
Malvidin 3-galactoside 3-galactoside acetylated 3-glucoside 3-glucoside acetylated 3-arabinoside 6-acetyl 3-glucoside		

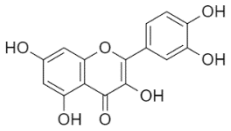
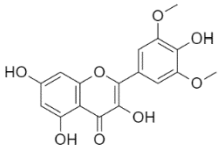
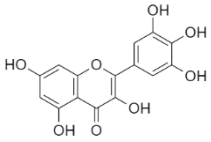
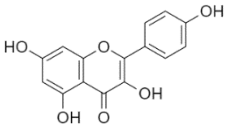
B. Flavan-3-ol compounds in blueberries.

Polyphenolic Compounds	Structure	References
Flavan-3-ols		(Arts, van de Putte, and Hollman 2000) (Grace et al. 2014) (Sellappan, Akoh, and Krewer 2002)
Epicatechin		
Catechin		

C. Phenolic acids in blueberries.

Polyphenolic Compounds	Structure	References
Phenolic Acids		(Correa-Betanzo et al. 2014) (Schuster and Herrmann 1985) (Wei Zheng and Wang 2003) (Gao and Mazza 1994) (Grace et al. 2014) (Sellappan, Akoh, and Krewer 2002)
Chlorogenic Acid		
Caffeic Acid		
P-Coumaric Acid		
Gallic Acid		
Ferulic Acid		
Syringic Acid		
Vanillic Acid		

D. Flavonols in blueberries

Polyphenolic Compounds	Structure	References
Flavonols		(Correa-Betanzo et al. 2014) (Wei Zheng and Wang 2003) (Cho et al. 2004) (Grace et al. 2014) (Sellappan, Akoh, and Krewer 2002) (Kader et al. 1996)
Quercetin 3-galactoside 3-glucoside 3-arabinoside 3-rutinoside 3-xyloside 3-acetyl rhamnoside		
Syringetin 3-galactoside 3-glucoside arabinoside		
Myricetin 3-galactoside 3-glucoside 3-arabinoside 3-rhamnoside		
Kaempferol 3-glucoside		

With interest in enhancing the potential of polyphenols to deliver on these benefits, phenotyping, including phenolic and micronutrient phenotyping, have been conducted on select germplasm collections (Kader et al. 1996; Howard, Clark, and Brownmiller 2003; Kalt et al. 1999; Connor et al. 2002; Timmers et al. 2017; Grace et al. 2014). Such insights have proven useful to blueberry breeding programs, a relatively modern field, which has developed great interest as of late. Common breeding objectives revolve around producing higher yields by improving overall plant strength, in terms of disease resistance and inclement weather, as well as expanding the harvest season. In addition to improving plant vigor, blueberry breeders aim to enhance characteristics like flavor, size, and overall fruit quality (Moore 1965; Retamales and Hancock 2018; Prodorutti et al. 2007; Hancock et al. 2008). However, more recent strategies have narrowed their focus on consumer-oriented fruit quality with the primary goal of improving the nutritional value (Brennan 2008; Graham et al. 2004). Such an objective requires the in-depth investigation of varied germplasms of blueberries to identify traits related to enhanced bioactivity (Scalzo et al. 2005; G. J. McDougall et al. 2005).

1.6. Bioavailability of polyphenols

Given the potential impacts of internal and external factors on the ultimate biological activity of any dietary polyphenol, it would be inaccurate to assume that the most commonly consumed polyphenols or foods that are the highest in polyphenol concentration are the most biologically relevant (Bravo 1998). In this regard, it is critical to consider the ultimate delivery of polyphenols from foods and as such introduce the concept of bioavailability as a factor when considering fruit, vegetables and other polyphenol rich foods and associations to health.

Bioavailability, in regards to phytochemicals from the diet, is defined as the rate and extent to which the active ingredient or active moiety is absorbed from the ingested matrix and becomes

available at the site of action (Neilson and Ferruzzi 2013). In the case of polyphenols bioavailability is reportedly influenced by several factors including both food and host related factors. While an individual's genetics, microbiota, background diet and nutritional and pathophysiological status have all been shown to impact bioavailability of polyphenols (Fairweather-Tait and Southon 2003), food related factors are also critical to consider as they are perhaps most modifiable. In the case of food, factors influencing bioavailability include fruit and vegetable genetics (van der Sluis et al. 2001; DuPont et al. 2000), food formulation (Neilson and Ferruzzi 2011; Tamura et al. 2007; Lesser, Cermak, and Wolffram 2004), as well as type and extent of food processing (Ferracane et al. 2008; Miglio et al. 2008). Additionally, polyphenol chemical structure, intestinal pH, interaction with other compounds, food matrix, environmental factors and availability of digestive enzymes further impact potential release from the food matrix, stability to the gastrointestinal conditions and ultimate absorption and metabolism (Scholz and Williamson 2013; Holst and Williamson 2008). In this context, *bioaccessibility* is defined as the fraction of polyphenols released from the food matrix by normal digestion, are solubilized and recovered in the intestinal lumen and thereby made available for absorption in the intestine (Saura-Calixto, Serrano, and Goñi 2007). This metric encompasses release, stability and absorption potential of polyphenols and is often used as a surrogate for bioavailability in the context of describing food and matrix influences on bioavailability.

1.6.1. Digestion, absorption and metabolism of polyphenols

An overview of polyphenol bioaccessibility and bioavailability/metabolism adapted from Ferruzzi and Neilson (2011) (Neilson and Ferruzzi 2011) can be seen in Figure 1.3. The process of absorption of polyphenols from foods begins with mastication of the food within the oral cavity and the initiation of the digestion process. The combination of chewing (mechanical tissue

disruption) and the introduction of digestive enzymes, primarily alpha amylase, allows for both reduction of food particle size and initiation, to a small degree (Lewandowska et al. 2013), of polyphenol release. Following this initial oral phase, the digesta (DG) enters the gastric phase where a majority of polyphenols will ultimately be released (Neilson and Ferruzzi 2013).

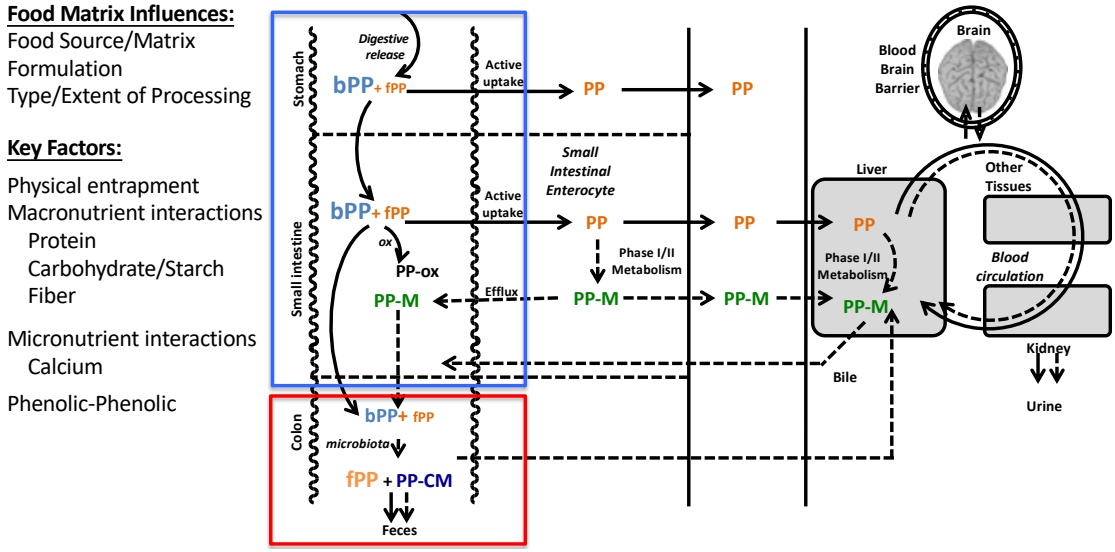


Figure 1. 3. Schematic of the processes that affect systemic bioavailability and metabolism adapted from Ferruzzi and Neilson (2011). [1]

[1]*Abbreviations: bound polyphenols (bPP), free polyphenols (fPP), polyphenol metabolite (host-derived) (PPM), polyphenol colonic metabolite (PPCM)*

Following oral processing and the initiation of digestion, the food bolus is transferred to the stomach where the gastric phase of digestion is initiated. During the gastric phase, polyphenol rich food bolus is acidified to a pH range of 2-4 slowly and exposed to pepsin, the chief digestive enzyme in the stomach (Bohn 2014). The enzyme addition, acidic pH (2-4) and mixing in the stomach further accelerates the breakdown of the food matrix resulting in further release of the polyphenols and exposure to gastric juices and formation of gastric chyme. Most polyphenols are generally considered stable in this phase of digestion, as evidenced by minimal amounts of

degradation present in the literature (Y. Liu et al. 2014; Bermúdez-Soto, Tomás-Barberán, and García-Conesa 2007; Tagliazucchi et al. 2010; Pérez-Vicente, Gil-Izquierdo, and García-Viguera 2002).

Following gastric digestion, the polyphenol rich gastric chyme is slowly released through the pyloric sphincter to the upper small intestine (duodenum) where small intestinal phase of digestion is initiated, including neutralization of the environment to pH of 5.5-7.4. At this stage the largest extent of food disintegration takes place by the actions of enzymes secreted by the pancreas and the liver including phospholipase, sterol esterase, amylase, carboxypeptidase, trypsinogen, chymotrypsinogen, lipase, and bile salts (Bohn 2014). With complete digestion of the food matrix to absorbable macronutrient units, further release of polyphenols will follow, as will increased exposure to the elevated pH of the small intestinal lumen. Within the small intestine, factors regarding chemical structure, degree of glycosylation acylation, derivative type, conjugation with other phenolic acid, molecular size, degree of polymerization, and solubility will heavily influence the stability of polyphenols, their release from undigested plant materials (e.g. cell walls), and their ultimate rate of intestinal absorption (Bravo 1998).

Polyphenols, anthocyanins in particular, are noted to be highly unstable in the intestinal phase, due to the weakly alkaline environment (Correa-Betanzo et al. 2014; Tagliazucchi et al. 2010; Bermúdez-Soto, Tomás-Barberán, and García-Conesa 2007). According to Liu et al. (2014), blueberry anthocyanin content decreased by approximately 42% during simulated small intestinal phase (Y. Liu et al. 2014). Notably, degree of glycosylation impacts potential absorption by affecting chemical, physical and biological properties of the polyphenol. While aglycones can be readily absorbed in the small intestine, most flavonoids are glycosylated or attached to sugars (Tsao 2010). Here the glycosides must be hydrolyzed or cleaved from their sugar-portion to their

corresponding aglycones. Once in a simpler form, polyphenols may be taken up into the enterocytes of either the small or large intestine by means of passive diffusion or facilitated transport (Neilson and Ferruzzi 2013). This process is rather inefficient with typical absorption rates ranging from 0.3-43% (Manach et al. 2005), while a significant portion of polyphenols from fruits and vegetables remain in the gut lumen. The remaining unabsorbed phenolics continue to the lower intestinal compartments (colon), where they are subject to interactions with native microbiota which are capable of metabolizing polyphenols to a broad series of metabolites including smaller molecular weight phenolic derivatives that are readily absorbed and metabolized.

Once absorbed either in the upper or lower intestines, polyphenols or their microbial metabolites, can be further metabolized by host systems to form conjugates including methyl, glycine, glucuronic acid, or sulfate (Manach et al. 2004). The formation of metabolic conjugates can dramatically alter the biological properties of the circulating metabolites. These metabolites can move within bloodstream or be transported to reach target tissues via plasma proteins (Dangles et al. 2001), or lipoproteins (Meng et al. 1999). Following transport in the bloodstream, polyphenols can be distributed in most tissues and can cross the blood-brain barrier (Andres-Lacueva et al. 2005). Ultimately, phenolic metabolites are excreted by means of the urinary route or the biliary route. As such urine is a common target to assess ultimate flux of polyphenols through humans.

1.7. Current models for assessing bioavailability

Various techniques to evaluate the bioavailability of polyphenols are leveraged today. However, the best estimate is likely derived by combining the results from several different reliable methods (Fairweather-Tait and Southon 2003). In order to precisely evaluate bioavailability

directly, researchers utilize human subjects and clinical testing as part of a comprehensive *in vivo* approach. However, given their associated high cost and logistical challenges or experimental/mechanistic questions not possible or ethical to conduct in humans, investigators do choose alternative animal models as they have repeatedly proven valuable as a predictive measure of bioavailability, metabolism and tissue distribution of polyphenols within humans.

In vivo bioavailability studies commonly incorporate pharmacokinetic (PK) analyses, which encompass the absorption, distribution, metabolism, and excretion of a compound over time (Turfus et al. 2017). Common parameters of PK are utilized in bioavailability research include maximal plasma concentration (Cmax), time to reach Cmax, area under the plasma concentration-time curve, elimination half-life, and relative urinary excretion (Manach et al. 2005). Many researchers have investigated the combination of PK and extent of polyphenol absorption by assessing direct measurements of a specific compound or its metabolites after the ingestion of a single dose, provided as pure compound, plant extract, or whole food/beverage (Manach et al. 2005; Fairweather-Tait and Southon 2003).

1.7.1. *In vivo* approaches to measure polyphenol bioavailability (Human Clinical and Preclinical Animal models)

Human clinical studies remain the "gold standard" for assessment of polyphenol bioavailability and metabolism. In assessing bioavailability, human clinical trial designs leverage two general approaches that rely on assessing response to an oral challenge in an acute or chronic paradigm followed by collection and assessment of polyphenol metabolites in blood or urine (Fairweather-Tait and Southon 2003; Manach et al. 2005). This allows for acute (0-48h) pharmacokinetic responses to oral challenges to be constructed facilitating comparisons between food matrices and formulations (Manach et al. 2005). Longer more chronic feeding trials offer the

possibility to assess changes in pharmacokinetics as well as metabolism through monitoring of blood or urinary responses to long term feeding. Tissue distribution is not commonly seen in human clinical trials, although reports of phenolics in human tissues beyond blood and urinary include: human milk (Franke, Custer, and Tanaka 1998; B. J. Song, Jouni, and Ferruzzi 2013), prostate tissue (Henning et al. 2006), breast tissue (Maubach et al. 2003), and colorectal tissue (Garcea et al. 2005). In many trials, participants are often controlled in terms of factors that can impact absorption and metabolism. These factors include nutrition status, body weight, presence of disease or other metabolic disorder, typical polyphenol dietary exposure, supplement and drug use as well as exercise (Ting et al. 2015). While clinical trials are regarded as the best method of assessing human bioavailability, they remain expensive and time consuming. Furthermore, in the context of screening large germplasm collections and proving phenotypic data on bioavailability, they remain limited in their practical application as only a small number of samples can effectively be run at a time without complicating the study design and eventual statistical comparisons.

As an alternative to human clinical settings, animal models remain widely applicable and relevant as a model for human bioavailability and metabolism of polyphenols (Hunt and Roughead 2003). The literature is rich in animal models for polyphenol bioavailability studies, most commonly including rodents (rat or mice), pig, dog, and rabbits (KARAKAYA 2004; Crespy et al. 2002; Breinholt et al. 2000; Demrow, Slane, and Folts 1995; Nielsen et al. 2003; Wu et al. 2005; Hole et al. 2013; Cladis et al. 2019; Janle et al. 2014; Lesser, Cermak, and Wolffram 2004; Chen et al. 2015; Ferruzzi et al. 2009; Pawlowski et al. 2014). Of these the rodent and porcine models are most often used to assess both pharmacokinetic and tissue distribution of polyphenol metabolites (KARAKAYA 2004; Janle et al. 2014). Animals offer the advantages of highly defined nutritional and pathophysiological status, genetic uniformity and full control of

background and challenge diets (Hunt and Roughead 2003). Their relevance to humans and human systems for absorption and metabolism, including digestive processing and transport/metabolism of polyphenols is critical to consider. While the correlation between animals and human trials can be tenuous at times (Shanks, Greek, and Greek 2009), valuable insights into tissue distribution including to liver, brain, intestinal tract and other relevant targets remain perhaps one of the most important aspects of animal research models as they place polyphenol metabolites at the target tissues enabling mechanistic linkages between phenolic forms and disease prevention (Ferruzzi et al. 2009; J. Wang et al. 2013; Chen et al. 2015). Therefore, advantages of animal models include the fact that they offer a living system imitating the full dynamic physiological and physiochemical events during the absorption, distribution, metabolism, and elimination of ingested polyphenols without the cost associated with human clinical trials (Ting et al. 2015). However, differences in body weight, organ size, hepatic/renal blood low, metabolism, distribution and elimination rate have caused significant variation amongst these different animal species that must be accounted for (Ting et al. 2015; Hunt and Roughead 2003). Further, while relevant animal models such as rodents have been used to screen for genotypic differences in bioavailability including for blueberries (Maiz et al. 2016), these models are still limited in throughput, similarly to human clinal trials. While cost may not be as high, ethical considerations in the use of animals must be included as a limitation.

