CHEMICAL CHARACTERIZATION AND ABSORPTION OF PHYTOCHEMICALS FROM *MANGIFERA INDICA* L.

A Dissertation

by

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ABSTRACT

It was hypothesized that ester-linked gallic acid glycosides could be absorbed and metabolized *in vivo* due to the instability of an ester-linked glycosides at neutral pH. To evaluate *in vivo* absorption of *Mangifera Indica,* L. var. Keitt polyphenolics, it was first necessary to chemically characterize the compounds present using HPLC-MS^n analysis. Mango pulp and extracts were also incubated with a pectinase, cellulase, pectinase with ß-glucosidase activity, and a pure ß-glucosidase to learn the extent of hydrolysis with potential application to enhancing bioavailability as a result of incubation to increase mango juice yield. After which the same extracts were assessed in differentiated Caco-2 cells to discern stability at physiological pH and to characterize metabolite formation *in vitro*. Finally, human urinary metabolites were characterized after 10 day consumption of 400 g in 11 individuals. Mass spectroscopic characterization and HPLC quantification of mango pulp revealed for the first time two monogalloyl glucosides (MGGs) with distinct differences in their glycoside linkages, with the ester form dominating, as well as the presence of five other phenolic acid glycosides; hydroxybenzoic acid glucoside, courmaric glucoside, ferulic acid glucoside, and sinapic acid mono and di-glucosides. Six oxygenated carotenoid derivatives were identified for the first time in a phytochemical extract, namely, a phytohormone, abscisic acid and its glycoside, two catabolism products of abscisic acid, dihydrophasic acids, and two hydroxy-dimethyl decadiene-dioic acid glucopiranosylesters. Gallotannins ranging from tetra-galloyl glucosides to nona-galloyl glucosides were also identified in the pulp, but

not quantified. Clearzyme 200XL and Rapidase AR2000 were the most effective at increasing juice yield of mango pulp due to their pectinase action. Cz reduced the amount of ester-linked MGG by 70% after 4 hours of incubation while Rap hydrolyzed the ether linked MGG. The instability of ester-linked galloyl-glycosides at pH 7.4 was characterized by HPLC-MS and after only four hours of incubation a shift from HWM tannins (>8GG) to LMW (<8GG) occurred and resulted in 25 mg/L free gallic acid. Caco-2 cells metabolized gallic acid, MGG from a mango extract, and a gallotannin extract into *O*-methyl gallic acid indicating catechol-*O*-methyl transferase (COMT) as a major metabolizing enzyme for gallic acid. Urinary metabolites were identified by HPLC-MS^n in dependant scans. *O*-methylgallic acid-*O*-sulfate was identified as the major metabolite 0-6 hours post consumption, followed by *O*-methylgallic acid at a lower concentration. The presence of gallic acid metabolites in the urine indicates absorption of ester-linked glycosides. Colonic metabolites were detected beginning 3-6 hours after consumption of mango, and were identified as pyrogalloyl derivatives. They are hypothesized to be the products of microbial breakdown of gallotannins. Daily consumption of mango for 10 days increased the concentration of *O*-methylgallic acid-*O*-sulfate, but was not significant.

DEDICATION

To my parents, Gerard and Theresa Krenek, for their unconditional love and support of my dreams.

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Ad maiorem Dei gloriam

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CHAPTER I

INTRODUCTION

Mango (*Mangifera Indica* L.) is traditionally grown in tropical regions of the world but has since gained popularity in the U.S. where it has become a major import crop with over 360,000 metric tons imported in 2012 (USDA 2012). Mangos are reported to be good sources of vitamin C, dietary fiber, phenolic compounds, carotenoids and are eaten for their fragrant aroma and tropical flavor (Mahattanatawee et al., 2006; Mercadante, Rodriguez-Amaya, & Britton, 1997; Perkins-Veazie, 2007). The bark, peel, leaves, and seed kernel of the mango contain high concentrations of phytochemicals which together comprise the formula for many traditional medicines to treat dysentery, asthma, and a host of other ailments (Barreto et al., 2008; Sairam et al., 2003; Singh, 1986). Barreto et al. (2008) reported xanthone C-glycosides, gallotannins, and benzophenone derivatives while (Berardini, Carle, & Schieber, 2004) found eighteen gallotannins and five benzophenone derivatives in the peel of Tommy Atkins variety. The pulp of mango fruit, although consumed worldwide has not been chemically characterized in its entirety and to a lesser extent in comparison to the seed kernel, peel, leaves, and bark however, gallic acid and gallotannins were identified (Kim, Lounds-Singleton, & Talcott, 2009). In plant extracts, gallic acid esters of glucose have been reported to range from one to twelve degrees of polymerization (DP) and most research suggests that gallotannins are not absorbed intact. However, research has not been conducted on higher molecular weight gallotannins that occur in diverse ranges of

molecular weights and in the company of other polyphenols and even less is known about the metabolic fate of gallotannins in humans. The degradation of pentagalloyl glucoside in slightly alkaline pH was reported by (Krook & Hagerman, 2012) suggesting that gallotannins could be degraded when exposed to intestinal conditions and could potentially serve as a great reserve for free gallic acid. Gallic acid is absorbed paracellularly due to its polarity and hydrophilicity, and both gallic acid and gallic acid metabolites, such as 4-*O*-methyl gallic acid, have been detected in the plasma and urine of humans and rats following the consumption of gallic acid rich food sources, suggesting gallic acid is absorbed *in vivo* and further metabolized. However, it is not known if gallic acid glycosides are hydrolyzed *in vivo* and if subsequent free gallic acid can be absorbed and metabolized.

The objectives of this work were to:

1. Extensively characterize and quantify the phytochemical compounds in Keitt mango pulp.
2. Determine the effects of commercial enzymes used in juice production on the phytochemical profile of Keitt mango and additionally determine if ß-glucosidase (an enzyme found in the small intestine) is capable of hydrolyzing gallotannins bound to protein.
3. Characterize metabolites produced from mango extracts using differentiated Caco-2 cells and determine stability of compounds in a pH 7.4 environment.

4. Identify, characterize, and quantify urinary metabolites from 11 participants following the consumption of 400 g mango over 10 consecutive days.

CHAPTER II

LITERATURE REVIEW

Mango: The King of Fruits

Mango (*Mangifera Indica* L.) is arguably the most popular fruit consumed world-wide and has gained popularity in the United States recently with increased imports from Central and South America providing abundant, inexpensive fruit to service these demands. Over 1000 mango varieties have been identified and commercial production has been cited in 87 countries (Iyer, 1989; Tharanathan, Yashoda, & Prabha, 2006). They are reported to be good sources of vitamin C, dietary fiber, phenolic compounds, carotenoids and are eaten for their fragrant aroma and tropical flavor (Mahattanatawee et al., 2006; Mercadante et al., 1997; Perkins-Veazie, 2007). Mangoes are cultivated in tropical and subtropical regions of the world, with a large majority of the cultivation taking place in India, China, Mexico, Thailand, and Brazil (FAOSTAT 2012). In the last decade, however, mango production has increased in areas that were not typical for mango cultivation such as South and Central America, Egypt, and Southeast Asia. Many South American and Southeast Asian countries rely on the commodity for exportation profits which has a large impact on their local economy, yet still less than 3% of the global production of mangos are traded (Evans, 2008; Tharanathan et al., 2006).

Mango's world-wide popularity did not begin to reach the United States until the 1990s and due to climatic reasons, Florida was the only state that produced mangos for

meaningful distribution. Prior to Hurricane Andrew, Florida reported close to 3,000 acres planted for mango production, yet many trees were lost to the storm and never replanted. Florida now boasts less than 1700 acres planted for commercial production and a modest harvest of 3,000 metric tons per year (Evans, 2008; Mossler & Nesheim, 2001). It is not a surprise then that U.S. production alone was not able to keep up with the growing demand for fresh, reasonably priced fruit, and has become the largest importer of mango fruit at approximately 360,000 metric tons per year (USDA 2012). Between the years 2003-2005, the U.S. captured 32.7% of the import market, sourcing most mangos from Mexico, Peru, Brazil, and Ecuador (FAOSTAT 2007) and American's mango consumption has steadily increased over the last 14 yrs driving a 56% increase in the amount of fresh mango fruit imported from 1998 to 2012 (USDA Market News 2013). Despite the small amount of mango cultivation in the US, many of the world's most popular commercial varieties were actually developed in Florida and include: Haden, Kent, Keitt, Palmer, Tommy Atkins, and van Dyke. They are cited as superior mango cultivars for exportation due to their firmer texture, less fibrous pulp, and suitability to long-distance travel than other mango varieties (Evans, 2008; Galán Saúco, 2002). The most popular global commercial variety, Alphonso, is native to India and is liked for its thin skin, soft flesh, low fiber content, and sweet aroma (Tharanathan et al., 2006). This variety was not available in the U.S until 2007 and despite much popularity among internationals, the price has remained too high for this variety to become a major contender in the U.S. fresh mango market (Ferrier, Petersen, & Landes, 2012; Sharp, 1993).

The fresh fruit market in the U.S. (and world-wide) is limited by the shelf-life of mangos. A physically mature, green mango will ripen in 6-7 days and will become overripe in 15 days after harvest (Mitra, 1997). The use of exterior coatings have been suggested as shelf-life extenders, and research has proven that a polysaccharide based coating was superior to a wax coating at delaying ripening and concentrating volatile components (Baldwin et al., 1999). Yet still, the most employed way of delaying ripening as with most produce, is the use of cold storage (Sivakumar, Jiang, & Yahia, 2011). However, because mangos are tropical, they are particularly susceptible to chill injury which has been shown to increase their oxidative stress and negatively affect their sensory properties (Dat et al., 2000). Prior to entering the U.S, mangos must first be treated to ensure the death of invasive insects such as the Mediterranean fruit fly and the Mexican fruit fly (Jacobi, MacRae, & Hetherington, 2001). Irradiation and hot water treatment (HWT) are currently the only methods approved for post-harvest decontamination of mangos, with HWT serving as the most common method (Kim et al., 2009). Current regulations require complete immersion treatment in water at 46.1 °C over times that range from 65-110 min depending on cultivar and fruit size (USDA-APHIS 2002). Following HWT, mangos are typically stored at 5°C to extend shelf life which causes chilling injuries to the fruit and negatively affects aesthetic qualities. Phytochemical and carotenoid losses were also observed following HWT and cold storage, but were reported to be only minor (Talcott, Moore, Lounds-Singleton, & Percival, 2005). Since the development of shelf-life extension processes for mango and

high quality exportation varieties, mango has become more common place in the American diet.

Composition and Physiology

Mangos are members of the Anacardiaceae family and are kin to both the cashew and poison ivy. They are drupes, possessing a waxy endocarp (peel) and a fleshy mesocarp (pulp) that surrounds a shell with a seed inside; similar to what is found in fruits such as a cherry, peach, and apricot. Mangos are climacteric fruits indicated by their ability to produce ethylene gas and ripen post-harvest from a mature, green stage. Along with ethylene, the presence of abscisic acid (ABA) and the catabolism products of ABA, phaseic acid, dihydrophaseic acid (DPA), and epi-DPA in mango pulp increase during the storage of mangos. ABA was shown to play a role in mango ripening and senescence, and is rapidly synthesized when stress is added to a plant (heat, salinity, chilling) as part of its defense mechanism (Kondo, Sungcome, Setha, & Hirai, 2004). ABA is believed to be produced by the oxidative cleavage of the C_{15} carbon skeleton from a C_{40} carotenoid as shown in (Figure 1). A recent review article overviewed the possible roles that ABA might play as a universal signaling molecule and noted the production of ABA in mammalian tissues and cells (leukocytes, pancreatic cells, and mesenchymal stem cells) and the potential application to many human diseases. ABA was shown to regulate cell growth, development and immune responses through a signaling pathway similar to that found in plants which makes it relevant for characterization and quantification in foods that are consumed (H.-H. Li et al., 2011).

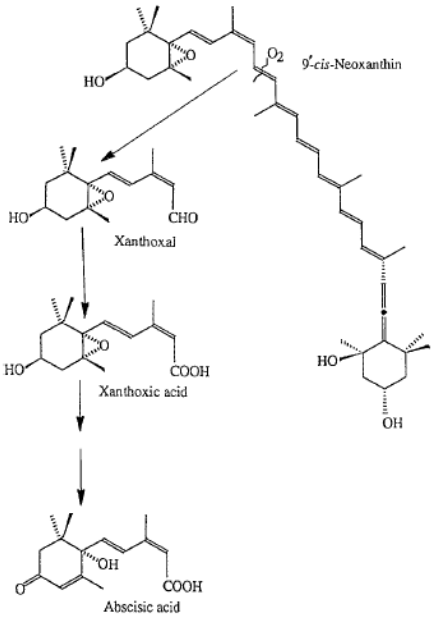

Figure 1. Proposed pathway of ABA in chloroplasts (Milborrow, 2001).

In an unripe state, mangoes are higher in starch, titratable acidity (TA), total soluble solids (TSS), chlorophyll and are lower in carotenoids as compared to their ripened counterparts. Climacteric fruits show a sudden, marked increase in their respiration rate when ripening occurs and can use organic acids like citric acid as substrates. The decrease in citric acid is responsible for the appreciable increase in pH seen upon ripening (ovar, arc a, Mata,). Amylase production is increased simultaneously with ethylene, accounting for the decrease in fruit firmness and by the subsequent hydrolysis of starch (White, 2002). The sugar content of mangos varies with each stage of ripening, but generally sucrose predominates accompanied by the reducing sugar, fructose with total sugar concentrations as high as 10% of fruit weight (Medlicott & Thompson, 1985). Mango pulp is also rich in dietary fiber, which includes both pectin and cellulose. One study reported 1.4 and 0.51 g per 100 g fruit of dietary fiber and

pectin, respectively in Keitt mangos, with no changes associated with fruit ripening (Mahattanatawee et al., 2006). Vitamin C content varies between cultivars, but all analyzed (Tommy Atkins, Atualfo, Kent, Keitt, and Haden) contained at least 20% of the recommended dietary intake of 60 mg/100 g, making the mango an excellent source of vitamin C per serving (Perkins-Veazie, 2007). Mangos are a staple part of the diet of many developing countries which raises this source of Vitamin C to a higher level of importance in its ability to impact health.

Carotenoid biosynthesis is responsible for the characteristic yellow color of mango flesh and the change of the pulp from white to yellow, to orange, has been shown to be a good indication of ripeness (Ornelas-Paz, Yahia, & Gardea, 2008). Carotenoids from mangos have been extensively studied and unanimously report *all-trans-ß*-carotene as the predominant carotenoid in most ripe varieties followed closely by a xanthophyll, *all-trans*-violaxanthin (Hewavitharana, Tan, Shimada, Shaw, & Flanagan, 2013; Manthey & Perkins-Veazie, 2009; Mercadante et al., 1997; Ornelas-Paz et al., 2008). *ß*-carotene is a precursor to vitamin A which has been associated with many health benefits and is especially important in impoverished areas where mangos make up a staple part of the diet (Edem, 2009). Because of the importance of pro-vitamin A, a considerable amount of research has been conducted discerning the stability of *ß*-carotene during differing storage treatments, hot water treatments, and over its ripening period. In general, carotenoids were shown to increase after initial hot water treatment after 10-20 days storage, but were negatively affected by the combination of cold storage (5°C) and hot water treatment (Talcott et al., 2005). *ß*-carotene specifically

was also reported to rise in concentration as a mango ripened and was quantified in the Keitt and Tommy Atkins varieties as a 3-fold increase in ß-carotene from the mature green stage to the ripe stage (Mercadante & Rodriguez-Amaya, 1998).

Polyphenolics

All chemical compounds produced by a plant which are not products of their primary or intermediate metabolism, and which give plants their distinct color, taste, and smell are termed secondary plant metabolites (Bennett & Wallsgrove, 1994). Polyphenolics are secondary plant metabolites found in mangos and all higher plants, and have been extensively researched for their composition, stability, flavor, and health benefits and are understood to play an important role in the defense mechanisms of plants (Bennett & Wallsgrove, 1994; Ye, Li, Yan, Liu, & Ji, 2002). They are abundant and diverse, with over 9000 phenolic structures identified to date (Williams & Grayer, 2004). The Shikimic Acid pathway is the main metabolic pathway that leads to the synthesis of the aromatic compounds tryptophan, phenylalanine, and tyrosine, which serve as precursors to many phenolic compounds (Ghasemzadeh & Ghasemzadeh, 2011). There are four major classes of polyphenols, flavonoids, phenolic acids, tannins, and to a lesser extent, stilbenes and lignans. Phenolic acids (Figure 2) are the simplest group of phenolic compounds and contain two sub-classes, benzoic acids and cinnamic acids. Hydroxybenzoic acids are comprised of a benzene ring, a carboxylic acid group, and at least one hydroxyl group. They can be found in either their free (aglycone) form, or attached to a sugar such as glucose, and are direct derivatives of shikimic acid.

Cinnamic acids, while similar to benzoic acids in structure, are derived from the phenylpropanoid branch of the Shikimic Acid Pathway and can be esterified to a sugar or in their aglycone from (Quideau, Deffieux, Douat-Casassus, & Pouysegu, 2011).

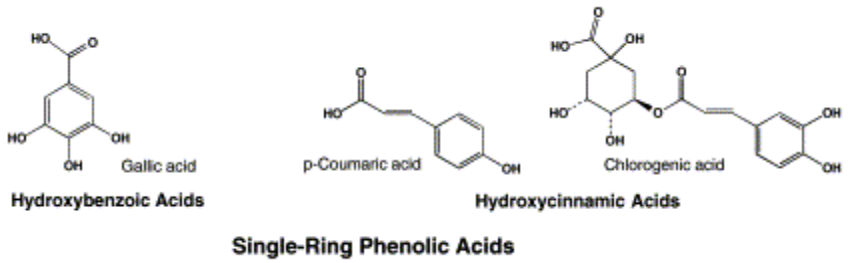

Figure 2. Common structures of phenolic acids

Flavonoids are the largest and most studied class of compounds and are derived from the Phenylpropanoid Metabolic pathway in which phenylalanine in combination with malonyl-CoA, produce the backbone of flavonoids. Subgroups of flavonoids include flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids. The aglycone structure (Figure 3) consists of a benzene ring (A) condensed to a six-membered ring (C), which in the 2 position, contains another benzene ring (B). The chemical substitution and placement of substituents is the determinant in which group a flavonoid is classified (Rathee et al., 2009).

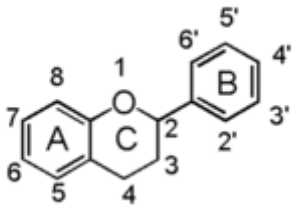

Figure 3. Basic flavonoid structure

The bark, peel, leaves, and seed kernel of the mango contain high concentrations of phytochemicals which together comprise the formula for many traditional medicines to treat dysentery, asthma, and a host of other ailments (Barreto et al., 2008; Sairam et al., 2003; Singh, 1986). The interest in the peel, leaves, seed kernel, and bark sparked extensive chemical characterization of each. Barreto et al. (2008) reported xanthone C-glycosides, gallotannins, and benzophenone derivatives (Figure 4) while Berardini et al. (2004) found 18 gallotannins and five benzophenone derivatives in the peel of Tommy Atkins variety, 21 different gallotannins in the seed kernels, and eight in the pulp. The seed kernel remained the highest reservoir for gallotannins with 15.5 mg/g dry weight, proving to be a good source for isolation of large quantities of tannins as potential by-product application from juice production (Barreto et al., 2008; Berardini et al., 2004). Cyanadin-3-*O*- galactoside, an anthocyanin, was reported in select varieties of mango peel where a red color is characteristic to that specific variety (Berardini et al., 2005), but are non-existent and all other parts of a mango. The pulp of mango fruit, although consumed worldwide has not been chemically characterized in its entirety and to a lesser extent in comparison to the seed kernel, peel, leaves, and bark. This incomplete characterization in part is due to the differences in chemistry between varieties and the low concentrations that some compounds are present in (Manthey & Perkins-Veazie, 2009). In a mango fruit concentrate (variety not reported), *p*-hydroxybenzoic and cinnamic acids such as gallic acid, caffeic acid, protocatechuic acid, and *p*-coumaric acid were found along with the xanthone, mangiferin and both aglycones and glycosides of

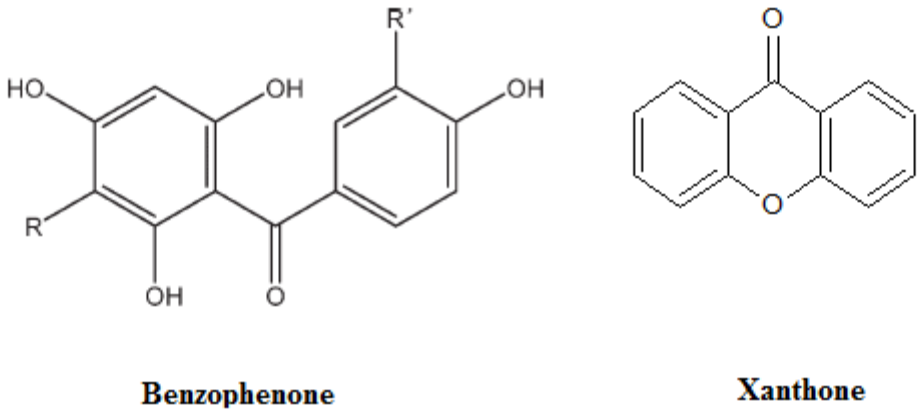

Figure 4. Basic benzophenone and xanthone structures of polyphenolics found in leaves, peel, seed kernal and bark of a mango plant.

the flavonoids kaempferol and quercetin (Schieber, Ullrich, & Carle, 2000). Nine different mango cultivars werescreened for flavonol *O*- and xanthone C-glycosides and despite high concentrations reported in the peels, only trace amounts were found in the edible flesh (Berardini et al., 2005). Free gallic acid and gallotannin were tentatively identified as the major polyphenolics in Tommy Atkins variety by HPLC analysis and the presence of *p*-hydroxybenzoic acid, *p*-coumaric acid, ferulic acid, and (+)-catechin were also reported, but combined, contributed less than 13% of the total phenolic content (Kim et al., 2009). Similarly, in the Kent cultivar, gallic acid predominated, followed by *p*-hydroxybenzoic acid, ellagic acid, quercetin, and kaempferol (Robles-Sánchez, Rojas-Graü, Odriozola-Serrano, González-Aguilar, & Martín-Belloso, 2009), however no reports were made of gallotannins, contrary to the findings of Manthey & Perkins-Veazie (2009) who reported an average of 117 mg/100 g fruit total gallotannin

concentration. Gallotannins are extracted more efficiently with acetone (Hagerman, 1988), therefore the use of methanol to extract polyphenolics by Robles-Sánchez et al. (2009) was likely the cause of the lack of gallotannin findings despite knowledge of their prevalence in mango pulp. In a recent publication, the fiber-bound phenolics of mango peel were identified and included gallic acid, protocatechuic acid, and syringic acid in a total concentration of 29.5 mg/g (Ajila & Prasada Rao, 2013). Gut microbiota have been reported to be capable of hydrolyzing polyphenolics that are bound to fiber (Högger, 2013; Russell & Duthie, 2011; Saura-Calixto, 2010) and while fruit have not historically had large amounts of fiber-bound phenolics, it could represent a potentially rich source of polyphenolics given mango's fiber and tannin content.

To date there is no comprehensive identification of polyphenolics found in mango pulp. As work with mango extracts in *in vitro* cell culture studies and *in vivo* animal studies begin to become prevalent, the importance of complete characterization and quantification has begun to take shape. Mechanisms of action cannot be attributed correctly to complex phytochemical mixtures if the compounds are not identified correctly and future metabolite work is impossible without knowing what compounds to focus on.

Tannins

Tannins are a class of high molecular weight polyphenolics that are structurally divided into two classes, hydrolysable and condensed tannins. Tannins were first appreciated for their ability to bind proteins, making them excellent agents for use in

preserving animal hides, thus vegetable extracts containing high amounts of these compounds were used for this purpose since ancient times (Serrano, Puupponen-Pimiä, Dauer, Aura, & Saura-Calixto, 2009). Condensed tannins, also called proanthocyanidins, are made of polymers of flavan-3-ols (tea) and flavan-3,4-diols (sorghum) (Chung, Wong, Wei, Huang, & Lin, 1998). Condensed tannins are more robust than hydrolysable tannins and only under extreme acid conditions can these compounds be oxidatively degraded into anthocyanidins such a cyanidin and delphinidin (Hagerman, Robbins, Weerasuriya, Wilson, & McArthur, 1992).

Hydrolysable tannins are further divided into two sub-classes, gallotannins and ellagitannins, however both are derivatives of gallic acid (3,4,5-trihydroxybenzoic acid). Ellagitannins, found in fruits such as strawberries, raspberries, muscadine, are made of highly polar hexahydroxydiphenic acid (HHDP) units esterified to a polyol (usually glucose) and once hydrolyzed, the HHDP undergoes lactonization to produce water-insoluble, free, ellagic acid (Chung et al., 1998). Ellagitannins and free ellagic acid have been reported is small quantities in mango (Manthey & Perkins-Veazie, 2009), but are hard to distinguish in mango extracts due to the large presence of gallotannins which typically elute in similar chromatographic conditions (Talcott, Krenek, Xu, & Howard, 2012). Fundamentally gallotannins are composed of gallic acid moieties esterified to a core polyol (Figure 5), also most commonly glucose, but gallotannins can also contain glucitol, hamamelose, shikimic acid, quinic acid, and quercitol (Ishimaru, Nonaka, & Nishioka, 1987; Masaki, Atsumi, & Sakurai, 1994). In plant extracts, gallic acid esters of glucose have been reported to range from one to twelve degrees of polymerization (DP).

