ABSORPTION AND METABOLISM OF MANGO (*MANGIFERA INDICA* L.) GALLIC ACID AND GALLOYL GLYCOSIDES

A Dissertation

by

RYAN CRISPEN BARNES

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Stephen Talcott
Co-Chair of Committee,	Susanne Talcott
Committee Members,	Nancy Turner
	Arul Jayaraman
Head of Department,	Boon Chew

December 2016

Major Subject: Food Science and Technology

Copyright 2016 Ryan Barnes

ABSTRACT

The composition, absorption, metabolism, and excretion of gallic acid, monogalloyl glucose, and gallotannins in mango (*Mangifera indica* L.) pulp were investigated. Each galloyl derivative was hypothesized to have a different rate of absorption, and their concentrations were compared in the pulp of five mango varieties. The cultivar Ataulfo was found to have the highest concentration of monogalloyl glucose and gallotannins while the cultivar Kent had the lowest. Enzymatic hydrolysis of gallotannins with tannase led to the characterization of six digalloyl glucoses and five trigalloyl glucoses that have the potential to be formed in the colon following gallotannin consumption.

The bioaccessibility of galloyl derivatives was evaluated in both homogenized mango pulp and 0.65 mm^3 cubes following in vitro digestion conditions. Monogalloyl glucose was found to be bioaccessible in both homogenized and cubed mango pulp. However, cubed mango pulp had a significantly higher amount of gallotannins still bound to the fruit following digestion. Gallic acid bioaccessibility significantly increased following digestion in both homogenized and cubed mango pulp, likely from hydrolysis of gallotannins. Additionally, for the first time, the absorption of monogalloyl glucose and gallic acid was investigated in both Caco-2 monolayer transport models and a porcine pharmacokinetic model with no significant differences found in their absorption or ability to produce phase II metabolites.

A method to extract free gallic acid and its metabolites using sodium dodecyl sulfate from plasma was developed, and the method extracted significantly ($p<0.05$) more pyrogallol, gallic acid, and their phase II metabolites compared to conventional methods that rely on solvent extractions or use of solid phase extraction. The method was reproducible and precise across three standard concentrations from 50 to 500 µg/L. The method is hypothesized to be more effective due to the ability of sodium dodecyl sulfate to denature proteins and inhibit polyphenol-protein interactions.

The pharmacokinetics of mango gallic acid and galloyl glycosides were evaluated in subjects who consumed mango daily for 42 days, and a significant ($p<0.05$) increase was observed in the urinary excretion of 4-*O*-methylgallic acid-3-*O*-sulfate, pyrogallol-*O*-sulfate, methylpyrogallol-*O*-sulfate, and catechol-*O*-sulfate. However, this same significant increase in metabolism was not observed in plasma concentrations. It was proposed that repetitive mango consumption created changes in the gut microbiota population increasing concentration of gallic acid and pyrogallol in the colon, but due to how quickly gallic acid is excreted from the body no accumulation in plasma was observed.

DEDICATION

For my mother, Tammy, who always told me, you get out of life what you put into it.

ACKNOWLEDGEMENTS

I would first like to thank Dr. Stephen Talcott, my committee chair. Without all your advice, mentoring, and friendship none of this research would have been possible. I have been truly blessed to have had the opportunity to work alongside you these past few years. To Dr. Susanne Talcott, my co-chair, thank you for all your help and support along this journey, and a special thank you for allowing me to be the teaching assistant for the study abroad course in Italy. It was the trip of a lifetime for me, and I was very grateful for the opportunity. Thanks also goes to my other committee members Dr. Nancy Turner and Dr. Arul Jayaraman who were helpful in the design of my experiments with publications in mind.

I want to give a special thank you to Kimmy who was such a great mentor when I first joined the lab as an undergraduate. You played a big part in my decision to go to graduate school, and helped give me the skills I needed to successfully complete my PhD. I would like to also thank my lab mates who were working on their PhDs alongside me, Hyemee, Paula, Maritza, Matt, and Chuo. It was great having y'all as coworkers and friends, and thank you for always lending a hand if I needed help with my experiments.

To my siblings, Dylan, Clinton, and Zoe, it has been great having y'all close by while I was in graduate school. I have loved each and every time we have all gotten to be together. To my roommates Brandon, Chadley, and Wes thank you for always having an open ear to listen to my research problems.

I would also like to thank all the nurses who assisted in the daily mango consumption clinical trial, and especially Cathy who would always be willing to make time for a study day. To my Dad and Laurie, thank you for all of your trips up to College Station and all the meals that came with them. To Joe, thanks for always giving a helping hand with any car troubles during my tenure at A&M. Finally, my deepest thanks goes to my mother, Tammy, who has sacrificed so much for all her children. This dissertation would not have been possible without all your love and support.

CONTRIBUTORS AND FUNDING SOURCES

The work for this dissertation was supervised by a dissertation committee chaired by Dr. Stephen Talcott, co-chaired by Dr. Susanne Talcott, and with support from Dr. Nancy Turner of the Department of Nutrition and Food Science and Professor Arul Jayaraman of Chemical Engineering. The clinical trials were performed at the facilities of Dr. Nicholaas Duetz with assistance from his staff. The clinical trials and Caco-2 monolayer studies were performed with assistance from Hyemee Kim.

This dissertation was funded fully by the National Mango Board (Orlando, FL). The National Mango Board sponsors research and promotion of domestic and imported mangos.

TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

CONTRIBUTORS AND FUNDING SOURCES....................vii

TABLE OF CONTENTS viii

LIST OF FIGURES....................x

LIST OF TABLES xiii

CHAPTER I INTRODUCTION 1

CHAPTER II LITERATURE REVIEW....................4

2.1 The Role of Fruits and Vegetables in Health 4
2.2 Polyphenolics: Synthesis, Nomenclature, and Properties 5
2.3 Mango History and Composition 10
2.4 Polyphenol Absorption, Metabolism, Distribution, and Excretion....................13

CHAPTER III QUANTIFICATION OF GALLIC ACID AND GALLOYL GLYCOSIDES IN FIVE MANGO CULTIVARS AND THE CHARACTERIZATION OF TANNASE HYDROLYSIS PRODUCTS 23

3.1 Introduction 23
3.2 Materials and Methods 26
3.3 Results and Discussion....................29
3.4 Conclusions 42

CHAPTER IV IN VITRO BIOACCESSIBILITY AND IN VIVO ABSORPTION OF MANGO GALLIC ACID AND GALLOYL GLYCOSIDES....................43

4.1 Introduction 43
4.2 Materials and Methods 44
4.3 Results and Discussion....................51
4.4 Conclusions 65

CHAPTER V A NOVEL PROTEIN PRECIPITATION METHOD FOR THE RECOVERY OF GALLIC ACID METABOLITES IN PLASMA 66

5.1 Introduction 66
5.2 Materials and Methods 68
5.3 Results and Discussion 73
5.4 Conclusions 85

CHAPTER VI DAILY CONSUMPTION OF MANGO CV. ATAULFO FOR 42 DAYS AND ITS INFLUENCE ON THE METABOLISM AND EXCRETION OF GALLIC ACID, GALLOYL GLYCOSIDES, AND GALLOTANNINS 86

6.1 Introduction 86
6.2 Materials and Methods 88
6.3 Results and Discussion 92
6.4 Conclusions 106

CHAPTER VII SUMMARY AND CONCLUSIONS 107

REFERENCES 110

APPENDIX 136

LIST OF FIGURES

Page

Figure 1. Sequential synthesis of gallic acid to pentagalloylglucose [34]. 7

Figure 2. Parent structures of polyphenols subclasses [37]. 8

Figure 3. Polyphenol absorption, metabolism, distribution, and excretion......................14

Figure 4. The current proposed mechanisms on the ADME of gallic acid and galloyl glycosides 18

Figure 5. Concentrations (mg/kg) of gallic acid (GA), ester-monogalloyl glucose (Ester-MGG), and ether-monogalloyl glucose (Ether-MGG) across five mango cultivars..31

Figure 6. Chemical structures for gallic acid (A), ester-monogalloyl glucose (B), and ether-monogalloyl glucose (C). 32

Figure 7. Chemical structures for trigalloyl glucose (A), tetragalloyl glucose (B), pentagalloyl glucose (C), and decagalloyl glucose..33

Figure 8. Composition of gallotannins ranging in degree of polymerization from trigalloyl glucose (3GG) to decagalloyl glucose (10GG) across five mango cultivars..35

Figure 9. Hydrolysis of pentagalloyl glucose (5GG) and subsequent formation of tetragalloyl glucose (4GG), trigalloyl glucose (3GG), digalloyl glucose (2GG) following 2 h incubation with tannase at 10^{-3} U/mL............................37

Figure 10. Chromatogram of five digalloyl glucoses and six trigalloyl glucoses at 280 nm (A) and extracted ion chromatograms at m/z 483.2 (B), and 635.2 (C) generated from hydrolysis of pentagalloyl glucose following 2 h incubation with tannase at 10^{-3} U/mL...38

Figure 11. Hydrolysis of monogalloyl glucose (MGG) and subsequent formation of gallic acid (GA) following 4 h incubation with tannase at 10^{-3} U/mL. 40

Figure 12. Hydrolysis of gallotannin isolate ranging in composition from tetragalloyl glucose (4GG) to undecagalloyl glucose (11GG) following 2 h incubation with tannase at 10^{-3} U/mL...41

Figure 13. Bioaccessibility of monogalloyl glucose in (A) mango extract, (B) homogenized mango pulp, and (C) cubed mango and free gallic acid in (D)

mango extract, (E) homogenized mango pulp, and (F) cubed mango under in vitro digestion conditions at 37 °C, pH 2.0 for 2 h and pH 7.1 for 8 h, with digestive enzymes. 54

Figure 14. Stability of gallic acid and monogalloyl glucose following in vitro digestion at 37 ºC pH 2.0 for 2 h and pH 7.1 for 8 h, with digestive enzymes. 55

Figure 15. Gallic acid, monogalloyl glucose, and gallotannins bound to cellular matrices of homogenized mango pulp and cubed mango following in vitro digestion at 37 °C pH 2.0 for 2 h and pH 7.1 for 8 h with digestive enzymes.. 57

Figure 16. Area under the curve (AUC) for the metabolites gallic acid, 4-*O*-methylgallic acid, and 4-*O*-methylgallic acid-3-*O*-sulfate after oral administration of gallic acid and monogalloyl glucose determined in a porcine model. 64

Figure 17. Comparison in extraction recoveries of pyrogallol (PG), gallic acid (GA), 4-*O*-methylgallic acid (MG), and ethyl gallate (EG) using precipitation with acetonitrile (ACN), SDS protein precipitation method (SDS), and solid phase extraction using Waters HLB cartridges (HLB) all at 500 µg/L. Different letters for each analyte denote a significant difference in recovery based on extraction method ($p < 0.05$). 77

Figure 18. Pharmacokinetics of 4-*O*-methylgallic acid (MG), 4-*O*-methylgallic acid-3-*O*-sulfate (MGS), following tentative characterization and semi-quantification in human plasma after consumption of 400 g of mango pulp (cv. Ataulfo) in healthy volunteers. Data represent the average and standard error of the mean (n=10). 80

Figure 19. Pharmacokinetics of pyrogallol-*O*-sulfate (PGS), methylpyrogallol-*O*-sulfate (MPGS), and catechol-*O*-sulfate (CS) following tentative characterization and semi-quantification in human plasma after consumption of 400 g of mango pulp (cv. Ataulfo) in healthy volunteers. Data represent the average and standard error of the mean (n=10). 81

Figure 20. Tentative chemical structures of phase II metabolites following consumption of gallic acid, galloyl glycosides, and gallotannins sourced from 400 g of mango cv. (Ataulfo) pulp. 93

Figure 21. The sum of urinary excretion in mg for the metabolites 4-*O*-methylgallic acid-3-*O*-sulfate (MGS), pyrogallol-*O*-sulfate (PGS), two isomers of methylpyrogallol-*O*-sulfate (MPGS), and catechol-*O*-sulfate (CS) after consumption of 400 g of mango cv. Ataulfo for Days 1 and 42 in the (A) Lean and (C) Obese Cohorts who consumed mango daily for 42 days and

the (B) Control Cohort who consumed mango only on Days 1 and 42. A (*) designates a significant difference between Days 1 and 42 for the respective metabolite. 96

Figure 22. Cumulative excretion in mg of the galloyl metabolites: 4-*O*-methylgallic acid-3-*O*-sulfate (MGS), pyrogallol-*O*-sulfate (PGS), two isomers of methylpyrogallol-*O*-sulfate (MPGS), and catechol-*O*-sulfate (CS) in the Lean Cohort following daily consumption of 400 g of mango cv. Ataulfo for 42 days on Days 1 and 42. 99

Figure 23. Sum of the area under the curve (AUC) for all galloyl metabolites in the plasma of subjects who consumed mango only on Days 1 and 42 (Control) and those who consumed mango daily for 42 days, on Days 1 and 42 (Lean and Obese). A (*) designates a significant difference between Days 1 and 42 for the respective cohort. 102

Figure 24. Plasma concentrations of 4-*O*-methylgallic acid-3-*O*-sulfate in human plasma on Days 1 and 42 following consumption of 400 g of mango (cv. Ataulfo) pulp in the Lean Cohort who consumed mango daily for 42 days. A (*) designates a significant difference between Days 1 and 42. 104

LIST OF TABLES

Page

Table 1. Plasma recoveries for gallic acid by use of solid phase and solvent extraction. 21

Table 2. Mass to Charge Ratios (*m/z*) and λ_{max} for gallic acid, galloyl glycosides, and gallotannins. 30

Table 3. Characterization and concentrations (mg/kg) of galloyl derivatives in mango (cv. Ataulfo) pulp. 52

Table 4. Transport of 50 µg/mL gallic acid, monogalloyl glucose, and pyrogallol across the apical to basolateral side of Caco-2 monolayers over 2 h. 59

Table 5. Pharmacokinetic parameters for gallic acid and monogalloyl glucose following oral and IV administration in a 10 h pilot porcine absorption study. 61

Table 6. Extraction recoveries, matrix effect, and inter-day precision of gallic acid metabolites (pyrogallol, gallic acid, 4-*O*-methylgallic acid, and ethyl gallate) in human plasma using sodium dodecyl sulfate at three concentrations (50, 250, and 500 µg/L). 74

Table 7. Characterization and pharmacokinetic parameters of gallic acid metabolites following human consumption of 400 g of mango pulp (cv. Ataulfo). 79

Table 8. Comparison of gallic acid and pyrogallol phase II metabolite plasma concentrations from pooled plasma using SDS protein precipitation method (SDS), acetonitrile protein precipitation (ACN), and solid phase extraction with HLB chemistry (HLB). 84

Table 9. Metabolites characterized and quantified in the plasma and urine of subjects who consumed 400 g of mango cv. Ataulfo pulp daily for 42 days. 94

Table 10. Area Under the Curve (AUC) from 0 to 8 h of phase II metabolites sourced from gallic acid and galloyl glycosides following daily consumption of mango cv. Ataulfo pulp for 42 days in Lean and Obese Cohorts, and compared against subjects who consumed mango only on days 1 and 42, Control Cohort. 101

CHAPTER I

INTRODUCTION

Mango (*Mangifera indica* L.) is perhaps the most popular fruit in the world. Historically enjoyed for its unique flavor and sweet taste, mango has also been used to treat asthma, dysentery, sinus troubles, and even dandruff [1, 2]. Mangos, while native to South Asia, are now grown in tropical and subtropical regions all over the globe with cultivars available for consumption throughout the year in most developed countries [3]. For example, the United States has seen a rise in mango popularity over the past decade, and mango can now be found in grocery stores all over the country. Central and South America supply the majority of fruit that is consumed in the US, and mango is enjoyed in all stages of ripeness in a wide variety of products. The recent growth in mango's popularity has given attention to the potential health benefits regarding mango consumption with specific regards to its fiber, vitamin C, and polyphenolic content.

Polyphenols are a group of plant secondary metabolites that are acclaimed for their potential role in the overall benefits of fruit and vegetable consumption [4-8]. All edible parts of the mango (peel, flesh, and seed kernel) have been found to contain galloyl derived polyphenols, which include gallic acid, mono-galloyl glucoses, and gallotannins ranging in degrees of polymerization from 4 to 12 galloyl substitutions [9-13]. The anti-inflammatory and anti-cancer activities of polyphenol extracts derived from mango flesh, peel, and skin have been reported in vitro [5, 6, 14, 15]. However, these studies did not take into consideration the role metabolism may have on

polyphenol consumption. The human super organism treats polyphenolics as xenobiotics, non-nutritive, non-endogenous compounds; which upon absorption in the GI tract polyphenolics will be conjugated with methyl, sulfur, or glucuronide functional groups by epithelial or hepatic enzymes. Polyphenols not absorbed in the small intestine will continue to the colon where they can be catabolized by the enzymes produced by the microbiota that reside there creating metabolites that were not initially present in the fruit itself. This makes the conclusions from prior in vitro work on polyphenol isolates inadequate in supporting the hypotheses that fruit and vegetable polyphenols positively contribute to human health as the polyphenolics found in fruit are unlikely to be the form exerting anti-inflammatory and anti-cancer activity in vivo. Additional studies are needed to characterize what metabolites are found after consumption of polyphenols as well as their concentration in plasma and tissues to allow for a deeper understanding on the mechanisms behind polyphenols and health.

Mangos' current global popularity and wide range of galloyl derivatives make it a practical and relevant medium for studying gallic acid metabolism. Gallic acid has been shown to possess both anti-cancer and anti-inflammatory activity in vitro [16], and if its metabolites display these activities as well it will aide in strengthening the hypothesis that polyphenols contribute to the health benefits of fruits and vegetables. The objectives of this work were as follows:

1. Quantify the gallic acid and galloyl glycoside content in five different U.S. mango cultivars, and characterize the effects of tannase on gallotannin hydrolysis.

2. Investigate and compare the absorption and metabolism of gallic acid and monogalloyl glucose through in vitro digestion and in vivo porcine models.

3. Develop a more efficient method for the extraction of gallic acid metabolites from human plasma.

4. Quantify the changes in gallic acid, galloyl glycoside, and gallotannin metabolism in subjects who consume mango daily for 42 days.

CHAPTER II

LITERATURE REVIEW

2.1 The Role of Fruits and Vegetables in Health

The consumption of fruits and vegetables has long been associated with health and longevity, and fruit and vegetable intake has been reported to reduce the risks of cancer and cardiovascular diseases in a number of meta-analyses [17-22]. Geographic regions such as the Mediterranean which includes Spain, Southern Italy, and Greece have lifestyles where fruits and vegetables are a predominate portion of their traditional diets and have historically had some of the highest rates of human life expectancy and lowest incidences of cardiovascular disease and cancer around the world [23].

Despite the suggested benefits of fruit and vegetable intake the United States reported no increase in their average consumption (3.43 servings of fruits and vegetables per day) in US adults from 1990 – 2010 [24, 25], and during the same time period the United States has observed an obesity epidemic [26]. This obesity epidemic has sparked educational movements for healthier lifestyles such as the national leadership's 'Lets Move' campaign, Healthy People 2020, and the USDA's 2010 MyPlate which recommends that half the plate of each meal should consist of fruits and vegetables [27]. While these campaigns have helped raise awareness for healthier lifestyles, a recent investigation reported a decrease in fruits and vegetables consumption from 2009 - 2014 [28].

Despite the reports that have associated fruit and vegetable consumption with reduced risk of cancer and cardiovascular diseases the exact mechanisms behind the benefits of fruits and vegetables are still unclear, but they have mainly been attributed to their phytochemical content. Phytochemicals, or compounds that naturally occur in plants, can be anything from vitamins, minerals, fatty acids, proteins, and dietary fiber. With such a wide range of different phytochemicals in all the fruits and vegetables it has been difficult for researchers to pinpoint the mechanism behind their benefits. Among the non-nutrient phytochemicals, polyphenolics are a group of compounds that have achieved notoriety in the biomedical and food sciences due to their in vitro antioxidant capacity, and are believed to be an important class of bioefficacious compounds present in fruits and vegetables.