1.7.2. *In vitro* screening of bioaccessibility as a surrogate for bioavailability

As previously mentioned, a prerequisite for bioavailability of phenolics is their bioaccessibility. Consideration of their release from a food matrix by the actions of normal digestion and stability/solubility in the gut lumen is both predictive of ultimate absorption in the

small intestine and also potential availability for interaction by gut microbial communities and conversion to relevant metabolites in the lower gastrointestinal tract.

Unlike bioavailability, bioaccessibility of polyphenols can and is often determined by use of *in vitro* methods. In particular, *in vitro* digestion models are designed to simulate the conditions of the human gastrointestinal tract as they provide a valuable tool for characterization and prediction of release, solubility and stability to physiological/digestive conditions that are relevant to *in vivo* pharmacokinetics (Hur et al. 2011). Based on predetermined conditions of the upper and lower gastrointestinal tract, these models range from relatively simple to complex, static to dynamic, and inexpensive to expensive. However, relative to animal or human studies these models are generally rapid, reproducible, and allow for comparative screening of food matrix and formulation effects (Bohn et al. 2015). Additionally, their design makes them well suited to screening larger sample throughput. However, as is the case with most models, the relevance to bioavailability in humans always requires some level of validation (Fairweather-Tait and Southon 2003).

In general, in vitro digestion models fall under two categories, static/compartmental and dynamic models. For the context of this review we will consider two common *in vitro* models used to measure bioaccessibility of phenolics, including a multicompartmental static model and the dynamic TNO gastrointestinal model (TIM-1). These models focus on the upper (oral, gastric and small intestinal phases of digestion and do not include microbial fermentation. While several in vitro models do focus on or include the lower intestinal phase of digestion (e.g. TIM-2, SHIME) (Van de Wiele et al. 2015), they will not be the focus of this review.

A. Multicompartmental static gastrointestinal models

Several multicompartmental static *in vitro* digestion models have been proposed for the investigation of micronutrient bioaccessibility. These models simulate the physiochemical condition commonly beginning within the mouth, followed by the stomach, and ending in the small intestine (Thakkar et al. 2007). Historically, a static *in vitro* model was developed and utilized to assess the bioavailability of minerals, such as iron (D. D. Miller et al. 1981; Gangloff et al. 1996) and phosphorous (J. Liu, Ledoux, and Veum 1998) from foods. To evaluate iron bioaccessibility, Miller et al. (1981) relied on gastric and small intestinal digestion followed by dialysis of digestate to resolve bioaccessible iron (as dialyzable iron (D. D. Miller et al. 1981). Having found good correlation to *in vivo* iron bioavailability (r=0.93-0.99) (D. D. Miller et al. 1981) interest in adaptation of these models to other micronutrients and phytochemicals expanded. In 1999, Garrett et al. adapted conditions in the model of Miller et al. (1981) to characterize bioaccessibility of fat-soluble carotenoids from fruits and vegetables (Garrett, Failla, and Sarama 1999). In their model, Garrett et al. (1999) focused on mimicking gastric and small intestinal conditions to digest fruits and vegetables in the presence of added dietary lipid to facilitate release and transfer of fat-soluble carotenoids to bile salt lipid micelles. This process of micellarization is key to solubilization of carotenoids in the small intestinal lumen and allows for easy determination of their fractional bioaccessibility (Garrett, Failla, and Sarama 1999). The model was further validated against human clinical responses and found to be highly correlated to the bioavailability of α- and γ-tocopherol, β-carotene, lycopene, and lutein (r=0.98) in humans (Reboul et al. 2006), therefore suggesting its potential use as a predictive tool to assess food matrix and formulation factors that could influence bioavailability of these fat solubles.

Over the years, the model has been further refined and adapted to successfully evaluate bioavailability of various sources (Table 1.2) of polyphenols. Parameters of the Garrett et al. (1999), model were successfully modified by our group to screen the effects of formulation factors on the bioaccessibility of flavan-3-ols from green tea (Green, Murphy, and Ferruzzi 2007). Compared to solubility, the main driver of bioaccessibility was observed to be modification of the chemical stability of flavan-3-ols to elevated pH in small intestinal phases that fostered auto-oxidative reactions even under low oxygen tensions (Neilson et al. 2007). These findings led to a comparative assessment in vitro and in vivo (in a rodent model) of green tea formulation factors whereby the in vitro model showed high predictability to in vivo plasma pharmacokinetics (Peters et al. 2010).

Subsequent adaptation of our manual three compartment static *in vitro* model (Figure 1.4, Table 1.3), described by our group to assess phenolics from fruits and juices was further adapted by Moser et al. (2016) (Moser et al. 2016). This model is essentially a derivative of previous models, including that of Garrett et al. (1999), but adapted for consideration of digestion of high carbohydrate foods and beverages. The models rely on commercially available enzyme sources and are executed in 15 or 50 mL test tubes. Inclusion of an oral phase and amylase treatment was a critical adaptation applied for assessment of starchy foods (Thakkar et al. 2007), such as cereal grains (Kean, Hamaker, and Ferruzzi 2008), and continued in the Moser et al. (2016) model. This ultimately provided a broader model suitable for screening of solid and liquid foods to assess the impact of food structure/form as well as formulation on polyphenol bioaccessibility (Moser et al. 2016).This model has been applied by our group for the assessment of these factors across a wide array of foods including tea (Moser et al. 2014; Green, Murphy, and Ferruzzi 2007), wholegrains (Li et al. 2016), and grape juice (Moser et al. 2016).

Table 1. 2. Various polyphenol sources subjected to a static, in-vitro gastrointestinal model.

Plant Source	Relative Bioaccessibility (%)	Reference
Blackberry (Rubus sp.)	Total phenolics: 27.5% Anthocyanins: 10% Quercetin derivatives: 40-80% ET: 30-40%	(Tavares et al. 2012)
Red Wine	Phenols: 39.7% Anthocyanins: 34.1%	(G. J. McDougall et al. 2005)
Orange Juice	Flavanones: 40-42%	(Gil-Izquierdo, Zafrilla, and Tomas-Barberan 2002)
Strawberry	Anthocyanins: 9.5-18.7% Phenolic acids: 46.1-827% Flavonols: 56.5-71.7%	
Strawberry Jam	Anthocyanins:15.9-24% Phenolic acids: 46.7-139.7% Flavonols: 39.7-84%	
Wild Blueberry	Chlorogenic acid: 118.6% Flavonols: 45.4-74.3% Anthocyanins: 4.4-37.3%	(Correa-Betanzo et al. 2014)
Grape	Total phenolics: 62.4% Total flavonoids: 56.1% Anthocyanins: 7.6% Non-flavonoids: 67.8%	(Tagliazucchi et al. 2010)
Wild Blueberry	Anthocyanins: 42.36-64.4%	(Y. Liu et al. 2014)
Cultivated Blueberry	Polyphenols: 141.7% Anthocyanins: 3.4%% Flavonoids: 161.4%	(Jiao et al. 2018)
Grape Juice	Anthocyanins: 3.7-37.2% Phenolic acids: 22.0-56.0% Flavan-3-ols:11.8-27.4% Flavonols: 1.7-21.7% Stilbenes: 8.1-26.6%	(Moser et al. 2016)
Chokeberry	Anthocyanins: 57.4% Flavonols: 73.5% Chlorogenic acid: 76.1% Flavan-3-ols: 80.7%	(Bermúdez-Soto, Tomás-Barberán, and García-Conesa 2007)
Pomegranate	Anthocyanins: 15.3% Anthocyanins (acidified): 48% Total phenolics: 61%	(Pérez-Vicente, Gil-Izquierdo, and García-Viguera 2002)
Andean Berry	Phenolic Acids: 25.85-2482.4% Flavonoids: 23.82-119.7%	(Agudelo et al. 2018)
Raspberry	Anthocyanins: 61.9% Phenols: 75.8%	(Gordon J. McDougall et al. 2005)
Green Tea	Flavan-3-ols: 80.4%	(Green, Murphy, and Ferruzzi 2007)
Whole Grain Oats	Phenolic acids: 0.3-6.3% Avenanthramides: 3.4-83.8%	(Li et al. 2016)
Red Cabbage	Phenols: 24.9% Anthocyanins: 79.3%	(Gordon J. McDougall et al. 2007)

Introduction to this model begins with the selection and processing of the polyphenol source, such as a fruit, vegetable, or beverage (ie. tea, wine, coffee, etc.). In the case of whole foods, they can either be mechanically processed orally (Walsh et al. 2003) or foods can be homogenized and introduced as semi solid or liquid aliquots to the oral phase of digestion. The oral phase includes exposure to the enzyme alpha-amylase, but also includes exposure to simulated saliva containing electrolytes and mucin (Oomen et al. 2003). The combination of oral phase and test material is then subjected to gentle mixing in a temperature-controlled incubator for 10 min to ensure proper exposure. The primary goal of this phase is not to mimic fully the conditions of the mouth, but rather the broader extent of oral digestion which continues with amylase activity following transit to the stomach for a period of time (Oomen et al. 2003; Thakkar et al. 2007).

Following oral digestion, the gastric phase is initiated by adjustment of the bolus pH to between 2 and 3.5 and addition of a porcine pepsin solution. Gastric digestion marks the beginning of protein breakdown and the initial disintegration of whole food matrices. Gastric phase can be adjusted to last between 30-120min, however 60 minutes is considered a standard by most models (Garrett, Failla, and Sarama 1999; Green, Murphy, and Ferruzzi 2007). Following gastric digestion, the small intestinal phase is initiated by pH adjustment of the chyme to 5.5-7.4 with NaHCO3. After, a cocktail containing small intestinal enzymes (pancreatin, lipase, and porcine bile salts) is added to the reaction. Sample volume is then standardized, and the intestinal phase of digestion is continued under gentle mixing for 2 hours at 37^0C. Following completion of the oral, gastric and small intestinal digestion, finished digesta is typically centrifuged and filtered to isolate the aqueous bioaccessible fractions. Bioaccessibility is then determined by quantification of the phenolics in the aqueous fraction relative to the starting content in the raw material (Green, Murphy, and Ferruzzi 2007).

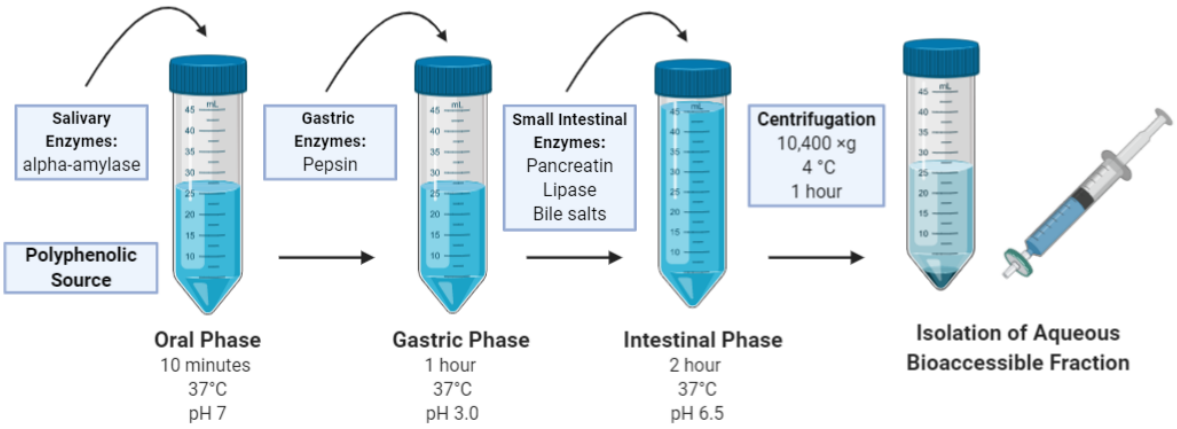


Figure 1. 4. Diagram of static, 3-stage in vitro gastrointestinal model adapted from Garrett et al. (1999) by Moser et al. (2016) for polyphenols.

As previously mentioned, the *in vitro* static model possesses several advantages. However, its inability to reproduce the dynamic *in vivo* physical conditions, including the peristalsis motion of the gastrointestinal tract, integration of all the influential physiological factors, transitional change in the physiological environment, and elimination from the absorption site, are amongst its disadvantages. Consequently, the bioaccessibility predicted by static models can often overestimate the oral efficiency (Ting et al. 2015) and, by extension, actual bioavailability. However, comparative assessment and use of control samples remains key to their ability to effectively screen and rank samples for changes in bioaccessibility.

B. TNO Gastrointestinal model (TIM)

The TNO gastro-intestinal model, commonly referred to as the TIM is a dynamic, continuous *in vitro* digestion process that is characterized the utilization of an automated computer system to accurately dictate physiochemical factors (Minekus et al. 1995). Similar to the static *in vitro* model, the TIM has proven high predictability as compared to *in vivo* data (Minekus et al. 1995), particularly enhanced when paired with transport data obtained with intestinal cells and/or in silico modeling. The TIM was created in the 1990's by TNO Nutrition and Food Research

(Zeist, The Netherlands) to fulfill the high demand for to study food products under more physiologically relevant conditions as compared to contemporary digestion models (Minekus 2015).

The most common configuration of the TIM platform currently utilized is the TIM-1 (Figure 1-5). The TIM-1 consists of four physiological compartments (stomach, duodenum, jejunum and ileum) that are inter-connected by peristaltic valve pumps that enable the transfer of appropriate of amounts of partially digested food and digestive secretions (Minekus et al. 1995; Minekus 2015). TIM fulfills all requirements necessary to simulate in vivo dynamic physiological processes, such as: consecutive addition of enzymes in proper physiological volumes and concentrations, adjustment of appropriate pH required for enzymes used of enzymes in physiological amounts and addition of relevant cofactors, removal of digestion products, proper mixing during digestion, and appropriate transit times for each step of digestion (Blanquet et al. 2004; Minekus 2015).

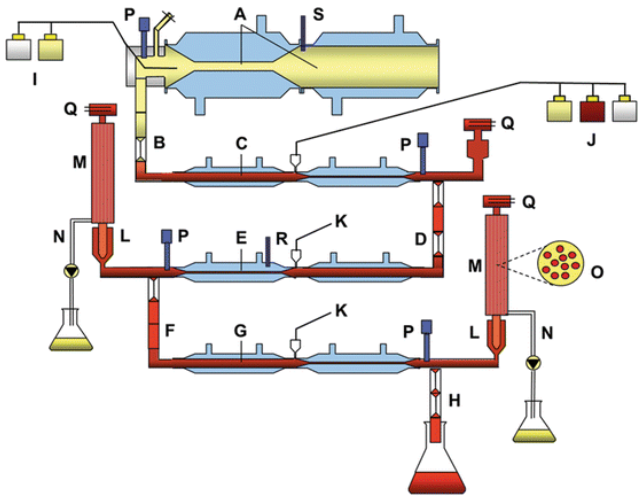


Figure 1. 5. Schematic presentation of TIM-1 adapted from (Minekus 2015). [1]

[1] *Diagram key: A. gastric compartment; B. pyloric sphincter; C. duodenal compartment; D. peristaltic valve; E. jejunal compartment; F. peristaltic valve; G. ileal compartment; H. ileal-cecal valve; I. gastric secretion; J. duodenal secretion; K. bicarbonate secretion; L. pre-filter; M. filtration system; N. filtrate with bio-accessible fraction; O. hollow fiber system (cross section); P. pH electrodes; Q. level sensors; R. temperature sensors; S. pressure sensor*

The TIM, pre-programmed *in vitro* process described by Minekus et al. (2015) and Anson et al. (2009) (Table 1.3) begins with the oral phase which includes the mastication of the food sample via a food processor and mixed with an artificial alpha-amylase-containing saliva before entering the gastric phase (Minekus 2015; Anson et al. 2009). Once in the gastric phase, a replicated gastric lipase (electrolytes, pepsin, fungal-lipase) is added to the digesta. To account for the acidic environment of the human stomach, the pH is adjusted with hydrochloric acid prior to transit to the intestinal phase. The intestinal phase is marked by the introduction of duodenal secretion (electrolytes, bile and pancreatin). Again, to account for the more basic intestinal environment, the pH is controlled at pre-set values for each compartment with sodium bicarbonate. Following the intestinal, water-soluble products are removed via dialysis membranes connected to the jejunal and ileal compartments, while lipophilic products are filtered to pass micelles yet retain droplets of fat. Ultimately, bioaccessibility of targeted nutrients is determined by measuring the fraction of a compound that has successfully passed through aforementioned dialysis or filtration membrane.