However peta-galloyl glucose (PGG), with a galloyl moiety esterified to each of the five available hydroxyls of glucose, is considered the standard for gallotannins and the smallest unit with protein-binding properties (Hagerman et al., 1992). Since glucose has a maximum of five aliphatic hydroxyl groups, larger gallotannins are only created by gallic acid linking via a *para* or *meta*-depside bonds on a phenolic hydroxyl of gallic acid (Hagerman, 2002). The *p*- or *m*-depside bond between gallic acid moieties is generally weaker than the ester bond between glucose and the first gallic acid moiety, which allows PGG to be easily synthesized using commercial tannic acid through the addition of a mild acid and methanol (known as methanolysis) which preferably cleaves the depside likages (L. Li et al., 2011).

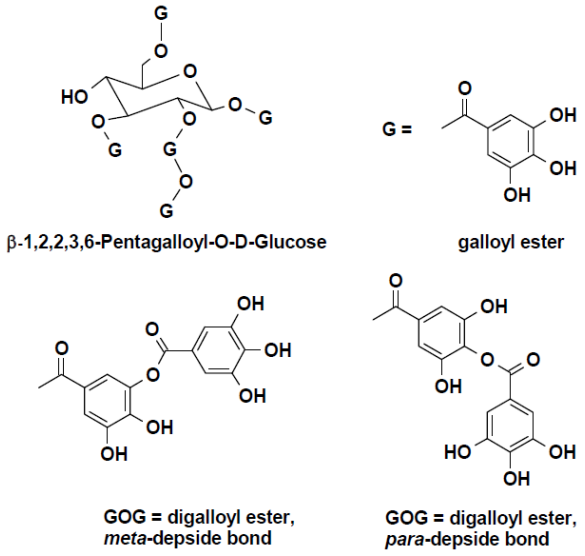

Figure 5. Examples of components that comprise gallotannins

Tannins characteristically cause an astringent taste when consumed which has principally been linked to the their interactions with proline-rich salivary proteins (Baxter, Lilley, Haslam, & Williamson, 1997; Jöbstl, O'Connell, Fairclough, &

Williamson, 2004; Luck et al., 1994). Early tannin research reported the negative of tannin-rich food consumption had on growth in both humans and animals. This anti-nutritional effect was attributed to the protein-binding affinity of tannins, but it was clear that the protein affected was not entirely dietary, but proteins in the body as well (Butler & Rogler, 1992). Conversely, it was hypothesized with much supporting evidence that salivary proteins may actually provide a defense against the negative effects of tannins by initially binding at the moment of ingestion, thus decreasing their availability later in the digestive tract. It was also reported by the same group that species who ingested tannins on a regular basis, produced more of proline-rich proteins (Shimada, 2006), suggesting that binding of tannins to protein at the moment of consumption could limit the determinative effects tannins impart on ruminant animals. However, it is still unclear if gallotannins bound to protein is an advantage or disadvantage in the human body.

While initial interest in tannins centered on their anti-nutritive effect, more recent interest is tied to their potential health-promoting effects. A metabolomic study using mice showed a positive correlation between the consumption of 80 and 100 mg/kg PGG by C57BL/6 male mice and the reduction in oncoproteins and upregulation of tumor suppressive proteins which may contribute to prostate cancer reduction (J Zhang et al., 2011).Cell-culture studies have also reported positive effects of gallotannins (specifically PGG) against prostate, lung, and breast cancer, along with a host of other health-related issues (Jinhui Zhang, Li, Kim, Hagerman, & Lü, 2009). However, most research suggests that PGG and other gallotannins are not absorbed intact. A pharmacokinetic study of PGG injected into the abdominal cavity of mice revealed 3-4

μM maximum absorption of P after hr s when a dose of 20 mg/kg was administered. These results indicate the poor bioavailability which they attributed to protein binding (L. Li et al., 2011). An *in vitro* absorption study using a Caco-2 cellular monolayer also reported that PGG introduced to a proline-rich-protein decreased absorption and that SGLT1, a sodium dependent glucose transporter, played a role in the absorption of PGG (Cai, Hagerman, Minto, & Bennick, 2006). The degradation of PGG in at pH 7.0 was reported by Krook & Hagerman (2012) who also showed that digestive fluids (lipase, pepsin, pancreatin, bile) and baby food seemed to stabilize PGG against degradation possibly due to the interaction with proteins and lipids. However, research has not been conducted on higher molecular weight gallotannins that occur in diverse ranges of molecular weights and in the company of other polyphenols. Still even less has been shown on the metabolic fate of gallotannins in humans.

Absorption and Metabolism by Caco-2 Cells

Caco-2 cells are popular for use in high-throughput drug metabolism studies because of their ease of growth, maintenance and their reliability of predicting drug absorption especially if said drug is passively diffused (Sun, Chow, Liu, Du, & Pang, 2008). When cultured and allowed to differentiate, Caco-2 cells adopt the morphology of the human small intestine endothelial cells by expressing similar transport and metabolizing enzymes and cell conformations. A confluent monolayer of Caco-2 cells will have columnar and polarized cells that produce microvilli on the apical side and tight junctions between cells (Hilgendorf et al., 2007). Absorption transport mechanisms

of compounds through Caco-2 cells are similar to those found in the small intestine, namely; passive diffusion through the membrane, paracellular diffusion between cells, transcellular diffusion through the cell, and active transport. Most polyphenolics are transported paracellularly due to their hydrophilic nature or by an active transport mechanism (Volpe, 2011). However, paraceullar absorption of polyphenolics was found to be up to 100 times slower than that of active transport (Konishi, Hitomi, & Yoshioka, 2004). Differentiated Caco-2 cell's transport and metabolic enzymes have been shown to be highly correlated with those present in the human small intestine (Hilgendorf et al., 2007; Sun et al., 2008) and thus are a useful tool for determining absorption and metabolism mechanisms. Those transporters found of the apical side include (Figure 6) the H^+/di-tripeptide transporter (PEPT1), organic anion-transporting polypeptide 2B1 (OATP-2B1), monocarboxylic acid transporter (MCT1), apical Na^+-dependent bile acid transporter (ASBT), and the organic cation/carnitine transpoter (OCTN2). Efflux transporters include the multidrug resistance protein 1 (MDR1), multi-drug resistance-associated protein 2 (MRP2), and the breast cancer resistance protein (BCRP). Metabolic enzymes have also been reported and characterized for the cell model. They include cytochrome P450 1A (CYP1A), sulfotransferases (SULTs), UDP-glucuronosyltransferases (UGTs), glutathione S-transferases (GSTs), and catechol –*O*-methyl transferases (COMTs) (Sun et al., 2008). Caco-2 cell models are so dependable for predicting drug permeability and drug-drug interactions as it relates *in vivo* that the FDA proposed a draft guidance for examination of compounds which might interfere with drug transporters via the use of Caco-2 monolayers, decreasing the need for clinical

testing in many cases (Huang, Temple, Throckmorton, & Lesko, 2007). The FDA also recognizes the model for classification of new drugs according to the Biopharmaceutics Classification System (Hu, Ling, Lin, & Chen, 2004). However, direct comparison of permeability rates of compounds completed between different laboratories is cautioned against (Ito, Okuda, Fukuda, Hatano, & Yoshida, 2007; Sun et al., 2008) due to high variability between laboratory groups.

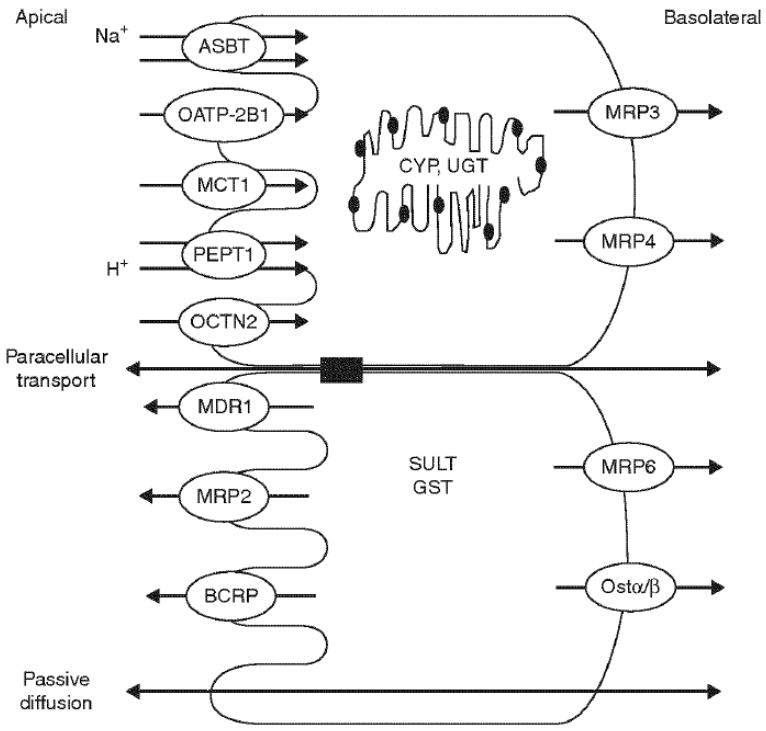

Figure 6. Drug transporters and metabolic enzymes present in the Caco-2 cell monolayer (Sun et al., 2008).

First used for drug pharmacology work, the Caco-2 cell models have since become a staple for the prediction of polyphenol intestinal absorption as greater interest rises for neutraceuticals (Langerholc, Maragkoudakis, Wollgast, Gradisnik, & Cencic, 2011). Early polyphenol bioavailability studies assumed polyphenols were all absorbed similarly, by passive diffusion which can be 100 time less efficient than active transport in cells (Camenisch, Alsenz, van de Waterbeemd, & Folkers, 1998). Compounds passively diffused across the cell membrane are also governed by partition coefficients (log octanol/water). The attachment of a glucoside greatly increases hydrophilicity of compounds and thus lowers its partition coefficient and diffusion rate (Brown, Khodr, Hider, & Rice-Evans, 1998). The work of Konishi, Kobayashi, & Shimizu (2003) studied the transport of gallic acid and *p*-coumaric acid, a hydroxy-benzoic acid and a cinnamic acid, respectively in side-by-side comparisons. They reported for the first time that *p*-coumaric acid was transported *via* the MCT while gallic acid was suggested to be transported paracellularly. The same group previously reported MCT as the primary transport mechanism for another cinnamic acid, ferulic acid (Konishi & Shimizu, 2003). Recent studies have shown flavonoid glycoside absorption *via* a sodium-dependent glucose transporter (SGLT1) (Walgren, Lin, Kinne, & Walle, 2000), however it is unclear if hydroxybenzoic acid glycosides are absorbed by the same mechanism.

Equally important is the ability of Caco-2 cells to produce enzymes capable of metabolizing compounds. It is becoming clear based on the work by many in the field, that polyphenols are extensively metabolized by the human body. The extent of metabolism and how this relates to health however is largely unknown and while *in vivo*

investigations would be ideal, Caco-2 serve as great screening process for potential metabolites. In one experiment, Caco-2 cells cultured for 14 days between passages 30-45 were able to methylate, sulfate, and glucuronidate cinnamic acid derivatives (Konishi et al., 2003). The cell metabolite results are in standing with those reported *in vivo* suggesting that a correlation does exist between the cell model and *in vivo* metabolism (Stalmach et al., 2009). In an additional study, punicalagin, a hydrolysable tannin, was hydrolyzed to yield free ellagic acid which was further metabolized to dimethyl-ellagic acid and conjugated to yield sulfite and glucuronide forms (H.-H. Li et al., 2011). Due to similarities that exist between ellagitannins and gallotannins (hydrolysable in the gut) this work suggests that gallotannins may be hydrolyzed and metabolized to some degree as well. Cai et al. (2006) also cited the action of esterase produced in the brush-border membrane of cells as likely responsible for the degradation of PGG in Caco-2 cells indicating that they could be made more bioavailable by action of enzymes in the small intestine. It should be noted however, that Caco-2 metabolism of polyphenolics does not occur as fast as would be seen *in vivo* as typical treatment times range from 8-48 hours to maximize concentration for detection (Larrosa, Tomás-Barberán, & Espín, 2006; Poquet, Clifford, & Williamson, 2008).

Absorption and Bioavailability of Polyphenols

Pharmacokinetics and bioavailability play an important role in determining which compounds to pursue in cell culture mechanistic studies as they relate to health. The trend today is to attribute a biological action to almost every plant extract based

solely on *in vitro* assays which often fails to account for absorption and metabolism of the compounds in the body, likely changing its biological activity (Gertsch, 2011). Human pilot studies have become increasingly important, especially in regard to revealing the absorption, metabolism, and excretion profiles of polyphenolics (Butterweck, Jürgenliemk, Nahrstedt, & Winterhoff, 2000).

Secondary plant metabolites, like most nutrients, are absorbed in the small intestine epithelial cells (Figure 7) due to the high surface attributed to the small villi on the circular folds of the small intestine that increase the surface area to approximately 400 m^2 (Donovan, Manach, Faulks, & Kroon, 2006; Langerholc et al., 2011). Unless the consumption of a single compound occurs, polyphenolics are delivered in complex mixtures of multiple classes of polyphenolics and absorption may be influenced by that matrix. For example, in order to be available for absorption, polyphenolics must first be released from interactions with food components, they must be soluble in the bulk aqueous phase in the small intestine, and they must be stable in gastrointestinal conditions. The term bioaccessibility was thus created to describe the fraction of polyphenols available at initial stages of absorption in the small intestine (Neilson & Ferruzzi, 2011).

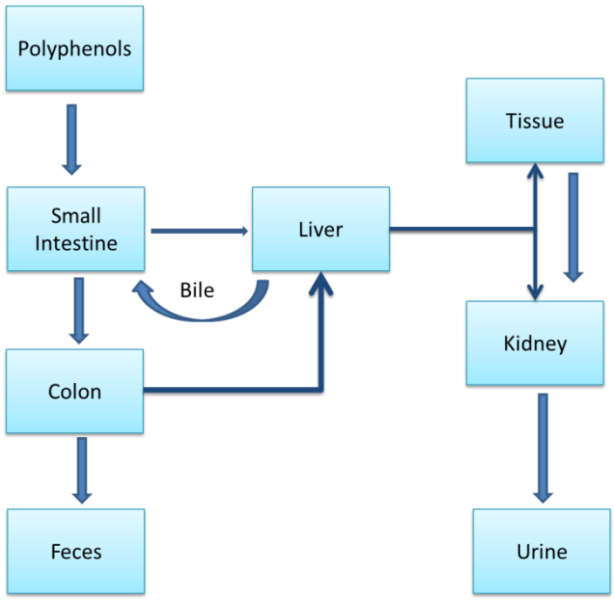

Figure 7. Simplified schematic showing metabolism of polyphenols.

Polyphenols can be found in their aglycone or glycosylated forms and while flavonoids can be transported *via* SGLT (Ader, Blöck, Pietzsch, & Wolffram, 2001), typically polyphenolics have to be deglycosolated prior to absorption due to the high polarity of glucosides and the increased size. Polyphenols can potentially be hydrolyzed in the three places; within the gut lumen, by brush border hydrolysates, and intracellular hydrolysates upon entering an enterocyte. Lactase-phlorizin hydrolase (LPH) is almost exclusively found in the small intestine and is responsible for a majority of polyphenol-glycoside hydrolysis prior to absorption into the enterocyte and has been shown to be essential in the deglycosylation of flavonoids (Brown et al., 1998).

Prior to entering blood circulation, extensive metabolism already occurs by action of SULTs, UGTs, and COMTs residing in the small intestine, resulting in sulfated, glucuronidated, and methylated forms of a deglycosylated parent compound.

This pathway of metabolism is common to drugs and most information known about polyphenol metabolism stemmed from this research. Often in drug metabolism, large doses are given (>100 mg) which completely saturate metabolic pathways, thus unconjugated forms are found in the blood. Concentrations of polyphenols from foods are usually found in much lower amounts and do not saturate pathways, therefore conjugated forms are predominately found in the blood and the small intestinal mucosa serves as a primary site of metabolism (Scalbert & Williamson, 2000). Even once absorbed, polyphenols are often effluxed back into the small intestine by MRPs and P-glycoproteins (P-gp), which are known to actively transport xenobiotics from inside a cell where they are viewed as toxic (Crozier, Del Rio, & Clifford, 2010). Unsurprisingly, those compounds with a higher affinity for the MRP and P-gp proteins are reported to have lower bioavailability (Yong Feng, 2006).

When in the bloodstream, further metabolism of absorbed compounds occurs by the same phase-II metabolizing enzymes also found in the liver, but where a proportionately higher amount of conjugating enzymes can be found (Nissinen, Tuominen, Perhoniemi, & Kaakkola, 1988; Yong Feng, 2006). From here, if not transported back into the small intestine via MRPs, conjugated and unconjugated polyphenols have the chance to finally interact with other bodily tissues. It is thought that many tissues either possess enzymes for the transport of conjugated polyphenols due to their high hydrophilicity, or they possess deconjugating enzymes such as ß-glucuronidase and sulfatase (Coughtrie, Sharp, Maxwell, & Innes, 1998; Paigen, Swank, Tomino, & Ganschow, 1975). Although conjugation of compounds is the body's way of

detoxification and elimination, conjugation has been found to increase the reactivity of some. Yet still relatively little is understood about this pathway especially in regards to secondary plant metabolites (Coughtrie et al., 1998; Glatt, 2000).

Studies involving ileostomy patients (those without a colon) have reported that a substantial amount of polyphenols (both metabolites and parent compounds) pass from the small intestine to the large (Marks, Mullen, Borges, & Crozier, 2009; Stalmach et al., 2010). This indicates the importance of the colon in polyphenol metabolism and until recently, a vastly under-represented portion of polyphenolic derived metabolites. The colon contains over 500 species of bacteria and a total of about 10^9-10^{12} cells/cm^2 capable of metabolizing polyphenol compounds by hydrolyzing them into smaller phenolics (O'Hara Shanahan, 7). The biodegradation of gallotannins (Figure 8) led to the production of gallic acid followed by the decarboxylated product, pyrogallol, and the dehydro product, resorcinol as well as a host of other smaller molecular weight compounds (Mingshu, Kai, Qiang, & Dongying, 2006). Following catabolism by microbiota, polyphenol derivatives can be re-absorbed into the blood stream, representing a large class of bioactive compounds largely under investigated (Gonthier et al., 2006; Williamson & Clifford, 2010). Circulating polyphenols are eventually filtered out of the blood by the kidney. Compounds found in the urine are predominately found conjugated with a sulfite, methyl, or glucuronide group. Work in this area and identification of metabolites is limited by analytical techniques and instrumentation. Prior to the affordability of high resolutions mass spectrometers, most absorption data was carried out using sulfatase and glucuronidase enzymes to produce parent compounds

and pharmacokinetic data presented as a total of all metabolites (Iswaldi et al., 2013; Lee et al., 2002). This underestimated the role of metabolites and produced falsely high concentrations of parent compound absorption (C. Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). With more sensitive LC-MS instruments available, metabolite identification has been steadily increasing. In tea alone, 39 catechin metabolites were found in the urine and plasma and the use of selected ion monitoring (SIM) and multiple reaction monitoring (MRM) have proved to be indispensable in metabolite research because quantities of compounds present is in the ng range and not detectable by UV analysis alone (Crozier et al., 2010; Del Rio et al., 2010; Masibo & He, 2008).

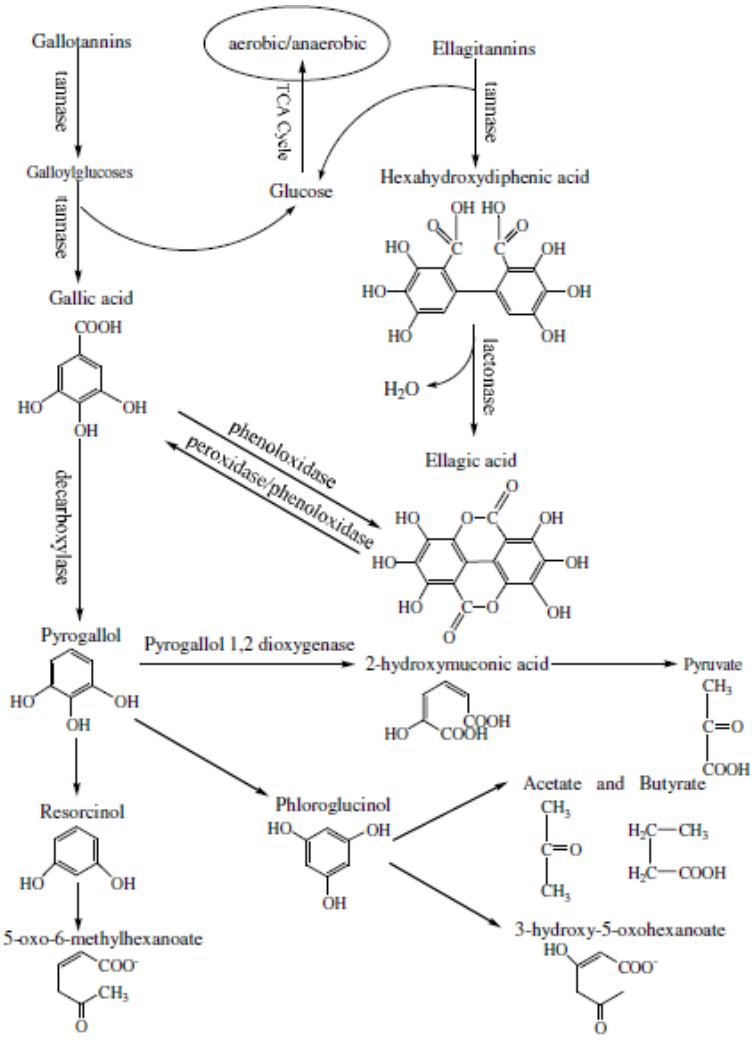

Figure 8. Biodegradation pathway for gallotannins and ellagitannins producing smaller, more absorbable molecules (Mingshu et al., 2006).

CHAPTER III

PHYTOCHEMICAL CHARACTERIZATION OF KEITT MANGO PULP

Introduction

Mangos are enjoyed world-wide for their exotic flavor and delicious taste and are referred to by many as the king of fruits. They grow in tropical and subtropical regions and originated in the Indo-Burmese region (Subramanyam, Krishnamurthy, & Parpia, 1975). Within the last 20 years, mango cultivation and exportation has increased in places of the world that are not considered traditional areas of mango production such as Central and South America, where Mexico is leading the exportation market (Galán Saúco, 2002). The close proximity to a major, growing exportation region has thus given the U.S. access to fresh mango for consumption where it did not have it before. Keitt mangos, specifically, are imported into the U.S. from Mexico for the fresh market consumption, with their peak availability between August and September, but unlike many other varieties, Keitt mangos can be bought year-round by consumers (National Mango Board, 2013).

The global consumption of mango places the fruit in a unique position to impact the health of many. Vitamin C and Vitamin A (derived from ß-Carotene) are important nutrients found in mango and are recognized as especially important in impoverished areas where mangos make up a staple part of a person's diet (Edem, 2009). Because of the RDI associated with Vitamin C and Vitamin A, extensive research has been conducted surrounding the impact cultivar, maturation, and various storage treatments

have on these nutrients. Mangos have also garnered much attention for their antioxidant capacity and promising results against cancer *in vitro* with many studies targeting mangiferin as a major bioactive constituent (Masibo & He, 2008). While phytochemical contents of mango fruit has been reported for the cultivars Tommy Atkins, Úba, Haden, Kent, Atualfo, and Francis most attention was given to gallic acid derivatives (gallotannins) and mangiferin, while phenolic acid glycosides and unidentified peaks were left uncharacterized (Kim et al., 2009; Manthey & Perkins-Veazie, 2009; Noratto et al., 2010; Ribeiro, Barbosa, Queiroz, Knödler, & Schieber, 2008). The aim of the present study therefore is to extensively characterize the polyphenolic extract from the pulp of the mango cultivar, Keitt, which is widely consumed in the U.S. The identification of compounds from this research will aid future *in vitro* and *in vivo* studies where knowing the phytochemical composition is importance for determination of potential metabolites and mechanisms of action in cell culture models.

Materials and Methods

Phytochemical Extraction

Mangos, cv. Keitt, were sourced from Mexico and allowed to ripen at ambient conditions. Upon ripening, whole fruits were peeled, deseeded, processed into cubes, and stored at -20°C until analysis. To quantify phenolics present in Keitt mango pulp, 500 g of thawed pulp was blended with an immersion blender to homogenize. Polyphenolic extraction was carried out on 10 g aliquots of homogenized pulp, in triplicate.

Polyphenolics from pulp were extracted by adding 30 mL (total volume) of a solvent mixture containing methanol, acetone, and ethanol 1:1:1. The mixture was stirred for 30 mins to facilitate extraction, subsequently filtered, and remaining solids were re-extracted following the same procedures for a total of 3 times. The liquid filtrates were combined and solvents evaporated under vacuum at 45°C and the extract brought to a known volume with water containing 0.01% HCl. The extracts were adjusted to a known volume and were centrifuged at 10,000 g for 5 min to remove insoluble solids (carotenoids and pectin) prior to filtering through a Whatman 0.45 μm PTFE membrane filter before immediate HLPC-MS quantification against authentic standards (gallic acid, *p*-hydroxybenzoic acid, sinapic, ferulic, *p*-courmaric acid; Sigma Aldrich, abscisic acid; Chromadex). All compounds were quantified with their corresponding aglycones unless otherwise indicated and data expressed as the mean of three independent extractions.