2.2 Polyphenolics: Synthesis, Nomenclature, and Properties

2.2.1 Plant Metabolism, Chemical Structure, and Physical Properties

Polyphenols are a class of phytochemicals in higher plants, and are well known for their prevalence in the human diet and potential health benefits [29]. A polyphenol is defined by its chemical composition of a benzene ring that contains multiple hydroxyl groups covalently linked. Polyphenols serve several roles in plants including providing color, antimicrobial properties, protection from reactive oxygen species, and protection from herbivores and insects [30].

Plant biosynthesis of polyphenols and other aromatic compounds such as the essential amino acids phenylalanine, tyrosine, and tryptophan begin with the precursor erythrose-4-phosphate and phosphoenolpyruvate as intermediates of glycolysis. Through

a series of enzymatic reactions known as the shikimic acid pathway these precursors produce shikimic acid. Shikimic acid then produces hydroxybenzoic acids through dehydrogenation to form gallic acid, or under a different path produce phenylalanine or tyrosine [31]. After production of phenylalanine, direct synthesis of other polyphenols can begin through the phenylpropanoid pathway, a series of enzymatic reactions which involve deamination proceeded by production of cinnamic acids, and the further conjugation to flavonoids and tannins [32, 33].

Biosynthesis of gallic acid occurs through dehydrogenation of shikimic acid, unique to other polyphenols, which are generally synthesized through deamination of the aromatic amino acids. Further synthesis of gallic acid to create more complex galloyl derivatives begins with esterification of glucose to gallic acid to form 1-*O*-galloyl-B-D-glucose or monogalloyl glucose; this is performed by a glucosyl transferase with UDP-glucose as the substrate. Monogalloyl glucose is unique in that it acts as both a reactant and substrate in sequential synthesis to di, tri, tetra, and pentagalloyl glucose [34] (Figure 1). Hofmann and Gross discovered that galloyl substitution past pentagalloyl glucose require site specific enzymes named galloyltransferases which can add galloyl moieties to specific a galloyl group on pentagalloyl glucose [35]. The site specific galloyl transferases may be a reason for gallotannins poor separation in high performance liquid chromatography since hexa to nonagalloylglucose can have galloyl units synthesized in five potential positions.

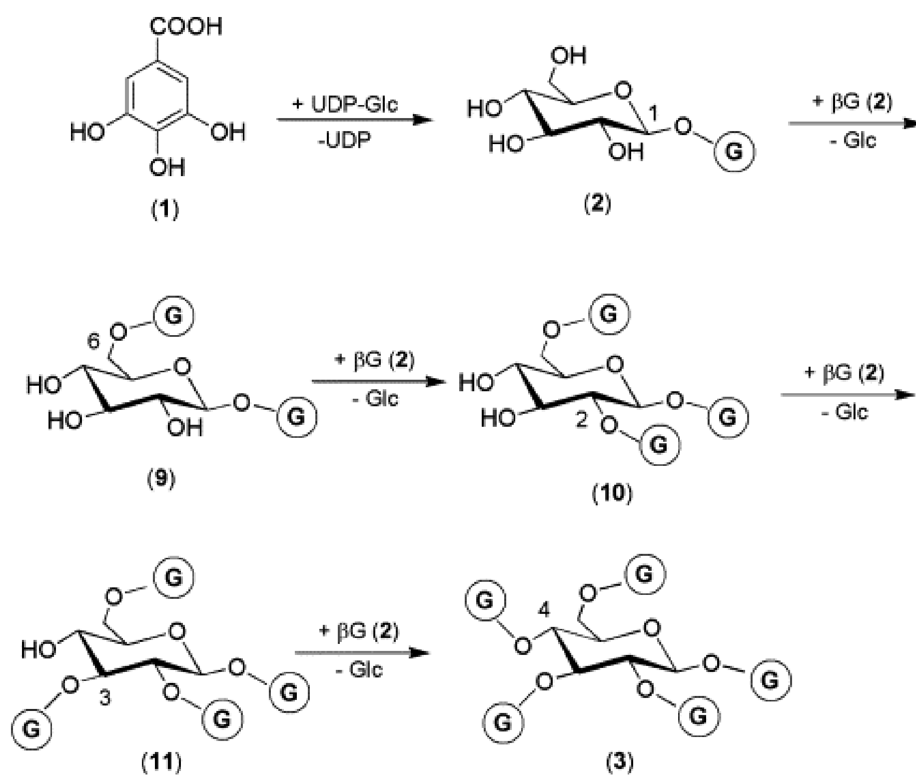

Figure 1. Sequential synthesis of gallic acid to pentagalloylglucose [34].

The pathways that generate polyphenols are upregulated by stresses occurring from plant damage, UV exposure, and temperature, thus affecting the quantity of polyphenolics are found in the plant [36]. Over 8000 different polyphenols have been identified, and as such contain a wide variety of structures and classifications [32, 33]. The major classes of polyphenols include the hydroxybenzoic acids, cinnamic acids, flavonoids, tannins, stilbenes, and benzoquinones (Figure 2).

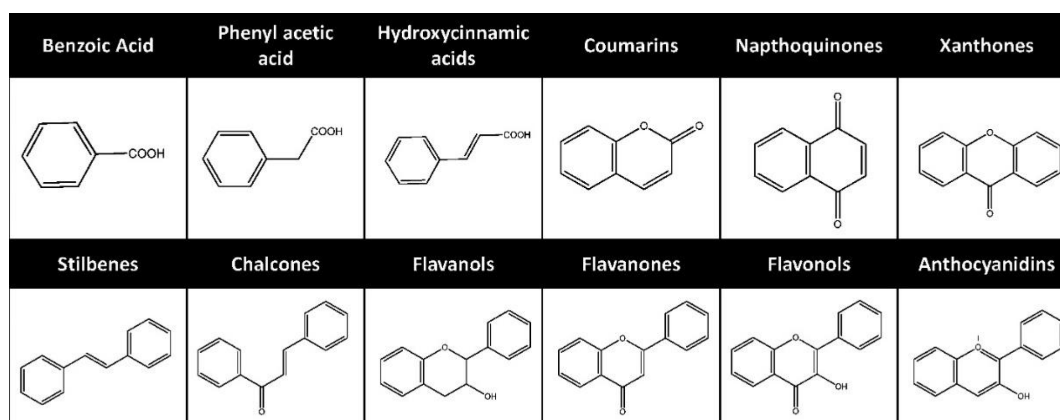

Figure 2. Parent structures of polyphenols subclasses [37].

2.2.2 Phenolic Acids

Phenolic acids include both the hydroxybenzoic and hydroxycinnamic acids. Both have a benzene ring base with a carboxylic acid and differ structurally by the number of carbons between benzene and the acid group (Figure 2). The most prevalent hydroxybenzoic acid is 3, 4, 5-trihydroxybenzoic acid more commonly referred to as gallic acid. Gallic acid can be found in its free form or covalently linked to glucose or flavonoids [11, 29, 38]. Other than mango, gallic acid and galloyl glycosides are naturally present in a variety of foods, beverages, and herbs/spices including gallated flavan-3-ols in green tea, gallotannins in chickpea, cow peas, persimmons, star fruit, pecans, and sumac, and gallated ellagitannins in berries [39-41].

The cinnamic acids are found more abundantly in the diet of Americans than the hydroxybenzoic acids in staples such as coffee and wheat. Chlorogenic acid, for example an ester of caffeic acid and quinic acid, is found extensively in coffee and one serving

can contain 70-350 mg [42]. Ferulic acid is another highly abundant cinnamic acid ranging from concentrations of 0.8-2.0 g/kg in wheat grains [43].

2.2.3 Flavonoids

Flavonoids are the most studied and largest classes of polyphenols with over 50% of the identified polyphenols belonging to this group. Flavonoids are widely distributed and are commonly found in the flowers and leaves of plants. Flavonoids can be further categorized into flavonols, flavanols, flavones, and anthocyanins with each of these groups having a similar backbone structure that includes three conjugated benzene rings with a C6-C3-C6 configuration with the differences between groups due to the presence of hydroxyl group and degree of saturation on C3 ring (Figure 2). Due to the large variety of flavonoids in fruits and vegetables, flavonoids are the most common polyphenol consumed in the human diet, and as such several reports are available on their potential health benefits [44].

2.2.4 Tannins

Tannins are a class of polyphenols that have high molecular weights (300-3000 Da) and the ability to precipitate proteins through hydrogen bonding due to the presence of several galloyl or trihydroxyl functional groups [45]. Tannins have been used historically for their ability to 'tan' animal hides into leather, and are divided into two subclasses the condensed and hydrolysable tannins. Condensed tannins are polymers of the flavanols, catechin and epicatechin, and are commonly found in green tea, grapes,

wines, and chocolate. Hydrolysable tannins are synthesized through esterification of gallic acid to glucose, but differ from condensed tannins in that they can be hydrolyzed by either alkaline or enzymatic mechanisms. Gallotannins and ellagitannins make up the hydrolysable tannin subclass with ellagitannins being formed from degradation and C-linkage of galloyl groups on gallotannins. Tannins are considered to be a double edge sword nutritionally as they have been found to have potent antimicrobial and antioxidant properties, yet they can also affect nutrient uptake and may potentially play a role in the formation of some cancers [46].

2.3 Mango History and Composition

2.3.1 The Mango Market

Mangos (*Mangifera inidica* L.) are grown all over the globe with varieties available for consumption all throughout the year [47]. Cultivated in tropical and subtropical regions the majority of cultivation takes place in India, China, Mexico, Thailand, and Brazil with India accounting for greater than 50% of worldwide mango production [3]. In the United States mango consumption has steadily increased over the last fourteen years with a remarkable 56% increase in the amount of fresh mango fruit imported from 1998 to 2012 [48]. In the last decade, mango production has increased in areas that were not typical for mango cultivation such as South and Central America, Egypt, and Southeast Asia. Mango is enjoyed for its flavor and exotic taste, and the fruit is consumed in all stages of its development. Unripe fruit is available in the form of

pickles and chutneys, and the fully ripened fruit is consumed raw and processed into purees, jams, and juices [3].

In 2014 the United States imported over 370,000 metric tons of fresh mango alone from Mexico, Central, and South America and domestically produced <100,000 metric tons [48]. As the majority of mango crop comes from the neighboring countries the cultivars that U.S. consumers are most likely to see in stores are Ataulfo, Haden, Keitt, Kent, and Tommy Atkins. Each of these varieties vary in flavor, size, and pulp consistency similar to the differences in apple varieties.

2.3.2 Mango Composition and Physiology

Fully ripened mango consists primarily of water and simple sugar; it contains approximately 1% combined fat and protein. The pulp of a ripe mango is yellow-orange in color due to the presence of carotenoids. Carotenoids are lipophilic hydrocarbons synthesized in organisms that utilize photosynthesis [49]. In these organisms, carotenoids function as aids to light harvesting, and protect the photo system from excess light and free radicals [50]. Only a few studies have been performed to characterize mango carotenoids in more than just a few varieties. Mercadante et al. found that the main carotenoids in two Brazilian mango varieties cv. Keitt and Tommy Atkins were *all-trans* β-carotene, *all-trans* violaxanthin, and *9-cis* violaxanthin. The same three carotenoids were found to be predominate in seven Mexican varieties (cv. Ataulfo, Haden, Tommy Atkins, Manila, Criollo, Kent, and Paraiso) as well [51].The quantity of carotenoids in the mango mesocarp has been reported to increase with fruit

ripening with a report by Mercandante et al. finding carotenoid levels in cv. Keitt increasing by over 200% in the transition from an un-ripened to fully ripened fruit [52, 53].

2.3.3 Mango Polyphenolics

The composition of mango polyphenols in the pulp, peel, and seed is well documented across several varieties, and like all fruits and vegetables the content and concentrations of mango polyphenols differ based on soil, growing conditions, variety, and part of the plant [10, 12, 13]. Consistently reported in mango though are the galloyl derivatives in the form of free gallic acid, gallic acid glycosides, and gallotannins [11]. Saleh et al. were the first to characterize phenolic compounds in mango pulp with their study across 20 varieties using thin layer chromatography [54]. Though the content of phenolic compounds in the pulp depends largely on variety, presence of gallic acid derivatives is a constant. Some cultivars (cv. Ataulfo) have high concentrations of gallotannins in the pulp while others do not. A recent focus has been to compare polyphenol content across varieties, an arduous task due to a large number of different varieties grown in various parts of the world [12, 13]. Knowledge of the differences in polyphenol content between varieties may be crucial to understanding mangos potential health benefits as different galloyl derivatives may have different bioefficacies. A similar but higher concentrated polyphenol profile is found in mango's peel. While not commonly consumed in the United States the peel is consumed in several parts of the globe where mango is grown. Gallotannins, flavonoids, and the unique polyphenol to

mango, mangiferin, have been reported in relatively high quantities in the peel [9, 10, 13].

2.4 Polyphenol Absorption, Metabolism, Distribution, and Excretion

Due to the proposed health benefits of polyphenolics the past decade has seen hundreds of investigations regarding their metabolic fate, where they have been found to be very poorly absorbed [55-68]. Once consumed polyphenols will be digested and undergo absorption, distribution, metabolism, and excretion (ADME) similar to how drugs would be metabolized. The human body recognizes polyphenols as xenobiotics, foreign compounds that the body will try to eliminate through metabolism and excretion. Xenobiotic metabolism is one of the bodies numerous defensive systems and specifically is utilized in detoxifying potential harmful compounds. Xenobiotic metabolism can occur in all cells however higher concentrations of phase I and II enzymes are found in the liver or small intestine. A summary of the pathways of polyphenol metabolism can be found in (Figure 3).

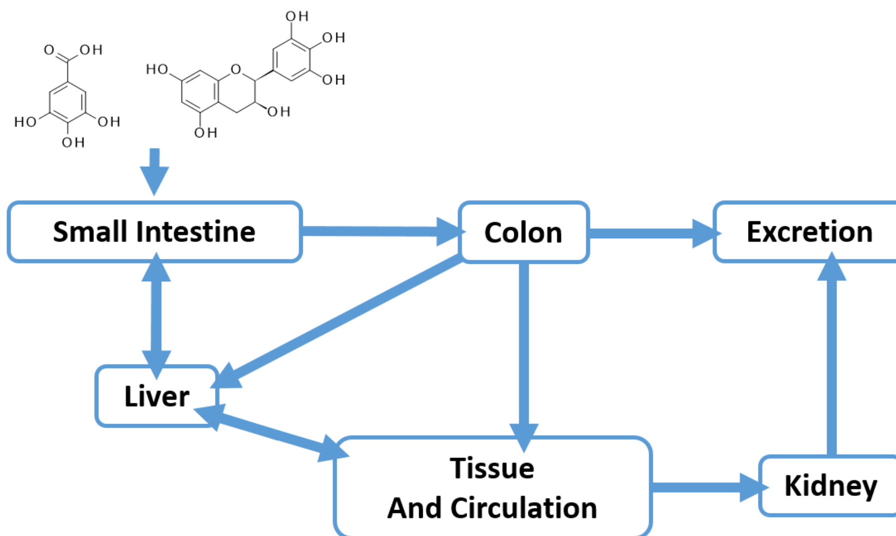

Figure 3. Polyphenol absorption, metabolism, distribution, and excretion

Before metabolism and distribution can take place, polyphenols must first be released from the food matrix and absorbed into the body. Absorption of polyphenols is complex due to the differences in bioaccessibility (the amount of a dose that is available for absorption), solubility, presence of glycosides and acylation's, and interactions with the food matrix [69]. Tannins are the conventional polyphenol that have been reported to have their bioaccessibility limited due to their ability to bind to proteins [70]. It is possible that polyphenols such as gallic acid and monogalloyl glucose that share the same galloyl or trihydroxyl functional group may have this same limited bioaccessibility. These interactions may limit the ability of some polyphenols to be absorbed in the small intestine, but could be beneficial in allowing them to reach the colon. Krook and Hagerman observed this *in-vitro* with epigallocatechin gallate and

pentagalloyl glucose, where each had higher stability at physiological conditions when paired with different food matrices [71].

Hydrophilicity and molecular weight are the most important factors when considering the absorption of a polyphenol into the enterocytes. Polyphenols are often glycosylated and are reportedly less absorbed than their aglycone counter parts [72]. Glycosylation of flavonoids increases their hydrophilicity and molecular weight and limits their absorption though passive diffusion. However the enzyme lactase phloridzin hydrolase found on the surface of epithelial cells is able to hydrolyze glycosides off some polyphenols allowing the now relativity hydrophobic polyphenols such as flavonoids to be absorbed through passive diffusion across the bi-lipid membrane [73].

Smaller polyphenols such as the phenolics acids are too hydrophilic for passive diffusion across the plasma membrane and must be absorbed through different mechanisms. One study using the Caco-2 absorption model reported that *p*-coumaric acid was 100 times more absorbed than gallic acid [74]. Follow up investigations hypothesized that this was due to some phenolic acids such as *p*-coumaric acid being able to be transported through the monocarboxylic acid transporter, an active transport found in cells that transports lactate, pyruvate, and ketone bodies across plasma membranes [75]. Polyphenols with trihydroxyl groups such as gallic acid and epigallocatechin gallate may have their bioavailability limited through due to their high protein binding affinity. This may diminish their bioactivity, however a study by Johnston et al. revealed that these polyphenols may limit the absorption of

macronutrients such as glucose through inhibition of α-amylase activity implying absorption and metabolism may not be needed for their bioefficacy [76].

Fruits and vegetables are whole foods and when consumed many phytochemicals other than polyphenols are consumed alongside which may inhibit or enhance absorption rates. Peters et al. found that formulations of tea with sucrose and ascorbic could increase the bioavailability of tea catechins [77]. They hypothesized that this was due to inhibition of efflux transporters which have been reported to reduce polyphenol bioavailability. This same phenomenon is likely to occur with other polyphenols in foods that share these ingredients and may indicate that polyphenols are best consumed as whole foods.

Once polyphenols are absorbed into a cell they will undergo xenobiotic metabolism similar to that of pharmaceuticals. The main function of xenobiotic metabolism is to assist in elimination of a foreign compound from the body and is divided into two phases, I and II. Phase I begins with oxidation reactions from the family of Cytochrome P450 oxidase enzymes. As non-polar compounds are more likely to be absorbed and distributed throughout the body Phase I reactions conjugate xenobiotics with polar functional groups (R=O, R-OH) which limits their ability to diffuse across cellular membranes [78]. Polyphenols by their nature already contain several hydroxyl groups, and have not been reported to participate in Phase I reactions. Metabolism proceeds with Phase II reactions where conjugations of methyl, glucuronide, and sulfur functional groups to the hydroxyl groups on polyphenols can occur with catechol-*O*-methyl transferase (COMT), uridine diphosphoglucuronosyl transferases (UGT), and

sulfotransferases (SULT), respectively [79]. Once metabolized, polyphenols will either efflux by multidrug resistance proteins back to the small intestine or through the basolateral side to circulation where it will procede to the hepatocyte for additional metabolism [80]. Finally, if polyphenols are not sent back to the small intestine through liver bile they will enter systemic circulation before ultimately being excreted through the kidney.