While the TIM is robust and efficient, there are associated disadvantages. Because conditions are pre-programmed into the TIM, there is much room for assumption and, therefore, associated error. However, conditions are completely dependent on published *in vivo* data for the meal type and can be adapted when necessary (Minekus et al. 1995). Additionally, the costly TIM is characterized by its inability to perform high throughput analysis compared to the static model. Lastly, compared to in vivo models, the TIM, although complex, is not fully predictive of bioavailability. This is due to its obvious lack of biological factors, including enterocyte absorption, systemic metabolism, and volume of body distribution (Ting et al. 2015).

Table 1. 3. Comparison of two in vitro models- static versus dynamic.

***In vitro* model**	**Multi-compartment static gastrointestinal model** *Adapted from Moser et al. (2016)*	**TNO dynamic gastrointestinal model (TIM-1)** *Adapted from Minekus (2015) and Anson et al. (2009)*
Brief description	• Static 3-stage digestion model carried out through manual liquid-handling via pipette into reaction test tube • Temperature is controlled during incubation periods • Mixing is achieved by utilizing vortex	• Dynamic 4-compartmental (connected by peristaltic valve pumps) digestion model, all execution and parameters controlled by computer • Liquid-handling achieved by computerized syringe-pumps • Mixing enabled by alternating pressure on flexible walls
Oral phase	• Addition of oral phase solution (containing alpha-amylase) to test material • Reaction tube vortexed, blanketed with nitrogen, placed in oscillating incubator (37 °C, 85 OPM, 10 min)	• Meal is mixed with artificial saliva (containing enzymes and alpha-amylase) • Temperature maintained at 37 °C
Gastric phase	• Addition of porcine pepsin solution • Adjustment of pH to 3.0±0.1 with 1.0 M HCl • Addition of 0.9% saline solution to volume • Reaction tube vortexed, blanketed with nitrogen, placed in oscillating incubator (37 °C, 85 OPM, 1 h)	• Addition of gastric secretion (electrolytes, pepsin, fungal lipase) • Adjustment of pH with HCl (1M) to follow a predetermined curve/or at a variable rate in time • Half-time of stomach emptying ranged between 35-70 min at 37 °C
Intestinal phase	• Adjustment of pH to 6.5±0.1 with 1.0 M NaHCO3 • Addition of small intestinal enzyme solution (pancreatin, lipase) and porcine bile salt solution • Standardized with 0.9% saline solution • Reaction tube vortexed, blanked with nitrogen, placed in oscillating incubator (37 °C, 85 OPM, 2 h)	***Duodenum:*** • Addition of duodenal secretion (electrolytes, bile, pancreatin) • Adjustment of pH with sodium bicarbonate (1M) at pre-set values (~6.5) for each compartment • Temperature maintained at 37 °C ***Jejunum and Ileum:*** • Compartments connected by hollow-fiber devices meant to absorb digestion productions and water • Water-soluble products are removed via dialysis through membranes • Lipophilic products filtered (50nm) filter • Half-time of ileal delivery ranged between 85-160 min)
Isolation of aqueous (AQ) bioaccessible fraction	• Reactions are centrifuged (10,400 ×*g*, 4 °C, 1 h) • AQ small intestinal digesta collected • Filtered AQ with PTFE 0.22um filter	• Collected aqueous fraction that passed through dialysis membrane and 50nm filter

[1]*Abbreviations: aqueous (AQ), oscillations per minute (OPM)*

C. Bioaccessibility as a phenotypic trait and potential of existing models for screening germplasm collections

With availability of *in vitro* digestion models, there has been interest in leveraging these techniques to develop insights into bioaccessibility of micronutrients and phytochemicals as

phenotypic traits appropriate to guide breeding and processing practices that could enhance bioavailability. Application of static models to screen variation in bioaccessibility has been noted in the identification of iron bioaccessibility as a genetically variable trait in maize (Šimić et al. 2009) as well as variations in beta-carotene bioaccessibility between biofortified cassava genotypes (J. Aragón et al. 2018). Similarly, Lipkie et al. (2013) assessed the delivery of provitamin A carotenoids from a limited set transgenic biofortified sorghum genotypes (Lipkie et al. 2013). These efforts served as a support to scientist breeding biofortified grains for at risk populations. Regarding polyphenols, such a model has recently been utilized to classify phenolic bioaccessibility as a genetically variable trait amongst 10 different oat cultivars (Li et al. 2016). Of these studies, the extent of throughput was seen to be relatively limited and not suitable for broader genomic work. The greatest application was observed within the assessment of phenolics in oats and the beta-carotene in the biofortified cassava as they both examined 10 different genotypes (Li et al. 2016; J. Aragón et al. 2018). Therefore, to gain a more comprehensive understanding of bioaccessibility as a genetically variable trait, more genotypes will need to be analyzed. Additionally, when observing variation in bioaccessibility, it is difficult to attribute variation to genotype solely, as processing and nutrient composition influence bioaccessibility as well.

While having proved promising for relatively limited comparisons and sample sizes, interest to expand to phenotyping of large germplasm collection suitable for genomic studies (100's to 1000's of genotypes) has grown. However, in practice, limitations of current digestion models remain. Specifically, while dynamic models such as the previously discussed TIM are most similar to humans, they remain very limited in throughput and high in cost. Considering the need for low cost, rapid, high-throughput models, the static models offers the potential for adaptation to

high throughput workflows. However as currently designed, they remain reliant on manual execution and are time-consuming. Furthermore, they typically require large quantities of sample and associated materials, thus constituting a challenge for germplasm collections and creating a bottleneck for high-throughput analysis (Hansen et al. 2009). While such models are considered relatively high-throughput, low cost system (Beiseigel et al. 2007), the turn-out of several hundred samples over a few months is limiting the potential to leverage variation of traits related to bioavailability. Therefore, to develop insights into genetic influences of bioaccessibility, a model able to screen larger germplasm collections is necessary.

1.8. Research Objectives

To advance our ability to improve delivery of fruit and vegetable bioactives, our ***overall objective*** is to develop high throughput *in vitro* digestion model suitable for screening of large germplasm collections of target plant foods for phytochemical bioaccessibility. We propose to develop and validate an automated static three compartment in vitro gastrointestinal model that has high correlation to established models and in vivo bioavailability with a focus on blueberry and blueberry phenolics.

Aim 1: Adapt an existing in vitro digestion model for high throughput screening of polyphenol bioaccessibility from blueberry fruit.

Working Hypothesis: *In vitro* digestive conditions previously developed for the screening of polyphenols by Moser et al. (2016) can be adapted to semi-automation by use of a liquid-handling robot (Tecan Evo 150) and sample size and volume reductions to increase throughput from approximately a maximum of 24 samples per day to 96+ samples per day while maintaining similar predictive bioaccessibility capacity.

General approach: Bioaccessibility of blueberry polyphenols will be assessed using a three-stage *in vitro* digestion with compartmental oral, gastric and small intestinal conditions previously described by Moser et al. (2016).This model will be adapted for automation and volumes using the Tecan EVO 150 fluid handling robot for semi-automation and enhanced throughput. Current static model throughput is typically in the 10's per day, while our goal is to automate 100+ per day.

Aim 2: Assess the potential for variation in phenolic bioaccessibility from a limited selection (n=33) of blueberry genotypes derived form a mapping population DraperXJewel (DxJ).

Working hypothesis: The developed adapted model will serve as an efficient method for screening large populations of blueberry enabling the potential to leverage variation of traits related to bioavailability.

General Approach: Phenotyping of bioaccessibility traits will be analyzed in a small population (n=33) of blueberries chosen from a select mapping population (DXJ) by applying the best developed modified high-throughput method, based on the information discussed in Aim 1. Variation between blueberry genotypes as well as differences in phenolic profile and bioaccessibility data (relative and absolute) will be assessed.

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CHAPTER 2. DEVELOPING A HIGH THROUGHPUT IN VITRO DIGESTION MODEL TO PHENOTYPE BIOACCESSIBILITY OF HIGHBUSH BLUEBERRIES

2.1. Introduction

The Dietary Guidelines for Americans has consistently based recommendations for consumption of plant foods including fruit and vegetable on evidence for their role in meeting nutrient needs and based on the growing evidence of their association with chronic disease prevention (USDA. 2015 – 2020 Dietary Guidelines for Americans. 8th Edition, 2015). While micronutrient content of fruits and vegetables is critical to meeting select nutrient needs, phytochemicals including (poly)phenolics found in many plant foods have also been associated with prevention of cardiovascular disease (Basu et al. 2010; Bazzano et al. 2002; Curtis et al. 2019), neurodegenerative disorders (Joseph et al. 1999; Tavares et al. 2012; Papandreou et al. 2009; Krikorian et al. 2010), cancer (Bagchi et al. 2004; Bunea et al. 2013; Damianaki et al. 2000; Yi et al. 2005), obesity (Stull et al. 2010; Prior et al. 2010; Vuong et al. 2009), and diabetes (van Dam and Feskens 2002; Grace et al. 2009; Fan et al. 2013). Interest in so called "superfoods" has been driven by a combination of micronutrient and phytochemical content and the notion that phytochemicals, such as phenolics, may have the ability to alter both oxidative and inflammatory stress associated with these disorders (Triebel, Trieu, and Richling 2012; Zheng and Wang 2003; Ghosh et al. 2006).

Blueberries (*Vaccinium Sp.)* are one such "superfood" that have received much attention by virtue of their diverse array and relatively high concentration of phenolic compounds including anthocyanins (ANC), phenolic acids (PA), flavonols (FLAV) and flavan-3ols (F3L) (Wilhelmina Kalt, Joseph, and Shukitt-Hale 2007; Smith et al. 2000; Kong et al. 2003; W. Kalt, McDonald, and Donner 2000). Blueberries belong to three distinct species within the genus *Vaccinium* section (or sub-genus) *Cyanococcus* (Ericaceae family); the *vaccinium angustifolium* (lowbush blueberry),

the *vaccinium virgatum* (rabbiteye blueberry) and the *vaccinium corymbosum* (northern highbush blueberry) (Retamales and Hancock 2018). All species are native to North American and have been commercially produced for many years (Retamales and Hancock 2018) Specifically, lowbush blueberries are produced mostly in eastern Canada and the northeastern United States (US), rabbiteye are localized in the southern parts of the US, while highbush are found in temperate regions throughout North America (Retamales and Hancock 2018). Lowbush "wild" blueberries are composed of many genetically and phenotypically different varieties (W. Kalt, McDonald, and Donner 2000) and are known to have a greater antioxidant capacity and a naturally smaller and more compact (less moisture) composition compared to its cultivated counterpart (Wild Blueberries of North American, 2019).

While blueberry consumption specifically is associated with a reduced risk of diabetes (Martineau et al. 2006; Grace et al. 2009), cardiovascular disease (Basu et al. 2010; Curtis et al. 2019), cancer (Bunea et al. 2013; Kraft et al. 2005), as well as Alzheimer's disease and other neurodegenerative processes (Papandreou et al. 2009; Brewer et al. 2010), less is known regarding the individual differences in potential health benefits between unique blueberry genotypes. With growing interest in understanding the underlying mechanisms by which blueberries deliver on these benefits, phenotyping including phenolic and micronutrient phenotyping have been conducted on select germplasm collections (Howard, Clark, and Brownmiller 2003; Wilhelmina Kalt et al. 2001; Connor et al. 2002; Bunea et al. 2013; Grace et al. 2014). In general, insight provided from phenotyping select germplasms have proven useful to breeding programs interested in enhancing the phenolic content and potential health benefits of various plant materials, including berries (Brennan 2008; Graham et al. 2004).

While promising, health benefits from polyphenol consumption are dependent not only on the qualitative and quantitative profiles in the fruit, but perhaps most critically on their efficiency of absorption and metabolism (bioavailability) by the human body. Enhancement of phenolic content does not always translate to higher bioavailability (Scalzo et al. 2005; G. J. McDougall et al. 2005). Maiz et al. (2016), recently reported that bioavailability of phenolics differed significantly between nine individual blueberry genotypes in a preclinical rodent model (Maiz et al. 2016). The reason for these differences remains unclear but could be critical to understanding distinct bioactivity differences between these berries.

While existing breeding programs seek to enhance our understanding of factors impacting fruit phenolic profiles and content (Prodorutti et al. 2007), little is known regarding traits that are associated with phenolic bioavailability. One reason for this remains the notion that bioavailability is a "trait" best screened using costly and time-consuming *in vivo* models that can compare only small numbers of genotypically distinct fruits (G. J. McDougall et al. 2005). Therefore, the potential to leverage variation in traits related to bioavailability remains untapped due to a lack of physiologically relevant phenotyping methods appropriate for screening large populations.

The use of *in vitro* digestion models to estimate nutrient and phytochemical bioavailability from foods has been previously established by our group and others (Lipkie et al. 2013; Moser et al. 2016; G. J. McDougall et al. 2005; Tagliazucchi et al. 2010; Bermúdez-Soto, Tomás-Barberán, and García-Conesa 2007). Relying on predetermined human physiological conditions, these *in vitro* models measure bioaccessibility, the digestive release and solubilization of individual micronutrients or phytochemicals (Garrett, Failla, and Sarama 1999; Miller et al. 1981). In this fashion they estimate the amount of micronutrient or phytochemical made available for absorption and have demonstrated high correlation to bioavailability in humans (Reboul et al. 2006; Peters et

al. 2010). With the potential to screen matrix and processing factors that impact on digestion and intestinal absorption of nutrients and bioactives (Neilson and Ferruzzi 2011; Neilson et al. 2009), these models have more recently been used to identify iron bioaccessibility as a genetically variable trait in maize (Šimić et al. 2009) and variations in beta-carotene bioaccessibility among biofortified cassava genotypes (J. Aragón et al. 2018). These efforts served as a support to scientist breeding biofortified grains for at risk populations (J. Aragón et al. 2018; Lipkie et al. 2013; Pixley, Palacios-Rojas, and Glahn 2011).

While promising, current digestion models remain dependent on manual processing with larger sample volumes, thus making them somewhat inefficient for screening larger germplasm collections required to develop insights into genetic influences of bioaccessibility. Therefore, given the interest in developing a bioaccessibility phenotype across larger germplasm collections, a need exists to improve the throughput of existing *in vitro* digestion models. With this in mind, the main objective of the current study was to adapt and semi-automate an existing static three-compartment *in vitro* gastrointestinal digestion model for high throughput (HT) phenotyping of the bioaccessibility of blueberry phenolics. Following validation of the HT model against the standard, low throughput *in vitro* digestion models (LT), the HT model was applied to a subset of 33 individual blueberry genotypes from a mapping population currently used in phenolic research.

2.2. Materials and Methods

2.2.1. Chemicals, solutions and standards

Chromatography solvents (acetonitrile, methanol, water and formic acid) and phenolic standards (cyanidin 3-O-glucoside, delphinidin 3-O-glucoside, peonidin 3-O-glucoside, petunidin 3-O-glucoside, malvidin 3-O-glucoside, catechin, epicatechin, caffeic acid, vanillic acid, chlorogenic acid, syringetin and quercetin 3-O-glucoside) were purchased from Sigma Aldrich (St.