It was necessary to achieve a concentrated sample for extensive characterization of polyphenolics that occur in smaller quantities in mango pulp. To do so, 1 kg mango was prepared as described, and polyphenolics were isolated in order to aid in identity. Mango extracts were applied to a 25 g C18 Sep Pak cartridge (Waters Corp. Milford, MA, USA) to remove organic acids and sugars in order to concentrate the polyphenolics. The C18 unbound elution, polar compounds with weak or no affinity to C18 under these conditions, was further extracted by liquid-liquid partitioning into two volumes of ethyl acetate to capture those unretained polyphenolics. Compounds adsorbed to the C18 were eluted with 100% methanol and combined with the ethyl acetate extract and solvents

removed under vacuum at 45°C and re-dissolved in a known volume of acidified water. The extract was filtered through a Whatman 0.45 μm P F E membrane filter prior to subsequent analysis.

HPLC-PDA-ESI-MS^n Analysis

Polyphenolics were characterized and quantified using a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source. Separations were in reversed-phase using a Finnigan Surveyor HPLC coupled to a Surveyor PDA detector and gradient separations were performed using a Dionex Acclaim™ (Bannockburn, Il) C_{18} column, (250 x 4.6 mm, 5 μm) at room temperature. Mobile phase A was 0.1% formic acid in water (Sigma-Aldrich) and mobile phase B was 100% MeOH containing 0.1% formic acid (Sigma-Aldrich). The mobile phases pumped at 0.4 mL/min and the gradient began initially with 0% Phase B for 3 min, a changed to 21% Phase B in 20 min, from 21 to 35% Phase B in 30 min, 35 to 49% Phase B in 50 min, and 49% to 70% Phase B in 70 min before returning to initial conditions. The electrospray interface worked in negative ionization mode. Source and capillary temperatures were set at 300°C, source voltage was 3.50 kV, capillary voltage was set at -42.00 V, and collision energy for MS/MS analysis was set at 35.0 eV. The instrument operated with sheath gas (N_2) and auxillary gas (N_2) flow rates set at 40 units/min and 5 units/min, respectively. In addition to column separations, the mango concentrate was infused to capture MS^n fragmentation patterns of poorly ionized compounds using instrument tuning specific to gallic acid, methyl gallate, and gallotannins.

Alkali Hydrolysis

Alkali hydrolysis was emplored to distinguish glycosidic moiety linkages between two dominant gallic acid monoglucosides in the mango extracts. To the extract, 1M NaOH was added drop-wise until a pH of 10 was achieved. The mixture was allowed to react for 0, 1, 5, 10, and15 min. The reaction was stopped with the addition of M HCl , and filtered through a .45 µ m filter prior to HPLC-MS analysis.

Similarly, alkali hydrolysis was utilized to liberate cell-bound phenolics present in the mango fiber following the polyphenolic extractions in an attempt to characterize a potentially under-represented portion of mango polyphenolics (Parr, Waldron, Ng, & Parker, 1996). The solids remaining after solvent extraction of mango pulp from the phytochemical quantification were extracted four times with a 4:1 ratio of ethanol to mango fiber. Following the first two additions of ethanol, the solids were tissumized and stirred at room temperature for 30 min. The third extraction with ethanol was allowed to stand for 10 hrs at 4°C followed by a fourth extraction with ethanol at 70°C to ensure complete precipitation of soluble fibers. The resulting insoluble fiber was dried at 50°C and amount of alcohol insoluble fibers, representing total fiber of the mango, were determined gravimetrically. Cell wall bound phenolics were assessed by reaction of the insoluble solids with with 1M NaOH for 1 hr followed by acidification with 1M HCl prior to HPLC-MS analysis.

Total Soluble Phenolics

Total soluble phenolics were measured as the total metal reduction capacity of polyphenolics present in C18 isolates of mango extracts were as analyzed by the Folin-Ciocalteu assay (Singleton & Rossi, 1965) and quantified in gallic acid equivalents.

Results and Discussion

Mango polyphenolic profiles may differ based on variety, maturity, and post-harvest handling; however all varieties that have reported polyphenolic characterizations have identified gallic acid and derivatives of gallic acid in the form of hydrolysable tannins as the predominant polyphenols in the fruit. For the first time, a detailed characterization of Keitt mango pulp was accomplished. Separations were performed on the mango extract (Figure 9) and structural characterization of polyphenolics were made based on their UV absorption and mass fragmentation patterns, leading to the identification of 18 compounds in the pulp extract (Table 1).

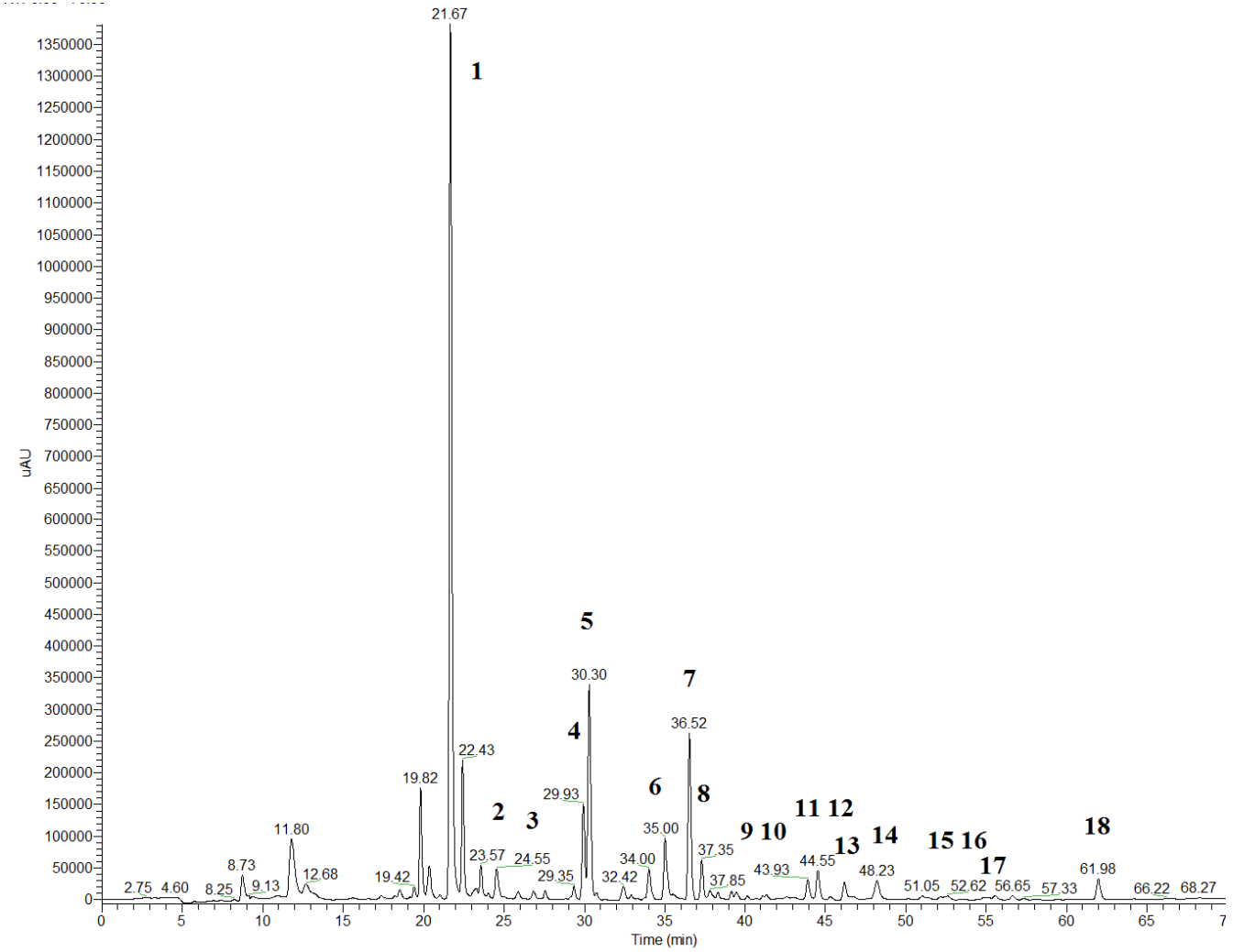

Figure 9. HPLC chromatogram of compounds present in mango (Mangifera India L. var. Keitt) pulp at 280nm. Peak assignments are shown in Table 1.

Table 1. Characterization of compounds present in Mangifera Indica L. var. Keitt, pulp

Peak no.	RT (min)	Compound	λ_{max} (nm)	$[M-H]^-$ (m/z)	MS/MS (m/z)	Concentration (mg/400 g MP)[1]	Concentration (mg/kg MP)
1	21.67	EL[2]-mono-galloyl glucoside	278	331.2	271.1, 169.2, 211.2, 125.3	15.07 ± 0.23	37.67 ± 0.56
2	24.55	gallic acid	271	169.2	125.3	0.7 ± 0.04	1.74 ± 0.10
3	27.55	galloyl di-glucoside	274	493.1	313.22	0.18 ± 0.01	0.45 ± 0.01
4	29.93	GL[2]mono galloyl glucoside	254, 298	331.2	169.2, 125.4	1.53 ± 0.05	3.82 ± 0.13
5	30.3	OH-benzoic acid glycoside	256	299.1	239, 179, 137, 209	7.40 ± 0.29	18.49 ± 0.72
6	35.00	coumaroyl glucoside	295	324.8	163.17	0.64 ± 0.02	1.61 ± 0.05
7	36.52	dihydrophaseic acid glucoside	265	443.5	237.0, 219.1, 281.1, 425.3	1.04 ± 0.02	2.60 ± 0.04
8	37.35	ferulic acid hexoside	290, 314	355.0	193.2, 149.3	0.36 ± 0.01	0.91 ± 0.02
9	41.17	sinapic acid hexoside-pentoside	251, 283, 328	517.1	385.1, 205.3, 223.3	0.052 ± 0.005	0.13 ± 0.01
10	41.42	sinapic acid hexoside	247, 327	385.1	223.0, 153.2, 205.3, 161.0	0.07 ± 0.01	0.164 ± 0.01
11	43.93	dihydrophaseic acid glucoside	267	443.4	263.1, 219.1, 143.2	0.189 ± 0.012	0.47 ± 0.03
12	44.55	hydroxy-dimethyl decadiene-dioic acid glucopiranosylester*	275	403.2	241.04, 343.02, 197.2	0.71 ± 0.04	1.78 ± 0.09
13	46.22	(hydroxy-dimethyl decadiene-dioic acid glucopiranosylester*	274	403.2	241.1, 343.1, 197.1	0.50 ± 0.05	1.25 ± 0.13
14	48.23	abscisic acid glucoside	273	471.0	263.0, 219.2, 189.1	0.23 ± 0.01	0.57 ± 0.02
15	52.02	eriodictyol-*O*-dihexoside*	nd	611.4	449.2, 287.2, 389.1	trace	trace
16	52.62	eriodictyol-*O*-hexoside*	nd	449.3	287.2, 389.1, 269.2, 227.1	trace	trace
17	55.57	unknown-glucoside	314	429.2	267.0, 223.2	trace	trace
18	61.98	abscisic acid	265	263.2	219.2, 153.2, 151.2	0.22 ± 0.02	0.55 ± 0.04

1Values represent the means and standard errors from three replicates.

2 EL, ester-linked, GL, glycosidically-linked

*Tentative identification based on spectral characteristics and similarities of ESI-MS^n fragmentation.

Compound 1 and **Compound 4** (Figure 10) were identified as mono-galloyl glucosides (MGG) as each compound showed a parent mass of 331 m/z showed a dominant ion at *m/z* 271 consistent with a loss of 60 Da (1/12 the weight of a carbon) and a secondary fragment with a loss of 162 Da indicating the loss of glucose to leave the aglycone, gallic acid. The characteristic loss of m/z 60, 90, or 120 Da in fragmentation experiments has long been associated with ring cleavage of sugar moieties and is a good indication of C-linked glycosides (Cuyckens & Claeys, 2004). However, since C-linked glycosides are not plausible for a gallic acid glycoside, the dissociation of a C6 sugar is an indication of a stronger bond on this compound, more resistant to MS fragmentation. Compound 4 also exhibited a characteristic loss of m/z 162 and 206 Da [M-H-162-44] indicating the relative ease of loss of both glucose and/or a carboxylic acid. Based on these fragmentation patterns, there is a strong indication that the MGG represented by Compound 1 was linked by an ester bond *via* the carboxylic acid of gallic acid, while MGG of Compound 4 was linked via an ether (glycosidic) bond at hydroxyl positions 3, 4, or 5 of gallic acid. To further investigate the chemical linkages that exist between these two forms of MGG, an alkali hydrolysis experiment was carried out using the same mango extract. An ester bond is formed between a hydroxyl group and a carboxylic acid, which makes this specific type of bond more susceptible to shifts in pH (Figure 111). Within 5 minutes of exposure to pH 10 conditions, a complete degradation of Compound 1 occurred while Compound 4 remained unchanged at 5 mg/L throughout the entire alkali exposure time. The hydrolysis of Compound 1 was strong, additional evidence to the mass spectral data for the presence of an ester linkage. This ester linked

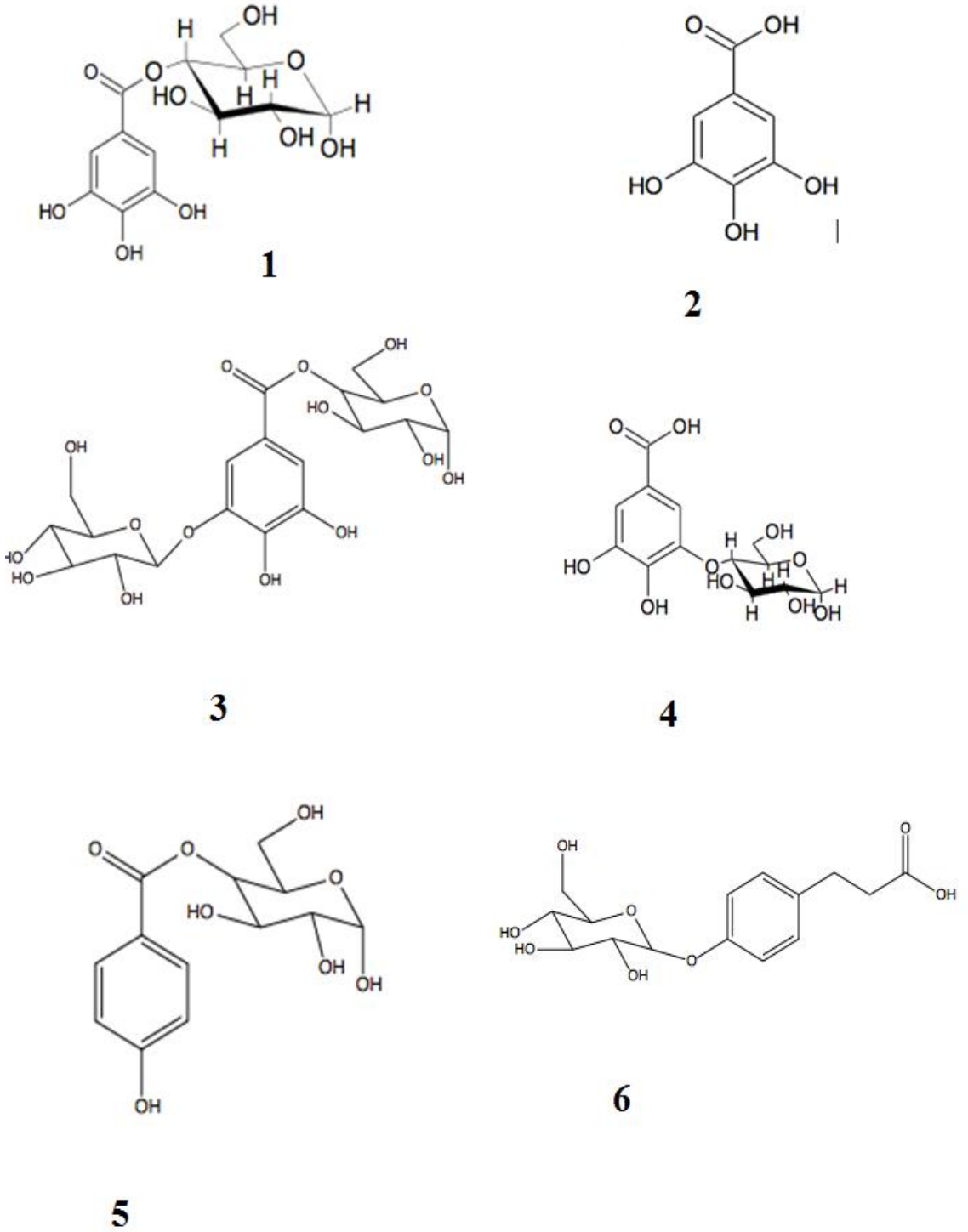

Figure 10. Structures of identified polyphenolics in Kiett mango pulp.

MGG accounts for 50% of the quantifiable polyphenolics in the extracted mango pulp with 37.67 ± 0.56 mg/kg, quantified as gallic acid. The ether-linked or glycosidic-linked MGG was less abundant at 3.82 mg/kg, and is the same chemical configuration for glycosides on compounds such as flavanols and anthocyanins. The type of linkeage between gallic acid and glucose is important in terms of bioavailability because while the ester linkage is more susceptible to pH environments about 7 (Hagerman, 2002), glycosidic bonds are capable of hydrolysis by ß-glucosidase (Brown et al., 1998).

Compound 2 was identified as free gallic acid based on a λ_{max} of 271 nm, a m/z $[M-H]^-$ of 169.2, and the subsequent loss of CO_2 under MS/MS conditions to yield pyrogalloyl at *m/z* 125.3. Free gallic acid was quantified as 1.74 mg/kg, substantially lower than other mango varieties that reported up to 14 mg/kg gallic acid (Kim et al., 2009). Therefore, the presence might indicate hydrolysis in the fruit or even during the extraction procedures. **Compound 3**, a gallic acid derivative, showed a pseudomolecular ion of m/z 493.4 while MS/MS experiments gave fragments of *m/z* 313 [M-H-180] and 169 [M-H-324] corresponding to the loss of two glucose moieties from gallic acid. This compound was identified as galloyl di-glucoside and is in agreement with mass spectral properties of this compound reported in logan seed (Soong & Barlow, 2005).

Compound 5 was tentatively identified as a hydroxy-benzoic acid glucoside at *m/z* 299.1 that has a 6-carbon sugar acyl linked to its carboxylic acid, similar to the acyl-linked mono-galloyl glucoside (Compound 1). Compound 5 produced fragments typical of a tightly bound glucose, m/z 239, 209, and 179 corresponding to the ring-fission of glucose and the resulting 60, 90, 120 Da neutral fragments. The corresponding aglycone

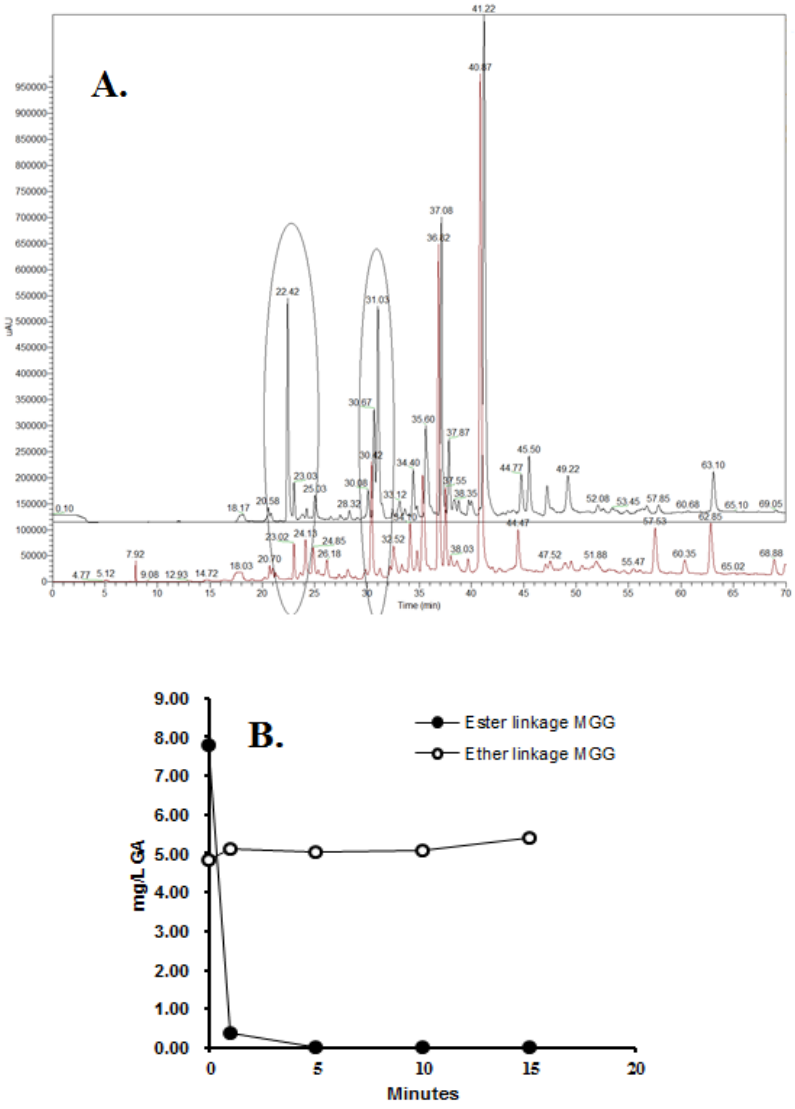

Figure 11. (A) HPLC chromatogram showing the disappearance of ester-linked MGG and the stability of ether-linked MGG at pH 10 (shown in red). (B) Complete disappearance of ester-linked MGG occured after 5 minutes at pH 10.

at m/z 137 was produced upon energy induced collision and corresponded to a monohydroxy benzoic acid, most likely *p*-hydroxybenzoic acid based on its characteristic UV spectra absorption bands at 256 and 299 nm. Compound 5 was the second most abundant polyphenolic found in Keitt mango extract at 18.49 mg/kg quantified in *p*-hydroxy benzoic acid equivalents.

Compounds 6, 8-10 were all characterized as cinnamic acid derivatives based on typical absorbance bands between 300-320 nm, typical for coumaric, ferulic and sinapic acids, and their mass fragmentation pathways. All cinnamic acid derivatives identified were found in concentrations less than 1.6 mg/kg, indicating that the biochemical pathway leading to these compounds are not as active, but could be enhanced by future breeding efforts (Krishna & Singh, 2007). **Compound 6** (Figure 10), a coumaroyl acid glucoside, showed a pseudomolecular ion of m/z 324.8 and a corresponding fragment of m/z 163, indicating the loss of a C6 sugar moiety resulting in coumaric acid. **Compound 8** (Figure 12) was identified as ferulic acid hexoside, m/z 355.0. When subjected to MS/MS experiments, the corresponding agylcone, ferulic acid, with an m/z of 193 was formed as well as the decarboxylated ferulate ion at 149 Da [M-H-162-44]. Compounds 9 and 10 were both identified as glycosylated sinapic acid derivatives. **Compound 9** gave three spectral bands at 251, 283, and 328 and a parent mass of m/z 517.1. In the MS^2 event, fragments of m/z 385 [M-H-132] corresponding to the loss of a pentoside, 205 [M-H-132-162-18] corresponding to the loss of a pentoside, hexoside, and water, and 223 [M-H-132-162] corresponding to the aglycones, sinapic acid, were recorded. Therefore this compound was tentatively identified as sinapic acid pentoside-hexoside as

reported in eucalyptus and jocote (Boulekbache-Makhlouf et al., 2010; Engels, Schieber, & Gänzle, 2012). **Compound 10**, m/z 385.1, similarly produced a fragment of m/z 223 [M-H-162] and 205 [M-H-180] by the loss of a C6 sugar, likely glucose, and was identified as a sinapic acid hexoside. Interestingly, all cinnamic acids were found glycosylated in Keitt mango pulp; inconsistent with previously published reports of other mango varieties where only aglycones were reported. However, detailed mass spectroscopic work was not conducted in these studies. Rather, identification was carried out using HPLC-PDA where glycosylated compounds are impossible to elucidate and the studies were not focused on phenolic acids that occur in low concentrations (Kim et al., 2009; Manthey & Perkins-Veazie, 2009; Ribeiro et al., 2008; Schieber et al., 2000).

Compounds 7, 11, 14, and 18 (Figure 12) share similar UV spectral properties (265 nm), and share similar fragmentation pathways when subjected to MS^n analysis. Compounds 7 and 11 both produced pseudomolecular ions of *m/z* 443.5. **Compound 7** produced fragments of m/z 237 [M-H-162-44], 219 [M-H-162-44-18], 425 [M-H-18] and are based on similarities between previously reported MS data, this compound was identified as dihydrophaseic acid glycoside (del Refugio Ramos et al., 2004). **Compound 11** was identified as an isomer of dihydrophaseic acid glycoside and produced fragments consistent with the loss of glucose (m/z 263) [M-H-180], carboxylic acid, and water, 219 [M-H-162-44-18]. The compound that eluted earlier in the chromatographic run was in abundance five times more (2.60 mg/kg) than compound 11 (0.47 mg/kg). **Compound 14** was identified as abscisic acid glycoside, m/z 471.0.