Due to its high potential to be consumed in the human diet the ADME of gallic acid has been extensively studied. Figure 4 illustrates the proposed metabolism of galloyl derivatives. In 1959 Booth et al. analyzed rat urine after ingestion of tannic acid, and with the use of paper chromatography were able to identify gallic acid in the urine along with a methylated gallic acid [81]. Yasuda et al. performed the first study to truly begin to characterize the metabolites of gallic acid in rats with modern analytical techniques and were able to characterize two phase II metabolites in urine, 4-*O*-methylgallic acid and 4-*O*-methylgallic acid-3-*O*-sulfate, after acute consumption of GA; these metabolites were characterized by a combination of their mass spectrum and NMR after acute consumption [82]. These studies report absorption and excretion happening quickly, with gallic acid and its metabolites being eliminated from the body within 2 h after consumption [65]. The ADME of monogalloyl glucoses has yet to be investigated, however, due to its higher molecular weight and size it is expected to have a lower bioavailability compared to gallic acid. Some reports are available on the bioavailability of pentagalloylglucose where low bioavailability is to be reported likely

due to a combination of its relatively large molecular weight, hydrophilicity, and protein binding capacity [58, 83].

2.4.1 Polyphenol Colonic Metabolism

Trillions of bacteria are estimated to be in the human body with the majority residing in the GI tract, and is commonly referred to as the gut microbiota. The gut microbiota has been hypothesized to play a large role in the health of humans. The majority of polyphenols are not absorbed in the small intestine, meaning most will reach the colon where enzymes synthesized by microorganisms can catabolize polyphenols to produce a number of different hydrolytic products that will be sequentially available for absorption and metabolism.

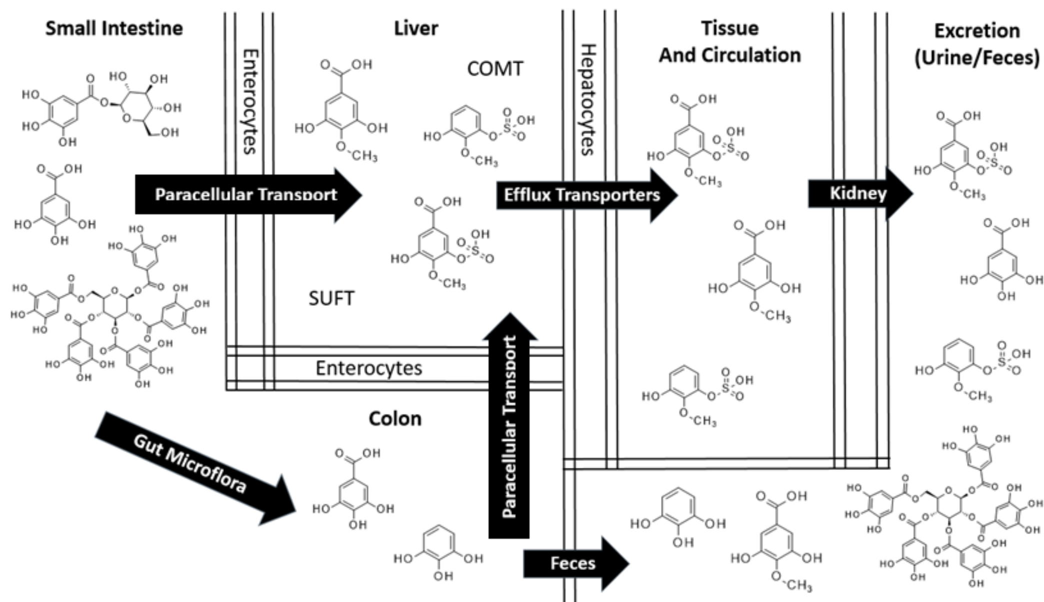

Figure 4. The current proposed mechanisms on the ADME of gallic acid and galloyl glycosides

The growing importance of the gut microflora in metabolomics has led recent investigations to analyze their role in gallic acid metabolism. In two recent studies performed by Pimpão et al., 500 mL of a berry puree containing 425 mg gallic acid was fed to humans. Methyl and sulfated forms of pyrogallol, a decarboxylated gallic acid hypothesized to be sourced from the gut microbiota, were observed in human plasma and urine at higher concentrations compared to their source gallic acid [38, 55]. This was also observed after consumption of black tea extract where high concentrations of pyrogallol were detected up to 30 h after consumption [84]. The anti-cancer and anti-inflammatory effects of pyrogallol metabolites should be investigated to determine if they have similar properties as gallic acid.

Gallotannins are another substrate for enzymatic hydrolysis by the gut microflora due to bacteria that are capable of producing tannase, an enzyme capable of cleaving *m*-depside and ester bonds. Tannase is produced by a number of bacteria where it is hypothesized to aid in microbial defense against tannins. *Streptococcus galloyticus* is one bacteria of note that can use tannins as a carbon source and one strain has been reported to live in concentration of up to 7% gallotannin [85]. Tannase has found industrial use for the production of gallic acid from tannic acid and aiding in the reduction of tea creaming. Release of gallic acid from enzymatic hydrolysis of gallotannins in vivo may give gallotannins additional bioefficacy as being a pool of pro gallic acid that can be released in the colon for absorption and metabolism.

2.4.2 Chronic Polyphenol Consumption

Several studies have investigated the influence of extended polyphenol intake on inflammatory and other health biomarkers [86-91], yet only a few have looked at how chronic polyphenol consumption affects concentrations of polyphenols in blood and tissues. To accurately understand the mechanisms behind the health benefits of polyphenol consumption it is necessary that their metabolites be characterized and quantified in plasma and other sites of action. Conflicting results are reported, but indicate that habitual consumption will lead to higher levels of polyphenol metabolites in vivo. Ferruzi et al. have reported rats that consumed grape seed extract for 10 days were found to have a significantly higher concentration of gallic acid and catechin in plasma with repeated consumption [92]. However this trend was not observed for tea catechins in the plasma of humans who consumed green tea for 4 weeks [93]. The reports of habitual polyphenol consumption and its effects on colonic metabolism are even fewer given its only recent interest. However significant increases in excretion have been reported for colonic metabolites from both mango and cranberries [94, 95].

2.4.3 Polyphenol Metabolite Analysis

Investigations characterizing and quantifying polyphenol metabolites in biological matrices cite the use of LC-MS as the instrument of choice. However, analysis of polyphenol metabolites contrast to that of their parent compounds present in fruits and vegetables due to conjugation of methyl, sulfate, and glucuronide functional groups as well as sample concentrations that are often at the nM level [96].

Table 1. Plasma recoveries for gallic acid by use of solid phase and solvent extraction.

	Method	**Concentration**	**% Recovery**
Gasperotti et al. [97]	Solid Phase Extraction	20 µg/L	53
de Farrars et al. [98]	Solid Phase Extraction	17 mg/L	<50%
Ma et al. [99]	Solvent	30 - 3000 µg/L	78-82
Margalef et al. [61]	Solid Phase Extraction	50 -500 mg/L	36-97
Sun et al. [100]	Solvent	40 – 8000 µg/L	80-88

Sample preparation methods vary depending on the class of polyphenol being analyzed with most methods having a concentration step in hopes of increasing analytical sensitivity. The two most common methods for metabolite extraction in the literature are extraction of plasma with an organic solvent (acetonitrile, methanol, and or ethyl acetate) and micro solid phase extraction (SPE) with reverse phase chromatography [59, 61, 66, 101]. The recovery of gallic acid from these methods is mixed with recoveries ranging from 36 - 99% (Table 1). Greater recoveries are reported for the extraction of gallic acid from plasma by use of solvents for high concentrations of analyte in the sample, yet as those concentration are unlikely to be found in vivo they aren't truly applicable for method development. Gallic acid has also been reported as one of the least recoverable polyphenolics most likely due to its protein binding affinities and high hydrophilicity compared to other phenolics making them difficult to partition with solvents or SPE.

No reports are available on the recovery of gallic acid's phase II metabolites from plasma likely due to the lack of analytical standards. It is possible that the

metabolites may have even lower recovery than the parent compound because of the additional methyl and sulfur groups, which may increase their ability to bind to proteins in plasma. Methylated epigallocatechin-gallate was found to have a higher plasma protein binding affinity than its non-conjugated form, and this same principle may carry over to gallic acid [102]. A method with increased efficiency for recovery of gallic acid and its metabolites is needed for accurate analysis in biological matrices.

In summary, while many absorption studies are available for gallic acid, little work has been performed on the ADME of monogalloyl glucose and gallotannins other than pentagalloylglucose to see what differences if any exist. A few studies have looked into the bioavailability of pentagalloylglucose, but with the growing importance of colonic microflora on polyphenolic metabolism there is a gap in knowledge with regards to how different galloyl derivatives are metabolized. As aforementioned, mango is unique in that it contains galloyl derivatives ranging from gallic acid all the way to the highly polymeric gallotannins making it the perfect vessel to study the ADME of different galloyl derivatives in a food matrix.

CHAPTER III

QUANTIFICATION OF GALLIC ACID AND GALLOYL GLYCOSIDES IN FIVE MANGO CULTIVARS AND THE CHARACTERIZATION OF TANNASE HYDROLYSIS PRODUCTS

3.1 Introduction

Elevated consumption of fruits and vegetables is correlated with a reduction in the rates of cancer and cardiovascular diseases [17, 21], and has largely been attributed to the polyphenols, a class of phytochemicals found in higher plants that have a polyaromatic core attached with multiple hydroxyl groups. As a result, understanding the mechanisms behind polyphenols preventative effects is currently of great scientific inquiry. Due to the diversity in chemical structures among polyphenolics it is important to individually investigate each compound to understand underlying mechanisms behind their potential health benefits. Mangos are a unique fruit as the polyphenolic content in their mesocarp is composed primarily of gallic acid, galloyl glycosides, and the polymerized gallotannins [11, 13, 103, 104]. Mango has had a 120% increase in the value of its imports to the U.S. over the past decade, and is perhaps one of the most popular fruits in the world, second only to bananas in global tropical fruit production [48]. Previous investigations have found significant differences in the composition of polyphenolics between various mango cultivars [12]. As the popularity of mango continues to increase and different varieties are brought to market, there is a need to characterize the phytochemical differences among cultivars that may relate to potential

health benefits. For example, gallic acid and pentagalloyl glucose are reported to have significantly different absorption rates despite similar functional groups [105]. For other polyphenolics, even the addition of functional groups such as hydroxyl or methoxy groups or the presence of a glycoside can affect absorption rates, as found by Hollman et al. (1995) who observed quercetin glycosides had higher absorption rates than quercetin aglycone [72, 106].

Tannins are a class of polyphenols found in a wide array of fruits, vegetables, and legumes that have been used for centuries as preservation agents in the process of turning animal hides into leather, and are additionally known for their ability to form complexes with protein and cause astringency in foods [39]. Tannins are categorized into two subtypes, condensed and hydrolysable. Condensed tannins are oligomers of flavan-3-ols that are linked carbon to carbon via an interflavan bond. The hydrolysable tannins are further divided into gallotannins and ellagitannins. Gallotannins are polymers of gallic acid esterified to a polyol core, and ellagitannins are made from both gallic acid and hexahydroxydephenic acid moieties esterified to a polyol. In contrast to condensed tannins, hydrolysable tannins are polymerized through esterification of the monomeric units, and these ester bonds are capable of being hydrolyzed through both enzymatic and non-enzymatic means [71, 107]. Historically, tannins have been difficult to analyze due to poor separation even with high performance liquid chromatography, however some successful attempts have recently been made [108].

Tannase (tannin acyl hydrolase, EC 3.1.1.20) is an enzyme capable of hydrolyzing ester and depside bonds of hydrolysable tannins [109]. Tannase is produced

by several genera of fungi including but not limited to: *Aspergillus*, *Penicillium*, *Fusarium*, *Trichoderma* and bacteria, *Lactobacillus spp* and *Streptococcus gallolyticus*, and commercially is used to prevent creaming in instant tea, clarification of beer and fruit juice, and the production of acorn wine and coffee flavored beverages [110, 111]. Recent interest has been given to the tannase produced in the colon of individuals and its potential impacts on the digestion of foods, mainly through the release of gallic acid, which has been shown to possess anti-cancer and anti-inflammatory properties [16, 112]. However, inter-individual differences in tannase activity will affect the rate at which gallic acid is hydrolyzed and the efficiency of hydrolysis *in vivo* is unknown. The production of gallic acid by hydrolysis from tannase has been monitored in numerous studies alongside the characterization of the hydrolysis of tannic acid. However, the characterization of solely pentagalloyl glucose and monogalloyl glucose has not yet been investigated. Additionally, tannase has been reported to have different enzymatic activities for different galloyl substrates [113]. Investigating the effect of tannase on galloyl derivatives is of special interest for mango since tannase is found environmentally, in the leaves of mango trees, and is present innately in the gut microbiota of some individuals [114, 115]. Therefore, it was hypothesized that the rate and byproducts of hydrolysis with tannase may affect each distinct galloyl glucose differently.

In this work, five mango cultivars were investigated for their gallic acid, galloyl glycoside, and gallotannin content. Additionally, enzymatic hydrolysis with tannase was performed on monogalloyl glucose, pentagalloyl glucose, and a gallotannin isolate

extracted from mango in an effort to characterize and quantify hydrolysates over time. Results from these studies are aimed to quantify and characterize possible galloyl derivatives in mango pulp before and after enzymatic changes.

3.2 Materials and Methods

3.2.1 Fruit

Mangos (cv. Ataulfo, Tommy Atkins, Keitt, Haden, and Kent) were sourced from Mexico through Frontera produce, and were allowed to ripen at room temperature. Fruit were manually peeled, deseeded, and vacuum sealed under good manufacturing practices. Mango pulp was held at -20°C until used for experiments.

3.2.2 Chemicals

Standards for gallic acid and pentagalloyl glucose were acquired from Sigma Aldrich (St. Louis, MO), and monogalloyl glucose acquired from CarboSynth (Berkshire, UK). Citric Acid and sodium carbonate were acquired from Fisher Scientific (Hampton, NH). Tannase (E.C. 3.1.1.20) sourced from *Aspergillus oryzae* was obtained from Kikkoman (Chiba Prefecture, Japan). HPLC grade 0.1% formic acid water and 0.1% formic acid methanol were acquired from Sigma Aldrich.

3.2.3 Mango Extracts and Tannin Fraction

Polyphenol extracts for mango quantifications were prepared from 10 mg of homogenized mango pulp extracted with 30 mL of a 1:1 ratio of acetone and methanol in

triplicate. Solvents were evaporated under vacuum at 45 °C, and re-dissolved in a known volume of water acidified with 0.1 M HCl. An isolate consisting of only gallotannins was prepared from Ataulfo mango pulp and isolated as previously described by Hagerman (2011) [116]. Briefly, 500 mL of reagent alcohol was added to 500 mL of the polyphenol mango extract, and loaded on to a column filled with 20 g of Sephadex LH-20 that was previously conditioned with 20 column volumes of reagent alcohol. Once the sample was loaded tannins were eluted using 80% acetone in water acidified with 0.1% formic acid. The eluted gallotannin isolate was evaporated under vacuum, and stored at -20 °C until use. Conformation that only gallotannins remained in the isolate was performed by HPLC.

3.2.4 Enzymatic Hydrolysis

Standard solutions of monogalloyl glucose, pentagalloyl glucose, and a gallotannin isolate were incubated with tannase at 10^{-3} U/mL to characterize the byproducts and relative rates of galloyl derivative hydrolysis, and 250 µL of 200 ppm monogalloyl glucose or pentagalloyl glucose were incubated with 650 µL of buffer set to the enzyme's optimum conditions (pH 5.5, 0.1 M citric acid buffer, 30ºC), and 100 µL of Tannase 10^{-3} U/mL for final standard concentrations of 50 ppm. For the gallotannin isolate, 250 µL of a 969 ± 31 ppm gallic acid equivalents determined by the Folin-Ciocalteu method was used [117]. Experiments were performed in a static water bath and prepared in triplicate for each time point at 0, 1, 2, 3, 5, 10 min and 0.5, 1, 1.5, 2, 3, 4 h. To end enzymatic activity solutions were immediately diluted with 1000 µL 0.1%

formic acid methanol. Lastly, samples were filtered with a Whatman 0.45 μm syringe filter prior to LC-MS analysis.

3.2.5 LC-MS

Galloyl derivatives were characterized and quantified by use of LC-MS on a Thermo Finnigan HPLC. Separations were in reversed-phase using a Finnigan Surveyor HPLC coupled to a Surveyor PDA detector and gradient separations were performed using a Phenomenex Kintex™ (Bannockburn, Il) C_{18} column, (150 x 4.6 mm, 2.6 μm) at room temperature. Injections were made into the column by use of a 50 μL sample loop. For separation of gallic acid and galloyl glycosides, mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in methanol run at 0.45 mL/min. A gradient was run of 0% Phase B for 2 min and changed to 10% Phase B in 4 min, 10% Phase B was held to 10 min, 10 to 40% Phase B in 25 min, and 40% to 65% Phase B in 35 min, 65% to 85% Phase B in 41 min, 85% was held to 49 min before returning to initial conditions. The electrospray interface worked in negative ionization mode. Source and capillary temperatures were set at 325°C, source voltage was 4.0 kV, capillary voltage was set at -47 V, and collision energy for MS/MS analysis was set at 35 eV. The instrument operated with sheath gas and auxiliary gas (N_2) flow rates set at 10 units/min and 5 units/min, respectively. The instrument was tuned specifically for pentagalloyl glucose. Gallic acid and both monogalloyl glucoses were quantified at 280 nm with their respective standards. Di- and trigalloyl glucoses were quantified at 280 nm and reported

as equivalents of monogalloyl glucose. Tetragalloyl glucose and higher were quantified and reported as pentagalloyl glucose equivalents.

3.3 Results and Discussion

3.3.1 Quantification of Gallic Acid and Galloyl Glycosides in Five Mango Cultivars

The polyphenolic content of fruits can differ based on ripeness, variety, and harvest conditions, however, previous investigations have all reported the polyphenolic content of mango pulp to be composed primarily of gallic acid and galloyl glycosides [9, 12, 104, 118]. Historically, investigations on mango pulp have focused primarily on quantification of the total polyphenolic content, and characterization of flavonoids and gallotannins leaving the majority of the phenolic acids unquantified [10, 13]. A recent investigation by Krenek, Barnes, and Talcott (2014) characterized two monogalloyl glucoses which accounted for over 50% of the cv. Keitt mango's non-tannin polyphenolic content [11]. Quantification of these two monogalloyl glucoses in other popular mango cultivars are needed as they likely attribute to mangos overall bioefficacy. In this work, the concentrations of gallic acid, monogalloyl glucoses, galloyl glycosides, and gallotannins were evaluated in the pulp of five mango cultivars, Ataulfo, Tommy Atkins, Keitt, Haden, and Kent. These five mango varieties are commonly found in grocery stores across the United States, and can be sourced from Mexico, Brazil, Ecuador, and Florida [3]. Characterization of the mango galloyl derivatives was performed from previously reported spectra and *m/z* (Table 2) [9, 11].

Table 2. Mass to Charge Ratios (*m/z*) and λ_{max} for gallic acid, galloyl glycosides, and gallotannins.