Louis, MO, USA). Additional standards, ethyl gallate and taxifolin, utilized for control purposes were purchased from TCI (Portland, OR, USA) and Selleckchem (Houston, TX, USA), respectively. Materials required for the simulated digestion including mucin (M2378), a-amylase (A3176), pepsin (P7125), lipase (L3126), pancreatin (P7545), bile (B8631) were purchased from Sigma Aldrich, while urea (U15-500) and uric acid (A13346-14) were purchased from Fisher Chemical (Fair Lawn, NJ, USA). Materials used for adjusting pH during in vitro digestion model, including HCl and NaHCO3 were purchased from Fisher Chemical. NaCl for adjusting volume throughout the digestion was purchased from Fisher Chemical.

2.2.2. Blueberry material

Initial *in vitro* digestion method adaptation and validation was completed with commercial store-bought, cultivated blueberries obtained from a local market in Kannapolis, NC. Following adaptation and validation, the HT method was applied to a subset of 33 individual blueberry genotypes derived from a mapping population generated by crosses of two elite, highbush blueberry cultivars, Draper and Jewel (DxJ). Select DxJ genotypes were harvested in June of 2017 (Corvallis, Oregon) and shipped frozen to the North Carolina State University Plants for Human Health Institute in Kannapolis, NC. All materials were stored frozen at -80ºC until further processing prior to digestion.

2.2.3. Blueberry processing for digestions

Frozen blueberries (85-100g) were thawed for approximately 30 minutes and coarsely blended (Waring Commercial Laboratory) for 2 minutes followed by a more complete homogenization using a VWR 250 homogenizer equipped with a VWR 20mmx200mm Saw-Tooth Generator Probe for 1.5 minutes to generate a smooth puree-like consistency. Once processed, blueberry purees were aliquoted stored at −80 °C until further analysis.

2.2.4. Established, low throughput (LT) in vitro gastrointestinal digestion

Phenolic bioaccessibility of fresh weight (f.w.), frozen blueberry plant material was assessed utilizing a modified version of a traditional, static, LT three-phase *in vitro* digestion model as described by Moser et al (2016) (Moser et al. 2016). Briefly, a simulated saliva or oral phase solution (6 mL) was added to the processed blueberry aliquots (3.3 g/50 mL reaction tube). The reactions were individually vortexed and blanketed with nitrogen prior to being placed in an oscillating incubator (37 °C, 85 opm, 10 min). Following, samples were diluted to a volume of 30 mL with 0.9% saline. Gastric phase was then initiated by the addition of porcine pepsin solution (2 mL, 20 mg/mL in 0.1 M HCl). The reactions were adjusted to pH 2.5.0 ± 0.1 with 1.0 M HCl and brought to 40 mL with saline solution before being vortexed, blanketed with nitrogen and placed into the incubator (37 °C, 85 opm, 1 h). The small intestinal phase began with pH adjustment of gastric digesta to 6.5 ± 0.1 with 1.0 M NaHCO3. Small intestinal enzymes (2 mL, 20 mg/mL pancreatin, 10 mg/mL lipase in 0.1 M NaHCO3) and porcine bile salts (3 mL, 30 mg/mL bile extract in 0.1 M NaHCO3) were added and samples were ultimately standardized to a final volume of 50 mL with saline. Each reaction was finally vortexed, blanketed with nitrogen and placed within the incubator (37 °C, 85 opm, 2 h). Following the intestinal phase, samples were centrifuged (10,400×g, 4 °C, 1 h). The centrifuged aqueous (AQ), or bioaccessible fraction, was collected, filtered (0.22 um; (Macherery Nagel, Bethlehem, PA, USA)) and stored (−80 °C). until further analysis.

2.2.5. High throughput (HT) in vitro gastrointestinal digestion

Development of a HT *in vitro* digestion model required manipulation of parameters set by the aforementioned “gold standard” model. Such modifications included proportionally reducing starting material and total digestion volume, as similarly conducted by Lipkie et al. (2014) where

carotenoid bioaccessibility with slight overall volume reduction accurately replicated that of *in vivo* analysis (Lipkie et al. 2014). Additional adaptations included modified mixing conditions, filtering enzyme solutions to avoid particulate, as well as integrating the adjusted model with an automated liquid-handling robot (Tecan EVO 150, Tecan; Mannedorf, Switzerland). Utilizing the Tecan as opposed to manual execution promoted increased speed while providing more precise reagent distribution, throughput and walkaway time. Aliquots (1.1 g/15 mL reaction tube) of processed blueberries were placed into Tecan sample racks (16 tubes/rack) and loaded into defined Tecan columns. Reaction tubes were subjected to a simulated 1.8mL oral phase containing a-amylase that had been previously centrifuged at 10,600 x g for 10 minutes to remove any potential coagulates that may disrupt aspiration of the fine-tipped Tecan syringes. Tube racks were removed from Tecan and reaction tubes were individually vortexed, placed back within racks and blanketed with nitrogen. Tube racks were then stacked into a closed, oscillating incubator (37 °C, 155 OPM, 10 min). Once removed from the incubator, reactions were diluted with 0.9% saline solution and introduced to the gastric phase by the addition of 0.6 mL porcine pepsin solution (10 mg/mL in 0.1 M HCl). A control sample (wild blueberry freeze-dried powder or 2% coffee solution) and a random blueberry sample were selected and removed from each Tecan rack and adjusted to desired pH (pH 2.5 ± 0.1 with 1.0 M HCl). Control samples were expected to be consistent to evaluate consistency of enzyme solution preparation and Tecan function between separate runs. HCl addition to randomly selected blueberry samples was averaged, determining the appropriate amount of the 1.0 M HCl for application to the entire blueberry subset. Following pH adjustment, 0.9% saline solution was added to sample tubes bringing the volume total to 12 mL prior to blanketing with nitrogen and incubating (37 °C, 155 OPM, 1 hr). The intestinal phase was initiated by the sequential addition of a combination of 0.6

mL small intestinal enzymes (20 mg/mL of pancreatin and 10mg/mL of lipase in 0.1M NaHCO3 centrifuged at 10,400xg rpm for 10 minutes) and 0.9 mL porcine bile salts (30mg/mL bile extract in 0.1M NaHCO3). Reactions were ultimately adjusted to pH 6.5 ± 0.1 with 1M NaHCO3, brought up to ultimate digestion volume of 15 mL with 0.9% saline solution, blanketed with nitrogen and incubated (37 °C, 155 OPM, 2 h). Following intestinal incubation, racks were immediately removed from the incubator and placed back in the Tecan where the robot aspirated a total of 3 mL of the digested material sample, known as digesta (DG), from each reaction tube and dispensed 1.5 mL into 2 separate 96-well (2 mL) collection plates (Waters US, Milford, MA, USA), consecutively, the second plate intended as a back-up. Plates were blanketed with nitrogen and covered with 96-well square plug silicone/PTFE cap-mats (Waters US, Milford, MA, USA) and secured within the centrifuge (2988xg, 1hr). Post-centrifugation, the 96-well plates are immediately placed on ice where the separated aqueous (AQ) fraction was removed, filtered (0.45 um PTFE; (Macherery Nagel, Bethlehem, PA, USA)) and stored in a −80 °C freezer until further analysis.

2.2.6 Low throughput (LT) phenolic extraction

Extraction for the initial method adaptation and validation was completed by combining 500 mg of processed blueberry with 5 mL of 2% formic acid in 80% methanol. The combination was vortexed for approximately 1 minute before being centrifuged at 3600 x *g* for 4 minutes. The supernatant was removed and dried under nitrogen. This was repeated a total of 3 times, however, the second and third round utilized 2% formic acid in methanol as the primary extraction solvent. Dried supernatant was further resolubilized in 5 mL of 0.1% formic acid in 50:50 methanol and water and filtered through a 0.22 um PTFE membrane. After samples were resolubilized, they were vortexed for a period of 2 minutes per sample until all residue was removed from sides of

test tube. Samples were further extracted by means of solid phase extraction (SPE) to additionally concentrate and purify samples, prior to analysis, using a modified method previously reported by Song et al. (2013) (Song et al. 2013). Briefly, cartridges were conditioned first with 1% formic acid in methanol followed by 1% formic acid in water. Once conditioned, 1 mL of sample was loaded onto the SPE cartridge (Oasis® HLB 1cc (30 mg)). Samples were spiked with 50 ul of an internal standard (200uM ethyl gallate) to determine extraction recovery. Samples were then rinsed with 0.1% formic acid in water and 1% formic acid in water. Elution of phenolics was completed with 0.1% formic acid in methanol. Eluates were completely dried down under nitrogen manifold. Extracted samples were then resolubilized with 0.1% formic acid in 50:50 methanol and water before being filtered through .45 um PTFE membrane and analysis by LC-MS**.**

2.2.7. High Throughput (HT) Phenolic Extraction

For analysis of the DxJ genotypes, a similar extraction method by (de Ferrars et al. 2014) was modified to account for the increase in throughput using a polymeric reversed-phase, 96-well plate (Phenomenex, Strata™-X 33 um, 10 mg/well). While cartridge preparation remained the same, sample loading volume was decreased to 500 ul sample (750 uL raw extracted sample diluted in 750 ul of 1% formic acid in water, loaded 1 mL) and approximately 583 ul aqueous fraction (700 ul filtered aqueous fraction diluted in 500 ul of 1% formic acid in water, loaded 1 mL) accounting for the smaller cartridge size. Simultaneously, samples were spiked with a reduced volume of ethyl gallate (200 uM) to later determine extraction recovery. Once rinsed, samples were reconstituted in 0.1% formic acid in methanol into a 96-round well, polypropylene 350 ul collection plate (Waters). Because this extraction procedure did not require a dry-down step following elution, taxifolin (200 uM) was utilized as a volume control to adjust for any variation

in volume between wells following SPE. Once spiked with taxifolin, the 96-well collection plate was placed into the LC-MS for analysis.

2.2.8. Analysis of polyphenolic compounds by LC-MS

Blueberry samples were characterized by LC-MS using a method adapted from that of Song et al., (2013) (Song et al. 2013). Samples were injected into a Waters ACQUITY *UPLC® H-Class* equipped with an ACQUITY *UPLC®* BEH C18 column (1.7 um, 2.1 mm x 150 mm). Phenolic compounds were separated using a gradient elution using a binary mobile phase composed by 0.1% formic acid in acetonitrile as solvent A and 2% formic acid in water as solvent B. Following separation, individual phenolics were detected using a QDA mass selective detector by means of individual single ion response (SIR) in both negative (phenolics and flavonoids) and positive ion (ANCs). A total of 28 phenolic compounds found to be representative of blueberry, based on previous literature (Grace et al. 2014; Correa-Betanzo et al. 2014; Sellappan, Akoh, and Krewer 2002), were targeted for analysis (Table 2.1). Authentic standards of each compound, with limited exception, were used to determine existing compounds within samples and were further used to quantify such compounds via calibration curves. Concentration of all ANC compounds were based upon the -3-O glucosidic form of each parent compound, while other glycoside peaks (galactoside, arabinoside, “other”) were identified based off elution order determined by previous reports (Kader et al. 1996; Wu et al. 2005; Wilhelmina Kalt et al. 1999; Grace et al. 2014). Furthermore, all quercetin glycosides were estimated utilizing only quercetin-3-O- glucoside.

Table 2. 1. Representative blueberry phenolics targeted for bioaccessibility screening [1]

Target Compound	Molecular Weight (g/mol)	SIR (m/z)	Ionization Mode +/-
Anthocyanins			Positive
Delphinidin glycosides	303.2 g/mol	304.2 m/z	
Cyanidin glycosides	287.2 g/mol	288.2 m/z	
Peonidin glycosides	301.3 g/mol	302.3 m/z	
Petunidin glycosides	317.3 g/mol	318.3 m/z	
Malvidin glycosides	331.3 g/mol	332.3 m/z	
Phenolic Acids			Negative
Chlorogenic Acid	354.3 g/mol	353.3 m/z	
Vanillic Acid	168.2 g/mol	167.2 m/z	
Caffeic Acid	180.2 g/mol	179.2 m/z	
Flavonols			Negative
Quercetin glycosides	463.4 g/mol	463.4 m/z	
Syringetin 3-glucoside	508.4 g/mol	507.4 m/z	
Flavan-3-ols			Negative
Catechin Epicatechin	290.3 g/mol	289.3 m/z	

[1]*Note: Acylated ANCs (other) were estimated based on response of corresponding glycosides.*

2.3. Data Analysis

Data for polyphenol content of blueberry samples, aqueous digesta and raw material are represented as mean ± standard deviation. Relative (%) bioaccessibility is the percentage of recovered polyphenols in the digested (AQ) relative to that in the starting fresh, frozen blueberry. Absolute bioaccessibility (mg/100g) is defined as the amount of phenolic compounds found in the digested (AQ) material compared to the amount found in the original raw blueberry material, thus calculated by multiplying the found relative (%) bioaccessibility by the concentration determined to be in 100 grams of the starting raw material. Store-bought, cultivated blueberry phenolics were expressed by individual compound and as sum of total content between classes. Phenolic content

of DxJ blueberries were solely expressed by class due to their minimal overall content. To assess variation between models and blueberry genotypes as well as differences in phenolic profile and bioaccessibility data (relative and absolute) JMP (version Pro 14, SAS Institute, Cary, NC, USA) was utilized. Statistical analysis was done with one-way analysis of variance (ANOVA) and Tukey test or t-test.

2.4. Results and Discussion

2.4.1. Phenolic profiles of commercial blueberries

Phenolic content including ANCs and non-ANC phenolics in commercial, cultivated blueberries obtained locally in Kannapolis, NC can be seen in Table 2.2. Among the ANCs (ANC) in the blueberry raw material, malvidin glycosides were shown to be the main compounds contributing approximately 43% of the total ANC content which is comparable to previous reports (Liu et al. 2014; Grace et al. 2014; Yousef et al. 2013). In addition, petunidin and cyanidin glycosides accounted for about 24% and 16% of the total ANC content, respectively. Delphinidin and peonidin contributed the lowest at approximately 12% and 11% of the sum-total ANC content. Chlorogenic acid was found to be the major phenolic acid (PA) found in blueberries, contributing significantly to the total sum of phenolic acids. These results were consistent with previous reports (Howard, Clark, and Brownmiller 2003; Grace et al. 2014; Gao and Mazza 1994; Correa-Betanzo et al. 2014; Yousef et al. 2013). Low amounts (0.2 and 0.3 mg/100g f.w.) of vanillic acid and caffeic acid (<1%) were also noted, similarly reported by (Willy Kalt and McDonald 1996). Relatively low levels of epicatechin and catechin (0.3 and 0.2 mg/100g f.w.) were observed resulting in low overall content of F3Ls (F3L). Comparable to that previously reported (Grace et al. 2014) quercetin was found to be the predominant FLAV (FLAV) contributing roughly 77% of the sum-total FLAV content, while syringetin contributed approximately 23%.

Table 2. 2. Content (mg/100g f.w.) of individual marker (poly)phenolic compound in commercial store- bought blueberries used for method adaptation and validation. [1,2]

Anthocyanins	**Delphinidin**	**Cyanidin**	**Peonidin**	**Petunidin**	**Malvidin**	**Sum of Anthocyanins**
3-galactoside	11.6±2.7	21.2±3.4	16.6±2.6	35.8±6.6	60.3±8.1	145.5
3- glucoside	0.2±0.0	1.1±0.1	1.5±0.1	1.1±0.2	2.1±0.2	6.0
3-arabanoside	10.7±2.2	16.9±2.5	11.2±1.2	25.0±4.0	44.6±5.3	108.4
Other forms	11.2±1.43	5.3±0.3	0.7±0.1	5.6±0.7	0.9±0.0	23.7
Total	33.7	44.5	30.0	67.5	107.9	283.6
Phenolic Acids	**Chlorogenic**	**Vanillic**	**Caffeic**			**Sum of Phenolic Acids**
Total	83.1±1.0	0.2±0.0	0.3±0.1			83.7
Flavonols	**Quercetin**	**Syringetin**				**Sum of Flavonols**
3-glucoside	38.4±2.9	12.4±1.0				50.8
3-arabinoside	4.0±0.3					4.0
Total	42.4	12.4				54.8
Flavan-3-ols	**Catechin**	**Epicatechin**				**Sum of Flavan-3-ols**
Total	0.2±0.1	0.3±0.0				0.5

[1]*Identification based on published data and chromatographic standards.*
[2]*ANC "other forms" includes all acylated compounds.*

2.4.2. Comparison of Manual (LT) Model vs Automated (HT) Tecan Model

Polyphenol bioaccessibility from commercial blueberries was determined both through a LT and HT three stage in-vitro digestion model. Following in vitro digestion, phenolic content was assessed and compared to that seen in the raw material extraction to derive both relative and absolute bioaccessibility values. The modified, HT model was compared to the traditional, LT model to better understand the relative alignment between these results (Table 2.3) and potential predictive power of the HT model.