Energy induced fragmentation resulted in the formation of the agylcone, abscisic acid (ABA), m/z 263 [M-H-162] and m/z 219 by the neutral loss of 162 Da (glucose) and 44 Da (carboxylic acid). **Compound 18** was confirmed as ABA based on UV and MS data that matched an authentic standard. ABA has previously been reported as a phytohormone responsible for ripening of mangos along with ethylene (Palejwala, Amin, Parikh, & Modi, 1985; Parikh, Nair, & Modi, 1990; Zaharah, Singh, Symons, & Reid, 2012), however, derivatives of ABA have never been reported in the phytochemical composition of mango pulp. **Compounds 12 and 13** each had a derivatives. The fragments 241 m/z [M-H-162], 343 [M-H-60], and m/z 197 [M-H-162-44] were identical to those reported for the compound (2E, 4E)-8-hydroxy-2,7-dimethyl-2,4-decadiene-1,10-dioic acid 6-*O*-ß-D glucopiranosylester as identified in walnuts (Gómez-Caravaca, Verardo, Segura-Carretero, Caboni, & Fernández-Gutiérrez, 2008). In the mango extract two compounds with similar masses were identified, however, the MS fragmentations alone were not enough to determine positioning as both sugars could be attached as esters on either carboxylic acid. Compound 12 and 13, were identified as hydroxy-dimethyl decadiene-dioic acid glucopiranosylesters and were quantified in equivalents of abscisic acid. The discoveries of C12 dicarboxylic acid derivatives are not unusual in plants which are known to synthesize oxygenated carotenoids such as violaxanthin and as reported by (Ito et al., 2007; Zeevaart, Heath, & Gage, 1989) stem from a similar biosynthetic pathway as abscisic acid with violaxanthin serving as the precursor molecule.

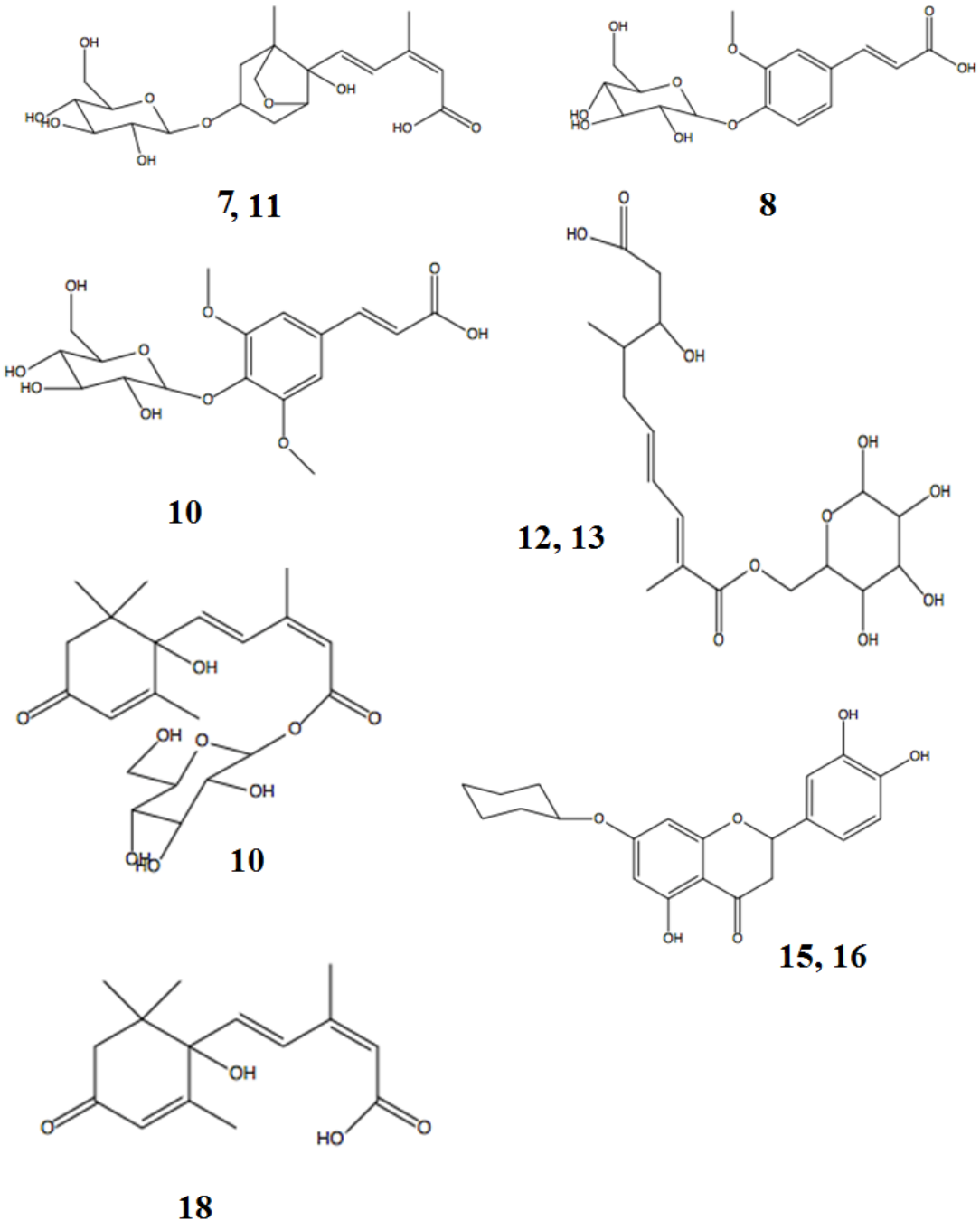

Figure 12. Structures of identified polyphenolics in Kiett mango pulp.
molecular ion of *m/z* 403.2 and were tentatively identified as C_{12} dicarboxylic acid

Flavonoids, such as quercetin and kaempherol, were reported in mango concentrate, peels, seed kernel, and leaves, (Barreto et al., 2008; Schieber et al., 2000), but were not identified in Keitt pulp in these studies. However, **Compounds 15 and 16** were tentatively identified as the flavonoids eriodictyol-*O*-dihexoside, eriodictyol-*O*-hexoside, respectively, based on the observed parent masses, m/z 611.4 and 449.3 and fragmentation patterns reported from eriodictyols in the dried roots of *Astragalus membranaceus*, a traditional Chinese medicine (Vallverdú-Queralt, Jáuregui, Di Lecce, Andrés-Lacueva, & Lamuela-Raventós, 2011). **Compound 17** could only be identified as a glycoside based on its observed *m/z* of 429.2 and fragmentations of *m/z* 267 and 223 and needs further characterization in MS^3 in a higher concentration to provide more structural information.

Mango fruit is generally known to contain gallotannins ranging from pentagalloyl glucoside to didecagalloyl glucoside, however the amount of gallotannins present in the pulp have been shown to differ based on cultivar (Manthey & Perkins-Veazie, 2009). In the extraction carried out to quantify the compounds present in Keitt mango, gallotannins were not found in high enough concentrations to give a noticeable peak in UV detection. The lack of gallotannins detected from this extraction does not indicate an absence of these compounds, but can instead be attributed to a chromatography issue as gallotannins tend to elute in broad bands (Mueller-Harvey, 2001). A confirmation of the existence of gallotannins in the pulp was accomplished when the more concentrated extracts were created from C18 partitions. As seen in Figure 13, the characteristic hump, or raise in the baseline towards the end of the

chromatographic run, in the HPLC chromatogram is indicative of gallotannins being present. A MS total ion count (TIC) averaged over RT42 mins to 70 mins (Figure 14), also revealed the occurrence and distribution of gallotannin species in the same C18 isolate. The m/z of 469 (939/2), 545(1091/2), 621(1243/2), and 697(1394/2), and neutral loss of 76 (152/2) Da points to the doubly charged (m/2z) gallotannins; penta-galloyl glucoside (5GG); hexa-galloyl glucoside (6GG); hepta-galloyl glucoside (7GG); and octa-galloyl glucoside (8GG), respectively as shown in (Table 2). Doubly charged gallotannins were also reported by (Engels, nzle, Schieber,) who attributed their presence to the large structure and molecular weight. Manthey & Perkins-Veazie (2009) reported Keitt variety as containing less (53 mg/100 g FW) gallotannins than other mango varieties such as Ataulfo (708 mg/100 g FW) and Haden (93 mg/100 g FW), but the gallotannins that are present could potentially serve as a large reservoir of gallic acid molecules.

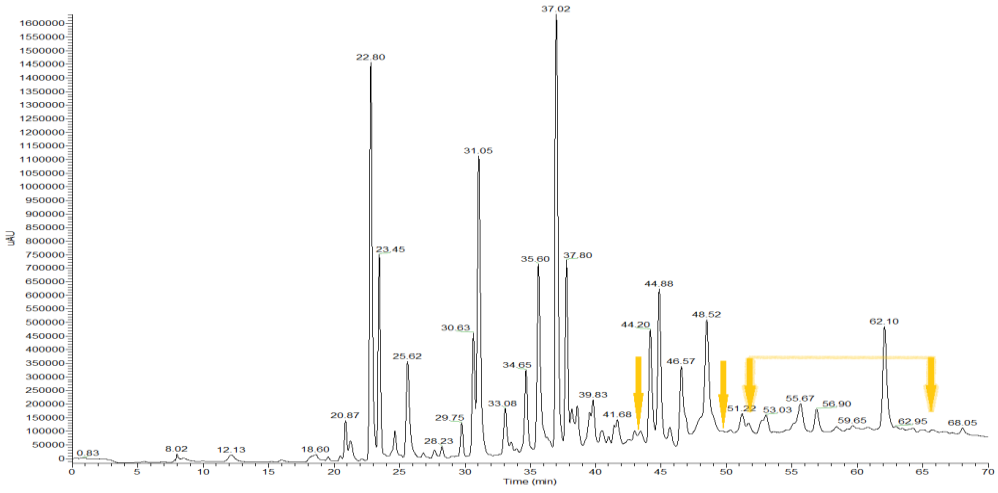

Figure 13. HPLC Chromatogram of C18 concentrated extract indicating the presence of gallotannins with the characteristic gallotannin hump.

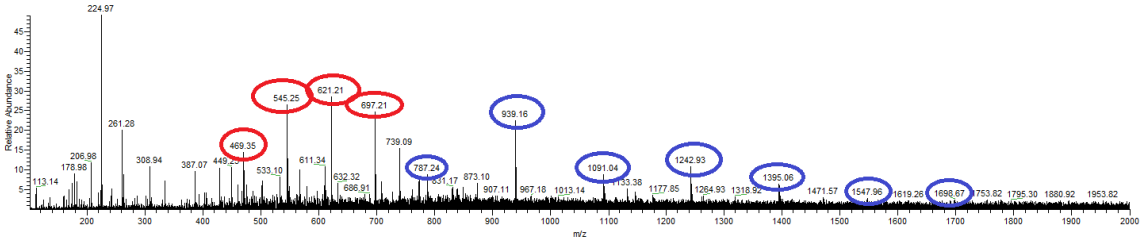

Figure 14. TIC averaged from 45-70 min in C18 isolate. Masses circled in red indicate doubly charged gallotannin while those circled in blue highlight the actual mass [M-H]- of gallotannin molecules indicate their presence despite bad chromatography.

Table 2. Gallotannin molecular weights reported as singly and doubly charged molecules.

Abb.	**Compound I.D.**	**$[M-H]^-$ (*m/z*)**	**$[M-2H]^{2-}$ (*m/2z*)**
4GG	tetra-*O*-galloylglucose	787	393
5GG	penta-*O*-galloylglucose	939	469
6GG	hexa-*O*-galloylglucose	1091	545
7GG	hepta-*O*-galloylglucose	1243	621
8GG	octa-*O*-galloylglucose	1395	697
9GG	nona-*O*-galloylglucose	1547	773
10GG	deca-*O*-galloylglucose	1699	849
11GG	undeca-*O*-galloylglucose	1851	925
12GG	dideca-*O*-galloylglucose	2003	1002

Total soluble phenolics were quantified from the 10 g aliquot of pulp to serve as a reference to evaluate against when concentrated extracts used for mass spectrometric evaluation and future cell culture experiments are made. During the concentration and purification step where solid phase extraction is utilized, polar compounds such as gallic acid and the MGG generally do not have a high affinity to Sep-Pak C18 cartridges. The loss of MGG is especially critical considering that roughly it comprised 50% of mango pulp polyphenolics. The pulp extract was found to contain 238.7 ± 5.7 mg/kg GAE which is consistent with the findings of Manthey & Perkins-Veazie (2009) who reported

260-270 mg/kg GAE in Keitt pulp. Alcohol insoluble fiber was experimentally determined to be 4.72 g/100 g pulp which was 1 g higher than the total dietary fiber analyzed according to the AOAC official method for this variety (Gorinstein et al., 1999) and may indicate natural fruit differences or an over-estimation of total dietary fiber from alcohol insoluble solids. The alcohol insoluble fraction was further subjected to alkali hydrolysis in an attempt to characterize any fiber-bound polyphenolics that may have been esterified, but only di-gallic acid was identified by HPLC-MS analysis. This finding could indicate that the hydrolysis procedure was too harsh and bound gallotannins were hydrolyzed to free gallic acid di-gallic and subsequently oxidized to quinone, as the extract was green, indicative of oxidation; or mango fiber is not a significant source of polyphenolics.

Conclusion

Mass spectroscopic characterization and HPLC quantification of mango pulp revealed for the first time two MGGs with distinct differences in their glycoside linkages, with the ester form dominating, as well as the presence of five other phenolic acid glycosides; hydroxybenzoic acid glucoside, courmaric glucoside, ferulic acid glucoside, and sinapic acid mono and di-glucosides. Six oxygenated carotenoid derivatives were identified for the first time in a phytochemical extract, namely, abscisic acid and its glycoside, two catabolism products of abscisic acid, dihydrophasic acids, and two hydroxy-dimethyl decadiene-dioic acid glucopiranosylesters. Gallotannins ranging from tetra-galloyl glucosides to nona-galloyl glucosides were also identified in

the pulp, but not quantified. The extensive identification of polyphenolics in Keitt mango pulp will aid in assessing the biological activity of this mango both *in vitro* and *in vivo*.

CHAPTER IV

PHYTOCHEMICAL CHANGES IN MANGO PULP ASSOCIATED WITH DIFFERENT ENZYME PREPARATIONS

Introduction

Mangos are rich in pectic substances which serve as the major polysaccharides in their cell wall and are in turn responsible for the high viscosity of mango purees. In an unripe mango, pectin tends to be bound to microfibrils in the cell wall and is generally water insoluble, but as a mango ripens endogenous pectinases release pectin from the cell walls and depolymerize the molecules and the fruit slowly softens (Kashyap, Vohra, Chopra, & Tewari, 2001). When the pulp of a mango is crushed, both soluble and insoluble pectin can bind water, often aided by enzymes such as pectin methyesterase and natural metal ions to form a gel that makes juice extraction and juice clarification difficult. Under commercial processing conditions, mango juice may be subjected to the addition of exogenous enzymes such as pectinase, cellulase, and/or protease to help increase juice yield. Pectinases are widely used in the food industry to achieve higher juice extractability; however, some pectinases have known side activities that have been detrimental to the targeted hydrolysis reaction. For example, in bilberry extraction anthocyanin glycosides were hydrolyzed into their corresponding agylcones by Econase CE due to beta-glucosidase or anthocyanase side activity but not by four other commercial pectinases evaluated in the study (Buchert et al., 2005). Likewise cranberry anthocyanin glucosides were degraded after enzymatic treatment in 24 of 28 commercial

enzymes evaluated. Wightman & Wrolstad, 1995). However, side-activities can also be optimized to achieve specific and desirous end products. Pectinolytic and cellulolytic enzymes were evaluated for their ability to deglycosylate flavonoids glyocsides from citrus peels to yield products that were more likely to be absorbed *in vivo* (Mandalari et al., 2006). Many of the side activities of commercial enzyme preparations are derived from the lack of purification of the enzymes, usually due to costs, that are used in an effort to depolymerize the fiber present in many fruits. When applied to mango pulp as a processing aid, the presence of beta-glucosidase as a side activity enzyme in pectinase could impact the resultant phytochemical composition of the fruit. Mango pulp contains mainly glycosylated phenolic acids and hydrolysable (gallo)tannins that could potentially be altered by this side-activity, and in turn impact the color, stability, and bioactivity of these compounds.

How gallotannins behave *in vivo* is still largely unknown by researchers. It is known that they bind preferentially to proline-rich proteins (Soares, Mateus, & de Freitas, 2012), but if they can be hydrolyzed when bound to a protein and by what mechanism is still not well defined. Lactase-phlorizin hydrolase and cystolic ß-glucosidases are both found in the small intestine where they function as deglycosylating enzymes very similar to plant or fungal ß-glucosidase. The objectives of this research were to evaluate changes to Keitt mango polyphenolics, known to be low in gallotannins and high in MGG, following reaction with three processing enzymes (a pectinase, cellulase, and a pectinase with known ß-glucosidase side activity) and to contrast these

differences against a mango variety (cv. Ataulfo) known to contain high gallotannin concentrations in the presence of ß-glucosidase.

Materials and Methods

Enzyme Use as a Processing Aid for Juice Extraction

Mango pulp, var. Keitt was thawed, homogenized and aliquoted into 44 g samples for enzyme treatment. Validase TRL (Val), a cellulase (DSM, USA), Crystalzyme 200XL (CZ), a pectinase (DSM, USA), and Rapidase AR 2000 (Rap), a pectinase with known ß-glucosidase activity (>4,000 BDG) (DSM, USA) were evaluated. Each enzyme was applied at 0.05% v/w and incubated in falcon tubes at 50°C for 1 or 4 hrs in an effort to represent potential commercial enzyme reaction times. Following incubation, each sample was placed in boiling water until 95°C was reached to inactivate the enzyme, and samples immediately cooled on ice. Following inactivation, samples were centrifuged at 2000 x *g* to separate solids from the supernatant that was collected for subsequent analysis. Percent juice yield was determined based on weight of the supernatant compared to the initial sample weight. The supernatant was filtered through a 0.45 µm PTFE filter and analyzed by HPLC-MS for MGG and gallic acid as previously described in Chapter III.

ß-Glucosidase Treatments of High Tannin Extract

Mango pulp, var. Atualfo, previously reported for its high gallotannin content (Manthey & Perkins-Veazie, 2009; Noratto et al., 2010) was extracted as described in Chapter III using an activated C18 cartridge. Total soluble phenolics were determined by

the Folin-Ciocalteu assay (Singleton & Rossi, 1965) and quantified in gallic acid equivalents (GAE) to standardize the extract to 10,000 ppm for use in the experiments. Bovine serum albumin (BSA) was obtained from Thermo-Fisher Scientific and a stock solution of 100 mg/5 mL prepared in a 0.25 M citrate buffer, pH 5.0. Treatments were applied to the concentrated mango extract in the ratios described in Table 3 both with and without BSA addition, in a total volume of 10 mL of a 0.25 M citrate buffer, pH 5. The treatments were incubated at 37° for 12 and 24 hrs and were filtered through a 0.45 μm P F E filter prior to HPLC-MS analysis as described in Chapter III.

Table 3. Experimental plan to assay effects of ß-glucosidase on gallotannins and gallotannin-protein interactions.

	Mango Concentration mg/L GAE	**Enzyme Type and Concentration**	**Protein Addition BSA[1]**
1	250	Rapidase – 0.4% w/v	0.5% w/v
2	250	Rapidase – 4.0% w/v	0.5% w/v
3	2500	Rapidase – 0.4% w/v	2.0% w/v
4	2500	Rapidase – 4.0% w/v	2.0% w/v
5	250	ß-glucosidase 0.028% w/v	0.5% w/v
6	250	ß-glucosidase 0.028% w/v	0.5% w/v
7	2500	ß-glucosidase 0.028% w/v	2.0% w/v
8	2500	ß-Glucosidase 0.28 % w/v	2.0% w/v

[1]Protein addition served as a separate (and single) time point and was dosed based on mango concentration.

Statistics

Mango puree was evaluated in triplicate in independent reaction vessels. Data represents the mean triplicate analyses using ANOVA (analysis of variance) followed by Holm-Sidak post-hoc test using the SigmaPlot statistics software package (Systat Software, Inc. London, UK) which provided corresponding probability ($p<0.05$) values to indicate

significant differences between treatments. Paired t-test was used to evaluate enzyme treatments against the control.

Results and Discussion

Enzyme Use as a Processing Aid for Juice Extraction

Incubation of mango pulp with Val, CZ, and Rap each resulted in a significant ($p<0.05$) increase in juice yield compared to the control (Figure 15) with the exception of Val whose juice yield did not differ from mango pulp without enzyme after 4 hours of incubation. Rap, a pectinase, produced the highest juice yield, 59.9 ± 1.52%, after 4 hrs of incubation at 50°C, but did not differ significantly from CZ, also a pectinase, which

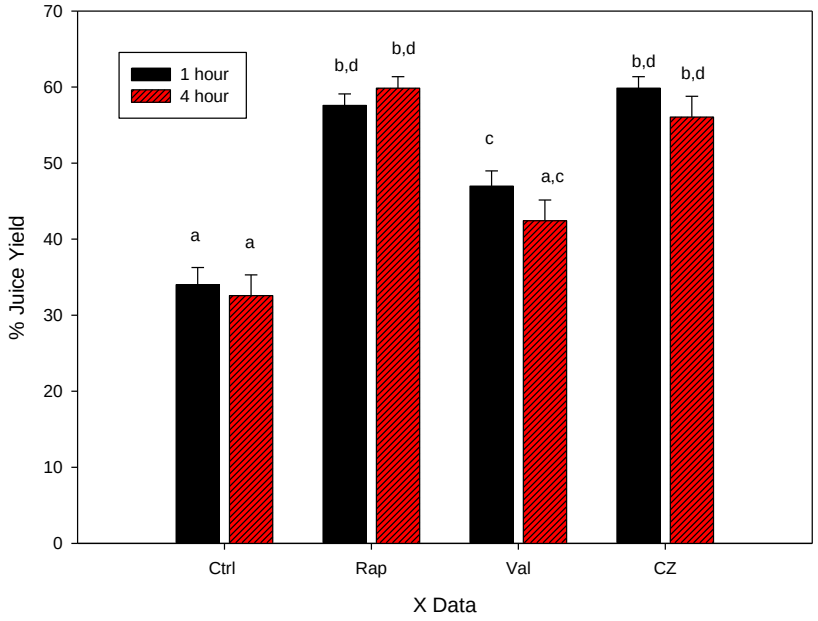

Figure 15. Percent juice yield of mango pulp following incubation with the enzymes Rap, Val, CZ for 1 or 4 hrs. Values with different letters are significantly different (Holm-Sidak, $p<0.05$).

gave a maximum juice yield of 56.1 ± 2.73%. Similar results were observed after 2 hrs of treatment with pectinase and cellulase on the Smith cultivar and the Amrapali variety after 7 hr of incubation with 1.2% of a pectinase to produce a maximum juice yield of 57% (Chauhan, Tyagi, & Singh, 2001; Ollé, Baron, Lozano, & Brillouet, 2000). The enzyme usage rate in this study was almost 3 times less than their reported dosage yet produced a higher juice yield suggesting that both CZ and Rap used at 0.5% v/w were more effective at breaking down pectin, but fruit ripeness was not mentioned and could have also played a major factor in juice yield. A mango that is riper would produce more juice naturally due to endogenous pectinases. Mango pulp is high in pectic substances, with up to 1.6% of the fruit weight comprised of these carbohydrates while cellulose is estimated to be around 0.2%, which could account for the effectiveness of pectinase enzymes over the cellulase enzyme treatment at increasing juice yield (Ollé, Lozano, & Brillouet, 1996). The time of incubation did not have a significant effect on juice yield as no difference was seen in percent juice yield produced by any enzyme between 1 and 4 hrs of incubation suggesting that a shorter incubation time is sufficient for complete hydrolysis of viscosity inducing cell wall material.

The alteration of phenolic acid glycosides by enzyme treatment was also investigated. Figure 16 illustrates the change in polyphenolic profile of mango juice following a 4 hr treatment with 0.5% v/w CZ. Ester-linked monogalloyl glucoside (MGG), the major polyphenolic in Keitt mango was significantly reduced following 1 hr incubation by CZ, only. After 4 hr of incubation however, all enzymes reduced the ester-linked MGG (Figure 17) by at least 30%, while treatment with CZ caused the maximum

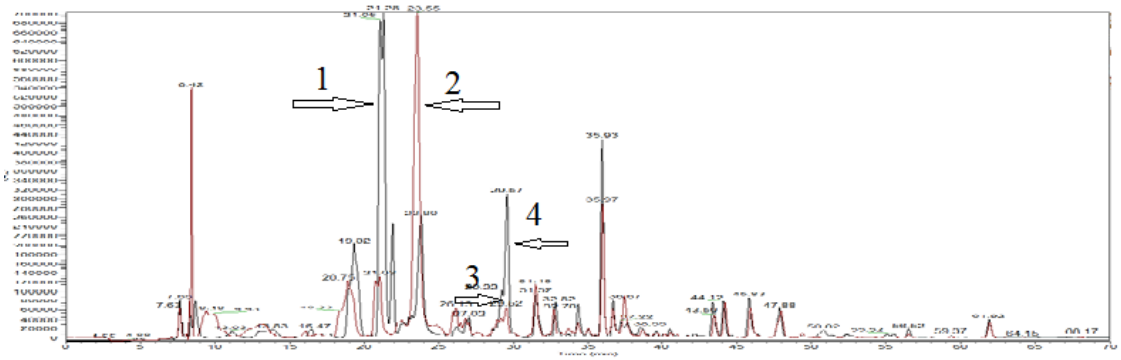

Figure 16. HPLC chromatogram at 280 nm of mango pulp incubated for 4 hr with CZ overlayed with the 4 hr control. Arrows indicate changes due to enzyme hydrolysis 1- ester-linked MGG 2-Gallic acid 3- ether-linked MGG 4- hydroxybenzoic acid glucoside.