Compound	λ_{max} (nm)	$[M-H]^{-}$ (m/z)	$[M-2H]^{2-}$ (m/2z)	MS/MS (m/z)
gallic acid	271	169	-	125
mono-galloyl glucose	278	331	-	271, 169, 125
di-galloyl glucose	272	483	-	331, 271, 169
tri-galloyl glucose	273	635	-	483, 422
tetra-galloyl glucose	275	787	-	635, 465
penta-galloyl glucose	279	939	469	-
hexa-galloyl glucose	279	1091	545	-
hepta-galloyl glucose	279	1243	621	-
octa-galloyl glucose	279	1395	697	-
nona-galloyl glucose	279	1647	773	-
deca-galloyl glucose	279	1899	849	-
unadeca-galloyl glucose	279	2003	1001	-

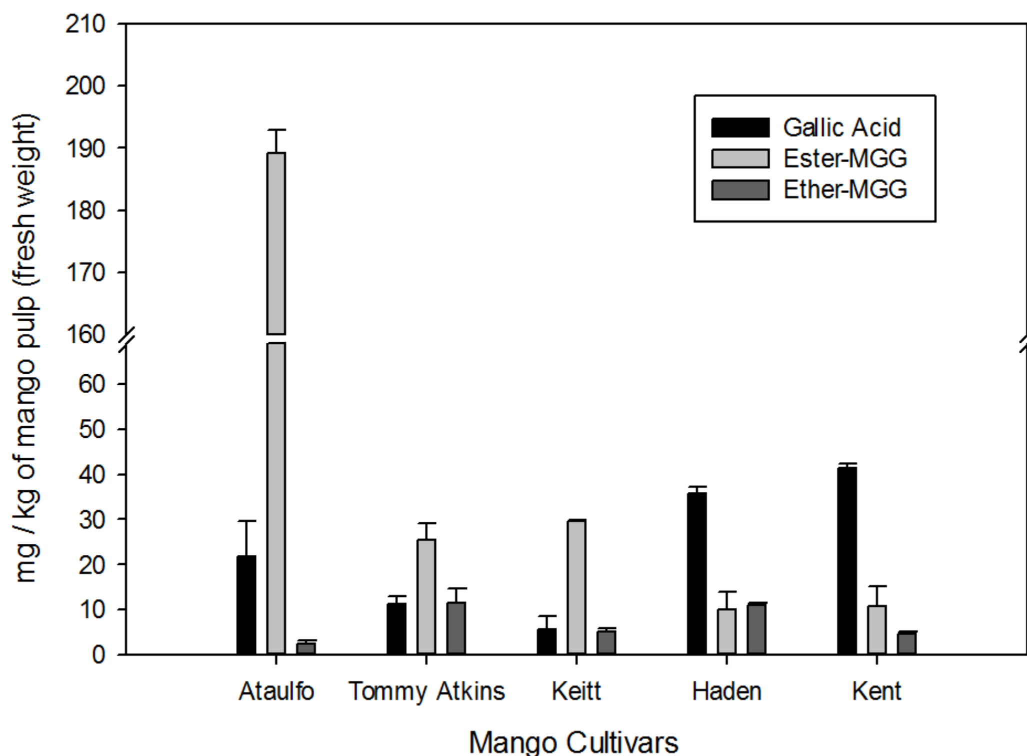

Figure 5. Concentrations (mg/kg) of gallic acid (GA), ester-monogalloyl glucose (Ester-MGG), and ether-monogalloyl glucose (Ether-MGG) across five mango cultivars.

Gallic acid was characterized from a *m/z* of 169 and λ_{max} of 271 nm, and concentrations in the mango pulp were found to be at 21.6 ± 4.53, 11.3 ± 0.98, 5.69 ± 0.11, 35.8 ± 0.81, and 41.3 ± 0.68 mg/kg for Ataulfo, Tommy Atkins, Keitt, Haden, and Kent, respectively (Figure 5). For the varieties, Haden and Kent, gallic acid was the highest concentrated non-tannin galloyl derivative in the pulp similar to report by Scheiber et al. where gallic acid was found to be the highest concentrated individual polyphenol at 6.9 mg/kg in a commercial mango puree [104]. The other predominate non-tannin galloyl glycosides were the two monogalloyl glucoses, ester-monogalloyl

glucose and ether-monogalloyl glucose which have previously been characterized by Krenek, Barnes, and Talcott (2014) with *m/z* of 331.2 and λ_{max} of 278 nm [11]. Ester-monogalloyl glucose has a glucose esterified to the acid group and fragmented into ions at *m/z* 271, 169, and 125 while the ether-monogalloyl glucose has a glucose covalently linked to a hydroxyl group on the 4 position and MS/MS fragments at *m/z* 169, 125 (Figure 6). The concentration of ester-monogalloyl glucose for the five mango varieties was 189 ± 2.09, 25.3 ± 2.16, 29.7 ± 1.59, 35.8 ± 2.25, and 41.3 ± 2.6 mg/kg, and ether monogalloyl glucose was 2.52 ± 0.35, 11.4 ± 1.89, 5.08 ± 0.37, 10.91 ± 0.28, and 4.63 ± 0.30 for Ataulfo, Tommy Atkins, Keitt, Haden, and Kent, respectively.

The remaining galloyl derivative content included the galloyl glycosides, trigalloyl glucose and tetragalloyl glucose, and the gallotannins ranging from pentagalloylglucose to decagalloyl glucose (Figure 7) for the five cultivars was 3,040 ± 962, 1,370 ± 58, 309 ± 36, 401 ± 41, and 127 ± 30 mg/kg for Ataulfo, Tommy Atkins, Keitt, Haden, and Kent, respectively.

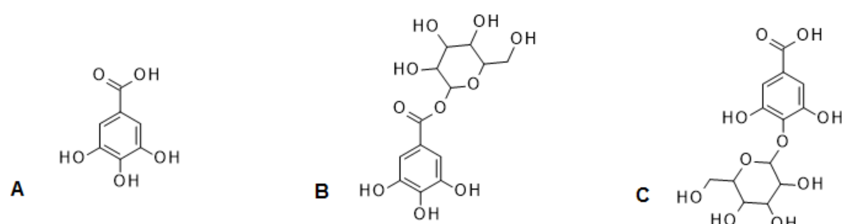

Figure 6. Chemical structures for gallic acid (A), ester-monogalloyl glucose (B), and ether-monogalloyl glucose (C).

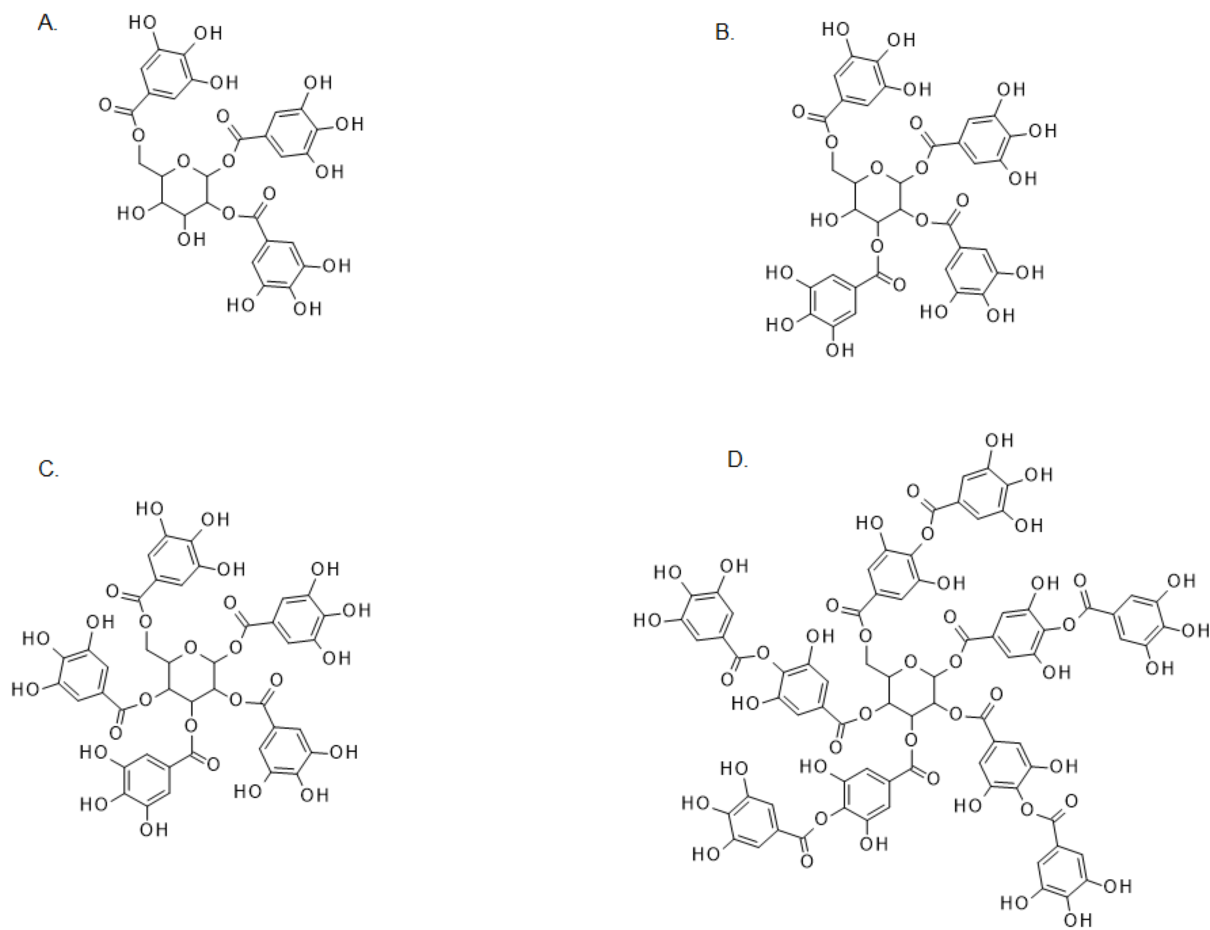

Figure 7. Chemical structures for trigalloyl glucose (A), tetragalloyl glucose (B), pentagalloyl glucose (C), and decagalloyl glucose.

Ester-monogalloyl glucose was significantly higher in concentration across all mango cultivars except Haden when compared to ether-monogalloyl glucose (Figure 5), and was hypothesized to be due to ester-monogalloyl glucose being the precursor required for synthesis of gallotannins [34]. The Ataulfo cultivar had both the highest concentration of ester-monogalloyl glucose and gallotannins in comparison to other cultivars. In contrast, the two cultivars with the lowest concentration of ester-monogalloyl glucose had the lowest total amount of gallotannins. Significant differences ($p<0.05$) were additionally found in the composition of gallotannins as well. Over 60% of the gallotannins in Ataulfo were heptagalloyl glucose (7GG) or larger. Conversely, Kent had the lowest concentration of total galloyl derivatives with over 60% of its gallotannin composition as pentagalloyl glucose (5GG) or smaller (Figure 8).The Ataulfo mango has previously been reported to be a superior cultivar in relation to its high polyphenolic concentration when compared to other mangos [119], and in this work it was the cultivar found to be the highest in total galloyl derivatives.

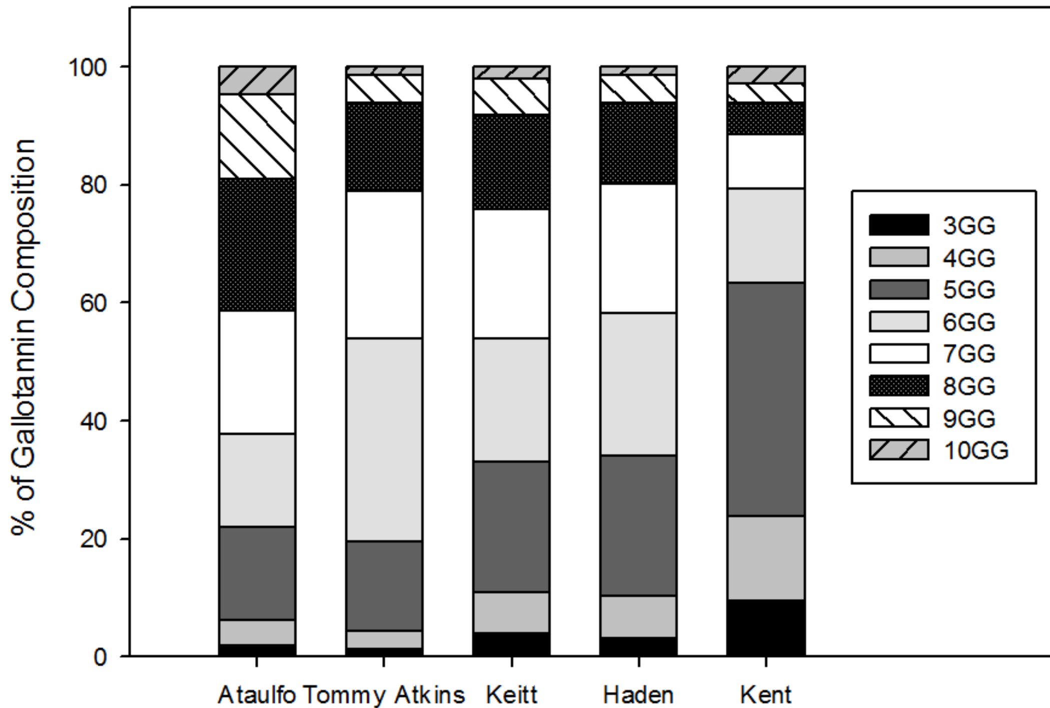

Figure 8. Composition of gallotannins ranging in degree of polymerization from trigalloyl glucose (3GG) to decagalloyl glucose (10GG) across five mango cultivars.

3.3.2 Characterization of Tannase Hydrolysis Intermediates

Tannase sourced from *Aspergillus oryzae* was incubated with standard solutions of monogalloyl glucose, pentagalloyl glucose, and a gallotannin isolate sourced from mango in an effort to characterize and quantify the intermediates formed during hydrolysis. Prior investigations using hydrolysis with tannase have focused primarily on the substrates, methyl gallate, propyl gallate, epigallocatechin-gallate, and tannic acid, and specifically only on the rate of gallic acid production [120, 121]. Characterization of the different galloyl derivatives that can be formed during tannase hydrolysis is critical

as they are likely to have different rates of absorption, which will ultimately affect their health promoting properties.

Hydrolysis of pentagalloyl glucose and other galloyl glycosides with tannase generates additional substrates upon hydrolysis, similar to the hydrolysis of glucose from maltodextrins with amylases. In this study after 2 h incubation of pentagalloyl glucose with tannase at 10^{-3} U/mL, 97.2 ± 0.27 % of the initial pentagalloyl glucose concentration had been hydrolyzed to create tertra-, tri-, digalloyl glycosides, and gallic acid (Figure 9). After 2 h, two tetragalloyl glucoses, six trigalloyl glucoses, and five digalloyl glucoses were characterized from *m/z* previously reported by (Table 2) [9]. Specifically, tetragallyol glucose was characterized from a parent ion at *m/z* 787 and fragment ions at *m/z* 635, 465 corresponding to the losses of galloyl moieties. Trigalloyl glucose at *m/z* 635 and fragments at *m/z* 483, 422, and 313, and digalloyl glucose was characterized from a parent ion at *m/z* 483 and fragment ions at *m/z* 331, 271, and 169.

Concentrations of tetra-, tri-, and digalloyl glucoses changed significantly over the course of 2 h. At 0.5 h, the sum of tetragalloyl glucoses reached a maximum concentration of 49.2 ± 2.22 % of the total galloyl glycosides content, but at 2 h decreased to 13.8 ± 0.68%. In addition, di- and trigalloyl glucoses were still being synthesized and made up 10.4 ± 0.65 and 73.7 ± 1.92% of the total galloyl glycosides content, respectively. The six trigalloyl glucoses and five digalloyl glucoses each had distinct retention times (Figure 10), and were hypothesized to each have unique stereochemistry. The presence of numerous di- and trigalloyl glycosides indicates tannase randomly hydrolyzes the ester linkages to glucose. The hydrolysis of

pentagalloyl glucose with tannase directly contrast with its synthesis in plants, which follows a specific enzymatic pathway. In vivo, pentagalloyl glucose synthesis begins with the formation of monogalloyl glucose where subsequent enzymatic condensation reactions add additional galloyl groups to create 1,6-digalloyl glucose, followed by, 1,2,6 -trigalloyl glucose, then 1,2,3,6-tetragalloyl glucose, and lastly 1,2,3,4,6-pentgalloyl glucose, as previously discussed in Chapter II [34].

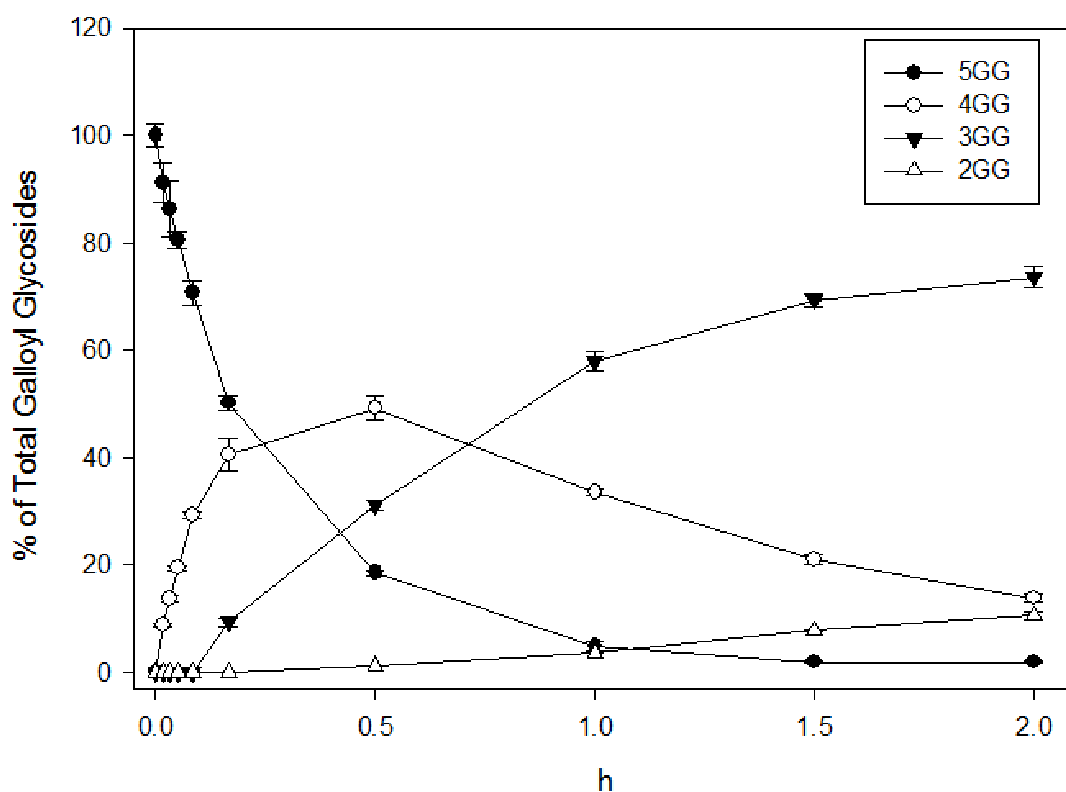

Figure 9. Hydrolysis of pentagalloyl glucose (5GG) and subsequent formation of tetragalloyl glucose (4GG), trigalloyl glucose (3GG), digalloyl glucose (2GG) following 2 h incubation with tannase at 10^{-3} U/mL.