While ANCs are the primary polyphenols within blueberry extracts, specifically the glycosidic derivatives of delphinidin, malvidin, cyanidin, peonidin and petunidin, they are

generally considered to be very chemically unstable compounds susceptible to in vitro digestive conditions (Correa-Betanzo et al. 2014; Liu et al. 2014; Tagliazucchi et al. 2010; Bermúdez-Soto, Tomás-Barberán, and García-Conesa 2007; G. J. McDougall et al. 2005). In the current study, ANCs were noted to compose roughly 67% of the total phenolic profile of the analyzed commercial blueberry. When assessed following digestion, the greatest absolute bioaccessibility in ANCs was observed in malvidin glycosides (LT; 41.1mg/100g, (43.3%), HT; 61.2mg/100g, (64.0%)). Following malvidin, the highest bioaccessible content was noted within peonidin glycosides (LT; 12.7mg/100g, (45.0%) HT; 19.2mg/100g, (60.8%)) cyanidin glycosides (LT; 11.8mg/100g, (32.0%), HT; 18.2mg/100g, (43.8%) and petunidin glycosides (LT; 7.3mg/100g, (20.0%), HT; 11.4mg/100g, (26.4%)). Of the ANCs analyzed, delphinidin glycosides exhibited the lowest absolute bioaccessibility (LT; 5.6mg/100g, HT; 56.9%, 6.4mg/100g). Detectable peaks consisted solely of acylated forms of delphinidin, while delphinidin 3-galactoside, 3-glucoside and 3-arabinoside proved too unstable for detection following digestion. Regarding relative bioaccessibility, LT and HT were consistent in terms of their rank order of highest to lowest bioaccessibility apart from peonidin having a slightly greater relative bioaccessibility compared to malvidin in the LT method.

Overall, the total sum of ANCs experienced a total digestive loss of 64.6% (LT) and 51.5% (HT), respectively, noting relative bioaccessibility ranging from 6.0-61.6% (average 35.4%) (LT) and 12.1-75.4% (average 48.5%) (HT). A similar study, conducted by Liu et al. (2014), investigated the stability of blueberry ANCs throughout a successive in vitro gastric and pancreatic digestion model and observed an average loss of 46% in ANC content (Liu et al. 2014). Likewise, additional consistencies in bioaccessibility were noted in chokeberry (57.4%) (Bermúdez-Soto, Tomás-Barberán, and García-Conesa 2007), grape juice (3.7—37.2%) (Moser et al. 2016),

pomegranate juice (48%) (Pérez-Vicente, Gil-Izquierdo, and García-Viguera 2002), red wine (34.1%) (G. J. McDougall et al. 2005) and raspberries (61.9%). Interestingly, in agreement with the current study, a study investigating wild blueberry (*Vaccinium angustifolium*) saw a range of bioaccessibility (4.4-37.3%) with an average around 17% following digestion (Correa-Betanzo et al. 2014), while significantly lower recovery was noted in a study conducted by Jiao et al. (2018) which found ANC bioaccessibility in cultivated blueberries to be only 3.4% (Jiao et al. 2018). In the same way, researchers determining the bioaccessibility of maqui berry detected very low ANC content, approximately 0.5-0.6% following the intestinal phase (Lucas-Gonzalez et al. 2016).

Table 2. 3. Comparison of absolute (mg/100g) and relative (%) bioaccessibility of individual phenolics from commercial blueberries determined by LT and HT models. [1,2]

Model / **Target Compound**	**LT** *(Manual)*	**CV** *(%)*	**HT** *(Tecan)*	**CV** *(%)*	**P-Value** *(Absolute)*	**P-Value** *(Relative)*
Anthocyanins						
Delphinidin other	5.6±0.3,(50.0%)	9.8%	6.4±0.4(56.9%)	6.4%	.0083	.0073
Cyanidin 3-galactoside	5.3±0.3,(25.1%)	4.9%	8.7±0.4(40.2%)	5.9%	<.0001	<.0001
Cyanidin 3-glucoside	0.3±0.0,(32.7%)	6.3%	0.5±0.1(49.3%)	12.5%	.0002	.0002
Cyanidin 3-arabinoside	3.6±0.3,(21.4%)	7.9%	6.4±0.5(37.3%)	9.5%	<.0001	<.0001
Cyanidin other	2.6±0.5,(48.7%)	20.4%	2.6±0.5(48.3%)	21.9%	.8436	.9332
Peonidin 3-galactoside	7.1±0.3,(42.6%)	4.8%	10.7±0.4(62.9%)	3.3%	<.0001	<.0001
Peonidin 3-glucoside	0.7±0.0,(49.2%)	4.1%	0.8±0.1(53.2%)	13.5%	.0714	.2291
Peonidin 3-arabinoside	4.9±0.1,(43.2%)	1.7%	7.7±0.2(66.3%)	1.3%	<.0001	<.0001
Petunidin 3-galactoside	2.2±0.2,(6.0%)	1.4%	4.4±0.4,(12.1%)	8.2%	<.0001	<.0001
Petunidin 3-glucoside	0.1±0.0,(6.0%)	16.2%	0.1±0.0,(12.2%)	4.6%	.3282	<.0001
Petunidin 3-arabinoside	1.6±0.1,(6.2%)	4.8%	3.1±0.3,(12.9%)	6.0%	<.0001	<.0001
Petunidin other	3.4±0.6,(61.6%)	16.1%	3.8±0.4,(68.2%)	10.7%	.1200	.1162
Malvidin 3-galactoside	21.5±1.2,(35.6%)	5.7%	32.0±1.1,(52.2%)	2.5%	<.0001	<.0001
Malvidin 3-glucoside	1.0±0.2,(47.0%)	22.9%	1.6±0.1,(75.4%)	8.2%	<.0001	<.0001
Malvidin 3-arabinoside	18.2±0.8,(40.8%)	4.7%	27.0±0.7,(59.3%)	1.3%	<.0001	<.0001
Malvidin other	0.4±0.1,(48.8%)	13.5%	0.6±0.1,(69.2%)	8.2%	<.0001	.0003
Sum of Anthocyanins	66.4±1.6,(29.7%)	7.8%	103.0±1.5,(44.4%)	6.5%	<.0001	<.0001
Sum of Other Anthocyanins	16.7±2.8,(52.5%)	14.9%	17.9±1.3,(60.7%)	11.8%	.2278	.2415
Total Sum of Anthocyanins	**83.1±4.2,(35.4%)**	**9.6%**	**121.0±2.8,(48.5%)**	**7.8%**	**<.0001**	**.0127**

Table 2. 3. (continued)

Model **Target Compound**	**LT** *(Manual)*	**CV** *(%)*	**HT** *(Tecan)*	**CV** *(%)*	**P-Value** *(Absolute)*	**P-Value** *(Relative)*
Phenolic Acids						
Chlorogenic Acid	27.8±5.6(33.4%)	20.2%	33.3±3.5(39.4%)	11.3%	.0298	.0540
Vanillic Acid	0.2±0.0,(71.1%)	7.1%	0.2±0.0(72.1%)	15.6%	.3676	.8703
Caffeic Acid	0.8±0.2,(230.7%)	20.6%	0.9±0.1(255.2%)	13.6%	.1536	.2513
Sum of Phenolic Acids	**28.7±5.8,(111.7%)**	**16.0%**	**34.4±3.6(122.2%)**	**13.5%**	**.0960**	**.4888**
Flavonols						
Quercetin 3-Glucoside	17.2±1.7,(42.3%)	7.6%	18.4±1.8(47.7%)	9.3%	.1746	.0111
Quercetin 3-Arabinoside	1.9±0.1,(39.4%)	10.8%	1.9±0.2(46.2%)	7.3%	.9280	.0082
Syringetin 3-Glucoside	6.1±0.6,(49.1%)	10.7%	6.9±0.7(55.6%)	9.8%	.0156	.0189
Sum of Flavanols	**25.2±2.4,(43.6%)**	**9.7%**	**27.2±2.6(49.8%)**	**8.8%**	**.0307**	**.2133**
Flavan-3-ols						
Catechin	0.1±0.0,(27.9%)	7.7%	0.1±0.0,(37.0%)	10.4%	.1522	.0019
Epicatechin	0.1±0.0,(19.5%)	23.2%	0.1±0.0,(21.8%)	12.8%	.4086	.2719
Sum of Flavan-3-ols	**0.1±0.0,(23.7%)**	**15.5%**	**0.1±0.0,(29.4%)**	**11.6%**	**.0284**	**.0306**

[1]*Values represent mean ± standard deviation of n=4 replicates digested per day for a total of 3 days. A total n=12 digestions per sample are represented.*
[2]*P-values (<0.05) denote a significant difference in bioaccessibility between models (LT v. HT).*

According to previous reports (Correa-Betanzo et al. 2014; Bermúdez-Soto, Tomás-Barberán, and García-Conesa 2007; Liu et al. 2014; Tagliazucchi et al. 2010) a majority of ANC loss can be attributed to their structural instability and ring cleavage within the weakly alkaline (pH 6.5) environment of the intestinal phase most likely initiated by the ionization of the hydroxyl groups. In general, the overall stability of ANCs is highly dependent on their chemical structure. It has been reported that ANCs containing aromatic acyl groups are more stable than non-acylated ANCs (Gordon J. McDougall et al. 2005; Stintzing et al. 2002; Mazza 2018; Giusti and Wrolstad 2003). This is evident in the current study as average ANC acylated form (LT; 52.5%, HT; 60.7%) reported higher relative bioaccessibility compared to the average of the ANC glycosides (galactoside, glucoside, arabinoside) (LT; 29.7%, HT; 44.4%). Additional factors of degradation include exposure to light, high temperatures, oxygen, as well as method and duration of storage (Patras et al. 2010; Alighourchi and Barzegar 2009; Reque et al. 2014; Rubinskiene et al. 2005; Buckow et al. 2010). However, in the current study, preventative measures were taken to limit such effects, including covering light sources with UV protective shields, blanketing the reactions with nitrogen prior to incubation periods, keeping samples on ice during pH adjustment periods, and storing samples within a -80^{o}C freezer prior to extraction and digestion.

Following digestion, it was noted that phenolic acid bioaccessible content exceeded 100% in both models (LT; 111.7%, HT; 122.2%), similarly seen in Andean berry (Agudelo et al. 2018). Such values can be attributed to the high relative bioaccessibility of caffeic acid (LT; 230.7%, HT; 255.2%). A possible explanation for the high relative bioaccessibility can be a result of possible caffeic acid derivatives, such as neochlorogenic acid or chlorogenic acid. In fact, high levels of chlorogenic acid were seen in chokeberry and Andean berry (Bermúdez-Soto, Tomás-Barberán, and García-Conesa 2007; Agudelo et al. 2018). Whereas much lower bioaccessible content of

caffeic acid was observed in grape juice which was noted at ranging from 24.8-44.7% (Moser et al. 2016), as well as Andean berry which was 26.9% (Agudelo et al. 2018). Though, according to a study by Tagliazucchi et al. (2010), homogenized red grapes were found to have a bioaccessibility of 75.1% (Tagliazucchi et al. 2010). In the current study, chlorogenic acid contributed to over 95% of the total PA content and approximately 20% of the sum-total polyphenolic content. This was in agreement with previous work on blueberries which found chlorogenic acid to be the predominant phenolic acid (96.5%) across several blueberry genotypes (Yousef et al. 2013; Connor et al. 2002; Willy Kalt and McDonald 1996). In general, blueberries have highest chlorogenic content of all fruits containing 0.5-2 g/kg f.w. (Macheix et al. 1990).

Within the targeted FLAVs, syringetin reported the highest relative bioaccessibility (LT; 49.1%, HT; 55.6%), followed by quercetin 3-glucoside and quercetin 3-arabanoside (LT; 42.3%, 39.4%, HT; 47.7%, 46.2%), comparable to that previously reported in blackberry, chokeberry and wild blueberry (Tavares et al. 2012; Bermúdez-Soto, Tomás-Barberán, and García-Conesa 2007; Correa-Betanzo et al. 2014). Additionally, quercetin 3-glucoside contributed the highest bioaccessible content (LT; 17.3mg/100g, HT; 18.4mg/100g). Catechin had the greatest relative bioaccessibility (LT; 27.9%, HT; 37.0%) within the targeted F3Ls, while the combined absolute bioaccessibility (0.2mg/100g) insignificantly contributed to the total sum of phenolics. Results obtained for F3L stability are similar to that observed in grape juice and chokeberry (Moser et al. 2016; Bermúdez-Soto, Tomás-Barberán, and García-Conesa 2007).

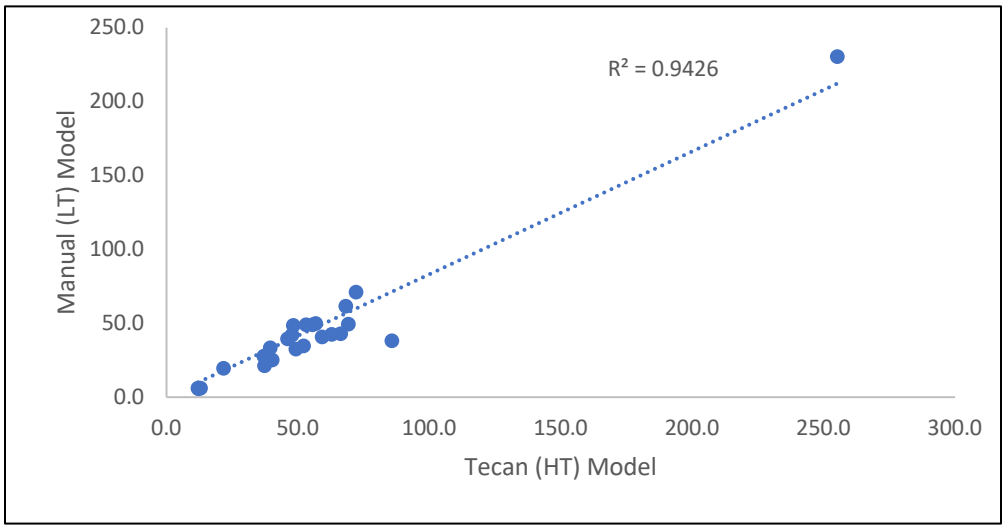
Higher absolute bioaccessibility was consistently observed within the HT method, not including acylated forms of cyanidin which showed higher absolute bioaccessibility content within the LT method compared to HT. Equivalent values noted in petunidin 3-glucoside, acylated forms of cyanidin, vanillic, quercetin 3-arabinoside and all targeted F3Ls (catechin and epicatechin).

While mechanisms are unclear, consistently higher bioaccessibility within the HT, Tecan samples could be attributed to a couple of reasons including: the smaller surface area of the 15 mL digestion test tubes as opposed to the 50 mL test tubes, more thorough mixing of the Tecan digestions by means of higher oscillation speeds and more compact rack placement within the incubator.

Despite differences in the bioaccessibility values obtained for the 24 targeted phenolic compounds between HT and LT models, ultimately the two models of *in vitro* digestion were determined to be highly correlated in both relative (r=0.97) and absolute bioaccessibility (r=0.98), as shown in figure 2.1. While highly correlated, model (LT v. HT) proved to have a significant effect on bioaccessibility values. Significant differences were observed in all compounds as a result of assessment by differing model with the exception of the sum-total of acylated forms of cyanidin and petunidin (p=0.84, p=0.12), petunidin and peonidin 3-glucoside (p=0.33, p=0.07), vanillic and caffeic acid (p=0.37, p=0.15), quercetin glycosides (3-glucoside; p=0.17, 3-arabinoside p=0.93), and catechin and epicatechin (p=0.15, p=0.41) where model-type did not significantly influence bioaccessibility. By class, the sum of ANCs (p=<.0001), FLAVs (p=0.03) and F3Ls (p=0.03) were all significantly affected by throughput of model. Conversely, model did not significantly affect sum of acylated ANCs (p=0.23) or phenolic acids (p=0.96). LT and HT models were analyzed for intra-day where two sets (n=4/set) of digestions were ran consecutively to analyze consistency and variation amongst sets. Similarly, both models were tested for inter-day variation, where digestion methods were tested across 3 different days to ensure consistency and account for variation of the LT and HT between days. Both models proved to have good reproducibility and relatively low coefficient of variance (CV%) both between sets and between days. Intra-day showed the HT model to be slightly more consistent (CV; 1.3-19.5%, mean 7.5%) compared to the LT model (CV; 3.2-39.3%, mean 9.4). HT model was shown to be more consistent

inter-day variability (CV; 2.7-21.9%, mean 8.8%), while LT model showed insignificantly more variability (CV 0.1-23.2%, mean 10.8%).