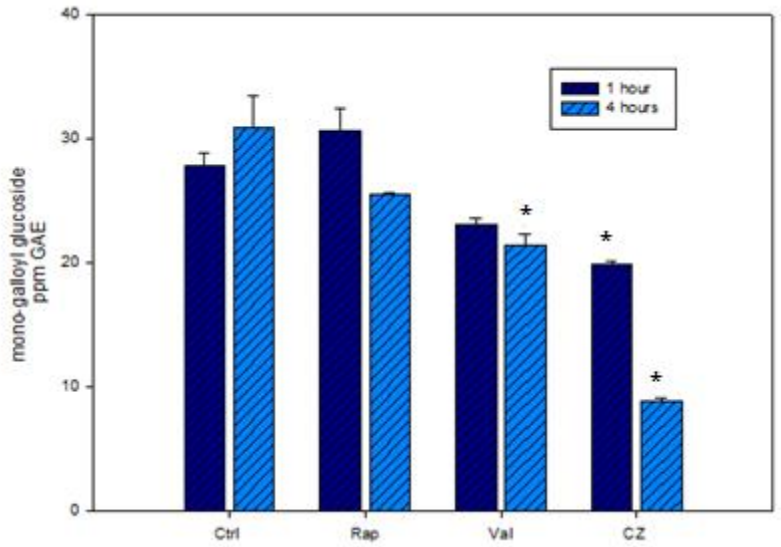

Figure 17. The effect of incubation with the enzymes Rap, Val, and CZ on ester-linked mono-galloyl glucosides in mango pulp. Data comparisons were made to the control at each time point. An (*) indicates a significant difference (paired t-test, $p<0.05$).

reduction of MGG at 70%. Rap, a pectinase with ß-glucosidase side activity, showed the least amount of esterase activity and reduction in MGG did not differ significantly from the control. The application of CZ is therefore cautioned against if a juice truly

representative of the polyphenolics in Keitt is wanted. However, the application of enzymes such as pectinases which naturally hydrolyze ester bonds in pectin could prove to be a useful addition in juice processing to obtain juice, free of ester-linked compounds.

The amount of free gallic acid significantly increased (Figure 18) during juice extraction with the use of all enzymes at both 1 and 4 hr of incubation indicating the hydrolysis of galloyl-glycosides. A Pearson's correlation analysis, showed that M 's decrease was correlated to gallic acid increase ($r=0.833$, $p<0.0001$), only with CZ data, indicating the 4-fold increase in gallic acid was likely only attributed to the same decrease in ester-linked MGG. Rap also significantly increased the amount of free gallic acid by 2-fold compared to the control after 4 hr of incubation, but could not be explained by a simultaneous decrease in ester-linked MGG. Further investigation revealed complete hydrolysis of the ether-linked MGG (true glycoside linkage), suggesting ß-glucosidase activity was responsible for the increase in free gallic acid which was also reported for Rap used on grapes for the purpose of releasing aromatic terpenes from terpene glycosides (Fundira, Blom, Pretorius, & Rensburg, 2002). Like ether-linked MGG, a terpene glycoside is linked hydroxyl group to hydroxyl group and confirms that the side-activity of Rap was capable of hydrolyzing the ether-linked MGG. The hydrolysis of ether-linked MGG is important because it could be good predictor of hydrolysis by cystolic ß-glucosidases *in vivo* which could potentially make the compound more bioavailable (Knaup et al., 2007).

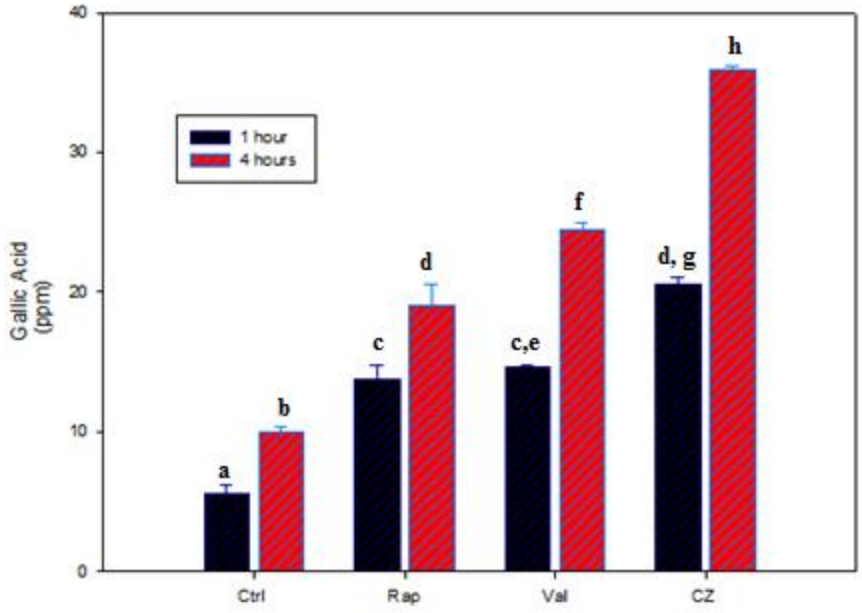

Figure 18. The effect of incubation with the enzymes Rap, Val, and CZ the quantity of free gallic acid in mango pulp. Values with different letters are significantly different (Holm-Sidak, $p<0.05$).

ß-Glucosidase Treatments of High Tannin Mango Pulp + BSA

In the previous study, gallotannins were not evaluated due to low concentrations extracted in the juice. But, to make predictions if ß-glucosidase is capable of hydrolyzing gallotannins *in vivo,* gallotannins isolated from mango pulp and gallotannins pre-bound to protein (to represent what might occur upon injestion), were utilized. Two different concentrations of gallotannins were also used in an effort to understand if enzyme inactivation would be achieved due to tannin-protein interactions. Two sources of ß-glucosidase were evaluated, one stemming from the side-activity of Rapidase, a previously described commercial pectinase, and the other, a purified ß-glucosidase from almonds. Regardless of mango concentration, Rapidase released more, free gallic acid

than treatments with purified ß-glucosidase, 205 mg/L vs. 82 mg/L (Figure 19). The largest increases in free gallic acid, 196% and 279%, were produced at the lowest mango polyphenolic concentration (250 ppm), which was incubated with 4,000 and 40,000 ppm Rapidase, respectively. Exposure of 2500 ppm mango extract with ß-glucosidase resulted in 171 and 105% increases in free gallic acid, differing from what was observed when a high concentration was exposed to interaction with Rapidase. ß-glucosidase, a proline-rich enzyme, has a known affinity to gallotannins; therefore, a large concentration of gallotannins in the presence of ß-glucosidase was expected to inhibit the deglycosylating activity of the enzyme. The lack of visible precipitation in the treatments indicated ß-glucosidase remained soluble, but does not indicate if tannin-protein interactions occurred. The results did indicate however, that a higher concentration of gallotannins proved to be beneficial to the release of gallic acid. This could be attributed to the fact that tannins were in excess of the enzyme which allowed an unknown amount of tannins to remain in a state where ß-glucosidase could deglycosolated, producing free gallic acid. As Hagerman, Rice, & Ritchard (1998) demonstrated, when tannins are in excess, precipitation can only occur based on the number of binding sites of the protein, thus tannins not bound would be left in solution.

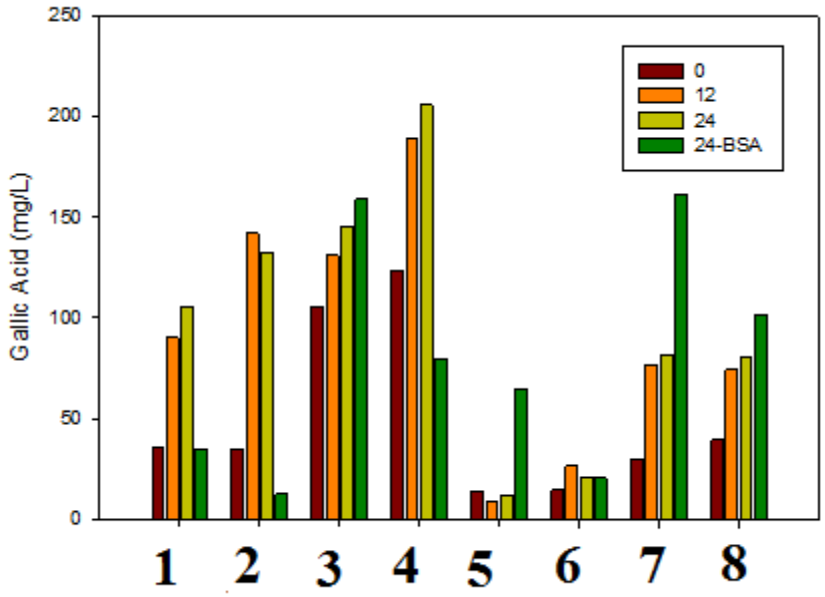

Figure 19. Mango extract (2500 ppm or 250 ppm) was incubated with Rapidase and pure ß-glucosidase at two different concentrations; both with and without BSA and the amount of gallic acid hydrolyzed was analyzed by HPLC for comparison. Numbered treatments correspond to those found in Table 3.

When tannins are consumed, they interact first with proline-rich salivary proteins which have been shown by many researchers to have a strong affinity to tannins, specifically PGG (Soares et al., 2012). The addition of BSA prior to addition of an enzyme treatment was done to mimic what might be actually observed *in vivo*. When BSA-bound tannins were treated with each enzyme preparation, a 500% increase in the amount of gallic acid hydrolyzed after incubation with 280 ppm ß-glucosidase was observed. This illustrates that gallotannins potentially bound to a protein (ß-glucosidase) were still capable of being hydrolyzed and ß-glucosidase was more effective at doing so under those conditions. Still, after 24 hr of incubation with each enzyme both with and

without BSA, a complete hydrolysis of all gallotannins did not occur, suggesting that neither enzyme system is efficient at hydrolyzing tannins.

Conclusions

CZ and Rap were the most effective at increasing juice yield of mango pulp due to their pectinase action. CZ reduced the amount of ester-linked MGG by 70% after 4 hr of incubation, indicating strong esterase activity, while no reports of ß-glucosidase side-activity were demonstrated. Rap did not hydrolyze ester-linked MGG, but showed ß-glucosidase side-activity by the complete hydrolysis of ether-linked MGG. High tannin extracts were hydrolyzed by both Rap and a pure ß-glucosidase, but pure ß-glucosidase worked more effectively when tannins were first bound to BSA prior to incubation which indicates that tannin-protein interaction between the enzyme and tannin is likely to cause it to lose activity. ß-glucosidase hydrolysis activity is important *in vivo* where it is thought to aid in the absorbability of glycosylated polyphenolics. The demonstration of the hydrolysis of gallotannins by enzymes *in vitro* could indicate the action *in vivo* as well, but more studies would need to be conducted to confirm.

CHAPTER V

IN VITRO METABOLISM, CELLULAR UPTAKE, AND STABILITY OF SELECTED MANGO POLYPHENOLICS IN CACO-2 CELLS

Introduction

Gallotannins are not a typical part of the diet due to their preponderance in the unedible portion of many plants; however mangos are unique in that they offer up to 7000 mg gallotannins/kg mango pulp. Pentagalloyl glucoside (5GG) is the prototypical gallotannin and serves as the central compound in the biosynthetic pathway of hydrolysable tannins as five galloyl groups esterified to gallic acid. Many researchers have used this compound as a model tannin in, *in vitro* work and has shown promising anti-cancer and anti-diabetic properites (Jinhui Zhang et al., 2009). Yet it is not likely that PGG reaches many target tissues intact as suggested by the low bioavailability in rats (L. Li et al., 2011). Gallic acid is the major byproduct of gallotannin ester hydrolysis, which could indicate gallotannins represent a potentially large reserve for gallic acid metabolites *in vivo*. Caco-2 cell models have been used for 20 yrs to model new drug absorption and have proved to be highly correlated to *in vivo* observances including expression of Phase II metabolizing enzymes such as (UDP)-glucuronosyltransferases, sulfotransferases catechol-*O*-methyl transferase (COMT), (Hilgendorf et al., 2007). Recently differentiated Caco-2 cells have been used to produce metabolites by incubating polyphenolics, individually or as a mixture, on Caco-2 cells for up to 48 hrs to ensure enough metabolite formation for detection. Extractions of cell

lysates after incubation for polyphenolics have also allowed researchers to determine additional mechanisms of cellular metabolism. It is hypothesized that ester-linked glycosides of gallic acid can be hydrolyzed at pH 7.4, intestinal pH, to yield free gallic acid that is capable of being metabolized by Caco-2 enzymes. The objectives of this research therefore were to characterize the stability of a gallotannin isolate to predict hydrolysis that might occur in similar pH conditions *in vivo,* and to determine the extent of metabolism on gallic acid derivatives due to the hydrolysis with the use of Caco-2 cells as a source of metabolizing enzymes.

Materials and Methods

Gallotannin Isolation and Stability

Mangos, cv. Ataulfo, were sourced from Mexico and allowed to ripen at ambient conditions. Upon ripening, whole fruits were peeled, deseeded, blended to homogenize, and stored at -20°C until analysis. A polyphenolic stock extract was made using 2 kg mango pulp following the procedures outlined in Chapter III. The extract was further concentrated by adsorbing polyphenolics to an activated, 25 g Sep-Pak C18 cartridge (Waters Corporation, Milford, MA). Metals, sugars, organic acids as well as polar compounds with weak or no affinity to C18 under these conditions were eluted from the column with water. The unbound fraction from C18 was extracted twice by liquid-liquid partitioning into ethyl acetate to capture polar polyphenolics that did not bind well to C18. Compounds adsorbed to the C18 were eluted with 100% methanol, combined with the ethyl acetate extract, and solvents removed under vacuum at 45°C. The extract was

re-dissolved in 500 mL deionized water and placed in a sonic water bath to ensure maximum solvation. From this extract, a 150 mL portion was once again adsorbed to a Sep-Pak C18 cartridge (6 cc) and was subsequently washed with a 20% methanol solution to elute non-gallotannin polyphenolics. The compounds remaining on the cartridge were eluted with 100% methanol and evaporated to dryness under vacuum at 45°C. The resulting gallotannin isolate was reconstituted in 15 mL of water acidified with 0.01% HCl and total phenolic content measured according to Singleton & Rossi (1965) and reported as GAE.

To assess the stability of gallotannins at physiological conditions, the gallotannin isolate was diluted to a final concentration of 8 mg/L A E in Hank's Balance Salt Solution (HBSS), Hyclone® Thermo-Fisher, and the pH was adjusted to 7.4 with 1.0 M NaOH to simulate the pH of the small intestine. A control sample (t=0) was immediately taken and the pH re-adjusted to 3.0 ± 0.2 with 1.0 M HCl. The stability of the gallotannin isolate was evaluated following incubation at 37°C and sampled at 0.5, 1, 2, 3, 4, 6, 12 hr, in triplicate. After each sampling, the pH was adjusted to 3.0 ± 0.2 as described and aliquots were centrifuged at 10,000 x g to remove particles and the supernatant frozen at -20°C until analysis.

Gallotannin stability was analyzed using a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source. Separation was achieved in reversed-phase using a Finnigan Surveyor HPLC coupled to a Surveyor brand PDA detector and gradient separations were performed using a Waters Sunfire™ (Milford, MA) C_{18} column (250 x 4.6 mm, 5 µm) at room temperature. Mobile phase A

was 0.1% formic acid in water (Sigma-Aldrich) and mobile phase B was 100% MeOH containing 0.1% formic acid (Sigma-Aldrich). The mobile phases pumped at 0.4 mL/min and the gradient began initially with 0% Phase B and changed to 10% Phase B in 5 min, from 10 to 30% Phase B in 10 min, 30 to 55% Phase B in 17 min, and run isocratically for an additional 13 min followed by another gradient from 55% to 100% phase B in 38 min before returning to initial conditions. The electrospray interface worked in negative ionization mode. Source and capillary temperatures were set at 300°C, source voltage was 4.0 kV, capillary voltage was set at -47.00, and collision energy for MS/MS analysis was set at 35.0 eV. The instrument operated with sheath gas (N_2) and aux gas (N_2) flow rates set at 40 units/min and 5 units/min, respectively. All quantification and characterization was performed using MS extracted ion chromatograms (EIC) and quantified in gallic GAE based on the response factor of gallic acid in EIC. Free gallic acid however was quantified based on its UV absorption at 280 nm.

Metabolism and Cellular Uptake of Gallic Acid Derivatives in Caco-2 Cells

In-vitro metabolism and cellular uptake was assessed using Caco-2 cell monolayers. Caco-2 colon carcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA), and cultured in Dulbecco's modified Eagle's medium (1X) high glucose (DMEM) containing 10% fetal bovine serum (FBS), 1% non-essential amino acids, 100 units/mL penicillin G, 100 µg/mL streptomycin, 1.25 µg/mL amphotericin B, and 10 mM sodium pyruvate. Cells were incubated at 37°C and 5% CO_2 (chemicals were obtained from Invitrogen Life Technologies, Grand Island, NY).

Cells between passages 22-28 were seeded at a density of $1.2x10^5$ cells/well in 12-well, 3.8 cm^2 transparent polyester cell culture plates (Corning Costar Corp., Cambridge, MA). Cells were allowed to grow and differentiate up to 8 days post-confluence in 10% FBS supplemented DMEM, with the media changed every second day. At this point the cells were polarized and differentiated, but had not begun to detach from the surface of the plate (Vachon & Beaulieu, 1992). However, if detachment occurred, the well was not used for experimental purposes.

Four different potential gallic acid sources were incubated with differentiated Caco-2 cells for metabolite determination. All sources were applied to cells in 1.5 mL of 10% FBS supplemented DMEM. Gallic acid was applied to cells at a concentration of 70 mg/L. Keitt mango extract (described in Chapter III) and an Ataulfo gallotannin isolate (described above) were applied at a concentration of 200 mg/L GAE each based on results from the Folin-Ciocalteu assay and the pH was subsequently adjusted to 7.4 after the addition to the media. Metabolism was studied by sampling supernatant media from each treatment at 0, 1, 2, 3, 8, 24, and 48 hr. Each sample (200 µL) was immediately acidified with 6 µL 1.0 M HCL, centrifuged at 10000 x g for 5 min and stored at -20°C until analyzed.

After differentiated Caco-2 cells were incubated with potential sources of gallic acid metabolites for 48 hrs, media was removed and cells were washed twice with PBS. Trypsin (0.25%) was added to each well to detach cells and lysed with 2 mL of methanol. Each methanol-cell mixture was pulse-sonicated for 30 sec on ice using a Model CV18 Ultrasonic Processor (Betatek Inc., Toranto, ON), centrifuged, and the

supernatant was evaporated under reduced pressure (Thermo-Scientific ISS110 SpeedVac Concentrator). The resulting cell concentrate was reconstituted in 100 uL of a 20% methanol 0.1% formic acid solution and centrifuged prior to LC-MS analysis.

LC-MS Analysis

Reversed phase HPLC and mass spectrometry analyses were conducted using a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source (Thermo Fisher, San Jose, CA). Chromatographic separations were performed using a Phenomenex Kinetex 26 μm C18, 100 X 3.0 mm column. Mobile phase A consisted of 0.1% formic acid in water (Sigma-Aldrich) and 100% MeOH containing 0.1% formic acid and flowed at 0.4 mL/min. Phenolics were separated in a gradient elution program in which mobile phase B changed from 0 to 21% in 4.61 min, 21 to 35% in 7.66 min, 35 to 49% in 13.77min, 49 to 70% in 19.88 min, followed by column equilibration with mobile phase A for an additional 3.6 minutes prior to the start of the next injection. Ionization was conducted in the negative ion mode under the following conditions: sheath gas (N_2), 40 units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 3.5 kV; capillary temperature, 300 °C; capillary voltage, -42.0 V; tube lens offset, -40.0 V. The instrument was set to operate in data dependent scan mode, with the following targets: m/z 169.2, 183.2, 197.2, 249.0, 263.0, 321.0, 331.0, 345.0, 359.0, 787.0, 1091.0, 1243.0, 1395.0, and 1547.0 corresponding to gallic acid derivatives and metabolites.

Statistics

Data were analyzed using a Student's t test from SigmaPlot statistics software package (Systat Software, Inc. London, UK) which provided corresponding probability ($p<0.05$) values to indicate significant differences between treatments.

Results and Discussion

Gallotannin Stability

The molecular weight distribution of gallotannins following incubation in cell culture and intestinal conditions, pH 7.4 and 37°C, was evaluated to determine the extent of gallotannin hydrolysis and to estimate the subsequent amount of liberated gallic acid that could potentially be included in the biologically available portion of mango polyphenolics. Prior to incubation, over 63% of the gallotannin isolate (Table 4) was

Table 4. Changes in the distribution of specific gallotannins ions expressed as a percentage of total ion counts when incubated for 4 hours at pH 7.4 and 37°C.

Abb.	Compound I.D.	$[M-H]^-$ (*m/z*)	$[M-2H]^{2-}$ (*m/2z*)	% Total Ion Signal[1] T_0	% Total Ion Signal[1] T_4
4GG	tetra-*O*-galloylglucose	787	393	0.94 ± 0.2 a[2]	9.44 ± 1.7 a
5GG	penta-*O*-galloylglucose	939	469	7.57 ± 1.2 a	14.82 ± 0.8 b
6GG	hexa-*O*-galloylglucose	1091	545	11.47 ± 0.8 a	19.55 ± 0.6 b
7GG	hepta-*O*-galloylglucose	1243	621	16.80 ± 1.1 a	21.20 ± 0.5 b
8GG	octa-*O*-galloylglucose	1395	697	20.67 ± 1.6 a	17.09 ± 1.2 a
9GG	nona-*O*-galloylglucose	1547	773	21.25 ± 1.8 a	9.77 ± 1.0 b
10GG	deca-*O*-galloylglucose	1699	849	15.27 ± 3.1 a	6.21 ± 0.9 a
11GG	undeca-*O*-galloylglucose	1851	925	4.44 ± 1.1 a	1.91 ± 0.3 a
12GG	dideca-*O*-galloylglucose	2003	1002	1.59 ± 0.5 a	nd b

[1] % Total ion signal is the summation of EICs for 4GG (m/z + m/2z) up to 12GG (m/z+m/2z). [2] Values with different letters within the same row are significantly different (student's t test, p< . 5)

comprised of compounds with a *m/z* of 1395, 1547, 1699, 1851, and 2003, corresponding to 8-GG, 9-GG, 10-GG, 11-GG, and 12-GG, respectively. The largest gallotannin identified, 12-GG, consists of 12 gallic acid moieties, seven of which are linked *via* an *m*-depside bond as shown in the proposed structure in (Figure 20). The complete

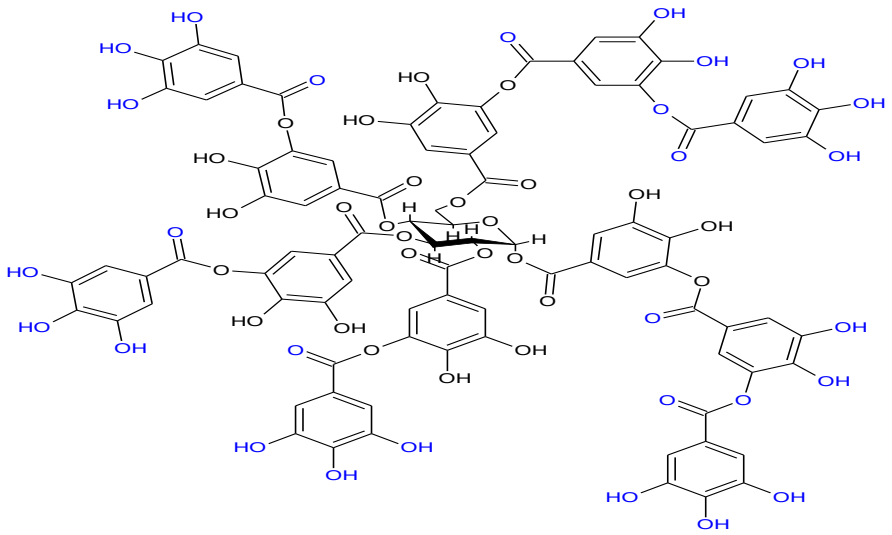

Figure 20. Proposed structure for dideca-O-galloylglucose, with m-depside linked molecules shown in blue.

disappearance of an ion at *m/z* 2003 and *m/2z* 1002 after 4 hr of incubation is an indication of the hydrolysis of at least one depside bond and thus a shift from *m/z* 2003 to a lower molecular weight. A depside-linkage is the name given to the ester bond between two gallic acid moieties and is formed between the carboxylic acid of one molecule and the hydroxyl group of the second. A study on the mechanism of alkali hydrolysis of ester bonds revealed that the presence of a hydroxyl group neighboring an

ester bond, such as that found in a depside linkage between gallic acid, increased the rate of ester hydrolysis by a factor of nine (Bruice & Fife, 1962). Mass spectroscopic studies performed by various groups (Berardini et al., 2004; Engels et al., 2009; Salminen, Ossipov, Loponen, Haukioja, & Pihlaja, 1999) using soft ionization procedures have also reported the fragmentation (loss) of *m*-depside bonds preferentially to the esters of gallic acid to glucose. The loss of *m*-depsides first during fragmentation logically points to the fact that it is the weaker of the two esters. A common assay to procedure PGG from high molecular weight (HMW) gallotannins at weakly basic conditions by addition of methanol utilizes the principle of depside bond weakness to produce hydrolyze the depside bonds of gallotannins 5 or more gallic acids esterified to glucose (Mueller-Harvey, 2001) and is further proof of the propensity of depside linkages to hydrolyze prior to ester linkages. After 4 hr of exposure to treatment conditions, the distribution of the gallotannin population transferred from HMW tannin (>8GG) dominance at 63% of the total ion count, to that of lower weight tannins (<8GG-4GG), 68%, presumably by the loss of depsidly-linked gallic acid which are abundant in HMW tannins. A statistical difference ($p<0.05$) was observed in the increase in percentage of total ion counts for 5GG (7.57% to 14.82%), 6GG (11.47% to 19.55%), and 7GG (16.8% to 21.2%), while a significant decrease was also detected simultaneously for 9GG (21.25% to 9.77%) and 12GG (1.59% to undetectable). This shift in molecular weight can be seen visually in Figure 21 as the HPLC chromatogram of the gallotannin isolate, which was once unresolved due to the presence of many isomers of galloylated polymers, has distinguishable earlier eluting peaks corresponding to the molecular weights of 3GG

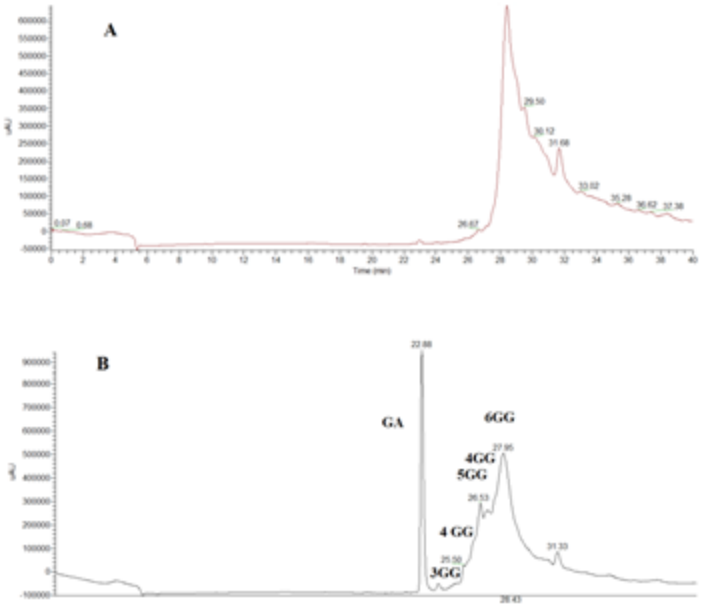

Figure 21. HPLC chromatogram of gallotannin extract at 280nm. (A.) Control (B.) 4 hrs after incubation at pH 7.4 and 37°C.