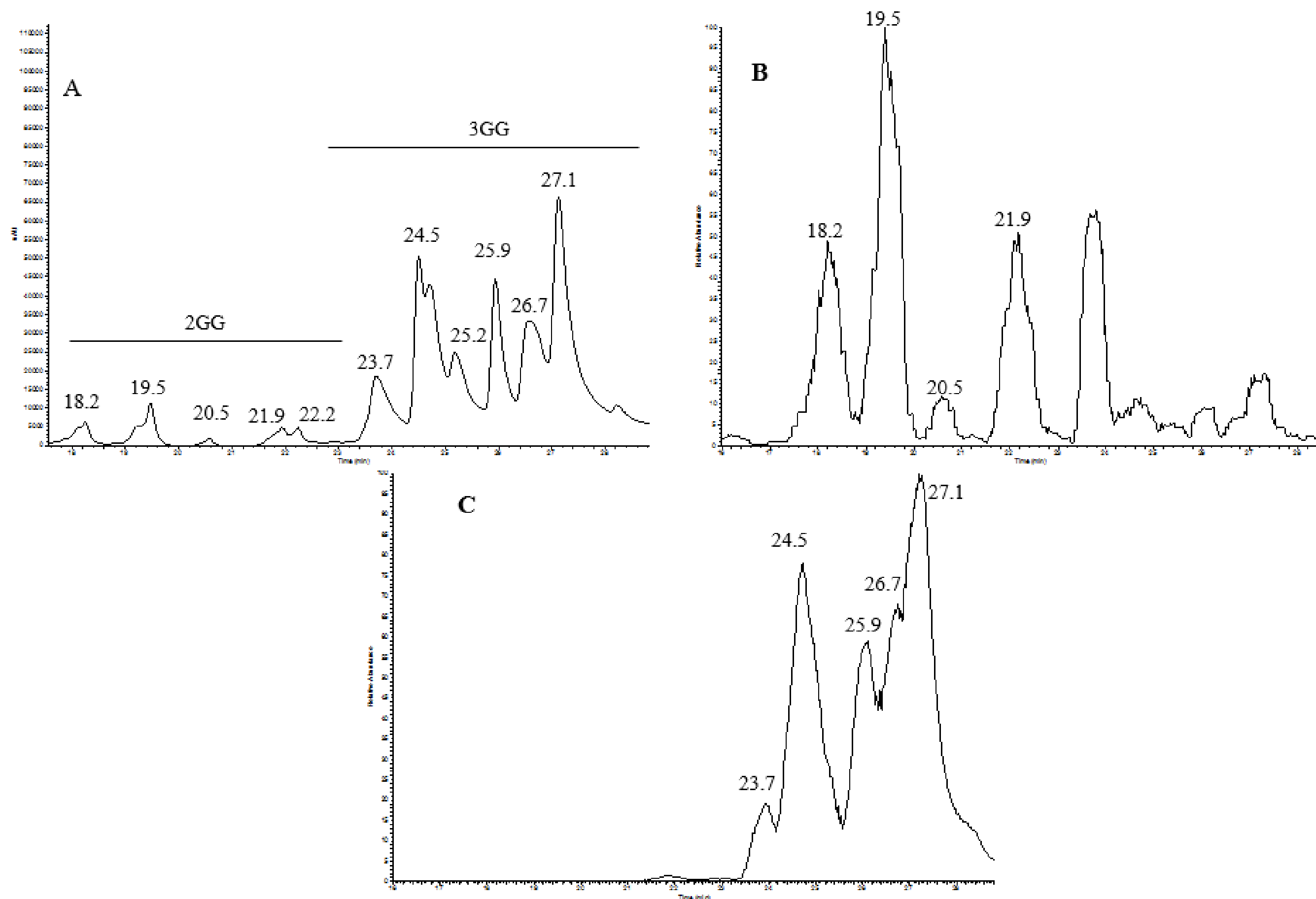

Figure 10. Chromatogram of five digalloyl glucoses and six trigalloyl glucoses at 280 nm (A) and extracted ion chromatograms at m/z 483.2 (B), and 635.2 (C) generated from hydrolysis of pentagalloyl glucose following 2 h incubation with tannase at 10^{-3} U/mL.

Thus, di- and trigalloyl glucoses are naturally found in plants that contain gallotannins as they are necessary for the formation of higher molecular weight gallotannins, but are observed in only two possible configurations, 1,6-digalloylglucose and 1,2,6-trigalloyl glucose [122]. The formation of numerous di- and trigalloyl glucoses from tannase hydrolysis has the potential to impact the bioefficacy of gallotannins as bacteria residing in the gut microbiota of humans have been reported to express tannase [115]. As a result, these newly characterized di- and trigalloyl glucoses are hypothesized to each have a distinct bioavailability which should be investigated to fully elucidate the potential health benefits of gallotannin consumption.

On a molar basis 45.5 ± 0.53% of the total potential gallic acid content was generated from hydrolysis of pentagalloyl glucose following 2 h of incubation. When an equivalent concentration of monogalloyl glucose (20 mg/L) was incubated with the same duration and tannase activity only 28.9 ± 0.26 % of the total potential gallic acid was generated, a significant ($p<0.05$) difference (Figure 11). It took a total of 4 h for monogalloyl glucose to reach the same amount of gallic acid generated by pentagalloyl glucose, despite there being more moles of monogalloyl glucose compared to pentagalloyl glucose. This observation could be due to differences in enzymatic activities at specific galloyl-glucose positions. Wu et al. (2015) previously demonstrated that tannase can have different activities for various galloyl substrates, and when tannase sourced from *Lactobacillus plantarum* was incubated with methyl gallate, digallic acid, and tannic acid it was found to have enzymatic activities of 272, 16.8, and 10.4 U/mg, respectively [113]. Therefore, results from these experiments suggest tannase has

different enzymatic activates for each position of a galloyl group on the glucose core of pentagalloyl glucose.

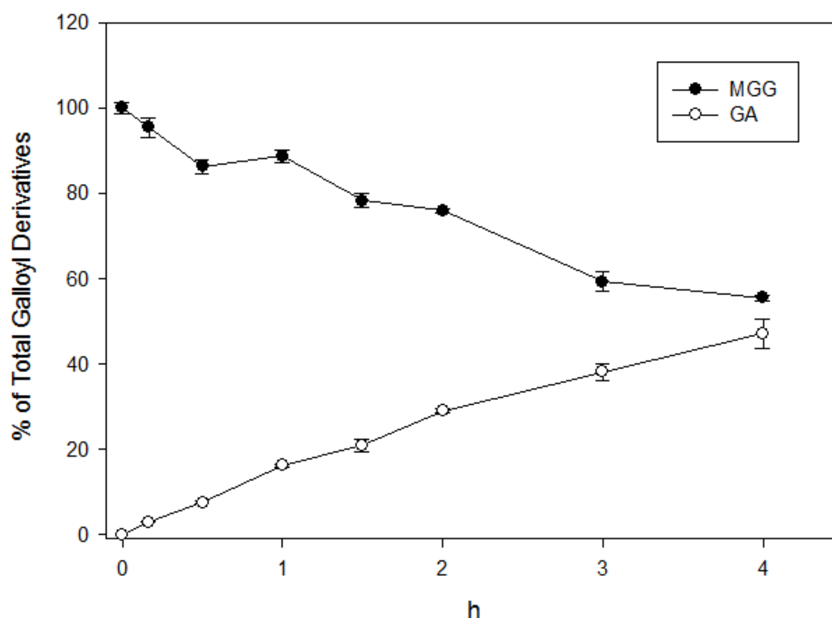

Figure 11. Hydrolysis of monogalloyl glucose (MGG) and subsequent formation of gallic acid (GA) following 4 h incubation with tannase at 10^{-3} U/mL.

A mango gallotannin isolate extracted from the Ataulfo cultivar containing tannins ranging in degree of polymerization from pentagalloyl glucose to undecagalloyl glucose was incubated with tannase at 10^{-3} U/mL (Figure 12). After 2 h, all gallotannins were still quantifiable indicating that there was not a preference for one gallotannin over others by the enzyme. Tetragalloyl glucoses and pentagalloyl glucoses all significantly increased in concentration to 3.22 ± 0.04 and 7.48 ± 0.13% of total galloyl derivatives, respectively. Trigalloyl glucoses and gallic acid were not detected initially and after 2 h

hydrolysis represented 1.31 ± 0.01 and 2.41 ± 0.02% of the total galloyl derivatives. No digalloyl glucoses or monogalloyl glucose were characterized or quantified within the 2 h hydrolysis. In addition, no digallic acid or trigallic acid were characterized whose presence would indicate direct hydrolysis of galloyl-glucose bonds of the larger gallotannins instead of just the depside bonds connecting the gallic acids together. Ren et al. (2013) previously described the structure and binding site of a galloyl in tannase produced by *Lactobacillus plantarum*, and found that there was only one binding site for both depside and esterase activities, and that only a single galloyl moiety enters the binding site at a time [123], which would explain the lack of digallic acid and trigallic acid.

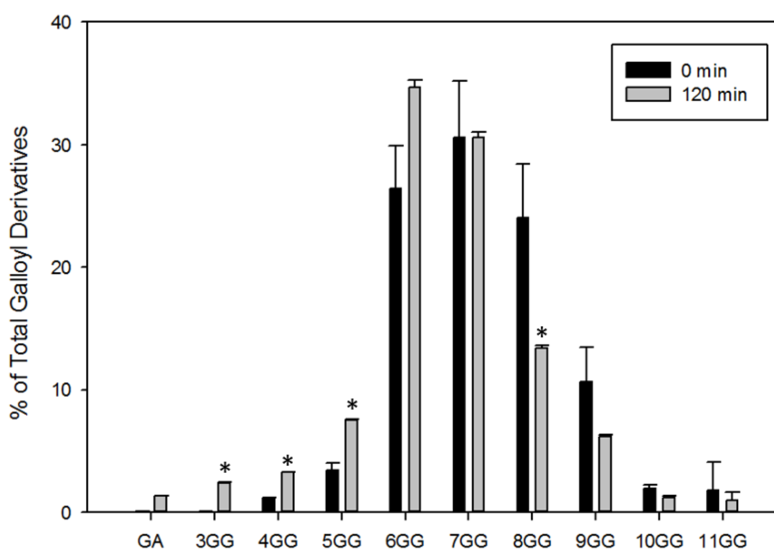

Figure 12. Hydrolysis of gallotannin isolate ranging in composition from tetragalloyl glucose (4GG) to undecagalloyl glucose (11GG) following 2 h incubation with tannase at 10^{-3} U/mL.

3.4 Conclusions

The pulp of Ataulfo, Tommy Atkins, Keitt, Haden, and Kent mango cultivars were investigated for their galloyl derivative content. Ataulfo had the highest among all cultivars with 21.6 ± 4.53 mg/kg of gallic acid, 189 ± 2.09 mg/kg of ester-monogalloyl glucose, and 3,040 ± 962 mg/kg of gallotannins while Kent had the lowest with 41.3 ± 0.68 of gallic acid, 41.3 ± 2.6 mg/kg of ester-monogalloyl glucose, 127 ± 30 mg/kg and of gallotannins. Additionally, the enzymatic hydrolysis of gallotannins with tannase was evaluated and the intermediates characterized, and when pentagalloyl glucose was hydrolyzed two tetragalloyl glucoses, six trigalloyl glucoses, and five digalloyl glucoses were characterized each hypothesized to have unique stereochemistry and as a result a unique bioavailability. Galloyl glycosides and other gallated species are found in many foods stuffs and have the potential to affect the diet of a large percent of the global population.

CHAPTER IV

IN VITRO BIOACCESSIBILITY AND IN VIVO ABSORPTION OF MANGO GALLIC ACID AND GALLOYL GLYCOSIDES

4.1 Introduction

Once considered an exotic delicacy, tropical fruits are gaining popularity in the United States for their sensorial properties, antioxidant capacity, and potential health benefits, and within the last decade, the mango imports to the United States have doubled in value to over $430 million [48]. Extracts from mango pulp, peel, and kernel have been shown to be an antagonist to chronic diseases such as diabetes, cancer, and inflammation and are rich in a wide array of phytochemicals including carotenoids, vitamin C, and polyphenols [124, 125]. The principal polyphenols in mango are the galloyl derivatives, that include gallic acid, gallic acid glycosides, and gallotannins that may range in polymerization from five to twelve galloyl moieties [12].

While several health benefits have been attributed to polyphenols it is possible that these benefits are limited due to low bioavailability [79], and this includes incomplete dissolution from foods at various stages of digestion. The bioavailability of a polyphenol is a result of its capacity to be transported across an enterocyte while its bioaccessibility is defined as the amount of compound in solution and available for absorption. Differences in bioavailability among polyphenolics are a result of a complex balance between a compound's size, polarity, presence of glycosides or acylation, and interaction with the food matrix [126]. The bioavailability, metabolism, and potential

health promoting properties of both gallic acid and pentagalloyl glucose have previously been studied [92, 112], however little is known regarding the metabolic fate of monogalloyl glucose that may account for more than 50% of non-tannin polyphenol content in some mango varieties [11]. In addition, the hydrolysis of galloyl derivatives through enzymatic and non-enzymatic reactions releases free gallic acid and shapes the metabolic reactions taking place upon digestion [127].

Mango is cultivated in tropical and sub-tropical climates and is arguably one of the world's most popular fruits [3]. As such, mango has the potential to affect the diets in many global regions. Understanding the digestion and absorption of galloyl derivatives is critical in the interpretation of potential health benefits of not only mango but of other fruits, nuts or botanicals rich in gallated polyphenolics. The aim of this work was to determine the bioaccessibility of mango galloyl derivatives using in vitro digestion conditions. Additionally, the absorption of monogalloyl glucose and free gallic acid was determined using a Caco-2 monolayer transport model and a pilot in vivo porcine absorption model.

4.2 Materials and Methods

4.2.1 Mango Fruit

Mature Mexican mangos (cv. Ataulfo) were kindly imported and donated by Frontera Produce (Edinburg, TX) and the National Mango Board and shipped refrigerated to the Department of Nutrition and Food Science at Texas A&M University. Fruit were allowed to ripen at ambient conditions, and fruit that exhibited uniform

ripeness based on skin color and manual texture determination were manually peeled, deseeded, cubed, and stored at -20°C in vacuum sealed bags under Good Manufacturing Practices.

4.2.2 Chemicals

Standards of gallic acid, ethyl gallate, pentagalloylglucose, porcine lipase, bile, and pancreatic enzymes were purchased from Sigma Aldrich (St. Louis, MO). A standard of monogalloyl glucose was purchased from Carbosynth Chemicals (Berkshire, UK). Methanol, ethanol, acetone, ethyl acetate, and gastric juice containing pepsin were purchased from Fisher Scientific (Hampton, NH). HPLC grade 0.1% formic acid water and 0.1% formic acid methanol were purchased from Sigma Aldrich (St. Louis, MO).

4.2.3 Quantification of Galloyl Derivatives

Galloyl derivatives from mango pulp were quantified by HPLC and served as a basis for comparison in bioaccessibility experiments. Homogenized mango pulp (10 g) was extracted with 50 mL of extraction solvent containing 1:1 methanol and acetone. The mixture was stirred for 30 min, filtered through Whatman #4 filter paper, and the remaining solids re-extracted twice under the same conditions. The solvents were evaporated under reduced pressure at 45°C and the concentrate dissolved in 30 mL of water with 0.01% formic acid. Samples were filtered through a Whatman 0.45µm PTFE membrane prior to characterization and quantification by HPLC-MS.

4.2.4 In Vitro Bioaccessibility

In vitro digestion conditions for assessing polyphenol bioaccessibility were similar to previously described models with some modifications [128, 129]. In triplicate, 10 g of homogenized mango pulp or 10 g of 0.65 mm^3 mango cubes were placed in 50 mL Falcon tubes. Deionized water (4 mL) was added along with 12 mL of commercial gastric digestion solution containing pepsin to reach a pH of 2.0 ± 0.1, simulating stomach conditions. Samples were held in a ThermoFisher SWB25 (Hampton, NH) shaking water bath at 37 °C. At 0, 1, and 2 h aliquots were removed, centrifuged for 2 min at 4,000 x g, and 1 mL of supernatant obtained for analysis. Following gastric digestion, 0.2 M Na_2CO_3 (16 mL) along with 0.5 mL of a digestive enzyme mixture (2.4 mg/mL bile, 0.4 mg/mL pancreatin, and 0.2 mg/mL lipase) were added to raise the pH to 7.1 ± 0.1 and simulate the conditions of the small intestine and colon. Samples were flushed with nitrogen to create an anaerobic environment and returned to the water bath. At 2, 4, 6, and 8 h aliquots were again centrifuged and 1 mL of supernatant collected. Each aliquot was immediately acidified with 0.1 mL of 88% formic acid, centrifuged at 10,000 x g for 5 min, and filtered through a 0.45µm membrane filter for HPLC-MS analysis. The bioaccessible fraction was calculated by the ratio of the amount (mg) of polyphenol in the supernatant to the initial amount (mg) in 10 g of mango pulp. As a control, a mango extract was likewise prepared from 1 kg of mango, using extraction procedures outlined in Section 2.3. This mango extract and a 10 mg/L standard solutions of gallic acid and monogalloyl glucose underwent the same in-vitro digestion procedures

as the homogenized cube and pulp and represented solutions containing 100% bioaccessible polyphenols.

Following the 10 h in vitro digestion, the amount (mg) of insoluble galloyl derivatives remaining in the digested homogenized mango pulp and cubed mango were additionally extracted and quantified. Galloyl derivatives were isolated by centrifuging remaining digests for 5 min at 4,000 followed by vacuum filtration using Whatman #4 Filter paper for 5 min. The insoluble material was extracted as previously described in Section 2.3, solvents evaporated, reconstituted into acidified water acidified, and filtered through a 0.45 μm membrane for HPLC-MS analysis.

4.2.5 Caco-2 Monolayer Transport

Gallic acid, monogalloyl glucose, and pyrogallol were evaluated in a Caco-2 monolayer trans-epithelial transport model. Caco-2 transport model procedures were based of procedures as previously described [130]. Caco-2 cells were acquired from ATCC (Manassas, VA), and cultured in media containing 84% fetal bovine serum, 4% penicillin, 4% glutamine, 4% sodium pyruvate, and 4% amino acids. Cells between 10-30 passages were seeded on to 2 mm transparent polyester cell culture insert well plates (Transwell, Corning Costar Corp., Cambridge, MA) at 1.0×10^5 cells per insert with 0.5 mL of medium in the apical side and 1.5 mL of medium in the basal side. Monolayers grew for 21 days and confluence was measured by resistance with an EndOhm Volt ohmmeter equipped with a STX-2 electrode (World Precision Instruments Inc., Sarasota, FL); monolayers with a resistance of ($> 450\ \Omega\ cm^2$) were used for experiments. Gallic

acid, monogalloyl glucose, and pyrogallol were applied to apical layer at 50 mg/L, and 200 µL aliquots were taken from the basal layer at 0.5, 1, 1.5, and 2 h, and replaced with 200 µL of media. Aliquots were acidified with 2.5 µL 88% formic acid prior to HPLC-MS analysis.

4.2.6 Porcine Model

In the porcine absorption model, a female crossbred piglet (32 kg, aged 10 wks) was obtained from a commercial breeder in Rosenbaum Farms, Brenham, TX, USA. Care of the animal in preparation for surgical procedures and post-surgical care was performed as previously described [131]. One week prior to surgery the piglet was adapted to its environment inside a 2 x 3 m kennel at 21-25 °C and fed a diet lacking polyphenols (Research Diets Inc, New Brunswick, NJ). Surgery was then performed to insert dwelling catheters into the stomach and caval vein to enable rapid infusion of polyphenols and blood sampling. Post-surgery, the pig was familiarized to a movable cage (0.9 m x 0.5 m x 0.3 m) with experiments beginning 1 wk later. The study protocol was approved by the Institutional Animal Care and Use Committee of Texas A&M University prior performing the animal experiment.