A. Correlation between LT and HT model in terms of relative bioaccessibility (%)



B. Correlation between LT and HT model in terms of absolute bioaccessibility (mg/100g f.w.)

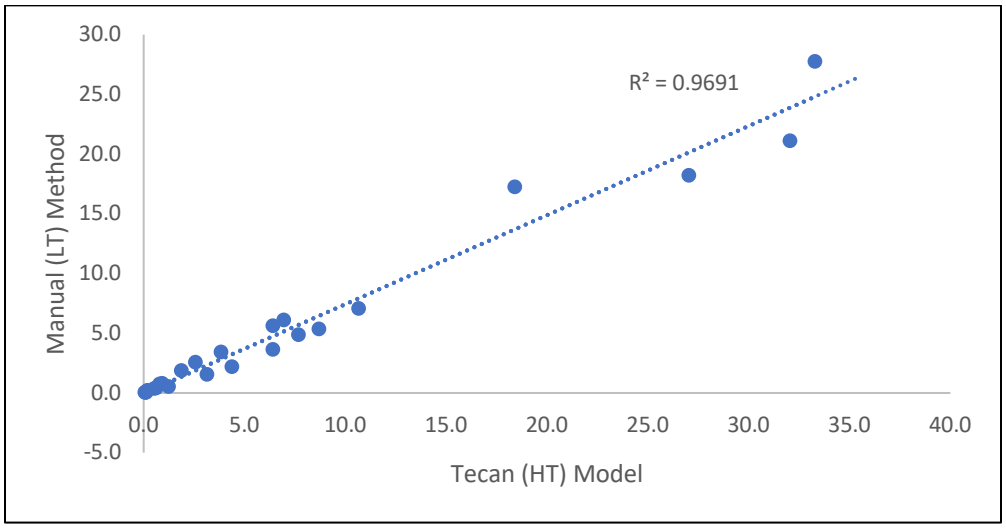


Figure 2. 1. Correlation between model throughput (LT v. HT) in terms of average inter-day bioaccessibility (relative (%) v. absolute (mg/100g f.w.). Circular markers indicate all targeted phenolic compounds within the varying phenolic classes (ANC, PA, FLAV, F3L).

2.4.3. Variability in bioaccessibility among a blueberry mapping population

Establishment of a correlation between a traditional LT model and the adapted HT digestion model enabled the application of the HT model to a subset of blueberries selected from the mapping population DxJ. The DxJ subset is considered a first-generation mapping population derived from a cross between two elite, highbush blueberry cultivars, Draper (D) and Jewel(J). The need for screening large populations of different blueberry genotypes is derived from the recent demand for healthier fruits and vegetables by consumers. Thus, the need for development of extraction and analysis and further, digestion, procedures was recognized to permit for the reliable and quick comparative screening for a large number of blueberry samples (Yousef et al. 2013; Howard, Clark, and Brownmiller 2003). Such comparative screening was conducted in the current study where 33 individual blueberry genotypes were selected for extraction and analysis to determine variation in phenolic content as a possible effect of genotype.

Average composition (Figure 2-2) of the 33 genotypes of blueberry making up the DxJ subset consisted largely of ANCs (59.7%), derived from 35.3% of ANC glycosides (3-galactoside, 3-glucoside, 3-arabinoside) and 24.4% of “other”, primarily acylated forms of ANCs. Additionally, phenolic acids made up 27.8% where approximately 99% is made up of chlorogenic acid, while the additional <1% consists of vanillic and caffeic acid. Lastly, FLAVs accounted for 12.3% of the total sum and F3Ls contributed 0.2%.

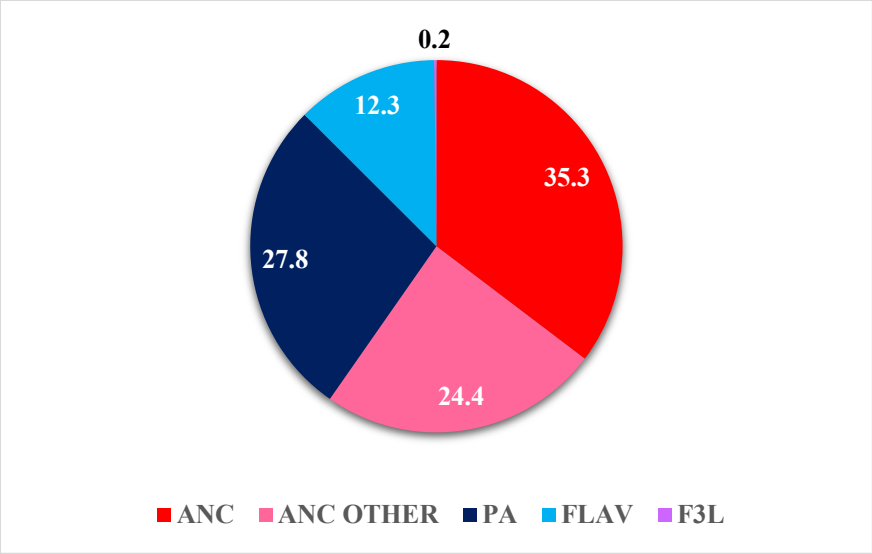


Figure 2. 2. Blueberry phenolic composition (%) based on the average of 33 individual selected genotypes within the Draper x Jewel (DxJ) mapping population. [1]

[1]*Composition (%) based off mean (n=3) replicates extracted across 3 days (1 extraction per day).*

In regards to content (Figure 2.3), results concluded that sum-total of phenolic content within the DxJ subset ranged from 31.7-81.1 mg/100g f.w. Significant variation of raw material content (figure 2-3) was observed within all phenolic classes. Specifically, ANC content ranged from 11.0-34.4 mg/100g f.w., while other ANC content varied between 8.2-21.1 mg/100g f.w.. It was concluded that within a majority of the analyzed genotypes, blueberries accumulated mostly glycosidic ANCs, whereas the acylated (other) ANCs constituted a smaller portion of the total ANCs, similarly observed in previous studies, where acylated anthocyanins constituted a minor portion of the total anthocyanins depending on the genotypes (Yousef et al. 2013; Wilhelmina Kalt et al. 2001). Additionally, results were determined to be in agreement with previous studies, where large variations in phenolic content were observed to be influenced by genotype (Yousef et al. 2013; Wilhelmina Kalt et al. 2001; Stevenson and Scalzo 2012; Connor et al. 2002). Meanwhile, the remaining ~40% of the of the overall phenolic content consisted of PA (3.4-28.0 mg/100g f.w.), FLAV (4.9-11.3 mg/100g f.w.) and F3L (0.03-0.2 mg/100g f.w.).

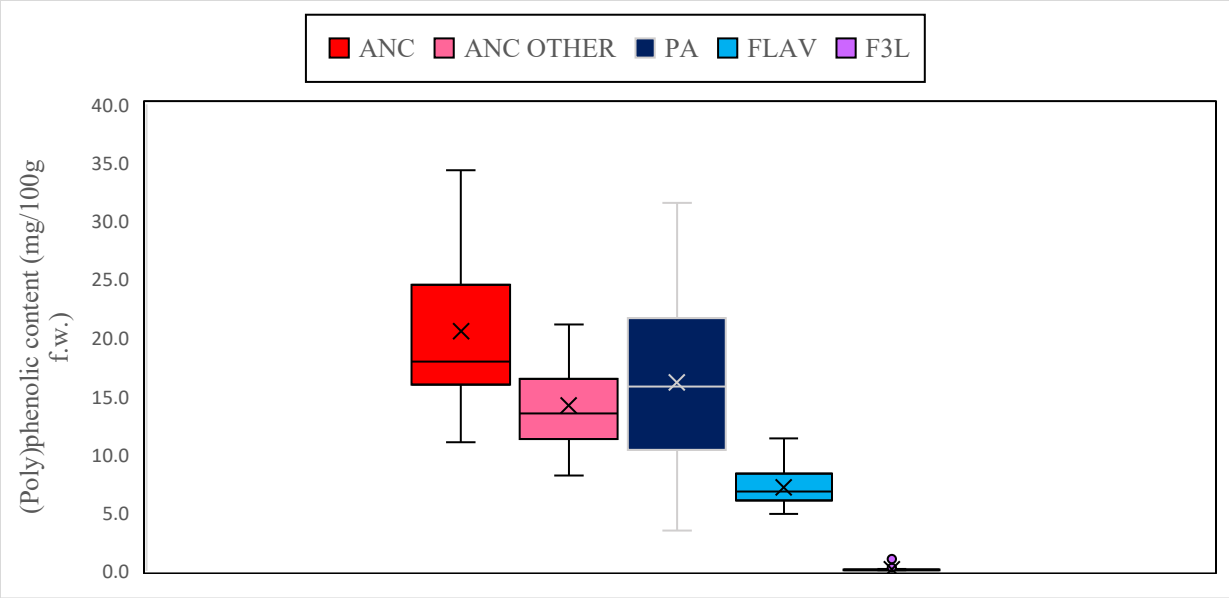


Figure 2. 3. Range of (poly)phenolic content (mg/100g f.w.), by class, found within 33 individual blueberry genotypes within the Draper x Jewel (DxJ) mapping population. [1,2]

[1]Content (mg/100g f.w.) values based off mean (n=3) replicates extracted over a course of 3 days (1 extraction per day).
[2] (*) denotes significant difference (P value (<0.05)) in (poly)phenolic content found within 33 individual genotypes making up the DxJ mapping population.

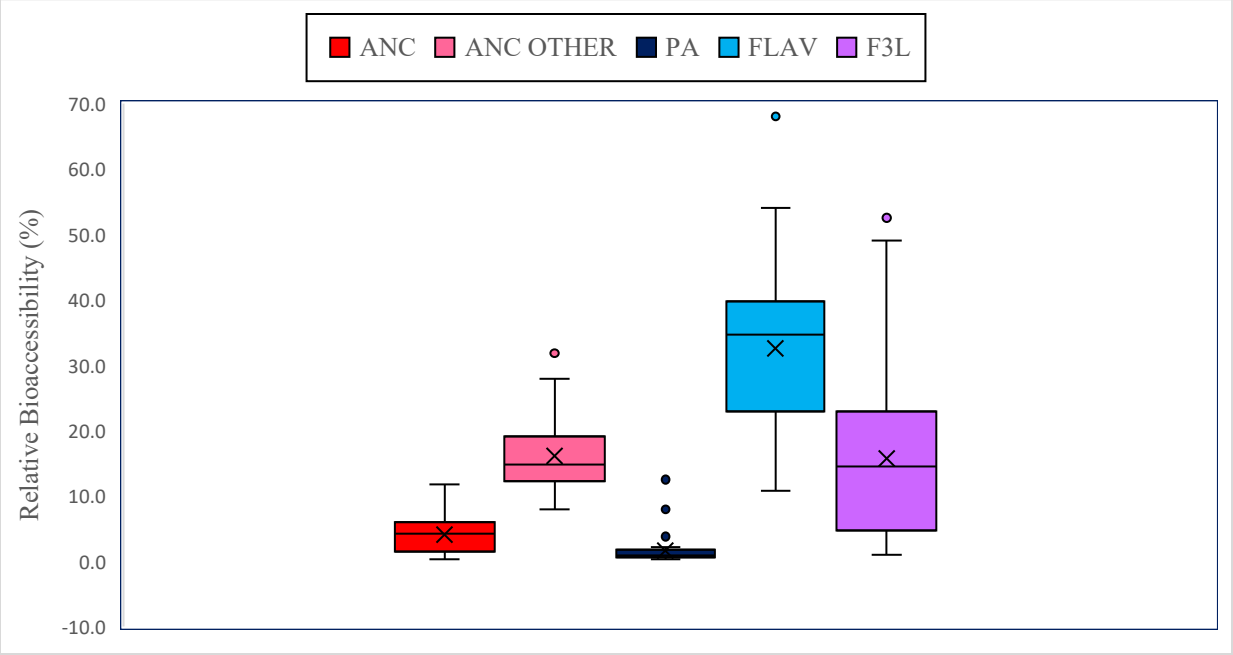


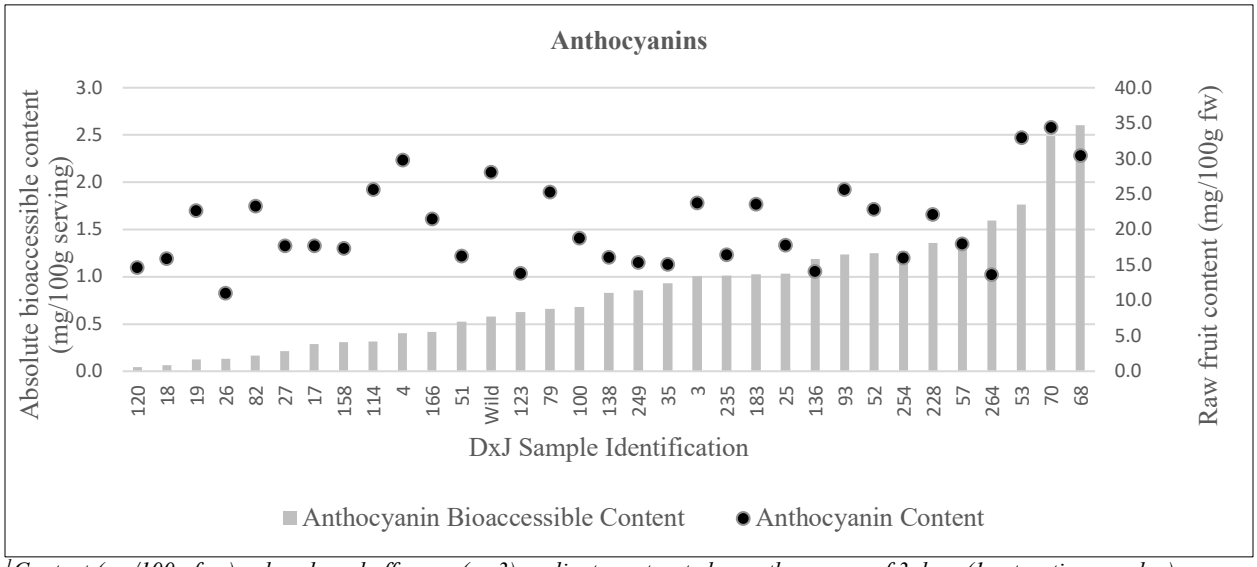
Figure 2. 4. Range of relative bioaccessibility (%) values observed for phenolic classes following HT digestion of 33 blueberries derived from Draper x Jewel (DxJ).[1,2]

[1]Content (mg/100g f.w.) values based off mean (n=3) replicates extracted over a course of 3 days (1 extraction per day).
[2] (*) denotes significant difference (P value (<0.05)) in (poly)phenolic content found within 33 individual genotypes making up the DxJ mapping population.

The wide range exhibited in ANC and phenolic acid concentrations observed in this study, suggests a possible direct correlation to *in vivo* bioactivity and therefore proposes a strong potential for selectively breeding blueberry for improved ANC and phenolic acid content. However, a recent study by Maiz et al. (2016), highlights the importance of the relation between total phenolic content and bioavailability as a result of varying genotype (Maiz et al. 2016). Within the study, investigators sought to characterize the phenolic content of 9 blueberry varieties of *Vaccinium spp.* in addition to determining whether there is a difference in their bioavailability after an acute dose in 84 ovariectomized rats. Urine and blood analysis revealed that total (poly)phenolic content and bioavailability of individual phenolics varied widely among blueberry varieties. Notably, varieties with significantly higher total phenolic content than other varieties did not have the highest have the highest bioavailability (Maiz et al. 2016), thus indicating that blueberry content may not be directly correlated to bioavailability, and by association, bioaccessibility. Therefore, to determine the blueberry genotype(s) with the highest bioactivity, it is essential to not only assess berry content, but the bioaccessible content following berry digestion.