(*m/z* 635), 4GG (*m/z* 787*)*, 5GG (*m/z* 939), and 6GG (*m/z* 1091). Although a decrease in 9GG and10GG occurred in 1 hr, all other individual galloylglucosides did not significantly differ from the control until hour 2 (Figure 22). After 30 mins of the gallotannin in treatment conditions, 2.63 mg/L gallic was observed. While not an appreciable amount of gallic acid was freed within the first 30 mins, the amount of liberated gallic acid increased linearly (Figure 23) by a factor of 2 until hour 3, producing 22.25 mg/L free gallic acid (Table 5) which followed the same trend observed in the change in weight distribution of gallotannins between 2 and 3 hrs of treatment. Between 3 and 4 hrs, the amount of gallic acid liberated did not change significantly ($p<0.05$) indicating bonds that were easiest to dissociate, were all dissociated by this point, and/or oxidation and subsequent loss of gallic acid began to occur. However, no

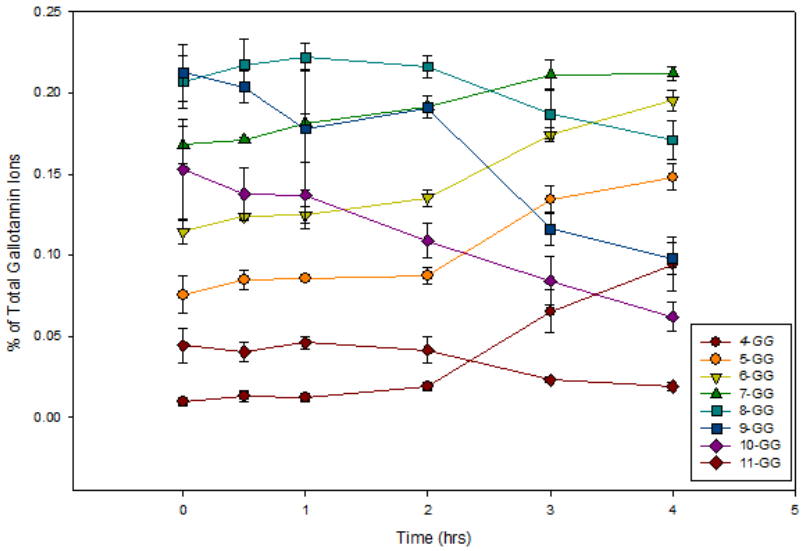

Figure 22. The percentage of high molecular weight gallotannins, 11-8 GG, decrease when incubated for 4 hours at pH 7.4 and 37°C, while lower molecular weight gallotannins, 4GG-7GG, increase in percentage of total gallotannin ion count.

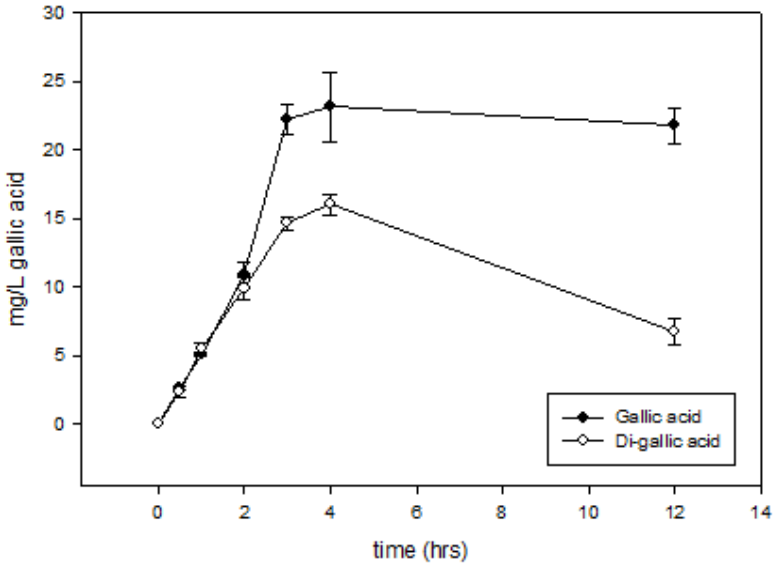

Figure 23. Liberation of gallic acid and digallic acid from a gallotannin isolate in a pH 7.4 buffer and 37°C.

Table 5. Changes in gallic acid, digallic acid, and total gallotannins following incubation in a pH 7.4 buffer at 37°C.

Time (hrs)	Gallic Acid (mg/L)	Digallic Acid (mg/L GAE)	Total Galotannins (mg/L GAE)
0	0.0	0.0	311.83 ± 29.3
0.5	2.63 ± 0.1	2.4 ± 0.4	229.4 ± 15.0
1	5.15 ± 0.1	5.56 ± 0.4	255.01 ± 9.6
2	10.87 ± 1.0	9.87 ± 0.8	253.27 ± 0.4
3	22.25 ± 1.1	14.65 ± 0.4	260.42 ± 14.05
4	23.16 ± 2.5	16.02 ± 0.8	243.90 ± 8.0
12	21.79 ± 1.3	6.76 ± 0.9	108.0 ± 4.78

dissociation products, pyrogalloyl or quinones, were found in the wavelengths and mass ranges scanned. Similarly, when 5-GG, a gallotannin often used as a standard, was incubated at pH 7.0 for 2 hrs, Krook & Hagerman (2012) observed an 80% reduction in the amount of recovered 5-GG and reported the detection of degradation products, 3-GG and 4-GG, as well as free gallic acid and undetectable products, assumed to be oxidation products, which were reported to be difficult to separate and detect by HPLC (Y. Chen & Hagerman, 2005). However, after 4 hrs of incubation, a 33% reduction in total gallotannins were observed, much less than that observed by Krook and Hagerman (2012). This difference could be explained by the imprecise integration and quantification of the gallotannin "hump" based on gallic acid's standard curve, compared to the standard 5GG (Mueller-Harvey, 2001). In addition, when a HMW compound was hydrolyzed to its LMW counterparts, it was still included in the total quantification and thus would not show up as a decrease in the amount of total gallotannins present. The amount of *m*-digallic acid, *m/z* 321, corresponding to the linkage of two gallic acid moieties $M\text{-}H^-$ (170+170-18 Da), was also monitored

throughout the treatment time and concentrations analyzed match closely the concentrations of gallic acid, 9.87 vs. 10.87 mg/L, at each sampling time point up to the second hour. The sharper decline in the amount of *m*-digallic found after 12 hrs could be another indication of the instability of *m*-digallic acid (and its depside bond) and its propensity to convert to two molecules of free gallic acid under these conditions. It is unclear based on the data if the free digallic acid reported is a product of a *m*-depside cleavage or of an ester-glycoside cleavage, but the lack of a prominent ion at *m/z* 473 which corresponds to trigallic acid is evidence that if trigallic acids were esterified to glucose, the *m*-depside bond was hydrolyzed preferentially.

The ability of free gallic acid to arise from mango gallotannins in conditions similar to *in vivo* pH and temperature conditions gives gallotannins an almost dual purpose of providing the potential for bioavailable compounds in the small intestine and as substrates for microbial fermentation as reported by Selma, Esp n, om s -Barber n (2009) in the colon. Gallotannin content of mangos range from 270 mg/kg fruit weight (FW) to 7000 mg/kg FW depending on variety, and based on HPLC analysis and quantification techniques similar to the ones for values reported, the gallotannin isolate used to model mild alkali hydrolysis is feasible at 311.8 mg/L. The degradation of the gallotannins used in the present study produced 23 mg/L of free gallic acid in 4 hrs, 60% of the amount of the most abundant compound, acyl-linked MGG, found in Keitt mango and therefore should not be strictly assumed to only exert an effect in the colon.

Metabolism and Cellular Uptake of Gallic Acid Derivatives in Caco-2 Cells

Three different potential sources for gallic acid metabolites were evaluated using a differentiated Caco-2 monolayer capable of expressing metabolizing enzymes such as SULT (sulfur transferases), UGT (gluuronosyltransferase), and COMT (catechol-*O*-methyltransferase) which are commonly found in varying levels in small intestinal enterocytes and the liver (G. Chen et al., 2003; Dihal, Woutersen, Ommen, Rietjens, & Stierum, 2006; Paine & Fisher, 2000). Pure gallic acid (170 mg/kg) was used as a control for this experiment, as 3-*O*-methyl gallic acid and 4-*O*-methyl gallic acid are known metabolites of gallic acid (Yasuda et al., 2000). After 2 hrs of exposure to differentiated Caco-2 cells, a portion of gallic acid was metabolized into *O*-methyl gallic acid, as detected by mass detection only, and was quantified as 0.3 mg/L (Figure 24). Prior to 2 hrs, if *O*-methyl gallic acid was produced, it was below the limit of detection. After 8 hrs of incubation in cell culture media exposed to Caco-2 cells, a parent ion of *m/*z 183.1, corresponding to *O*-methyl gallic acid, was observed and fragmented to m/z 168, 139, and 124, indicating the loss of a methyl group, a carboxylic acid, and the methyl + carboxylic acid, and was confirmed by use of a standard of 3-*O*-methylgallic acid (Figure 25). The amount of *O*-methylgallic acid was quantified as 0.7 mg/L. After 24 hrs of incubation, 2.26 mg/L *O*-methyl gallic acid was detected and remained constant over the next 24 hr period; however a 70% loss of gallic acid following 48 hr treatment was observed indicating that *O*-methylgallic acid was more stable than gallic acid in cell culture conditions. The instability of gallic acid has been reported in pH > 6.0, which often results in the irreversible loss of gallic acid and the subsequent

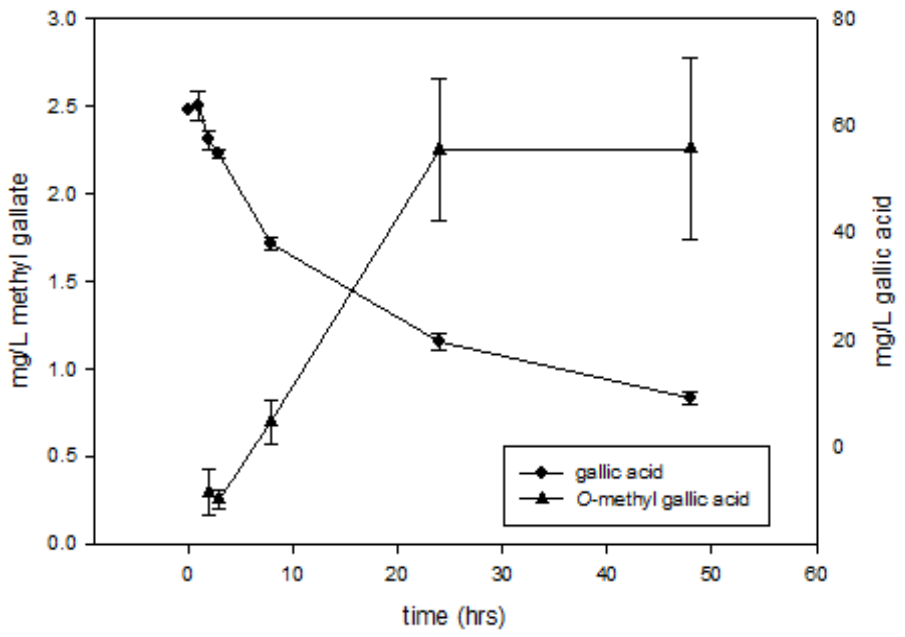

Figure 24. Simultaneous increase in methyl gallate and decrease of gallic acid over 48 hr. incubation with differentiated Caco-2 cells. (n=3).

production of a quinone (Friedman & Jürgens, 2000). Similarly, when 3 μ mol/L ellagic acid (oxidative dimer of gallic acid) was incubated with differentiated Caco-2 cells, the metabolite dimethyl gallic acid did not appear to increase after 24 hrs of incubation. A 70% loss in ellagic acid after 24 hr incubation, likely due to the oxidation of ellagic acid was also reported (Larrosa et al., 2006). Oxidation of (-)-epigallocatechin-3-gallate from green tea in cell culture media at pH 7.4 was also observed by the production quinones and the formation of dimers using HPLC-MS (Hong et al., 2002; Sang, Lee, Hou, Ho, & Yang, 2005; Vaidyanathan & Walle, 2003). The presence of the oxidation products of

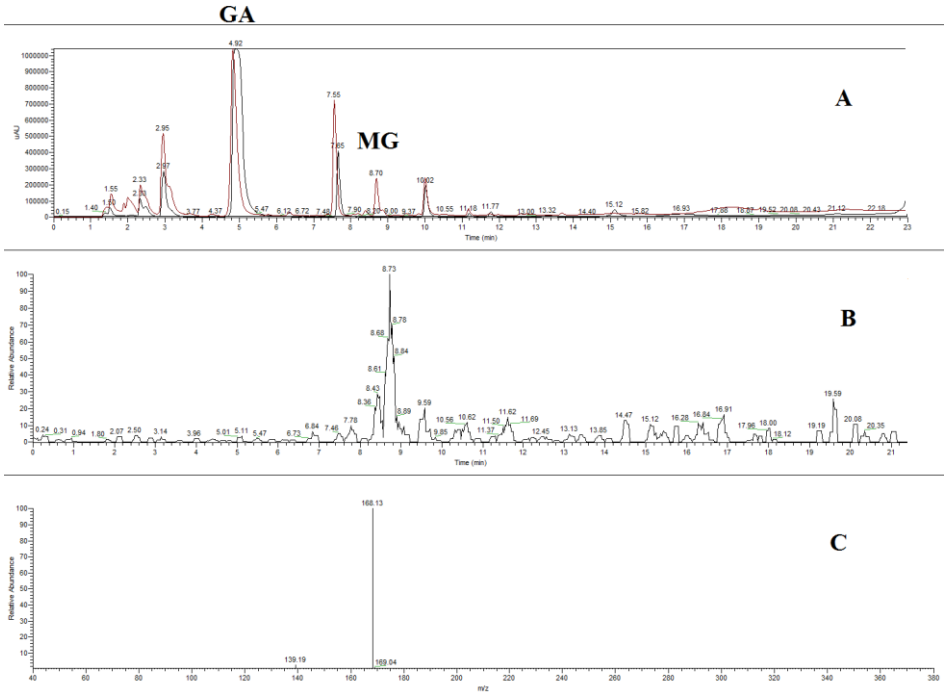

Figure 25. A.) HPLC chromatogram of gallic acid (GA) and its metabolite, methyl gallate (MG) at Time 0 and 24 hours. (B.) EIC m/z 183 (C.) MS2 fragment of m/z 183.2

EGCG were also shown to have a cell sealing effect which prevented the efflux of metabolites out of the cell and was observed by a colored cell surface, similar to the green surface of Caco-2 cells incubated with gallic acid (Hong et al., 2002; Kitano, Nam, Kimura, Fujiki, & Imanishi, 1997). The formation of quinones and ensuing cell sealing could explain the lack of increase in metabolite formation of gallic acid to *O*-methylgallic acid following 48 hr incubation.

Mango extract, var. Keitt, as characterized in Chapter III was also incubated in the presence of differentiated Caco-2 cells at a concentration of 200 mg/L GAE. For this analysis, only gallic acid derivatives were monitored. Actual quantities of MGGs and gallic acid were analyzed by HPLC and determined to be 10.6 mg/L acyl-linked MGG,

4.1 mg/L gallic acid, and 2.8 mg/L glycosidically-linked MGG. Total gallotannins were estimated as described previously in this section by integration of total peak area and totaled to be roughly 150 mg/L GAE. Figure 26 reports the stability of the individual compounds monitored over the 48 hr incubation period. Contrary to what was observed in the incubation of pure gallic acid, a 78% increase in free gallic acid, which correlated to a maximum of 19 mg/L was observed until a 24-hr period. A steady decrease in concentration of the ester-linked MMG also occurred, but only a 6 mg/L loss was recorded over the 24 hr period which does not account for the amount of free gallic released that must have been required to overcome the simultaneous degradation. The hydrolysis of gallotannins as reported earlier could make up the difference, as a 40% decrease in the amount of gallotannins present in the supernatant was detected after 24 hrs. It should also be noted that the affinity of gallotannins to proteins could also cause the decrease in total gallotannin content (Y. Chen & Hagerman, 2005) if bound to the Caco-2 cells. However it is not clear if a mixture of gallotannins bound to a protein (Caco-2 cells) could also release gallic acid as was observed with gallotannins at pH 7.4 in the absence of cells. However, Krook and Hagerman (2012) showed the stabilization of 5-GG with the addition of Cheerios and digestive components such as pancreatin, lipase, and bile, when incubated at pH 7.0 (30% reduction as compared to 80% reduction) suggesting a protective effect attributed to polyphenol-protein interaction. This could suggest that gallotannins bound to protein are more likely to remain intact in digestive conditions and would be an unlikely source of free gallic acid.

Methylgallic acid metabolites produced from gallic acid in a Keitt mango extract were only detectable after 24 hr incubation as shown in Figure 27, and was quantified by MS to be 2.1 mg/L. When cells were incubated with pure gallic acid, they were exposed to 70 mg/L; however at 24 hr the mango extract had a maximum of only 19 mg/L,

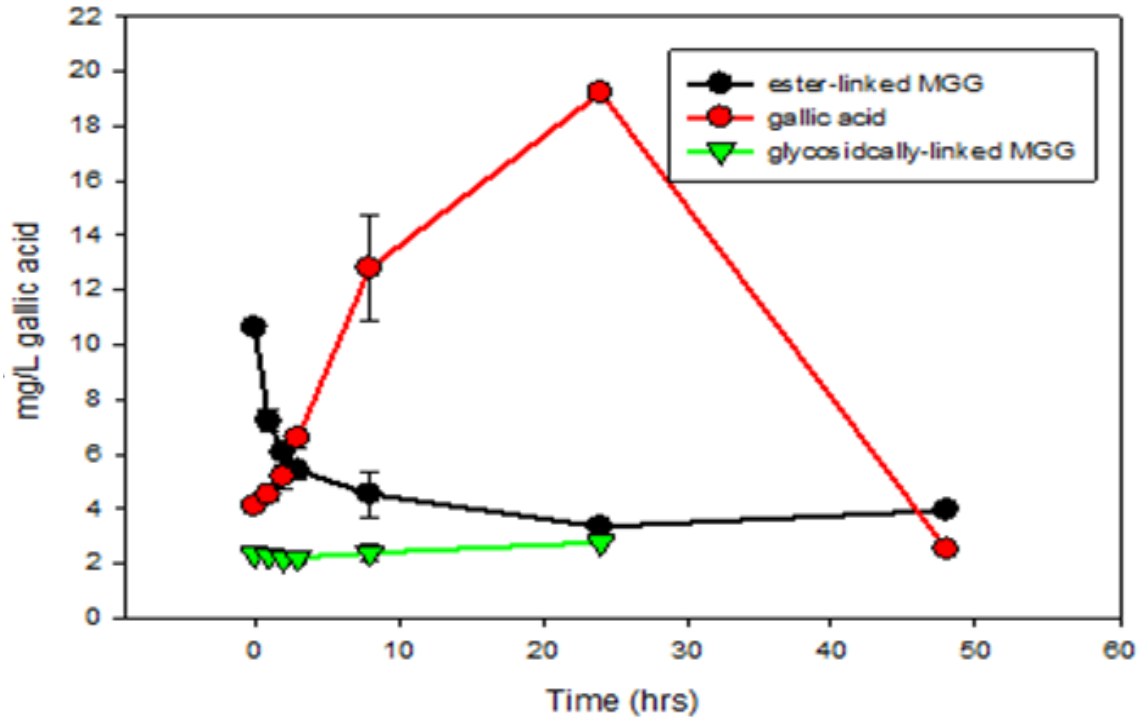

Figure 26. Stability of ester-linked MGG, glycosidically linked MGG and gallic acid from a mango pulp extract incubated with differentiated Caco-2 cells for 48 hrs.

significantly less substrate which could explain why methylgallic acid was not detected prior to 24 hrs. Similar results on the stability of gallotannins were observed as reported previously for both the mango extract and in the gallotannin stability study as illustrated in (Figure 28) with a maximum free gallic acid produced after 24 hrs of incubation with

a concentration of 10.31 mg/L. Trace amounts of methyl gallate were visible (data not shown) as ions in EIC of full MS scan, but were not abundant enough to unanimously identify as the formation of a metabolite from free gallic acid.