Gallic acid and monogalloyl glucose were administered orally in independent experiments via the stomach catheter at a dose of 32 mg /kg of body weight in a 100 mL 0.2 M citric acid vehicle at a pH of 3.5. Blood collection times were 0.25, 0.5, 0.75, 1, 1.5, 1.75, 2, 3, 4, 5, 6, 8, and 10 h post oral administration at 2 mL per collection through the venous port. Collected blood was centrifuged for 5 min at 3,500 x g, and 500 µL

plasma was aliquoted and acidified with 25 μL 88% formic acid. Prior to LC-MS analysis, 50 μL of ethyl gallate as the internal standard was infused with the plasma, and samples were precipitated with 150 μL of 10% SDS and 300 μL methanol. Samples were spun at 10,000 x g for 5 min and the supernatant filtered through a 0.45 μm filter. Additionally, gallic acid and monogalloyl glucose were administered intravenously in independent experiments via the dwelling catheter of the venous port at a dose of 48 mg. The doses were administered in a 50 mL vehicle containing sodium carbonate buffered to a pH of 7.0. Prior to administration vehicles were sterile filtered via a 0.22 μ filter. Following administration, the catheter was flushed with saline and a sample was immediately taken for a baseline concentration. Additional blood samples were collected at 2.5, 5, 7.5, 10, 15, 20, 30, 60, 90, and 120 min, and were processed in the same manner as the samples in the oral administration. Non-compartmental pharmacokinetic analysis was conducted for both oral and intravenous administrations with the PKSolver add-in for Microsoft Excel 2013 [132]. Absolute bioavailability was calculated using:

$f = \frac{AUC_{Oral} * Dose_{IV}}{AUC_{IV} * Dose_{Oral}}$ with f as absolute bioavailability and AUC as area under the curve. Results were calculated from two independent extractions of porcine plasma and reported as the mean ± standard error of the mean.

4.2.7 Chemical Analysis

Galloyl derivatives were quantified using a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI source. Separations were in

reversed-phase using a Finnigan Surveyor HPLC coupled to a Surveyor PDA detector and gradient separations were performed using a Phenomenex Kinetex™ (Bannockburn, Il) C18 column, (150 x 4.6 mm, 2.6 μm) at room temperature. Injections were made into the column by use of a 50 μL sample loop. For separation of gallic acid and monogalloyl glucose mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in methanol run at 0.45 mL/min. A gradient was run of 0% Phase B for 2 min and changed to 10% Phase B in 4 min, 10% Phase B was held to 10 min, 10 to 40% Phase B in 25 min, and 40% to 65% Phase B in 35 min, 65% to 85% Phase B in 41 min, 85% was held to 49 min before returning to initial conditions. The electrospray ionization interface was in negative ionization mode, source and capillary temperatures were set at 325°C, source voltage was 4.0 kV, capillary voltage at -47 V, and collision energy for MS/MS analysis was 35 eV. The instrument operated with sheath gas and auxiliary gas (N_2) flow rates set at 10 units/min and 5 units/min, respectively. The instrument was tuned for gallic acid and along with monogalloyl glucose was quantified at 280 nm and the gallic acid metabolites 4-*O*-methylgallic acid and 4-*O*-methylgallic acid-3-*O*-sulfate were quantified using extracted ion chromatograms.

A second analysis protocol was used to separate and quantify gallotannins using a 250 x 4.6 mm, 2.6 μm Waters Sunfire C18 column (Milford, MA), and the conditions of Luo et al. (2014) with modification [103]. Mobile phase A was 0.1% formic acid in water and Mobile phase B was 0.1 % formic acid in acetonitrile run at 0.6 mL/min. A gradient of 10% Phase B was run for 2 min and changed to 25% Phase B at 6 min, 35% Phase B was held to 30 min, 35 to 45% Phase B in 35 min, and 45% to 55% Phase B in

40 min then returned to initial conditions. In negative ionization mode, source and capillary temperatures were set to 325°C, source voltage to 4.0 kV, and capillary voltage at -43 V. Sheath gas and auxiliary gas were likewise set to 10 units/min and 5 units/min, respectively. The instrument was tuned for pentagalloyl glucose and gallotannins quantified using extracted ion chromatograms.

4.2.8 Statistics

In vitro digestion and Caco-2 experiments were evaluated in triplicate and in independent reaction vessels. For the porcine model, two independent plasma extractions were performed prior to LC-MS analysis. Statistical differences were compared using unpaired student's t-test in JMP software. All values are reported as the mean ± standard error the mean.

4.3 Results and Discussion

4.3.1 Galloyl Derivatives in Mango cv. Ataulfo

Previous reports have documented the high concentrations of gallated phenolics and gallotannins in the pulp of the Ataulfo cultivar compared to other mango varieties [118, 119]. Eight galloyl derivatives where characterized in the Ataulfo pulp used in these studies which included gallic acid, esterified monogalloyl glucose, and six tannins ranging in degree of galloyl conjugation from 5 to 10 galloyl groups (Table 3).

Table 3. Characterization and concentrations (mg/kg) of galloyl derivatives in mango (cv. Ataulfo) pulp.

Compound	λ_{max} (nm)	$[M-H]^-$ (m/z)	$[M-2H]^{2-}$ (m/2z)	MS/MS (m/z)	Concentration (mg/kg)
mono-galloyl glucose	278	331.2	-	271.1, 169.2, 125.3	229 ± 9.33[1]
gallic acid	271	169.2	-	125.3	9.09 ± 0.170
penta-galloyl glucose	279	939	469	-	6.56 ± 1.20
hexa-galloyl glucose	279	1091	545	-	38.9 ± 7.04
hepta-galloyl glucose	279	1243	621	-	86.9 ± 10.4
octa-galloyl glucose	279	1395	697	-	90.5 ± 9.94
nona-galloyl glucose	279	1647	773	-	118 ± 9.35
deca-galloyl glucose	279	1899	849	-	68.4 ± 7.34

1. Values are reported as the mean ± SEM

Characterizations were based on UV and mass spectra compared to published values for these compounds [9, 104]. Monogalloyl glucose was present in the highest concentration at 229 ± 9.3 mg/kg compared to 9.09 ± 0.17 mg/kg for free gallic acid. Cumulatively, the six gallotannins totaled 409 ± 45.4 mg/kg and individually ranged in concentration from 6.56 ±1.20 mg/kg for pentagalloyl glucose and 117 ± 9.34 mg/kg for nonagalloyl glucose when quantified in equivalents of pentagalloyl glucose.

4.3.2 In Vitro Bioaccessibility

The bioaccessibility of mango polyphenolics from homogenized mango pulp and cubed mango was compared to a 100% bioaccessible mango extract that served as a control under in vitro digestion conditions (Figure 13). Monogalloyl glucose was readily soluble in the gastric solution at 0 h in both homogenized mango pulp and cubed mango with an initial bioaccessibility of 75.1 ± 6.25%, and 61.0 ± 5.80%, respectively, and no significant ($p<0.05$) changes occurred during the first 2 h under gastric conditions. At 4 h, 2 h following the pH transition from the stomach to the intestines, the bioaccessibility of monogalloyl glucose significantly increased for homogenized and cubed mango to 98.0 ± 4.58% and 85.0 ± 10.58%, respectively, while the concentration of monogalloyl glucose in the mango extract decreased to 81.2 ± 2.49% of its initial concentration. This indicates that the mesocarp structure may be critical in stabilizing monogalloyl glucose in both digestive acidic or alkaline conditions. Krook and Hagerman (2012) have previously demonstrated the ability of a food matrix to increase the stability of a

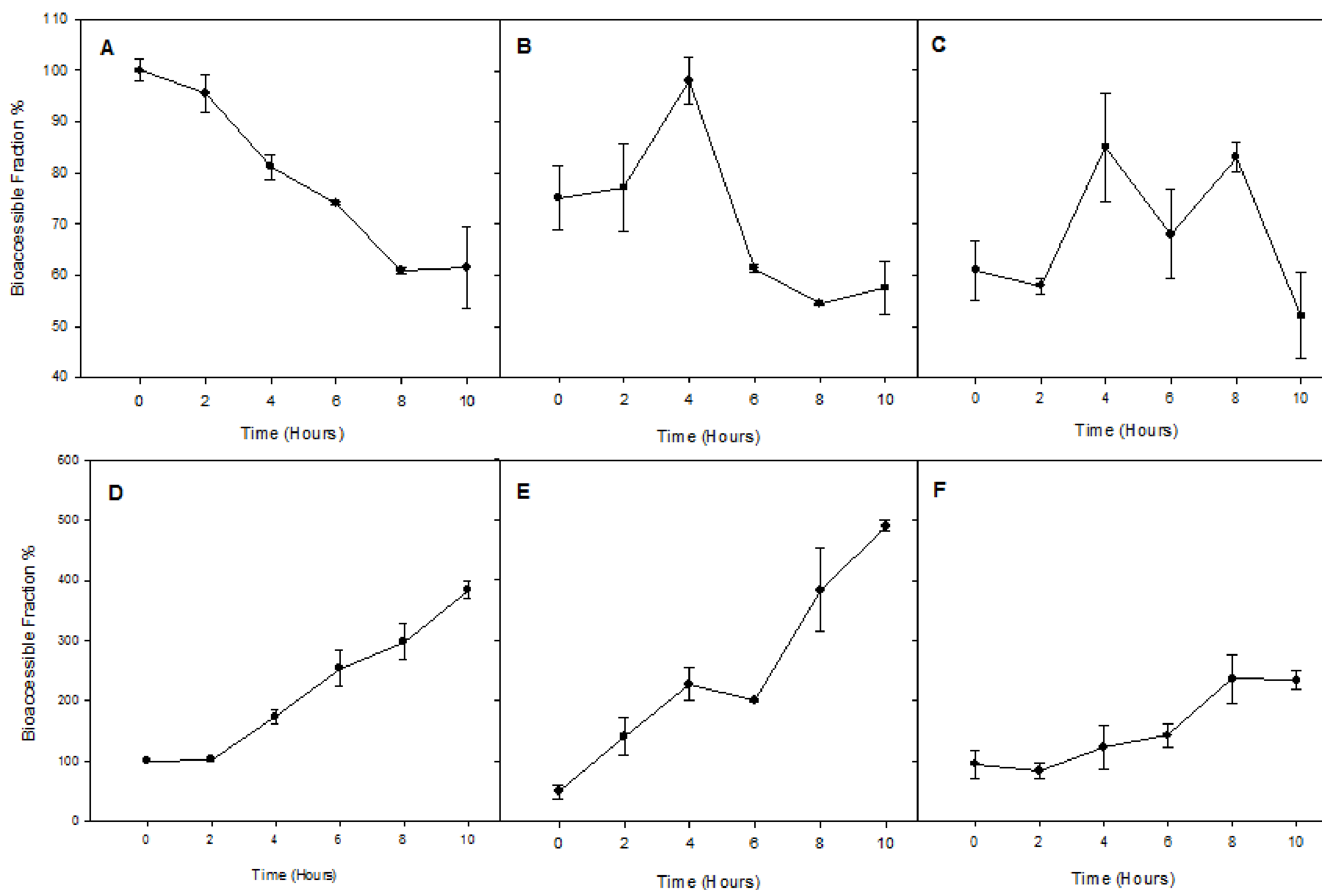

Figure 13. Bioaccessibility of monogalloyl glucose in (A) mango extract, (B) homogenized mango pulp, and (C) cubed mango and free gallic acid in (D) mango extract, (E) homogenized mango pulp, and (F) cubed mango under in vitro digestion conditions at 37 °C, pH 2.0 for 2 h and pH 7.1 for 8 h, with digestive enzymes.

polyphenol in intestinal conditions with standards of pentagalloyl glucose and epigallocatechin gallate when incubated with proteins, starches, and fiber [71]. After 10 h of digestion, a significant loss in monogalloyl glucose was observed and concentrations decreased to 61.6 ± 7.98%, 57.9 ± 5.23%, and 52.1 ± 8.41% for the mango extract, homogenized mango pulp, and cubed mango, respectively. Likewise, when a standard of monogalloyl glucose was held under in vitro digestion conditions only 60.3 ± 2.41% remained after 10 h (Figure 14). Polyphenols are prone to auto-oxidation under duodenal to colonic pH conditions, and this may lead to a progressive

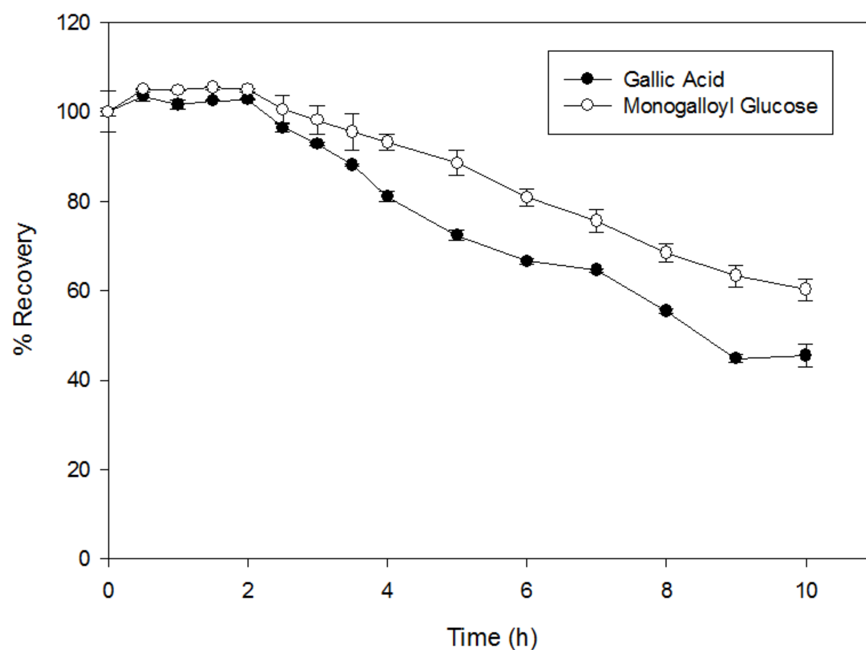

Figure 14. Stability of gallic acid and monogalloyl glucose following in vitro digestion at 37 ºC pH 2.0 for 2 h and pH 7.1 for 8 h, with digestive enzymes.

formation of oxidized and polymerized compounds in the small and large intestines [133]. Results suggest that when polyphenol bioaccessibility is increased during digestion, as observed for homogenized and cubed mango pulp, its concentration may rapidly decrease due to instability under elevated pH conditions.

As opposed to monogalloyl glucose, mango gallotannins were found to have limited bioaccessibility following in vitro digestion with no quantifiable amounts of gallotannins in the digestion solution. When post-digestion solvent extractions were performed on the fruit preparations, it was found that 51.5 ± 8.12% and 88.5 ± 1.03% of the initial amount of gallotannins remained in the fruit matrix for homogenized mango pulp and cubed mango, respectively (Figure 15). The amount of gallotannins remaining in the cubed mango matrix was significantly ($p<0.05$) higher compared to homogenized mango pulp and this mirrored a previous study with mango where the degree of fruit maceration was significantly correlated to increased carotenoid bioaccessibility [134]. Upon ingestion, gallotannins bound to the food-matrix in the fruit pericarp may become bioaccessible due to the microbial degradation of pectins and other cell wall materials by intestinal microflora [135]. The release of mango gallotannins in the colon could lead to potential health benefits; mainly through enzymatic hydrolysis to produce bioaccessible gallic acid and pyrogallol, the major microbial metabolite [136].

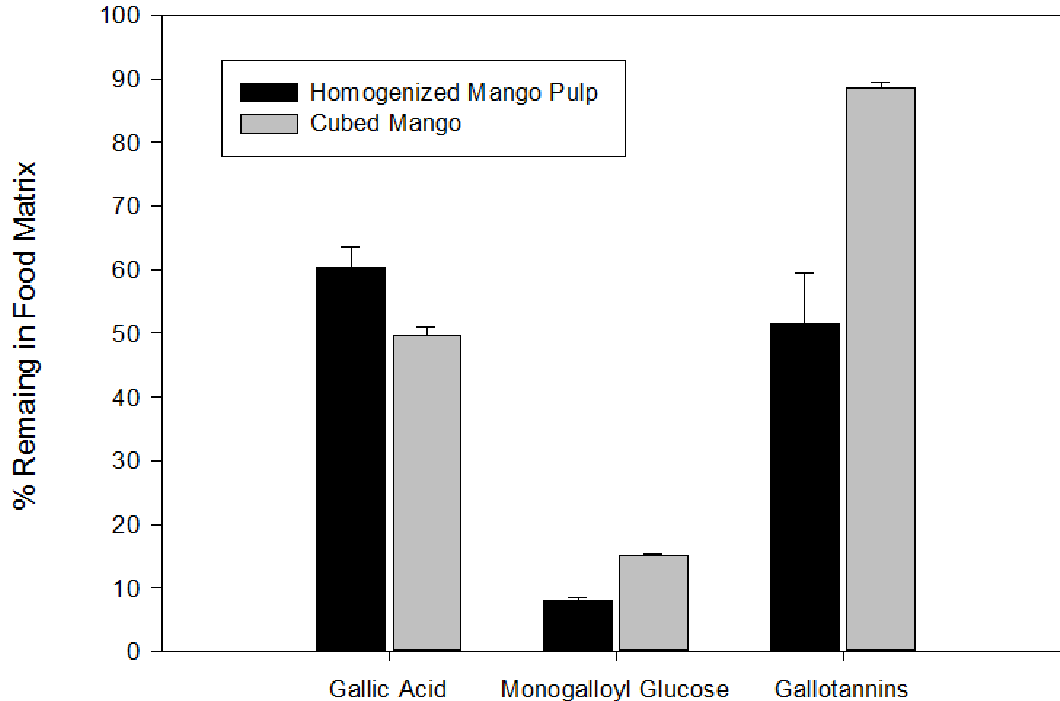

Figure 15. Gallic acid, monogalloyl glucose, and gallotannins bound to cellular matrices of homogenized mango pulp and cubed mango following in vitro digestion at 37 °C pH 2.0 for 2 h and pH 7.1 for 8 h with digestive enzymes.

Free gallic acid significantly ($p<0.05$) increased after 10 h of the in vitro digestion by 384 ± 14.9%, 490 ± 9.68% and 233 ± 15.8% for mango extract, homogenized mango pulp, and cubed mango, respectively (Figure 13). Gallic acid commonly experiences extensive degradation when exposed to physiological pH conditions and when an authentic standard was incubated under in vitro digestion conditions the amount of gallic acid decreased to 45.4 ± 2.51% of its initial concentration over 10 h (Figure 14). This indicates that the gallic acid concentration determined after 10 h is a result of simultaneously occurring generation from gallotannin hydrolysis and degradation of gallic acid under intestinal pH conditions [137]. In

contrast, monogalloyl glucose is not likely to be a source of hydrolyzed gallic acid as when monogalloyl glucose was incubated alone under in vitro digestive conditions free gallic acid was not produced. In vitro digestion of mango extract and homogenized pulp resulted in significantly higher amounts of bioaccessible gallic acid in conjunction with a greater concentration of gallotannins being released into solution while significantly less gallic acid was produced from cubed mango. This indicates that the majority of gallotannins in cubed mango may be able to reach the colon and be available for microbial metabolism, potentially resulting in higher production of pyrogallol. It is unknown how these differences might affect bioactivity, however pyrogallol the principle colon metabolite of gallic acid has been reported to have anti-carcinogenic properties [138, 139], and it may be beneficial to choose a food matrix that retains gallotannins throughout upper intestinal digestion to support its generation in the colon.

4.3.3 Caco-2 Monolayer Transport

For the first time the transport of monogalloyl glucose was evaluated in a Caco-2 monolayer model and compared to gallic acid and pyrogallol. Physiologically, the absorption of polyphenolics from the small intestine is governed by stereochemistry and polarity, and many higher molecular weight polyphenols or those with polar functional groups are less likely to be absorbed [72]. Gallic acid and monogalloyl glucose were separately applied to the apical layer at 50 μg/mL and their transport rates (apical to basolateral) were 160 ± 59.4 μg/ml·h and 257 ± 43.1 μg/ml·h, respectively (Table 4). The hydrophilicity of gallic acid decreases its ability to partition across the bi-lipid

Table 4. Transport of 50 μg/mL gallic acid, monogalloyl glucose, and pyrogallol across the apical to basolateral side of Caco-2 monolayers over 2 h.