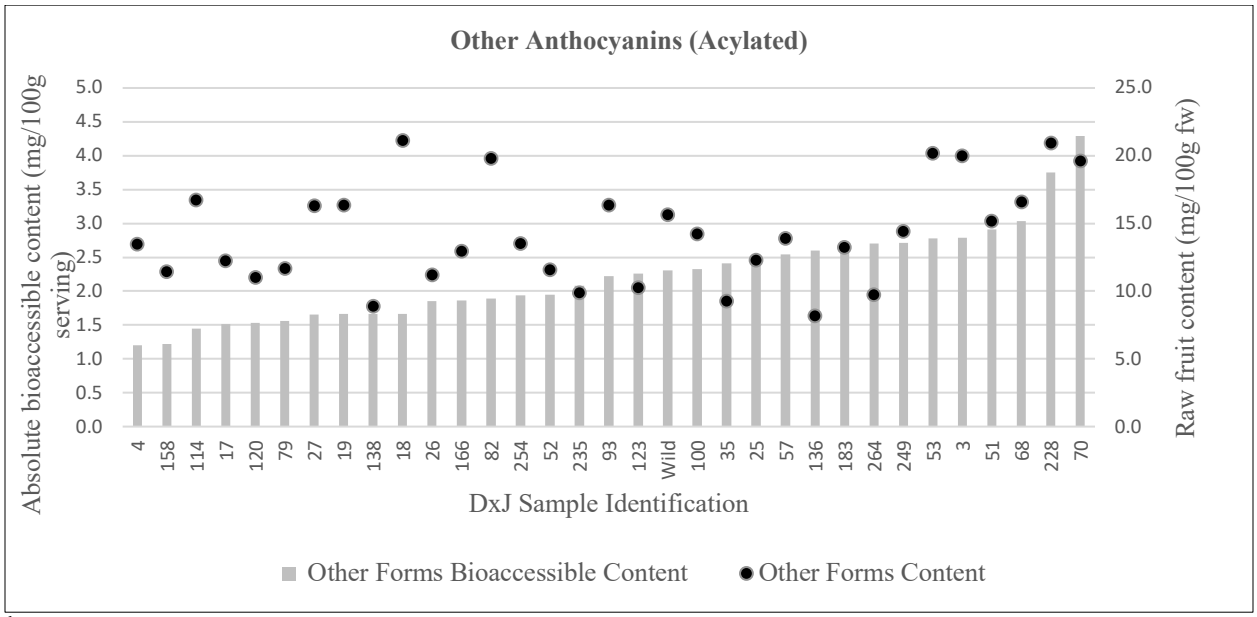
Figure 2.5. Mean phenolic content (mg/100g f.w.) and absolute bioaccessible phenolic content (mg/100g f.w.), by phenolic class, as seen in 33 selected blueberry genotypes from Draper x Jewel (DxJ) mapping population. [1]

A. ANC content and absolute bioaccessible content within DxJ subset.



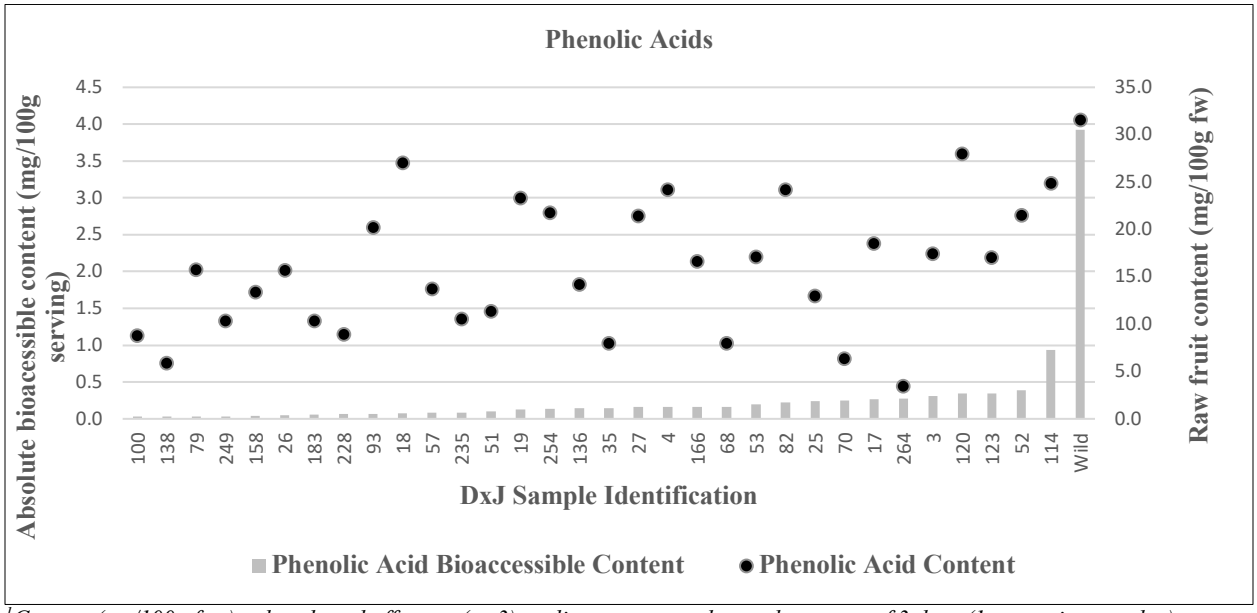
[1]*Content (mg/100g f.w.) values based off mean (n=3) replicates extracted over the course of 3 days (1 extraction per day).*

B. Other ANC content and absolute bioaccessible content within DxJ subset.



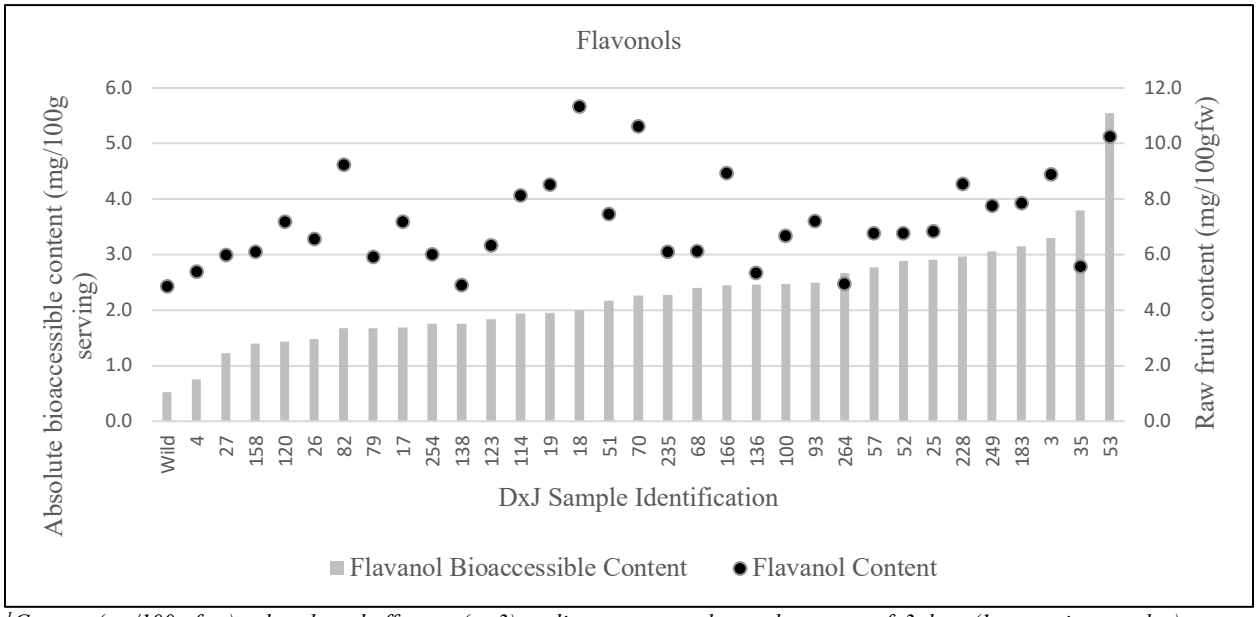
[1]*Content (mg/100g f.w.) values based off mean (n=3) replicates extracted over the course of 3 days (1 extraction per day).*

C. PA content and absolute bioaccessible content within DxJ subset.



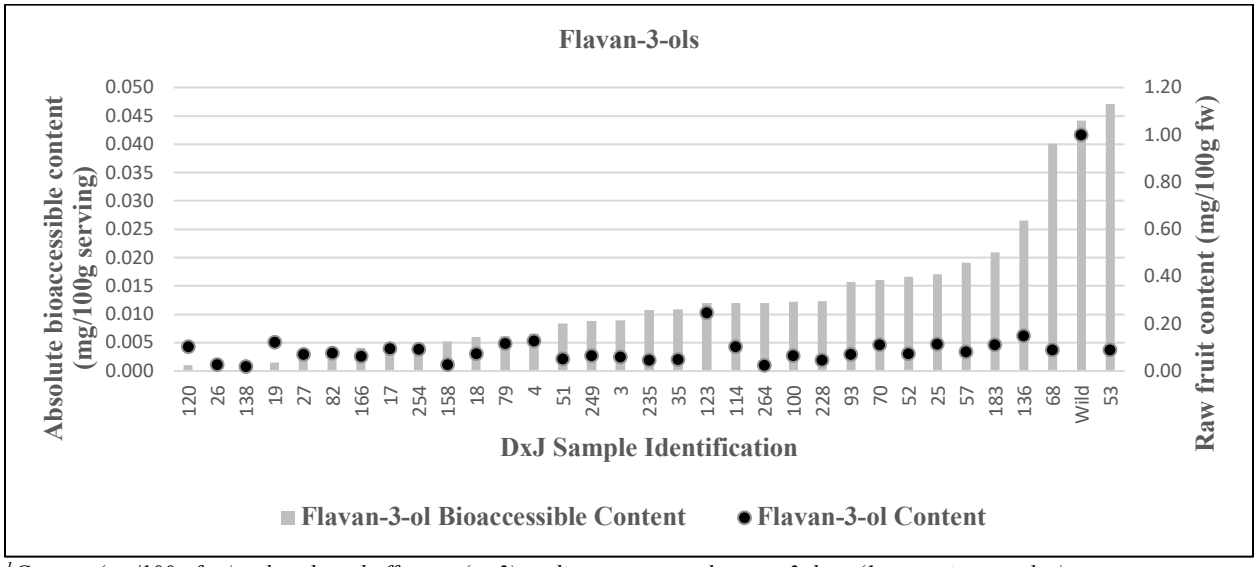
[1]*Content (mg/100g f.w.) values based off mean (n=3) replicates extracted over the course of 3 days (1 extraction per day).*

D. FLAV content and absolute bioaccessible content within DxJ subset.



[1]*Content (mg/100g f.w.) values based off mean (n=3) replicates extracted over the course of 3 days (1 extraction per day).*

E.F3L content and absolute bioaccessible content within DxJ subset



[1]*Content (mg/100g f.w.) values based off mean (n=3) replicates extracted across 3 days (1 extraction per day).*

2.4.4. Genotypic effect on phenolic bioaccessibility as determined by HT in vitro digestion within DxJ blueberry subset

With this in mind, in the current study, 33 individual blueberry genotypes were applied to the 3 stage HT in vitro digestion model to characterize variation in the phenolic bioaccessibility amongst the germplasm. Following digestion, significant variation was noted in bioaccessibility, both relative (Figure 2.4) and absolute (Figure 2.5), within all phenolic classes.

Amongst the 33 genotypes, ANC relative bioaccessibility ranged from 0.3-11.7% ($p < 0.0001$), while absolute bioaccessibility ranged from 0.1-2.6 mg/100g f.w. ($p < 0.0001$). Association between (poly)phenolic content and bioaccessible content was noted in DxJ sample: 070, 053 and 068 where individual samples were ranked in the top 15% within both ANC content within raw material and ANC content in the measured bioaccessible fraction. Conversely, DxJ sample: 026 and 120 were considered to be in the bottom 15%. While association between ANC content did exist, majority of the samples were assorted randomly with no observed relationship between content before and after digestion. Notably, DxJ 264 had the highest relative bioaccessibility (11.7%) of all digested samples as well as ranked within the top 15%, regarding greatest bioaccessible content (1.6mg/100g f.w.). Given its high bioaccessibility ranking, DxJ 264 had the second lowest ANC content in terms of undigested, raw material.

The relative bioaccessibility of other forms of ANC noted significant variation ranging from 7.9-27.9% ($p < 0.0001$), whereas DxJ 136 was observed as a statistical outlier (31.8%). Similar variability was noted in absolute bioaccessibility (1.2-4.3 mg/100g f.w., $p < 0.0001$). Sample association between raw material content and absolute bioaccessibility included DxJ 228 and 003, which were both found to be in the top 15**%.** Similar association were observed between

DxJ 114 and 004 in terms of relative and absolute bioaccessibility. Both were noted to be within the bottom 15% of both bioaccessibility values.

Phenolic acids exhibited relative bioaccessibility values that ranged from 0.3-2.1% ($p < 0.0001$) with outliers including DxJ 114 (3.8%), 264 (7.9%), and the freeze-dried blueberry control (12.4%). Absolute bioaccessibility ranged from 0.03-0.9 mg/100g f.w. ($p < 0.0001$). Specifically, DxJ 114 was highly ranked (top 15%) amongst all samples within absolute and relative bioaccessibility as well as raw material content. Likewise, DxJ 120 was ranked highly within absolute and raw material content, however not in relative bioaccessibility, while DxJ 138 was observed in both the bottom 15% of both raw material content and bioaccessible content. Looking into the relationship between relative and absolute bioaccessibility, DxJ 123 ranked was found to be highly ranked amongst all samples. Rankings for lowest relative bioaccessibility and absolute bioaccessibility were determined to be closely related as 80% of the bottom 15% were the same, including DxJ 100, 079, 249 and 158.

Bioaccessibility for FLAVs ranged from 10.7-54.1%, 0.8-5.5 mg/100g f.w. ($p < 0.0001$). In terms of relative bioaccessibility, DxJ 035 was noted to be an outlier (68.1%). Noteworthy samples include DxJ 053, 035 (top 15%) and 004, as they all were found to be associated between bioaccessible and raw material content, while DxJ 027 and 120 reported association between relative and absolute bioaccessibility only. Specifically, DxJ 053 and 035 are both ranked in the top 2 highest relative and absolute bioaccessibility where as DxJ 027 and 120 were observed to be within the lowest 15%. However, DxJ 053 is noted to be highly ranked in FLAV raw material content, while DxJ 035 is seen in to be the lowest ranking of all the samples. DxJ 004 had the lowest bioaccessibility (both relative and absolute) and was similarly ranked in the bottom 15% of FLAV raw material content.

Significant variation was observed amongst F3Ls relative bioaccessibility ranging from 1.0-49.1% ($p < 0.0001$), while DxJ 053 was deemed a statistical outlier at 52.5%. Specifically, DxJ 136 was found to rank highly in both raw material content and absolute bioaccessibility, while DxJ 138 and 026 fell into the bottom 15%. Majority of the lowest ranking samples in terms of relative bioaccessibility (lowest to greatest; DxJ 026, 120, 019, 027) were also found within the lowest 15% of absolute bioaccessibility. Additionally, DxJ 019 was observed to be highly ranked in raw material content, while DxJ 026 was considered one of the bottom 15% of raw material content. Both DxJ 053 and 068 are ranked in the top 15% for both absolute and relative bioaccessibility. Conversely, DxJ 235 was ranked in the bottom 15% in terms of relative bioaccessibility and raw material content.