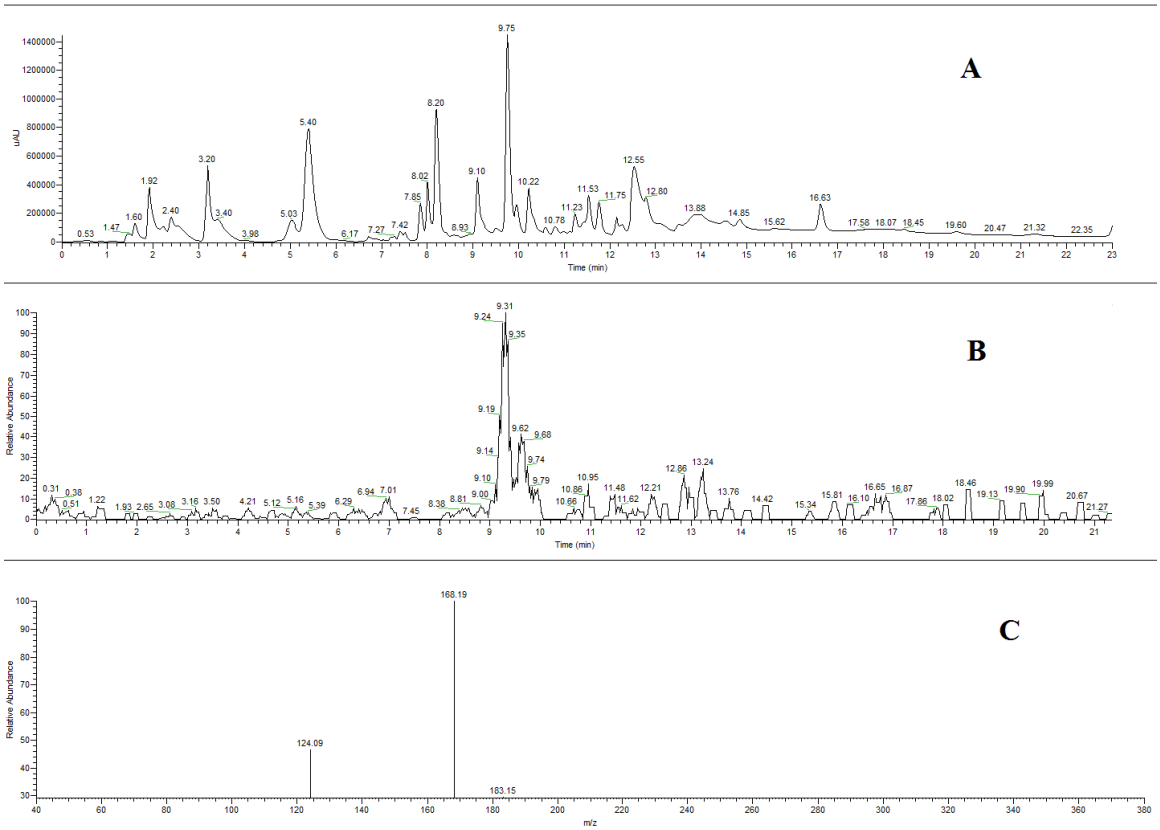

Figure 27. A.) HPLC chromatogram Keitt mango extract after incubation with Caco-2 cells for 24 hrs. (B.) EIC m/z 183 (C.) MS2 fragment of m/z 183.2

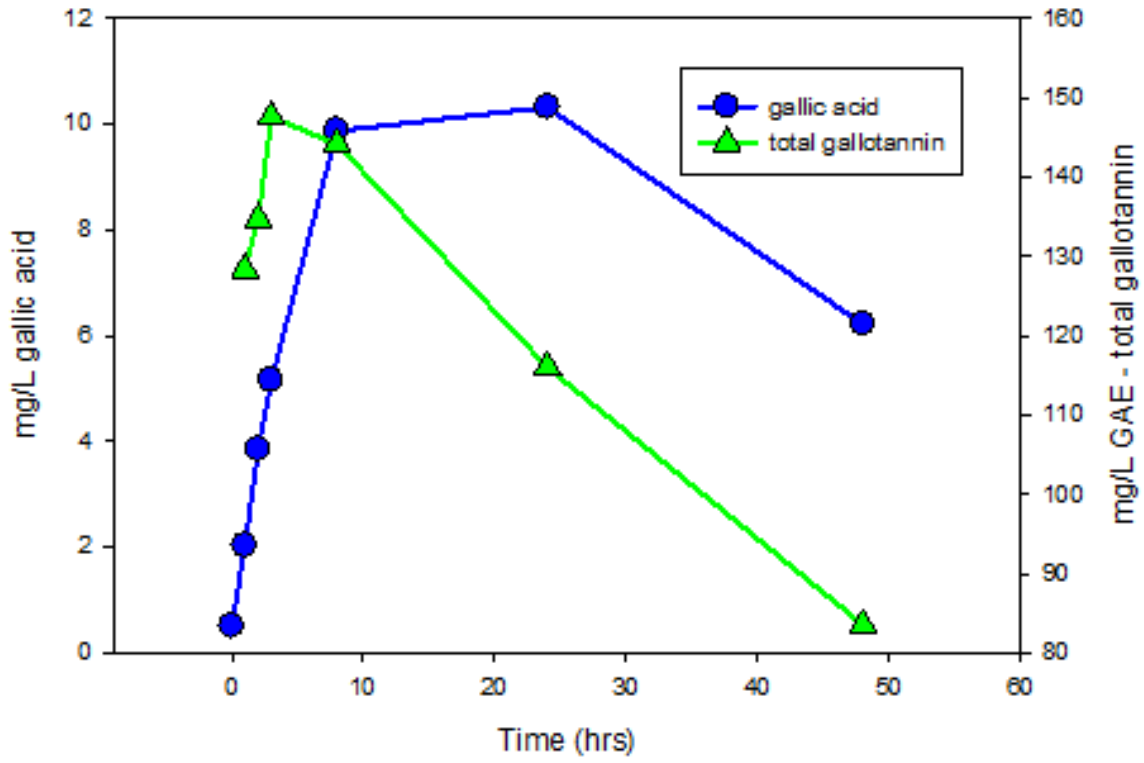

Figure 28. Stability of gallic acid and total gallotannins from a high tannin mango isolate extract incubated with differentiated Caco-2 cells for 48 hrs.

To determine cellular uptake of parent componds or formation of metabolites, Caco-2 cells were lysed and methanol extracted following the last sampling at 48 hrs post treatment with gallic acid, Keitt mango extract, and a high tannin mango isolate. Gallic acid or its metabolite, methylgallic acid were not detected by HPLC-MS in the cell extract of cells incubated with pure gallic acid. However, it was observed after two sequential extractions with methanol and sonication that the cell lysate pellet remained green, an idication that oxidized gallic acid remained bound to the cell. In the cellular extractions of those inicubated with Keitt mango extract, 0.11 mg/L methyl gallate was detected along with minor amounts of gallic acid, 0.02 mg/L and 4-GG, 5-GG, and 6-

GG (Figure 29). The detection of methyl gallate in the cellular extract is an indication that COMT activity originates intracellularly and methyl gallates are effluxed out based on their discovery in the media after sampling at different timepoints. A similar study demonstrating the metabolism of cinnamic acids also noticed the same occurance of intracellular enzymes while noting the lack of enzyme activity outside of the cell (Kern et al., 2003). The cellular extract of Caco-2 cells treated with the gallotannin isolate also showed a concentration of 0.1 mg/L methyl gallate and 0.3 mg/L 5GG but free gallic acid was not detected. It is unclear if the detection of galloyglucosides corresponds to the actual absorption of these higher molecular weight species or if they are a result of extraction of compounds bound to the cell membrane. 5GG was evaluated in a Caco-2 absorption model and it was discovered that the sodium-dependant glucose transporter (SGLT1) was able to transport this molecule and its degradation products, 4GG and 3GG, both apically and basolaterally, while the multidrug resistance-associated protein (MRP2) transported it out of the cell in the opposite direction. Still, there is more evidence that suggests the low absorption of gallotannins as indicated by 3-4 μM of PGG maximum absorption in mice after an abdominal injection of 20 mg/kg PGG (L. Li et al., 2011) likely due to their large size and protein binding capabilities. Therefore it is more probable that gallotannins were bound to the outside of the cell membrane and were extracted with methanol and sonication of lysed cells.

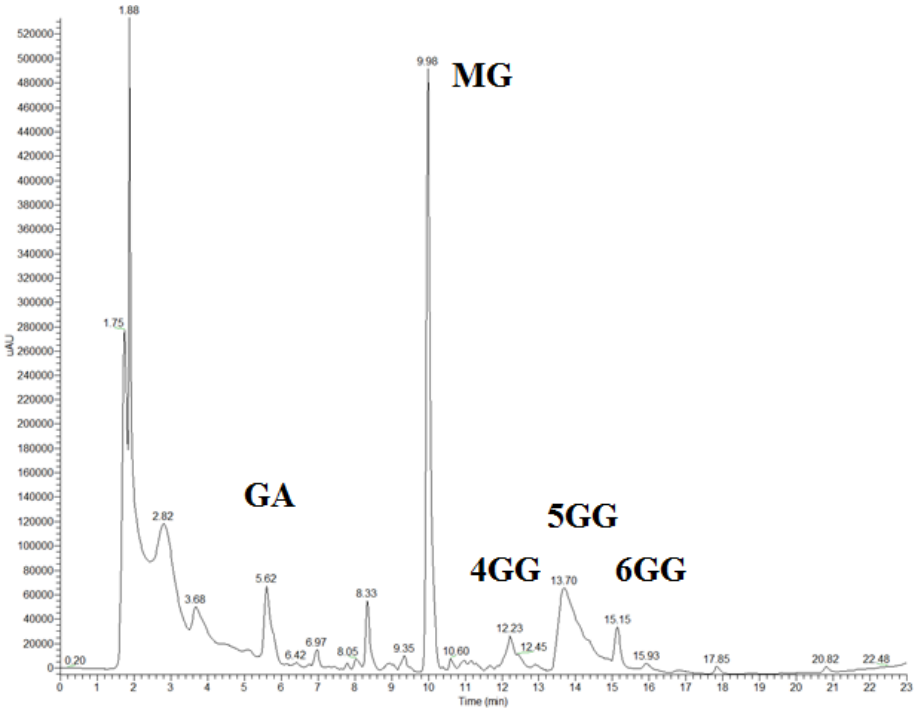

Figure 29. Caco-2 cellular extract after 48 hours post treatment with Keitt mango extract (200 ppm GAE) 15X concentrated.

Conclusions

The instability of ester-linked galloyl-glycosides at pH 7.4 was characterized by HPLC-MS and after only 4 hr incubation a shift from HWM tannins (>8GG) to LMW (<8GG) occurred, with LMW gallotannins representing 68% of the total ion count. This reduction in HMW gallotannins produced 23 mg/L of free gallic acid in 4 hrs without taking into account that lost by oxidation which suggests that gallotannins could potentially serve as a great source for gallic acid in the small intestine as well as the colon. The instability of the ester-linked galloyl-glucosides was also evaluated in the presence of differentiated Caco-2 cells to determine if they could also be metabolized to a known gallic acid metabolite, *O*-methyl gallic acid. Methyl gallate was detected in 24

hr incubation with a Keitt mango extract and in the cell lystate of the same cells after 48 hr as well as in the cell lysate of Caco-2 cells treated with a gallotannin-rich isolate of mango. These results indicate that ester-linked glycosides, once hydrolyzed can be metabolized similar to gallic acid.

CHAPTER VI

PROFILE OF URINE METABOLITES AFTER THE INTAKE OF MANGO (*MANGIFERA INDICA,* L.*) VAR. KEITT IN HUMANS*

Introduction

Mango is a rich source of polyphenolics which have been shown *in vitro* to possess anti-inflammatory and anti-carcinogenic properties (Percival et al., 2006; Taing et al., 2012). Mangos contain large amounts of sugar, organic acids, carotenoids, Vitamin C, ester-linked gallic acids, and many other phenolic acid glycosides. The world-wide popularity of this fruit suggests that it is capable of impacting the health of many and while *in vitro* cell culture reveals mechanisms for health-promoting properties, it often does not take into consideration the effect of the metabolism on phytochemicals. Based on studies where humans consumed relevant food doses, evidence suggests that virtually all circulating polyphenols are conjugated and found methylated, glucuronidated, and/or sulfated (Claudine Manach et al., 1998; Scalbert, Morand, Manach, & Rémésy, 2002). Gallic acid, a major polyphenolic in mango pulp, has been extensively studied in black tea (free form), and in green tea (esterified to catechins) where a majority of its pharmacokinetic and metabolite data stem; and all data suggest that gallic acid is absorbed *via* the paracellular route and the compound methylated upon absorption (Konishi et al., 2004; Konishi et al., 2003). Although there is a lack of data that confirms the hydrolysis of gallotannins by intestinal enzymes or colonic microbiota, reports of ellagitannin degradation and subsequent release of free ellagic acid after the

ingestion of 300 g raspberries (onz lez-Barrio, Borges, Mullen, & Crozier, 2010) is a good indication that gallic acid may also be released following consumption of gallotannins. Reports of the decomposition of 5-GG (40% loss) to gallic acid and lower molecular weight species in a simulated digestive system containing pancreatin, lipase, and bile at pH 7.0 for 2 hrs also point to the underrepresentation of the potential bioavailability of gallotannins (Krook & Hagerman, 2012). Keitt mango primarily contains ester-linked gallic acid glycosides which serve as the only sources of gallic acid upon consumption. Urinary metabolites of gallic acid have been reported following the consumption of black tea and a polyphenol juice drink and include methylated and sulfated conjugates of gallic acid (Mullen, Borges, Lean, Roberts, & Crozier, 2010; Shahrzad, Aoyagi, Winter, Koyama, & Bitsch, 2001). It is hypothesized that ester-linked gallic acid glycosides will be hydrolyzed *in vivo* by the action of pH or esterases, be absorbed, and metabolized before excretion in the urine. Therefore, the objective of this research was to characterize the urinary metabolites produced after the consumption of mango pulp in an effort to begin to characterize the bioavailability of the major group of polyphenolics in mango, ester-linked gallic acids, and to provide new insight into their *in vivo* behavior.

Materials and Methods

Whole mango fruits, var. Keitt were sourced from Mexico and allowed to ripen upon arrival. Ripened mangos were peeled, seed kernels removed and pulpy flesh was cubed and vacuumed sealed to be stored at -20°C until use.

Eleven healthy volunteers (2 male, 9 female) who had no medical history of digestive disorders or chronic diseases participated in a 10 d mango consumption pilot study. Subjects were aged between 21-38 yrs and with a weight between 45-95 kg. Participants were asked to stop taking dietary supplements for 1 wk prior to the start of the study begin and study duration and asked to avoid excessive exercise and alcohol consumption 3 d prior to the start of the study Day 1 and study Day 10. Participants were also required to fill out a 72-hr food diary that detailed their food consumption over the previous 3 d leading up to Day 1 and Day 10 and each diary was reviewed for dietary compliance. All participants fasted from fruit and vegetables, coffee, and tea 1d prior to study Day 1 and Day 10 and were asked to bring in a stool sample collected prior to consumption of mango on the first day of the study. After an overnight fast of at least 12 hrs, urine collections were made (baseline) and baseline blood samples were taken from each participant. Each participant was given 400 g of cubed mango pulp to consume and urine collections were made at 0-3, 3-6, 6-8, and 8-12 hrs. The volume of urine was recorded and an aliquot immediately frozen at -80°C until analysis. Blood samples were also taken at 0.5, 1, 2, 3, 4, 6, 8, and 12 hrs, collected in BD Vacutainer® Plastic Blood Collection Tubes with K_2EDTA tubes and centrifuged for 10 mins at 4000 x g, 4°C. Supernatant (plasma) was aliquoted into 2 mL samples, acidified with 5 µL 85% formic acid, and immediately frozen at -80 °C until analysis. Participants were given 400 g of mango to consume daily for the next 8 d and the study design was repeated again on Day 10. After completion of the tenth study day, stool samples were again collected and

immediately frozen at -20°C. The study protocol was approved by the Institutional Review Board (IRB) of Texas A&M University, IRB no. 2011-0735.

Aliquoted urine samples were thawed and centrifuged at 13,000 x g for 10 mins at 4°C immediately prior to analysis by HPLC-MS^n. Detection of urinary metabolites was accomplished using a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source. Separations were in reversed-phase using a Finnigan Surveyor HPLC coupled to a Surveyor brand PDA detector and gradient separations were performed using a Dionex Acclaim™ (Bannockburn, Il) C_{18} column, (250 x 4.6 mm, 5 μm) at room temperature. Separations and mass spectral detection was performed as described in Chapter III with modifications made to the data dependent collection of ions. The mass detector was set to scan for the following parent ions within a mass range ± 1 m/z: 137.0, 169.0, 183.0, 197.0, 205.0, 249.0, 263.0, 299.0, 301.0, 303.0, 313.0, 315.0, 331.0, 343.0, 345.0, 359.0, 403.0, 411.0, 439.0, 443.0, 507.0, 523.0, 533.0, corresponding to polyphenolics found in the highest concentration in mango and their potential methylated, sulfate, and/or glucuronidated metabolites. Quantification of metabolites was performed by integration of peak areas of EIC in full MS and expressed as gallic acid, pyrogalloyl, or 3-*O*-methyl gallic acid equivalents where applicable. To standardize the concentration of metabolites for the sake of comparison across varying volumes of urine collected by each participant; urinary creatinine concentrations were determined using a creatinine enzyme assay kit (Sigma-Aldrich). All values are expressed as mg of pyrogallol or mg 3-*O*-methylgallic acid/10 mg creatinine.

Bacterial DNA was extracted from 100 mg stool samples using the QIAmp® DNA Stool Mini Kit (Quiagen Ltd, UK) according to the manufacturer's instructions. qPCR assays for selected bacterial groups were performed: total bacteria, *Bacteroidetes, Fusobacteria, Blautia, Ruminococcaceae, Faecalibacterium* spp., *Turicibacter* spp., *Bifidobacterium* spp., *E coli*, *Lactobacillus*, and *Streptococcus*. The qPCR data were expressed as log amount of DNA (fg) for each particular bacterial group per 10 ng of isolated total DNA. Short chain fatty acid (SCFA) analysis of stool samples were performed using a gas chromatograph (HP 5890, Hewlett-Packard, Palo Alto, CA, USA) coupled to a quadrupole mass spectrometer (HP-5989A). Sample preparation for short chain fatty acids with GC/MS assay is as follows; feces (0.5g) were vortexed in 2N HCL for 30 min followed by centrifugation at 3000 x *g* for 20 min. The upper phase was transferred to a Waters Sep-Pak Vac C18 cartridge (100 mg) after adding 5 µL 200 mM internal standard (d7-butyric acid). The sample was then eluted with 2 mL diethyl ether and further purified by the addition of water and partitioning into diethyl ether and vortexing for 15 min, for a total of two extractions. After adding MTBSTFA to (N-tert-butyldimethylsilyl-N ethytrifluoracetamide) derivatize, samples were analyzed by GC/MS. Microbial analysis and SCFA analysis were performed by the Texas A&M Gastrointestinal Laboratory.

Results and Discussion

For the first time, six urinary metabolites of gallic acid were identified in participants who consumed 400 g of mango pulp. As reported in Chapter III and V,

mango pulp is a rich source of gallic acid in the form of hydrolysable ester-linked glycosides with the two mono-galloyl glucosides alone accounting for over 35% of the total phenolic content reported. For this reason, gallic acid and its metabolites were anticipated as the primary targets for potential metabolite identification. Metabolites derived from the action of the Phase II enzymes, (UDP)-glucuronosyltransferases, sulfotransferases, and catechol-*O*-methyltransferases (COMT) were first identified in urine by searching for a neutral loss of 176 Da (glucuronide), 80 Da (glucuronide), and 15 Da (methyl group) in each MS chromatogram as described by Yan, Chen, Li, Zhang, & Yang (2012). The phase II metabolites of gallic acid identified in urine from evaluation with a neutral loss scan and by comparison of an observable difference to baseline urine are listed in Table 6. Metabolite 1, or, **M1** was identified as

Table 6. Metabolites of gallic acid identified by MS/MS analysis in urine after consumption of 400 g Keitt mango pulp.

No.	RT (min)	Identification	$[M-H]^-$	MS/MS
M1	33.77	pyrogalloyl-O-glucuronide	301.2	175, 113, 125
M2	36.8	*O*-methyl-gallic acid	183.2	168, 124, 139
M3	41.58	*O*-methylgallic acid-*O*-sulfate	263.1	183, 168, 124
M4	43.0	*O*-methylpyrogalloyl-*O*-sulfate	219.2	139, 204, 80, 124
M5	43.7	pyrogalloyl-*O*-sulfate	205.3	125, 80
M6	44.2	deoxypyrogallol-*O*-sulfate	189.3	109, 80
M7	47.52	*O*-methylpyrogalloyl-*O*-sulfate	219.1	139, 80

pyrogalloyl glucuronide based on the predominant molecular ion at *m/z* 301.2 $[M-H]^-$ and fragments of *m/z* 175, 113, and 125 which correspond to dehydrated glucuronic acid, dehydrated glucuronate, and pyrogalloyl, respectively (Figure 30). This identification is consistent with the fragmentation pattern of a glucuronide conjugate (X. Chen, Zhong,

Jiang, & Gu, 1998) and were identical to the fragmentation reported for pyrogalloyl-1-*O*-glucuronide, a urinary metabolite derived from gallic acid metabolism in rats (Yan et al., 2012). **M2,** *m/z* 183.2 was identified by comparison to the fragmentation of an authentic standard of 3-*O*-methyl gallic acid as an *O*-methyl gallic acid, since the placement of the methyl group could not be distinguished. **M3** was identified as a sulfate conjugate based on the neutral loss experiment and further investigation revealed a predominant ion at *m/z* 263.1 $[M-H]^-$ and fragments corresponding to *O*-methylgallic acid, *m/z* 183 [M-H-80], gallic acid, *m/z* 168 [M-H-80-15], and pyrogalloyl, *m/z*, 124 [M-H-80-15-44]. Thus **M3** was identified as *O*-methylgallic acid-*O*-sulfate, in agreement with the mass spectral data and NMR confirmation reported for this metabolite (Yasuda et al., 2000). **M4** and **M7** both gave similar molecular masses at *m/z* 219.2 and 219.1, respectively, and nearly identical fragmentations at *m/z* 139, 124, and 80 which correspond to the loss of a sulfate conjugate, a methylated pyrogalloyl, and pyrogalloyl. **M5** was also first identified as a sulfate conjugate in an evaluation for neutral loss and gave a predominant molecular ion $[M-H]^-$ at *m/z* 205.3. Fragmentation of the parent compound produced ions at *m/z* 125 and 80 which correspond to the loss of a sulfate and pyrogallol, respectively. Therefore this compound was identified as a pyrogalloyl-*O*-sulfate. ***M6*** was identified by comparison to a published mass fragmentation pathway corresponding to 2-deoxy-pyrogallol-1-*O*-sulfate, *m/z* 189.3, a urinary gallic acid metabolite identified from rats fed a traditional Chinese medicine formula (Yan et al., 2012).

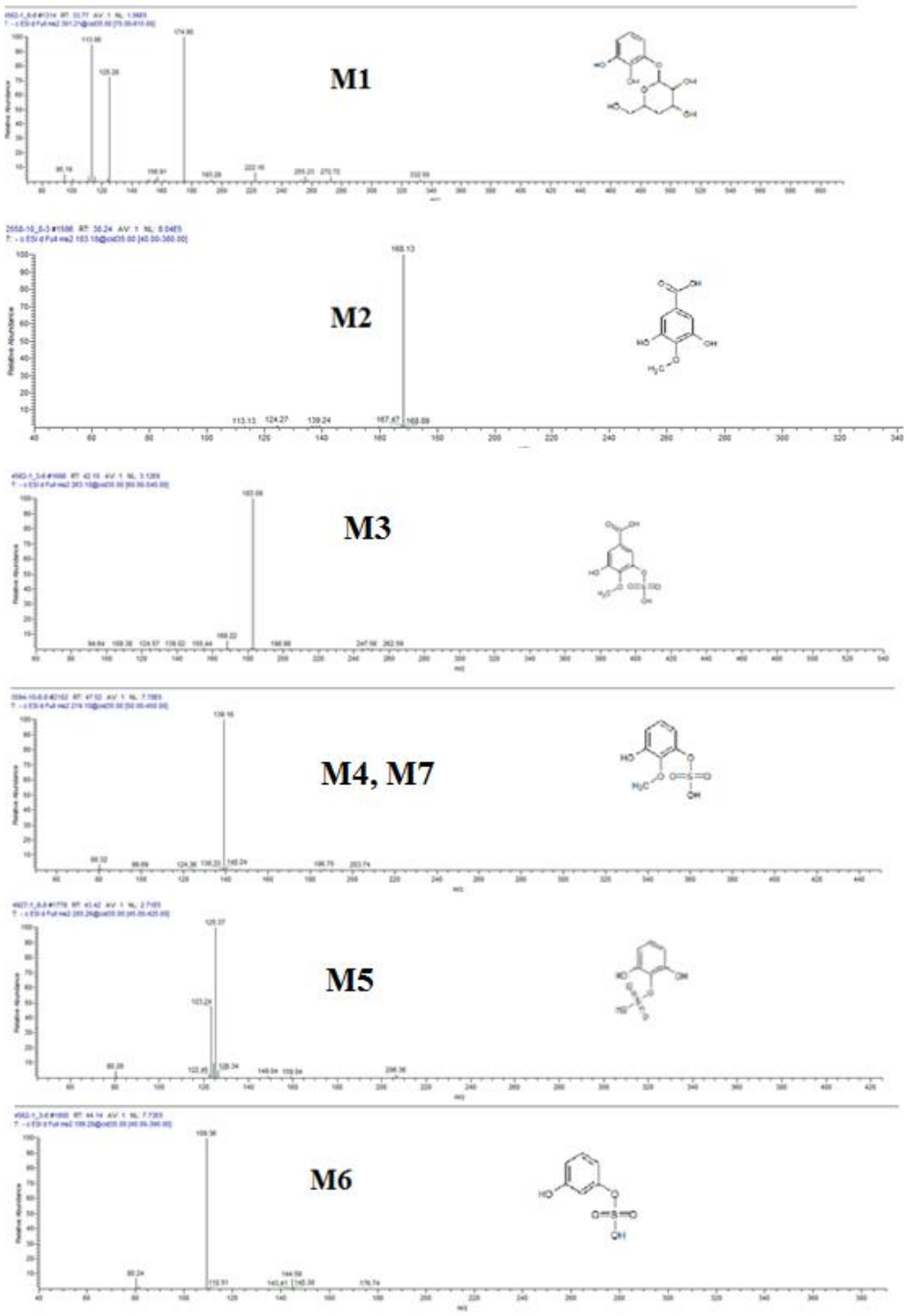

Figure 30. MS/MS fragmentation of human urinary metabolites for Keitt mango (**M1**) pyrogalloyl-O-glucuronide, (**M2**) *O*-methylgallic acid-*O*-sulfate, (**M3, M5**) *O*-methylpyrogalloyl-*O*-sulfate, (**M4**) pyrogalloyl-*O*-sulfate, (**M6**) *O*-methylpyrogalloyl-*O*-sulfate.