	% Transport				Transport Rate (μg/mL·h)
	0.5 h	1 h	1.5 h	2 h	
gallic acid	0.23 ± 0.05[1,a]	0.52 ± 0.15[a]	0.58 ± 0.15[a]	0.67 ± 0.13[a]	160 ± 59.4[a]
monogalloyl glucose	0.10 ± 0.01[a]	0.23 ± 0.02[a]	0.72 ± 0.10[a]	0.98 ± 0.14[a]	257 ± 43.1[a]
pyrogallol	1.55 ± 0.34[b]	3.51 ± 0.11[b]	5.55 ± 0.90[b]	4.19 ± 0.53[b]	1860 ± 341[2,b]

1. Values are reported as the mean ± SEM, and different letters in the same column are significantly different ($p < 0.05$).

2. Transport rate for pyrogallol was calculated only to 1.5 h.

membrane of an enterocyte and its proposed mechanism for absorption was hypothesized to be through para-cellular transport [74]. After 2 h of transport, $0.98 \pm 0.14\%$ of monogalloyl glucose had transported compared to the $0.67 \pm 0.13\%$ for gallic acid, a non-significant difference. With similar transport rates it is possible that monogalloyl glucose transports through the same mechanism as gallic acid, however given the glucose moiety there is potential for glucose transporter interactions [76]. Pyrogallol had a significantly ($p<0.05$) higher transport rate, $1{,}860 \pm 341$ μg/ml·h, and the amount transported after 2 h, $4.19 \pm 0.53\%$, when compared to either gallic acid or monogalloyl glucose. While not innately present in mango, pyrogallol has been reported to be the main colonic metabolite of gallic acid due to bacterial decarboxylase activity [140, 141]. From 1.5 to 2 h the amount of pyrogallol transported significantly ($p<0.05$) decreased from $5.55 \pm 0.90\%$ to $4.19 \pm 0.53\%$. Pyrogallol is exceptionally unstable compared to other polyphenolics especially under low acid conditions, and this may have resulted in an under-estimation of actual basolateral concentrations [142].

Table 5. Pharmacokinetic parameters for gallic acid and monogalloyl glucose following oral and IV administration in a 10 h pilot porcine absorption study.

	Oral			IV			
	C_{max} (mg/L)	T_{max} (h)	AUC_{oral} (mg/L*h)	AUC_{IV} (mg/L*h)	Half-life (h)	V_d (L)	Bioavailability $_{abs}$ [1] (%)
gallic acid	3.42 ± 1.07	0.5	3.57 ± 0.07	0.98 ± 0.20	0.10 ± 0.04	4.54 ± 1.1	19.3 ± 4.40
monogalloyl glucose	0.13 ± 0.05	0.5	0.09 ± 0.03	2.71 ± 1.31	0.34 ± 0.05	10.9 ± 2.18	0.28 ± 0.20

1. Absolute bioavailability was calculated using: $f = \frac{AUC_{Oral} * Dose_{IV}}{AUC_{IV} * Dose_{Oral}}$, with f as absolute bioavailability and AUC as area under the curve.

4.3.4 Bioavailability of Gallic Acid and Monogalloyl Glucose in a Porcine Model

The bioavailability of gallic acid and monogalloyl glucose was evaluated in a porcine model and qualitatively compared to transport in the Caco-2 cell monolayer. Using buffered solutions of gallic acid and monogalloyl glucose administered orally and intravenously, absorption was estimated by monitoring concentrations of gallic acid and monogalloyl glucose and key phase II metabolites. The compounds 4-*O*-methylgallic acid and 4-*O*-methylgallic acid-3-*O*-sulfate were previously identified as the predominant metabolites following gallic acid consumption [65, 82], and therefore were monitored in porcine plasma over a 10 h period through surgically inserted blood-draw catheters in this study. As an internal standard, ethyl gallate was recovered at 90.9 ± 0.23% across all samples. Non-compartmental pharmacokinetic analysis revealed the C_{max} for gallic acid and monogalloyl glucose as 3.42 ± 1.07 mg/L and 0.13 ± 0.05 mg/L and an AUC of 3.57 ± 0.07 mg/L·h and 0.09 ± 0.03 mg/L·h, respectively (**Table 5**). Despite a similar absorption rate in the Caco-2 model, monogalloyl glucose was found to be significantly ($p<0.05$) less absorbed than gallic acid. Gallic acid was quickly cleared from porcine plasma with a half-life of 0.10 ± 0.04 h (6 min). In contrast monogalloyl glucose had a half-life of 0.34 ± 0.05 h (20 min), and the absolute bioavailability for gallic acid and monogalloyl glucose was found to be 19.3 ± 4.40% and 0.28 ± 0.20%, respectively. The rapid elimination of gallic acid and monogalloyl glucose from plasma is significantly higher when compared to other polyphenolics such as epigallocatechin gallate which when intravenously administered in rats was reported to have a half-life of 51 min [143]. This suggests that gallic acid is more bioavailable than previously thought

and due to the short half-life any gallic acid that is absorbed will only be in circulation for a short time prior to renal excretion. Previous investigations that report the pharmacokinetics of gallic acid have only performed oral administration experiments which lacks data from intravenous administration needed to calculate absolute bioavailability, the relationship between oral absorption and intravenous clearance [84, 144]. This work indicated that in a porcine model, gallic acid has an absolute bioavailability near 20%, and supports previous investigations where high amounts of free and metabolized forms of gallic acid are found in urine but with low concentrations in plasma [38, 55, 145, 146].

Classic pharmacokinetic models, while useful in measuring the kinetics of the analyte of interest, do not take into consideration changes in analyte concentration due to phase II metabolism. Both gallic acid and monogalloyl glucose were converted to phase II metabolites with AUCs for gallic acid, 4-*O*-methylgallic acid, and 4-*O*-methylgallic acid-3-*O*-sulfate of 3.57 ± 0.07, 4.90 ± 1.30, and 7.86 ± 1.18 mg/L*h after oral administration of gallic acid and 2.73 ± 1.45, 5.08 ± 0.96, and 9.19 ± 2.38 mg/L*h after oral administration of monogalloyl glucose (Figure 16). Both gallic acid and monogalloyl glucose produced a similar concentration of gallic acid metabolites, but no methyl, sulfur, or glucuronide conjugates of monogalloyl glucose were detected. In context with their high bioaccessibility from mango fruit and Caco-2 transport, data suggest that monogalloyl glucose is absorbed in vivo but derived metabolites are not detected until deglycosylation occurs prior to being found in circulation, either in the intestines or liver. Monogalloyl glucose is the predominate polyphenol in several mango

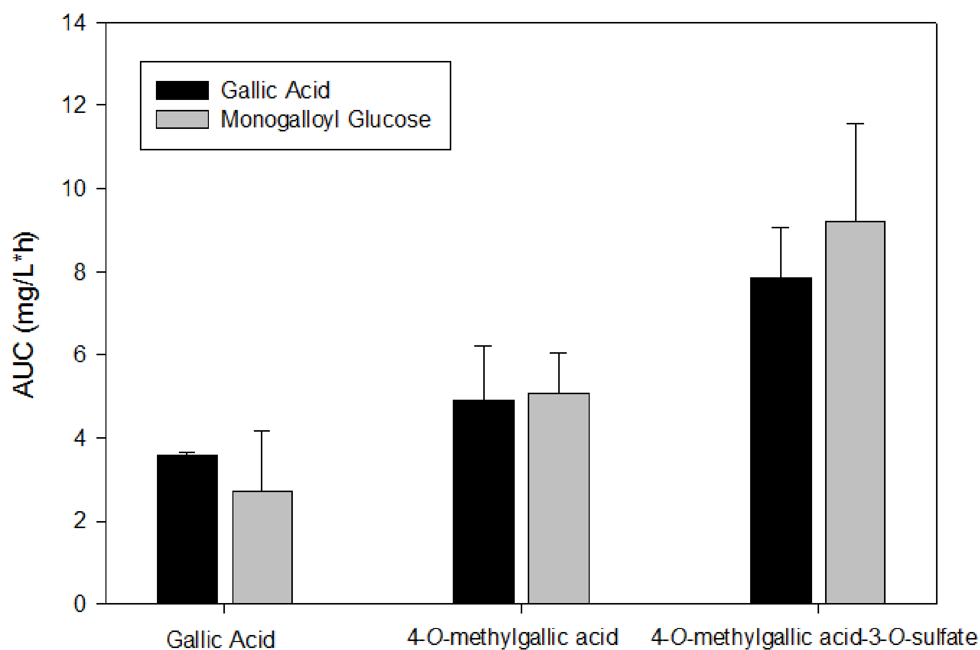

Figure 16. Area under the curve (AUC) for the metabolites gallic acid, 4-*O*-methylgallic acid, and 4-*O*-methylgallic acid-3-*O*-sulfate after oral administration of gallic acid and monogalloyl glucose determined in a porcine model.

varieties, and in the Ataulfo mango pulp analyzed in this study, it accounted for 35% of the total gallated polyphenolic content. The ability for monogalloyl glucose to have the same metabolic potential as free gallic acid is critical as the concentration differences between gallic acid and monogalloyl glucose among mango cultivars may not affect their bioefficacy.

Unexpectedly, no metabolites sourced from pyrogallol were identified in porcine plasma after oral administration trials. Pyrogallol metabolites were previously reported in trials where gallic acid containing foods were orally administered in humans [38, 55]. The lack of pyrogallol metabolites in the porcine model indicates that either the majority

of gallic acid and monogalloyl were absorbed in the small intestine or were degraded due to prolonged exposure in digestion conditions as previously discussed in Section 4.2, leaving only a limited amount available for colonic metabolism. In summary, both gallic acid and monogalloyl glucose are bioaccessible from mango fruit preparations, able to be absorbed in the small intestine, and able to produce similar amounts of the phase II metabolites, 4-*O*-methylgallic acid and 4-*O*-methylgallic acid-3-*O*-sulfate.

4.4 Conclusions

The polyphenol composition of mango pulp is comprised primarily of galloyl derivatives. In this study, the galloyl derivatives monogalloyl glucose and gallic acid were found to be bioaccessible in both homogenized and cubed mango pulp and have the potential to be absorbed and metabolized. A significant amount of gallotannins remained bound within the food matrix for both homogenized and cubed mango suggesting limited bioaccessibility in the small intestine and will transit to the colon. For the first time gallic acid and monogalloyl glucose were shown to be transported across Caco-2 monolayers at a similar rate. In a porcine model, similar amounts of phase II galloyl metabolites were found for gallic acid and monogalloyl glucose following oral administration, and this may suggest that the differences in composition of non-tannin galloyl derivatives between mango cultivars is not a major factor impacting the physiological production of gallic acid metabolites.

CHAPTER V

A NOVEL PROTEIN PRECIPITATION METHOD FOR THE RECOVERY OF GALLIC ACID METABOLITES IN PLASMA

5.1 Introduction

An inverse correlation between cancer and cardiovascular disease with increased levels of fruit and vegetable consumption is well established in a number of meta-analyses [17-20, 22]. This has largely been attributed to the phytochemical content of fruits and vegetables and specifically their polyphenols, secondary metabolites of all higher plants. Original hypotheses predicted the antioxidant properties of polyphenols were the source of their health promoting properties. However, numerous investigations report polyphenolics to have poor bioavailability, and any that are absorbed will undergo extensive xenobiotic metabolism or later experience catabolism by micro-organisms residing in the gastrointestinal tract that change their chemical properties [79]. This has led to the conclusion that the metabolites, not the precursors naturally found in fruits and vegetables, are likely a source of polyphenol's health benefits. Current research efforts create profiles of the potential metabolites that may occur after consumption of specific foods, which can then be tested for bioefficacy [38, 66, 147].

LC-MS is the method of choice for analysis of polyphenol metabolites in biological matrices. However, sample preparation techniques for analysis of polyphenol metabolites contrast to that of their parent compounds present in fruits and vegetables due to conjugation with methyl, sulfate, and glucuronide functional groups from phase II

enzymes. Additionally, analyte concentrations are inherently low, often at the nM level, and are complicated by protein and other matrix characteristics of biological samples. Currently there is no standard method for isolating polyphenols from biological matrices, and as the chemistries of the analytes differ depending on the class of polyphenol being analyzed sample preparation methodologies may differ for each investigation [59, 61, 66, 100, 101]. The two most commonly reported methods for metabolite extraction utilize an organic solvent such as acetonitrile, methanol, or ethyl acetate or solid phase extraction (SPE) using various stationary phases with reversed phase the most common. Gallic acid is reported as one of the least recoverable polyphenolics in biological matrices likely due to its innate protein binding ability and hydrophilicity compared to other polyphenolics and these physicochemical properties make it difficult to extract with solvents or partition with SPE [148].

No reports are available on the extraction and recovery of gallic acid's phase II metabolites from plasma, likely due to the lack of analytical standards. Metabolites may differ in extraction efficiency in relation to the parent compound with the addition of methyl and sulfur groups. For example, methylated epigallocatechin-gallates were found with enhanced plasma protein binding affinity compared to non-methylated forms [102]. In this study, a new method for extraction of gallic acid metabolites from plasma is described utilizing sodium dodecyl sulfate (SDS) as a protein denaturant and was evaluated against current state of the art methods in literature. The method was subsequently applied to an acute mango feeding trial with 10 subjects to investigate the pharmacokinetics of mango galloyl metabolites.

5.2 Materials and Methods

5.2.1 Chemicals and Supplies

Authentic standards for gallic acid, pyrogallol, and ethyl gallate were acquired from Sigma Aldrich (St Louis, MO). 4-*O*-methylgallic acid was acquired from Apin Chemicals (Oxfard, UK). Methanol, acetonitrile, and 88% formic acid were acquired from Thermo Fisher Scientific (Waltham, MA). HLB columns (100 mg) were acquired from Waters Corp. (Milford, MA). Prepared solvents for HPLC made of 0.1% formic acid water and 0.1% formic acid methanol were acquired from Sigma Aldrich, and 3M KCl solution was from Thermo Fisher Scientific.

5.2.2 SDS Plasma Precipitation

Recovery of pyrogallol, gallic acid, 4-*O*-methylgallic acid, and ethyl gallate in human plasma was performed by taking 475 µL of plasma acidified with 25 µL of 88% formic acid and mixing 50 µL of a standard solution containing pyrogallol, gallic acid, 4-*O*-methylgallic acid, and ethyl gallate in 1.5 mL Eppendorf tubes. Proteins were precipitated by adding 75 µL of 10% SDS and tubes gently mixed to ensure adequate binding to plasma albumins. Lastly, 400 µL of methanol acidified with 0.1% formic acid was added and tubes vigorously vortexed for 20 seconds followed by sonication in a Branson 3510 sonicator (Danbury, CT) for 10 minutes. Tubes were centrifuged at 10,000 x g for 5 min at 4 ºC and excess SDS in the supernatant removed by centrifugation after the addition of 50 µL of 3 M KCl. Samples were held at 4 ºC for 1 h prior to analysis.

5.2.3 Method Evaluation

The use of SDS to precipitate plasma proteins was evaluated for extraction recovery, matrix effect, and inter-day precision across three concentrations (50, 250, and 500 μg/L). Extraction recovery was calculated from polyphenolic-free plasma spiked with standard solutions and compared to aqueous standards under identical conditions. The matrix effect from plasma was calculated by recovery from SDS precipitated plasma spiked with standard solutions and compared to an aqueous standard of the same concentration. Inter-day precision was calculated from coefficient of variation between extractions of spiked standards performed across three different days. The limit of detection (LOD) and limit of quantification (LOQ) were found using aqueous standards and defined as the signal to noise ratio of 3/1 and signal to noise ratio of 5/1, respectively.

5.2.4 Plasma Extraction Comparisons

Two commonly reported methods to extract or concentrate polyphenolic metabolites from blood plasma are solvent precipitation with acetonitrile and SPE with reversed phase chemistry. Methods were based off prior studies that reported high recoveries of gallic acid [61, 99]. Polyphenol-free human plasma (200 μL) was spiked with 50 μl of a mixture containing standards of pyrogallol, gallic acid, 4-methylgallic acid, and ethyl gallate. Plasma was acidified to denature proteins with 800 μL of 0.1 % formic acid in acetonitrile added dropwise, sonicated for 10 minutes, centrifuged at 10,000 x g, and dried under vacuum at 65 °C in a Thermo Fisher SpeedVac. The extract

was reconstituted in 200 µL of 1:1 methanol and water containing 0.1% formic acid for HPLC-MS analysis. For solid-phase extraction, a 100 mg Waters Oasis® HLB cartridge was activated with 10 column volumes of methanol with 0.1% formic acid followed by 10 column volumes of water with 0.1% formic acid using a vacuum manifold. Polyphenolic-free plasma (500 µL) was diluted with 1,500 µL of water with 0.1% formic acid and dropwise passed through the column. The column was washed with 2 column volumes of acidified water and polyphenolics eluted with 2,000 µL of acidified methanol and evaporated to dryness under vacuum. Isolates were reconstituted into 200 µL of 1:1 methanol and water containing 0.1% formic acid for HPLC-MS analysis.

5.2.5 LC-MS Analysis

Spiked standards and phase II metabolites of gallic acid and pyrogallol were characterized and quantified using a Thermo Finnigan LCQ Deca XP Max MSn ion trap mass spectrometer equipped with an ESI source in TurboScan Mode. Separations were in reversed-phase using a Finnigan Surveyor HPLC coupled to a Surveyor PDA detector and gradient separations were performed using a Phenomenex Kintex™ (Bannockburn, Il) C18 column, (150 x 4.6 mm, 2.6 µm) at room temperature. Injections were made into the column by use of a 50 µL sample loop. For separation of galloyl metabolites mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in methanol run at 400 µL/min. A gradient was run of 0% Phase B for 5 min and changed to 5% Phase B in 10 min, 5 to 10% Phase B in 15 min, 10 to 20% Phase B in 25 min, and 20% to 80% Phase B in 35 min, and 80% Phase B was held to 39 min before

returning to initial conditions. MS analysis began 10 min into the run for quantifications and selected reaction monitoring (SRM) for characterization of metabolites in the absence of authentic standards. The electrospray interface worked in negative ionization mode tuned for 4-*O*-methylgallic acid with source and capillary temperatures set at 325°C, source voltage at 4.0 kV, capillary voltage at -47 V, and collision energy for MS/MS analysis at 35 eV. The instrument operated with sheath gas and auxiliary gas (N_2) flow rates set at 8 units/min and 0 units/min, respectively. All samples from a subject were run during the same set of runs. Following a set of runs the column was washed with 80:20 acetonitrile and methanol with 0.1% formic acid at 400 µL/min for 1 h.

5.2.6 Pharmacokinetics of Mango Gallic Acid Phenolics

Ten healthy human volunteers (aged 21-39) with no history of chronic diseases or digestive disorders were recruited for a mango consumption trial. Mature, unripened mangos (cv. Ataulfo) sourced from Mexico were imported by Frontera Produce (Edinburg, TX) and the National Mango Board, and shipped refrigerated to the Department of Nutrition and Food Science at Texas A&M University. Fruit were allowed to ripen under ambient conditions, and fruit that exhibited uniform ripeness, based on skin color and manual texture determination, were manually peeled, deseeded, cubed, and stored at -20°C in vacuum sealed bags under Good Manufacturing Practices.

Participants were asked to refrain from high polyphenolic foods and especially gallic acid containing foods such as berries, grapes, tea, and wine and filled out a 72 h

food survey prior to the study. After an overnight fast a baseline blood sample was collected into tubes containing EDTA then centrifuged at 3,000 x g for 5 min at 4°C. Participants then consumed 400 g of cubed mango pulp, and blood samples collected through a blood catheter at 0.5, 1, 1.5, 2, 3, 4, 6, and 8 h. Plasma was aliquoted and acidified with 20 µL of 88% formic acid for every 500 µL of plasma, and held at -80°C. This study was approved by the Institutional Review Board (IRB) at Texas A&M University.