Amongst the 33 individual genotypes, significant variation was noted in raw material content and both absolute and relative bioaccessibility. Although natural variation was expected, observed variability between days could potentially be attributed to points in the harvesting and processing steps prior to digestion. Specifically, during processing blueberry samples were blended separately, dependent on day rather than in bulk per genotype. It has been shown that reproducibility was significantly increased during bulk homogenization (Barnes et al. 2009). Similarly, studies have demonstrated the use of lyophilized or freeze-dried ground samples as they were shown to increase reproducibility as compared to ground fresh berries (Barnes et al. 2009; Grace et al. 2014) similar to those used in the current study. Additionally, prior to processing, the blueberries were photographed, post-harvest, to observe color and shape differences amongst varying blueberry cultivars. During this photographing process, blueberries were partially thawed and placed under bright lighting for different amounts of time before they were re-frozen. Similarly, during processing, the blueberries were thawed again for blending purposes. Likewise,

to prepare for digestion, the processed blueberries were thawed to be aliquoted for digestion. Ultimately, the aliquoted blueberries were thawed a last time the day of the digestion. Therefore, on average the blueberries were frozen and rethawed around four times throughout the entire process. Stability is greatly impacted by such influences and therefore could result in decrease in overall content (Patras et al. 2010; Alighourchi and Barzegar 2009; Reque et al. 2014; Rubinskiene et al. 2005; Buckow et al. 2010). Considering these are challenges often observed in a first-time application, it is expected that streamlining of these post-harvest processing and phenotyping would reduce the potential for this variability.

2.5. Conclusions

Overall, it was determined that the adapted HT *in vitro* digestion model does provide a new tool for phenotyping phenolic bioaccessibility in blueberry fruit germplasm collections as it was observed to be highly correlated to the existing model described by Moser et al. (2016). Application to a subset of 33 different blueberry genotypes further suggest that variation in phenolic bioaccessibility exist as a potential result of individual genetic makeup. Therefore, this supports the notion of broader application to diversity panels and other collections to inform for breeding programs and aid in the development of strategies to enhance the nutritional quality of fruits.

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CHAPTER 3. CONCLUSIONS AND FUTURE CONSIDERATIONS

The consumption of fruits and vegetables is based upon the wide array of evidence promoting the role of these nutrient and phytochemical dense foods (i.e. carotenoids and polyphenols) in satisfying nutritional requirements and in the prevention of select chronic diseases. However, intake of fruits and vegetables by the average American remains significantly below the recommended amounts. Novel strategies are needed to address the gap in delivery of fruit and vegetable benefits to consumers. Given that the associated health impacts from fruits and vegetables are ultimately dependent on their phytochemical density and their absorption and metabolism (bioavailability), it has been suggested that advances in breeding programs can be leveraged to provide fruits and vegetables with higher levels of these nutrients and phytochemicals. However, factors driving bioavailability of these compounds is equally as critical to consider in breeding for more effective fruits and vegetables. Critical to this goal is the development and implementation of novel phenotyping methods for functional traits including bioavailability that can in fact be combined with other "omic" technics techniques in a manner consistent with enhancement of existing breeding programs.

While existing breeding programs seeking to enhance our understanding of factors impacting nutrient and phytochemical profiles and content in fruits and vegetables, little is known regarding specific traits that are associated with bioavailability of target nutrients/phytochemicals. To date, it is known that nutrient and phytochemical bioavailability are best evaluated by *in vivo* models, including human clinal trials and animal studies. However, given the high cost, extensive time required, associated ethical considerations, and bottleneck for high throughput analysis, in *vitro models* of the gastrointestinal tract have proven valuable in the estimating the availability of nutrients and phytochemical for absorption, known as bioaccessibility, a precursor and predictor

of bioavailability. These models have demonstrated some practical applicability to screening of limited numbers of genotypes (J. Aragón et al. 2018). While promising, the implementation of current digestion models to screening of large germplasm collections and thereby enhancing the value of the "bioaccessibility phenotype" remains challenged by throughput constraints, due their dependence on manual processing and need for larger tissue samples/digestion volumes.

As a primary outcome of this thesis and associated work we aimed to adapt a current 3 stage *in vitro* digestion model used extensively in determination of food matrix factors impacting nutrient and phytochemical bioaccessibility from fruits and vegetables through semi-automation and sample reduction in the development of a new model suitable for higher throughput but without loss of predictive power. This required extensive consideration of the advantages and logistics of the existing model and consideration of the limitations, and potential for modification to allow for semi-automation an existing static three compartment *in vitro* gastrointestinal digestion model for high throughput (HT) phenotyping of the bioaccessibility of blueberry polyphenols. Following validation of the HT model against the standard in vitro digestion models (ST), the HT model was then applied to a subset of 33 individual blueberry genotypes from a mapping population currently used in polyphenolic research.

As described in Chapter 1 and 2, blueberries were selected as our target fruit based on their diverse array and relatively high concentration of polyphenols and wide variety of associated health benefits. Additionally, there are diverse public germplasm available as the US is the largest producer and processors of the fruit, therefore are broadly available. Utilizing previously frozen blueberries that underwent further processing, we were able to successfully adapt an existing in vitro digestion model adapted for polyphenol analysis, previously described 3 stage *in vitro* digestion model by Moser et al. (2016) (Moser et al. 2016). Specific modifications were applied

to make the model compatible with semi-automation and the limited tissue samples typical of germplasm collections. Initially, we established that scaling the protocol to lower digestion volumes was necessary to enable high throughput analysis. This meant reducing the overall volume from a total of 50 mL to 15 mL. By doing this, we further proportionally decreased the amount of blueberry starting material to be digested as well as volume of enzymes and saline solutions by a factor of 3.3x. Additionally, we adjusted enzyme concentrations to better apply to digestion of blueberries. A small but key adaptation in this case was the added step of enzyme solution clarification. Enzyme solutions required clarification by centrifugation in order not to clog the fine-tipped syringes of the Tecan. To further enhance efficiency of the HT model, we programmed a liquid-handling robot (Tecan Evo 150) to distribute all liquid solutions (enzyme solutions, 0.9% saline, pH altering solutions (HCl and NaHCO3) throughout the digestion. This semi-automation improved overall precision and allowed for streamlining of the process compared to manual digestions.

Comparison of the LT model to the adapted HT model showed significant differences in a majority of polyphenolic subclasses and individual compounds in both relative and absolute bioaccessibility. However, while statistically significant, these differences were mostly modest. Higher bioaccessibility values were consistent amongst blueberries applied to the HT model relative to the LT model. While the reason for this is unclear, we propose that the differing levels may be attributed the smaller surface area of the 15 mL digestion test tubes as opposed to the 50 mL test tubes, more thorough mixing of the Tecan digestions by means of higher oscillation speeds and more compact rack placement within the incubator. A second explanation could be the higher stability of the phenolics in the lower volumes. Despite a lack of headspace and flushing with N_2 in both systems, the potential for higher levels of dissolved oxygen in the 50 mL digestions could

be responsible, in part, for the outcomes observed. Despite these differences, the two models were found to be highly correlated in both relative and absolute bioaccessibility across all compound classes. While future work must consider validation by in vivo models to truly ensure the predictive nature of this model, our findings provided us the validation needed to move forward with applying the HT model to a subset of 33 individual blueberry genotypes from a mapping population in hopes of detecting variability in the bioaccessibility as a trait.

The DxJ mapping population was selected for our initial application because of their established diversity in anthocyanin profiles (Hancock et al. 2018). Consistent with these previous reports, the DxJ blueberries on average were found be largely composed of anthocyanins, followed by phenolic acids, flavonols, and insignificant amounts of flavan-3-ols. Initial analysis of the polyphenol profile and content of the 33 genotypes making up the DxJ subset showed significant variation amongst all targeted polyphenolic classes. This was in agreement with previously published literature where large variations in polyphenols as a result of different genotypes (Kalt et al. 2001; Yousef et al. 2013). Similar variation was noted in terms of both relative and absolute bioaccessibility. Flavonols were observed to consistently have the highest relative bioaccessibility, while the greatest average bioaccessible content was similarly noted within flavonols. When analyzing potential correlation between raw material content and bioaccessible content, it was concluded that few samples were determined to be correlated, thus confirming the importance of absorption (bioavailability) studies as the berries with the highest phenolic content were not always the most bioaccessible.

In conclusion the HT *in vitro* gastrointestinal digestion model adapted in the current work was determined to be novel tool for phenotyping phenolic bioaccessibility in blueberry fruit. This further establishes the need for continued screening of diverse blueberry germplasms and other

plant materials in combination with genomic approaches. This work is currently underway by our group with multi-year phenotyping to be completed by 2020 and subsequent alignment of this phenotypic data set with other “omic” datasets (genomic and metabolomic) expected to yield valuable insights in the genetic control of both polyphenol levels and their bioaccessibility. Overall, this will allow for greater understanding and investigation of specific genetic factors that influence polyphenolic density and bioavailability. The information gained from such advanced phenotyping and genetic techniques will ultimately allow for breeding of fruits and vegetables with higher nutritive content and bioavailability.

3.1. REFERENCES

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APPENDICES

Appendix A. Supplemental Materials for Chapter 2

Standard Operating Procedure:

Micro-Scaled, Three Stage In-vitro High-throughput Digestion Protocol for Blueberry

Ferruzzi Lab Protocol

Tecan Preparation: to be done before operating the machine for digestion

Maintenance Steps:

1. Replace DI Water in existing bottle with at least 2L of fresh DI water.
2. Gently move syringe tubing (located above the syringes) up and down to help circulate air bubbles throughout the tubing.
3. Using a chem-wipe with isopropanol to lightly wipe all syringe needles to remove any residual material.

Programming Steps:

1. Turn on Tecan by pressing and holding down the on/off button until hearing a beep.
2. Log-on to Standard EVO-Ware Standard
    a. EVO-ware standard is the user interface that controls the Tecan's ability to pipette, move well-plates, etc.
    b. Once logged into system, click "Run an existing script" followed by clicking "Start Your Selection".
3. To prepare the machine for use, click the "Initialize" (red X-shaped icon) on the main tool bar.
    a. During initialization, all 8 syringes and the single robotic arm will be assessed to ensure proper functioning. Tecan must complete initialization before utilized.
4. Once initialized, select the "open" file icon on the main tool bar. Within the folder, select the manuscript labeled "Pipette Rinse".
    a. This manuscript will wash and rinse each syringe a total of 3 times with DI water.

Digestion Preparation:

Stock Solutions:

0.9% NaCl
100mM NaHCO3
1.0M NaHCO3
100mM HCl
1.0M HCl

Gastric and Small Intestinal Phase Solution:

Pepsin Solution (0.6mL per reaction)
10mg/mL Pepsin in 0.1M HCl
Pancreatin-Lipase Solution (0.6mL per reaction)
20mg/mL Pancreatin in 100mM NaHCO3
10mg/mL Lipase in 100mM NaHCO3
Bile Solution (0.9mL per reaction)
30mg/mL Bile Extract in 100mM NaHCO3

Other Materials: 15mL tubes, 100 ul pipette, pH meter, incubator, 2mL micro-centrifuge tubes, micro-centrifuge

Preparation of Oral Phase:
Base Solution (q.s. to 1L with DI water):

Potassium Chloride 1.792g
Sodium Phosphate 1.776g
Sodium Sulfate 1.140g
Sodium Chloride 0.596g
Sodium Bicarbonate 3.388g

For high throughput (n=96 samples) Tecan run:

Prep 200mL oral phase solution
Need 1.8mL per digestion

1. Add 200mL base solution to beaker with stir bar
2. Add 80mg urea
3. Add 6mg uric acid
4. Add 10mg mucin
5. Add 6.36g a-amylase
6. Mix well (at least 15 min)
7. After mixing, utilize (6) 30mL centrifugation tubes and centrifuge at 10000xg for 10min

Start Up

1. Weigh out 1.0g of blended berry to be digested into 15mL tubes
2. Insert tubes into Tecan tube racks (n=16 tubes per rack)
3. Slide racks into appropriate columns within Tecan.
4. Pour oral phase (post-centrifugation) into labeled plastic Tecan troughs
    a. Place into appropriate well location, as indicated on Tecan Worktable visual

Oral Phase

1. Select "Open" file icon on the main tool bar. Within the folder, select the manuscript labeled "HLB_OralPhasePICollection". Select "Run" (green triangle icon) on the main tool bar.
    a. 1.8 mL of oral phase will be aspirated and dispensed into sample tubes according to location
2. Remove Tecan racks and begin capping samples. Once capped, vortex for 10 seconds and carry all racks to incubation. Insert into Lego rack holders within incubator.
    a. Let samples incubate at 37 degrees Celsius at 150 opm for 10 minutes.
3. Meanwhile, prepare pepsin solution and calibrate pH meter.

Gastric Phase

1. Remove all sample racks from incubation and uncap all samples. Slide back into appropriate columns within Tecan.
2. Pour saline and pepsin solution into individual labeled troughs.
    a. Place into well location.
3. Select "Open" file icon on the main tool bar. Within the folder, select the manuscript labeled "HLB_GastricPhasePICollection". Select "Run".
    a. 6.2 mL of saline will be aspirated and dispensed into sample tubes, bringing volume to 9 mL.
    b. 0.6 mL of pepsin will be aspirated and dispensed into sample tubes
4. Remove samples from Tecan and begin to cap. Once capped, invert each rack 3 times to mix and place immediately on ice.
5. Adjust pH to equal 2.5 +/- 0.1 1M using 1.0 M HCl.
    a. Adjust 1 sample out of each Tecan rack (column). Average adjustment volumes should be applied to the remaining samples. *Refer to step 6 to implement specific acid and calculated Saline volume on Tecan. Saline addition should bring volume to 12mL.*
6. Select "Open" file icon on the main tool bar. Within the folder, select the manuscript labeled "HLB_HClAdjust_SalineAdd_PICollcetion".
    a. To edit adjustment volume, right click the "aspirate" command within the established script labeled "HCl". Type in the selected HCl volume, click "Ok". Similarly, right click the "dispense" command within the established script labeled "HCl". Adjust the volume to reflect the aspiration volume, click "Ok".
    b. Repeat with Saline.

7. Uncap samples and place back in appropriate racks. Slide into Tecan columns.
8. Select “Run” to initiate adjustments.
    a. HCl will be aspirated and dispensed, followed by saline aspiration and dispensing.
9. Remove Tecan racks and begin capping samples. Once capped, invert each rack 3 times to mix and carry all racks to incubation. Insert into rack holders within incubator.
    a. Let samples incubate at 37 degrees Celsius at 150 opm for 1 hour.
10. Meanwhile, prepare bile extract. 30 minutes into incubation, prepare pancreatin-lipase solution and let stir for 12 minutes.
    a. Post-stirring, utilize 30mL centrifugation tubes and centrifuge at 10000xg for 10min

Intestinal Phase

1. Pour bile and pancreatin-lipase solutions into labeled plastic Tecan troughs
    a. Place into appropriate well location, as indicated on Tecan Worktable visual
2. Select “Open” file icon on the main tool bar. Within the folder, select the manuscript labeled “HLB_IntestinalPhasePICollcetion”. Select “Run”.
    a. 0.6 mL of (centrifuged) pancreatin-lipase solution will be aspirated and dispensed into sample tubes
    b. 0.9 mL of bile will be aspirated and dispensed into sample tubes
3. Remove samples from Tecan and begin to cap. Invert each rack 3 times to mix. Place immediately on ice.
4. Adjust pH to equal 6.5 +/- 0.1 1M using 1.0 M NaHCO3.
    Adjust 1 sample out of each Tecan rack (column). Average adjustment volumes should be applied to the remaining samples. *Refer to step 6 of “Gastric Phase” to implement specific base addition and calculated Saline volume on Tecan. Saline addition should bring volume to a final total of 15mL.*
5. Remove Tecan racks and begin capping samples. Once capped, invert each rack 3 times to mix and carry all racks to incubation. Insert into rack holders within incubator.
    a. Let samples incubate at 37 degrees Celsius at 150 opm for 2 hours.

Separation of Aqueous Fraction

1. Remove from incubation and uncap all samples. Slide back into appropriate columns within Tecan.
2. Place two 2mL 96-well microcentrifuge plates onto appropriate rack within Tecan.
3. Select “Open” file icon on the main tool bar. Within the folder, select the manuscript labeled “HLB_PreCentifugationPICollection”. Select “Run”.

   a. 3mL of each sample will be aspirated and 1.5 mL will be dispensed into each 2 mL 96-well microcentrifuge plate for centrifugation.
   b. Cap both 96-well plates and centrifuge at 4100 RPM for 1 hour.
4. Following centrifugation, syringe filter aqueous fraction into two new 2mL 96-well microcentrifuge plates through 0.45 mm filters.