Quantification of urinary metabolites and standardization to creatinine content allowed comparisons to be made across individuals. *O*-methylgallic acid-*O*-sulfate was determined to be the major urinary metabolites after consumption of 400 g Keitt mango. Similarly, investigators found *4*-*O*-methylgallic acid-*3*-sulfate metabolites in urine of rats following ingestion of 200 mg/kg gallic acid (Yasuda et al., 2000) and in rats fed 1000 mg/kg Si-Ni-San (a traditional Chinese medicine), but metabolite concentration was not determined in either study (Yan et al., 2012). In one pharmacokinetic analysis of gallic acid, HPLC-PDA was the only detection used to identify gallic acid from its metabolites following an enzymatic deconjugation step with sulfatase prior to analysis, eliminating the ability to identify the sulfated form (Shahrzad et al., 2001). The HPLC-MS analysis of human urine, following the consumption of a polyphenol rich juice drink with 5 μ mol gallic acid/35 m L reported for the first time *O*-methylgallic acid-*O*-sulfates, but again quantifications were not made (Mullen et al., 2010). Quantification of *O*-methylgallic acid-*O*-sulfate in urine after mango consumption revealed maximum excretion between 3-6 hrs at an average of 0.396 mg/10 mg creatinine on Day 1 and 0.746 mg/10 mg creatinine on Day 10 (Figure 31). Deposition of *O*-methylgallic acid-*O*-sulfate in urine within 3-6 hrs after consumption of 400 g mango was a good indication that gallic acid from mango was indeed absorbed in the small intestine as shown by (Borges et al., 2010) with the comparison of plasma pharmacokinetic profiles of both conjugated and unconjugated compounds to urine excretion profiles. An increase in maximum concentration *O*-methylgallic acid-*O*-sulfate detected in the urine collected

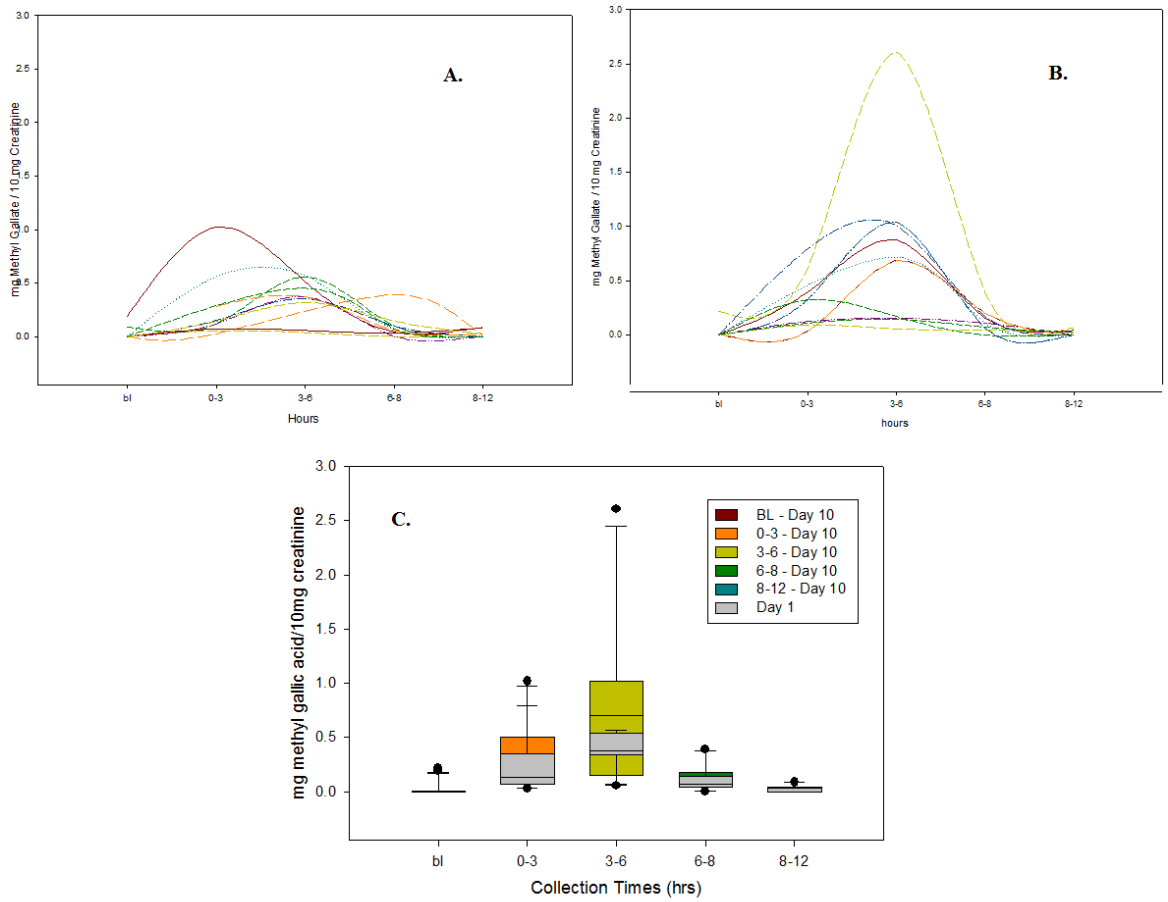

Figure 31. *O*-methyl gallic acid sulfate concentration (mg/mg creatinine) after consumption of 400 g. Keitt mango. **A.)** Excretion per individual, Day 1 **B.)** Excretion per individual Day 10 **C.)** Boxplot of combined data.

from 3-6 hr was observed following consumption of mango for an additional 9 d, but was not significant ($p<0.05$) due to high variability between individuals, a common observation in the metabolism of polyphenolics from foods (Cerdá, Tomás-Barberán, & Espín, 2005). In repeated dosing of rats over 10 d with grape seeds containing gallic acid an equivalent concentration of 483, 967, and 1451 mg in 60 kg humans, 4-*O*-methyl gallic acid levels were increased by 395% compared to absorption on Day 1 (Ferruzzi et al., 2009), suggesting that repeated exposures at high doses could alter the absorption

and metabolism of gallic acid. They proposed the action of increased enzyme expression to the enhanced absorption but cited that continued research would need to be conducted to determine mechanism.

Based on the excretion profile, the only other gallic acid-derived metabolite in urine that was hypothesized to be the result of absorption from the small intestine was *O*-methyl-gallic acid. Figure 32 shows the individual urinary excretion profiles for *O*-methyl-gallic acid. Maximum concentrations were calculated for Day 1 and Day 10 at the 3-6 hr time point and determined to be 0.012 and 0.027 mg 3-*O*-methyl gallic acid/10 mg creatinine, respectively; significantly lower than the concentration reported for *O*-methylgallic acid-*O*-sulfate.

Individual variability of *O*-methyl-gallic acid is particularly high on Day 1, (Figure 32a.) as two participants presumably absorbed, metabolized and excreted gallic acid at a faster rate compared to Day 10. One participant also excreted 40% more *O*-methyl-gallic acid the next highest individual on Day 10 and showed a second excretion maximum at the 6-8 hr collection range in both Day 1 and Day 10. It is possible based on the reappearance of metabolites 6-8 hrs post-consumption that it was a product of absorption from the colon as indicated by similarities in the findings of Crozier, Lean, Roberts, & Borges (2013) who cited a second peak in urinary excretion of metabolites in participants with a colon compared to those without. The lack of literature reporting sulfated *O*-methyl gallic acid in plasma, yet an abundance of citations relating to urinary

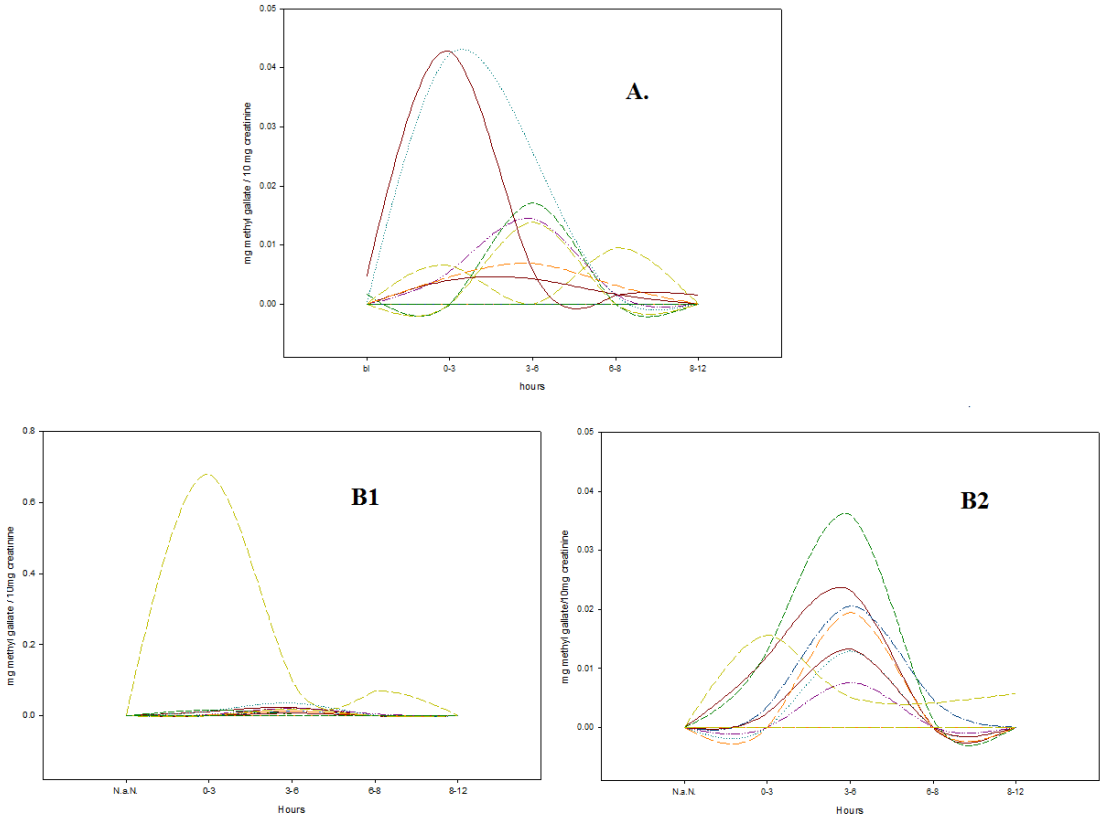

Figure 32. *O*-methyl gallic acid concentration (mg/mg creatinine) after consumption of 400 g. Keitt mango. **A.)** Excretion per individual, Day 1 **B.)** Excretion per individual Day 10 **C.)** Excretion per individual, Day 10 excluding the participant who showed highest absorption for the sake of comparison with Day 1 profiles.

excretion suggests that gallic acid is primarily circulated in its free or methylated form and is not sulfated by the action of sulfotransferases until its passage through the kidney (Borges et al., 2010; Mullen et al., 2010; Yan et al., 2012). COMT was shown by Galati, Lin, Sultan, & OBrein (2006) to be primarily involved in the detoxification of gallic acid in hepatocytes from the liver of rats, and is in agreement with the preponderance of literature reporting methylated forms of gallic acid in plasma. However it is still not entirely clear if this COMT predominantly acts on gallic acid in the liver or small

intestinal enterocyte, although it has been suggested that liver COMT appear to preferentially form 3-*O*-methyl derivatives over 4-*O*- methyl derivatives (Neilson & Ferruzzi, 2011). The site of conjugation is important because it could mean the difference between an unconjugated polyphenolic reaching a tissue or the conjugated form which could have major implications on mechanistic studies *in vitro*.

Five additional metabolites were characterized as conjugates of pyrogalloyl, the decarboxylated form of gallic acid. It was hypothesized that gallotannins and unabsorbed gallic acid would be degraded by colonic microflora possessing esterase activity (Yang, Zhao, Cui, & Guo, 2004) into low molecular weight compounds that could potentially be reabsorbed in the colon and enter into hepatic circulation. Because the microbial population of the colon is believed to directly influence metabolite formation, great variability is observed in metabolites produced and absorbed in the colon (Selma et al., 2009). For example, it was shown that only four out of ten individuals were able to metabolize the isoflavonoid, daidzein into its more active estrogenic forms, and each individual produced different combinations of metabolites (Rafii, Davis, Park, Heinze, & Beger, 2003). The pyrogalloyl metabolite, deoxypyrogallol-*O*-sulfate, also presented variability as shown in Figure 33a,b. This metabolite was detected in baseline urine of all individuals but levels increased following consumption of mango (0.651 mg pyrogalloyl/10 mg creatinine to 1.068 mg pyrogalloyl/10 mg creatinine). On Day 10 of the study, baseline concentrations were higher (1.351 mg pyrogalloyl/10 mg) than day 1 which could be attributed to the metabolism and absorptions of gallotannins from the consumption of mango the previous day. Pyrogallol sulfate showed less variability

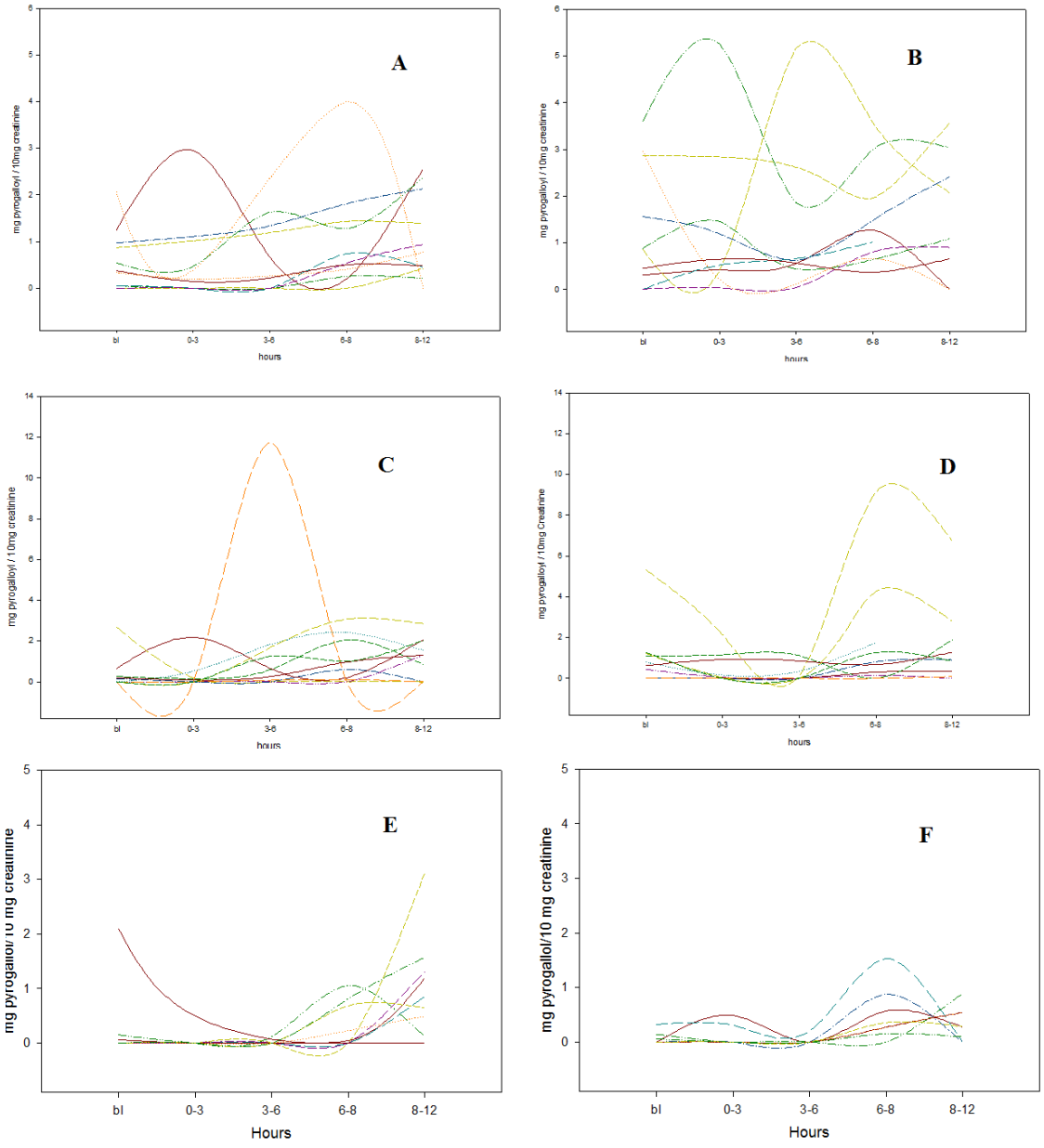

Figure 33. Pyrogallol conjugate concentrations (mg/mg creatinine) per individual after consumption of 400 g. Keitt mango. A.) deoxypyrogallol-O-sulfate Day 1 B.) deoxypyrogallol-O-sulfate Day 10 C.) pyrogalloyl-O-sulfate Day 1 D.) pyrogalloyl-O-sulfate Day 10 E.)

among individuals and was found in all participants 6-8 hrs post-consumption of 400 g. of mango, a good indication that the metabolite is a product of colonic metabolism and absorption. In agreement with results reported, pyrogallol sulfate was detected from the biotransformation of (-)-epicatechin 3-O-gallate by a human fecal suspension attributed to the gallic acid moiety of the compound (Meselhy, Nakamura, & Hattori, 1997) and has been reported elsewhere as a common fecal metabolite of gallic acid species of red wine (Dall'Asta et al., 2012). Two methyl-pyrogallol-sulfate metabolites were detected, however only **M7** was detected in all participants indicating that methylation and sulfation happens preferentially to specific hydroxyl groups during enzymatic conjugation. Methyl-pyrogallol-sulfate was also detected at maximum concentration from 6-8 hrs post consumption and based on the lack of detection for most individuals prior to 6 hrs, was rapidly hydrolyzed and absorbed from the large intestine. The last metabolite, pyrogalloyl-O-glucuronide, was identified in low concentrations (0.075-0.16 mg pyrogalloyl/10 mg creatinine) in only four of the eleven participants and was the only glucuronidated compound detected in urine. However, it is clear that colonic degradation and absorption of mango polyphenolics significantly contributed to the total metabolites detected from the consumption of mango. Based on urinary metabolite analysis, *in vitro* cellular experiments performed in Chapter V, and gallic acid metabolism reported in literature (as detailed in this chapter) a proposed metabolic pathway for gallic acid derivatives from mango pulp was created (Figure 34).

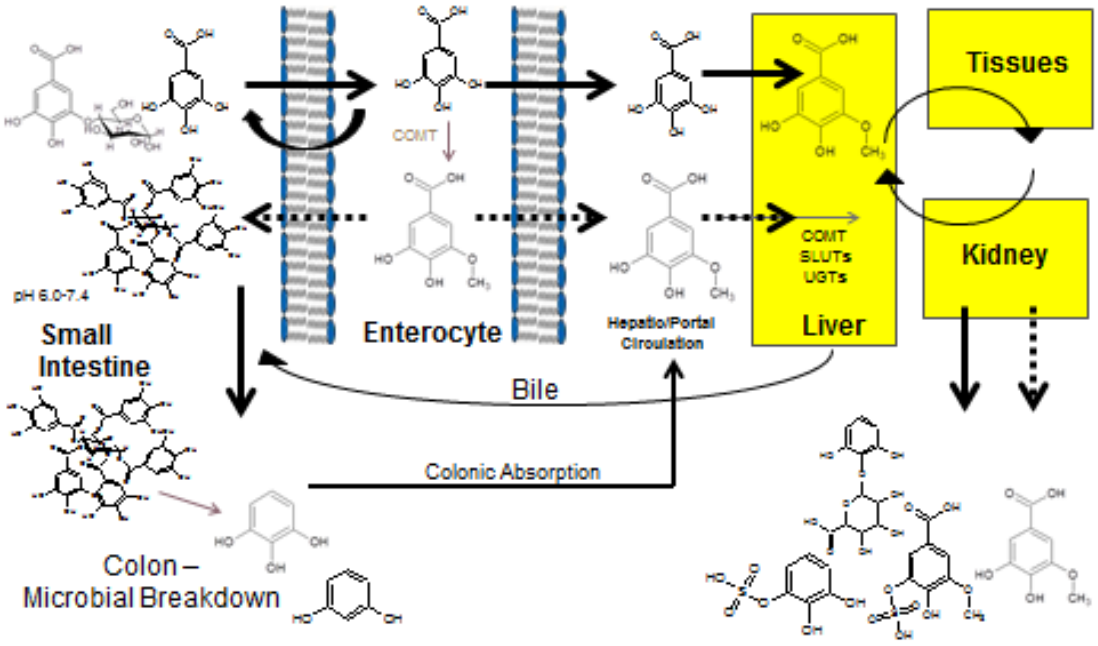

Figure 34. Proposed metabolic pathway for gallic acid derivatives from mango pulp.

Conclusions

The presence of gallic acid metabolites in human urine after the consumption 400 g of mango indicates the absorption of gallic acid derived from gallic acid glycosides. *O*-methylgallic acid-*O*-sulfate was identified as the major metabolite 0-6 hrs post consumption, followed by *O*-methylgallic acid at a lower concentration. Colonic metabolites were detected beginning 3-6 hrs after consumption of mango, were identified as pyrogalloyl derivatives and were hypothesized to be the products of microbial breakdown of gallotannins. Daily consumption of mango for 10 d did not significantly increase the concentration of metabolites found in urine.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Mass spectroscopic characterization and HPLC quantification of mango pulp revealed for the first time two MGGs with distinct differences in their glycoside linkages, with the ester form dominating, as well as the presence of five other phenolic acid glycosides; hydroxybenzoic acid glucoside, courmaric glucoside, ferulic acid glucoside, and sinapic acid mono and di-glucosides. Six oxygenated carotenoid derivatives were identified for the first time in a phytochemical extract, namely, abscisic acid and its glycoside, two catabolism products of abscisic acid, dihydrophasic acids, and two hydroxy-dimethyl decadiene-dioic acid glucopiranosylesters. Gallotannins ranging from tetra-galloyl glucosides to nona-galloyl glucosides were also identified in the pulp, but not quantified. The extensive identification of polyphenolics in Keitt mango pulp will aid in assessing the biological activity of this mango both *in vitro* and *in vivo*.

CZ and Rap were the most effective at increasing juice yield of mango pulp due to their pectinase action. Cz reduced the amount of ester-linked MGG by 70% after 4 hrs of incubation, indicating strong esterase activity, while no reports of ß-glucosidase side-activity were demonstrated. Rap did not hydrolyze ester-linked MGG, but showed ß-glucosidase side-activity by the complete hydrolysis of ether-linked MGG. High tannin extracts were hydrolyzed by both Rap and a pure ß-glucosidase, but pure ß-glucosidase worked more effectively when tannins were first bound to BSA prior to incubation

which indicates that tannin-protein interaction between the enzyme and tannin is likely to cause it to lose activity. ß-glucosidase hydrolysis activity is important *in vivo* where it is thought to aid in the absorbability of glycosylated polyphenolics. The demonstration of the hydrolysis of gallotannins by enzymes *in vitro* could indicate the action *in vivo* as well, but more studies would need to be conducted to confirm.

The instability of ester-linked galloyl-glycosides at pH 7.4 was characterized by HPLC-MS and after only 4 hrs of incubation a shift from HWM tannins (>8GG) to LMW (<8GG) occurred, with LMW gallotannins representing 68% of the total ion count. This reduction in HMW gallotannins produced 23 mg/L of free gallic acid in 4 hrs without taking into account that lost by oxidation which suggests that gallotannins could potentially serve as a great source for gallic acid in the small intestine as well as the colon. The instability of the ester-linked galloyl-glucosides was also evaluated in the presence of differentiated Caco-2 cells to determine if they could also be metabolized to a known gallic acid metabolite, *O*-methyl gallic acid. Methyl gallate was detected in 24 hr incubation with a Keitt mango extract and in the cell lystate of the same cells after 48 hrs as well as in the cell lysate of Caco-2 cells treated with a gallotannin-rich isolate of mango. These results indicate that ester-linked glycosides, once hydrolyzed can be metabolized similar to gallic acid.

The presence of gallic acid metabolites in human urine after the consumption 400 g of mango indicates the absorption of gallic acid derived from gallic acid glycosides. *O*-methylgallic acid-*O*-sulfate was identified as the major metabolite 0-6 hrs post consumption, followed by *O*-methylgallic acid at a lower concentration. Colonic

metabolites were detected beginning 3-6 hrs after consumption of mango, were identified as pyrogalloyl derivatives and were hypothesized to be the products of microbial breakdown of gallotannins. Daily consumption of mango for 10 d did not significantly increase the concentration of metabolites found in urine.

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APPENDEX

Plasma Extraction

Plasma samples were evaluated for metabolites using a SPE for sample clean up prior to analysis. Plasma (m L) was spiked with .47 µ g ethyl gallate as an internal standard for plasma extraction efficiency and diluted 1:1 with 4% phosphoric acid. The mixture was applied to a 30 g Oasis® MAX (Waters, Milford,MA) SPE cartridge previously activated and equilibrated with 4 mL methanol and distilled water. The cartridges were washed with 4 mL distilled water and compounds eluted with 100% methanol containing 3% formic acid. Extracted samples were evaporated under vacuum (Thermo-Scientific ISS110 SpeedVac Concentrator), residue dissolved in µ L of a % methanol solution containing . % formic acid, and an aliquot of 5 µ L analyzed directly by HPLC-MS^n analysis operating under the same conditions in SIM mode.