5.2.7 In Vivo Method Comparison

The ability of the SDS protein precipitation method to extract gallic acid and pyrogallol phase II metabolites was compared against the acetonitrile and SPE extraction methods. Plasma samples from the pharmacokinetics trial were pooled from 2 and 8 h post prandial and were extracted in triplicate with the SDS protein precipitation method, acetonitrile precipitation, and SPE with HLB chemistry outlined in the Method Comparison Section.

5.2.8 Statistics

Trials for method development were performed in triplicate in individual reaction vessels. All statistics were performed by the use of SigmaPlot 12.5. Significant differences were found by use of Student t-test. All results are reported as mean ± standard error of the mean. Non-compartmental pharmacokinetics were calculated using PkSolver previously developed by Zhang et al. (2010) with area under the curve being

calculated by the linear trapezoidal method [132]. The sample sizes for calculation of phase II metabolites standard error of the mean were the number of subjects with quantifiable amounts of metabolites.

5.3 Results and Discussion

5.3.1 SDS Plasma Precipitation

The ability of SDS to precipitate human plasma proteins and disrupt polyphenol-protein interactions for the analysis of pyrogallol, gallic acid, 4-*O*-methylgallic acid, and ethyl gallate was investigated. SDS is commonly used as a protein denaturant in SDS-PAGE applications, and has previously been demonstrated to separate tannin and protein complexes in numerous reports [149-151]. SDS is unique in its mechanism of protein precipitation as it unfolds proteins into their primary structure as opposed to solvent precipitation which denatures proteins by refolding amino acid side chains into a different tertiary structure [152]. Recovery from and matrix interactions in human plasma were determined by spiking polyphenolic-free human plasma with authentic standards of pyrogallol, gallic acid, 4-*O*-methylgallic acid, and ethyl gallate. LC-MS analysis allowed for detection of targeted analytes in aqueous solutions with a LOD for pyrogallol, gallic acid, 4-*O*-methylgallic acid, and ethyl gallate at 31.3, 3.90, 15.6, and 1.95 μg/L and LOQ at 62.5, 7.80, 31.3, and 3.90 μg/L, respectively. Gallic acid had the poorest recovery using SDS for protein precipitation at 79.6 ± 1.6, 79.9 ± 3.84, and 78.9 ± 4.10 % at, 50, 250, and 500 μg/L, respectively (Table 6). Recovery of 4-*O*-methylgallic acid and ethyl gallate exceeded 82% at all concentrations evaluated.

Table 6. Extraction recoveries, matrix effect, and inter-day precision of gallic acid metabolites (pyrogallol, gallic acid, 4-*O*-methylgallic acid, and ethyl gallate) in human plasma using sodium dodecyl sulfate at three concentrations (50, 250, and 500 μg/L).

Analytes	Concentration (μg/L)	Recovery[1]	Matrix Effect[2]	Inter-Day Precision (CV%)[3]
pyrogallol	50	ND[4]	ND	ND
	250	ND	ND	ND
	500	85.1 ± 5.0	0.85	10.2
gallic acid	50	79.6 ± 4.67	0.79	10.2
	250	79.9 ± 3.84	0.86	8.33
	500	78.9 ± 4.10	0.84	9.01
4-*O*-methylgallic acid	50	89.3 ± 5.97	0.89	11.57
	250	88.9 ± 6.79	0.89	13.24
	500	93.1 ± 5.10	1.10	9.50
ethyl gallate	50	102.9 ± 8.39	1.17	14.1
	250	93.1 ± 3.69	0.97	6.87
	500	82.4 ± 1.93	0.84	4.05

1. Recovery calculated as the ratio of analyte concentration spiked in plasma prior to extraction to a known standard with results reported as percent recovered ± standard error of the mean.
2. Calculated as the ratio of spiked analyte post extraction to a known standard.
3. Inter-Day Precision is expressed as the coefficient of variation between extractions of spiked plasma in triplicate on three different days.
4. ND, not detected.

Matrix effect is defined as the change in the ability of an analyte to ionize under electrospray ionization conditions due to co-eluting compounds from a complex sample matrix, and has been previously reviewed by Gleichenhagan and Schieber to be a current challenge in polyphenol analytical chemistry [80]. Using the SDS protein precipitation method, gallic acid had a fixed decrease in ion efficiency due to the plasma matrix with a decrease in ionization of 14 to 21%, which would cause an underrepresentation of actual concentrations. This was confirmed when the extracted ion chromatograms were compared to the chromatograms at 280 nm. A measurement of peak heights at 280 nm showed gallic acid recoveries were greater than 80% across all three concentrations. While the PDA can be used to quantify standards its application is limited in the analysis of unknown targets. 4-*O*-methyl gallic acid and ethyl gallate matrix effects were varied ranging from -11 to 10% and -15 to 17%, respectively. The coefficients of variation for inter-day precision was < 15% for all compounds and concentrations. Guidelines from the FDA regarding bioanalytical method development define <15% as acceptable [153].

Pyrogallol had an extraction recovery of 85.1 ± 5.0% at 500 µg/L, however, it was not able to be detected at lower concentrations. Free pyrogallol is known to be very unstable due to rapid reaction with oxygen and combined with concentrations near its LOD and a decrease in ionization efficiency by 15% due to the matrix effect for this compound made it difficult to recover [154].

In the method development for SDS plasma protein precipitation, it was found that plasma proteins only precipitated when plasma was acidified, and it was critical to remove excess SDS due to the known interferences with reverse phase chromatography

and ion suppression with an ESI source [155]. A simple solution was the addition of saturated KCl as a cationic counter-ion that adequately precipitated residual SDS. When samples were injected without the addition of KCl, the ability to ionize was substantially inhibited. Additionally, when SDS was applied in small increments 3 x 25 µL 10% SDS vs 75 µL of 10% SDS all at once, no significant ($p<0.05$) differences were found in the recovery of spiked standards which allowed for rapid processing of samples. This trait makes the SDS protein precipitation method a faster alternative to contemporary methods that rely on concentration steps. SDS protein precipitation was successful in recovering gallic acid and 4-*O*-methylgallic acid from human plasma and has the potential to be applied in the recovery of other polyphenols that may experience protein-polyphenol interactions.

5.3.2 Method Comparisons

The recovery of polyphenolic standards using SDS protein precipitation was compared to recoveries using more widely employed methods of acetonitrile protein precipitation and SPE with Waters Oasis HLB cartridges using spiked polyphenolic-free plasma at 500 µg/L. SDS protein precipitation was found to have a significantly ($p<0.05$) higher amount of recoverable polyphenolics compared to both acetonitrile and SPE. Extraction with SPE had the least amount of recoverable standards with extraction recoveries of 19.1 ± 3.41, 14.5 ± 3.74, 17.3 ± 6.78, and 6.46 ± 1.08% for pyrogallol, gallic acid, 4-*O*-methylgallic acid, and ethyl gallate respectively (Figure 17). Gasperotti et al. previously observed reverse phase SPE to be less efficient in retention of gallic

acid due to its polarity with a recovery of 53% in plasma while relatively more non-polar phenolics such as sinapic acid and coumaric acid had recoveries of over 87% [156]. Recovery of pyrogallol, gallic acid, 4-*O*-methylgallic acid, and ethyl gallate from

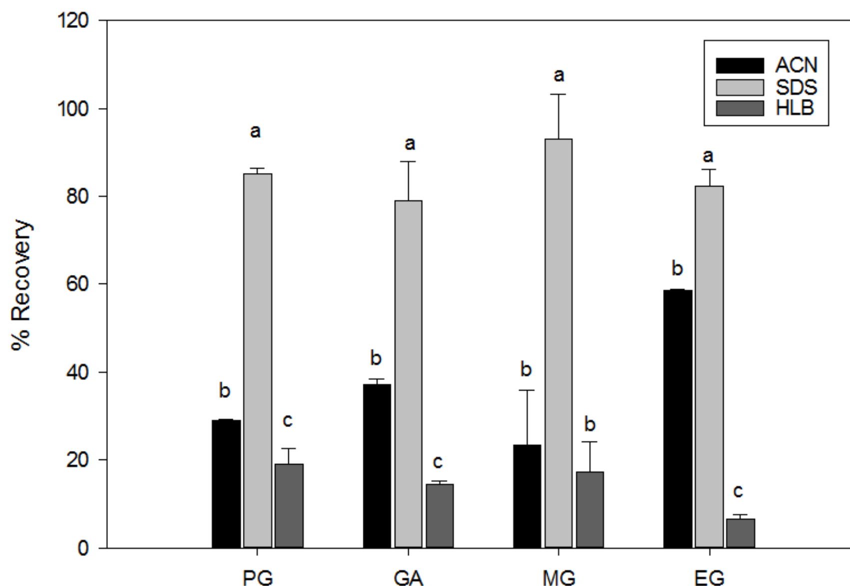

Figure 17. Comparison in extraction recoveries of pyrogallol (PG), gallic acid (GA), 4-*O*-methylgallic acid (MG), and ethyl gallate (EG) using precipitation with acetonitrile (ACN), SDS protein precipitation method (SDS), and solid phase extraction using Waters HLB cartridges (HLB) all at 500 µg/L. Different letters for each analyte denote a significant difference in recovery based on extraction method ($p < 0.05$).

plasma using acetonitrile precipitation was similarly low at 29.0 ± 1.20, 37.1 ± 9.04, 23.4 ± 10.1%, and 58.7 ± 3.72% for pyrogallol, gallic acid, 4-*O*-methylgallic acid, and ethyl gallate, respectively. Using the same ratio of plasma to acetonitrile, Ma et al. previously reported acetonitrile to isolate gallic acid with recoveries of 78-86% from 30-3000 µg/L, however such an efficient recovery was not replicated in this study [99].

Protein precipitation with solvent and SPE are the most cited methods to extract polyphenols from human plasma, yet the studies that have evaluated gallic acid report spiked concentrations ranging from 0.02 mg/L to 500 mg/L, with the later concentrations far higher than would be expected in plasma even with dietary supplementation [61, 98, 156].

5.3.3 Pharmacokinetics of Galloyl Metabolites

The use of SDS to rapidly denature serum proteins and extract polyphenolic metabolites was evaluated for its application in a human clinical trial, and the pharmacokinetics of gallic acid and galloyl glycoside phase II metabolites from acute mango consumption was evaluated from blood samples collected over an 8 h period. Both gallic acid and monogalloyl glucose were previously reported to be absorbed in the small intestine of humans and metabolized to produce 4-*O*-methylgallic acid and 4-*O*-methylgallic acid-3-*O*-sulfate in urine [74, 82]. Galloyl derivatives not absorbed in the small intestine are catabolized by microorganisms in the colon and converted to pyrogallol or even further into catechol that can be absorbed and also undergo phase II metabolism [38, 55]. The pro-gallic acid content of the mango pulp used in this study was previously reported in Chapter IV to contain 259 mg of total galloyl derivatives per 400 g of pulp that included 3.64 mg of free gallic acid, 91.7 mg of monogalloyl glucose, and 164 mg of gallotannins that ranged in degree of polymerization from 5 to 10 galloyl units.

Table 7. Characterization and pharmacokinetic parameters of gallic acid metabolites following human consumption of 400 g of mango pulp (cv. Ataulfo).

Metabolites	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)	T_{max} (h)	C_{max} (µg/L)	AUC_{0-8h} (µg/L·h)	Subjects[1] (n)
pyrogallol-*O*-sulfate	205	125	6	1,325 ± 437	5,990 ± 1,690[5]	9
catechol-*O*-sulfate	189	109	8	3,362 ± 1380	9,520 ± 3,360	10
methylpyrogallol-*O*-sulfate	219	205, 125	8	971 ± 391	4,020 ± 1,040	7
4-*O*-methylgallic acid-3-*O*-sulfate	263	183, 169	2	1,546 ± 291	6,030 ± 1,310	10
4-*O*-methylgallic acid	183	169, 125	1.5	611 ± 189	2,790 ± 1,190	7

1. The number of subjects whose plasma had quantifiable amounts of each metabolite.
2. Reported as the average concentration ± the standard error of the mean.

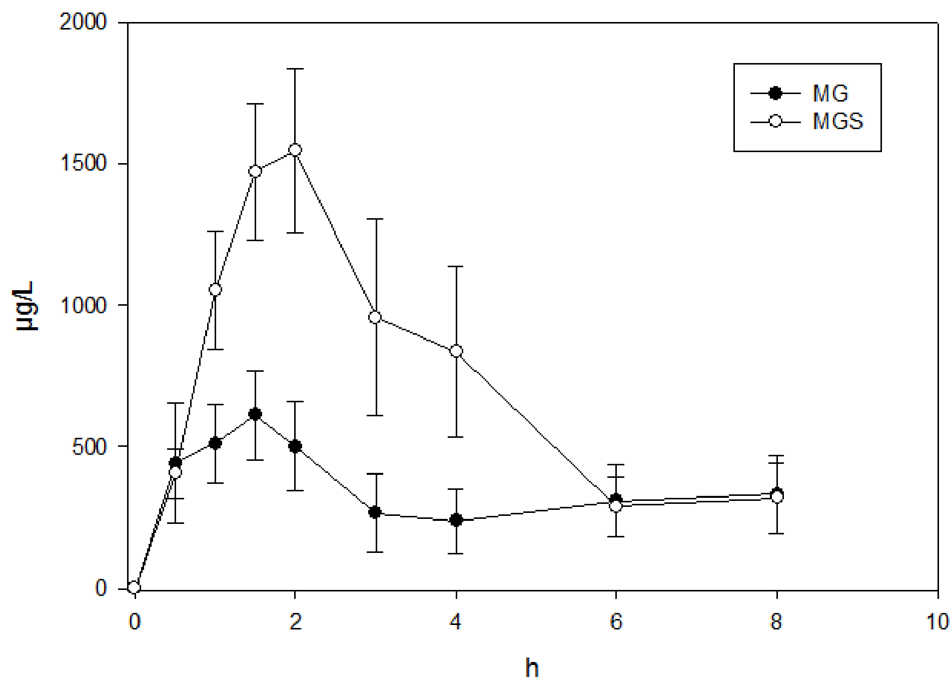

Figure 18. Pharmacokinetics of 4-*O*-methylgallic acid (MG), 4-*O*-methylgallic acid-3-*O*-sulfate (MGS), following tentative characterization and semi-quantification in human plasma after consumption of 400 g of mango pulp (cv. Ataulfo) in healthy volunteers. Data represent the average and standard error of the mean (n=10).

Five plasma metabolites sourced from gallic acid and galloyl glycosides were characterized based on fragmentation patterns previously reported, and semi-quantified using the SDS protein precipitation method (Table 7) (Figure 18) (Figure 19) [65, 82]. Average recovery of ethyl gallate as an internal standard across all samples (n=88) from all subjects was 87.7 ± 1.23%. Two metabolites with a base structure of gallic acid were characterized as 4-*O*-methylgallic acid and 4-*O*-methylgallic acid-3-*O*-sulfate. 4-*O*-methylgallic acid was compared to an authentic standard for retention time and its parent

ion at *m/z* 183 and MS/MS fragment ion at *m/z* 169. Likewise, 4-*O*-methylgallic acid-3-*O*-sulfate had a parent ion at m/z 263 that produced two major MS/MS fragment ions of *m/z* 183 and 169, which represented methylgallic acid and gallic acid respectively. Both 4-*O*-methylgallic acid and 4-*O*-methylgallic acid-3-*O*-sulfate had peak plasma concentrations within 2 h at a C_{max} of 611 ± 189 μg/L and 1,550 ± 291 μg/L, and AUC_{0-8h} of 2,780 ± 1,190 and 6,030 ± 1,310 μg/L·h, respectively. Free gallic acid was not found in any plasma samples in agreement with Pimpão et al. who fed 425 mg of free gallic acid from a mixed berry puree that contained, blueberries, blackberries, raspberries, and strawberries [55].

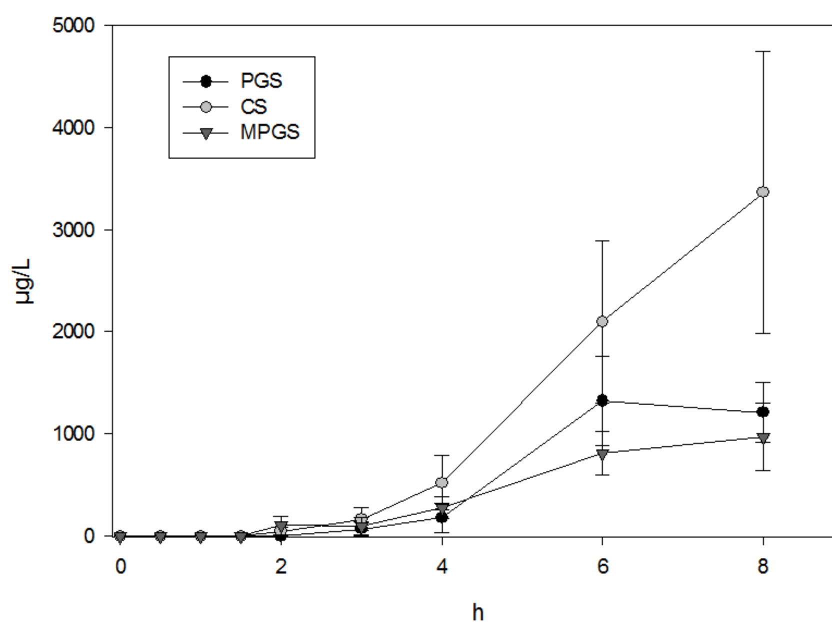

Figure 19. Pharmacokinetics of pyrogallol-*O*-sulfate (PGS), methylpyrogallol-*O*-sulfate (MPGS), and catechol-*O*-sulfate (CS) following tentative characterization and semi-quantification in human plasma after consumption of 400 g of mango pulp (cv. Ataulfo) in healthy volunteers. Data represent the average and standard error of the mean (n=10).

Three metabolites were generated from decarboxylated gallic acid in the GI tract and were tentatively identified as pyrogallol-*O*-sulfate, methylpyrogallol-*O*-sulfate, and catechol-*O*-sulfate. Pyrogallol-*O*-sulfate and catechol-*O*-sulfate were characterized based on their respective predominant parent ions at m/z 205 and 189 and fragment ions at *m/z* 125 and 109, which represent pyrogallol and catechol, respectively. Both produced neutral loss fragments of 80 Da corresponding to the losses of sulfate groups. Methylpyrogallol-*O*-sulfate had a parent ion of *m/z* 219 and produced fragment ions at *m/z* 205 and 125, which represented fragments of pyrogallol-*O*-sulfate and pyrogallol. The three metabolites had a T_{max} from 6 to 8 h postprandial, and C_{max} of 1,330 ± 437, 971 ± 391, and 3,360 ± 1,380 µg/L and $AUC_{0\text{-}8h}$ of 5,990 ± 1,690, 4,020 ± 1,040, and 9,520 ± 3,360 µg/L·h for pyrogallol-*O*-sulfate, methylpyrogallol-*O*-sulfate, and catechol-*O*-sulfate, respectively. Free pyrogallol and catechol were not found in any plasma isolates, it is possible that the body is adept at metabolizing and clearing these xenobiotics, however, as concentrations below 500 µg/L could not be recovered in the method development it is possible they were not detected. Absorption of pyrogallol and catechol from the colon was still occurring after 8 h indicating long-term production of key metabolites from mango consumption (Figure 19). A study by Van der Pijl et al. reported finding plasma pyrogallol metabolites from black tea 30 h after a single consumption [84]. Given the extended period at which polyphenol metabolites sourced from colon can be detected in plasma they could be useful biomarkers for fruit and vegetable intake, and are critical to properly assay in order to investigate their bioactive responses in vivo.

All metabolites were reported to have high coefficients of variation ranging from 69 to 113%. While this is not uncommon for polyphenol pharmacokinetic studies, mango in particular has an additional factor that can increase the variation of subjects' ability to produce gallic acid metabolites, the gallotannins. Gallotannins can freely hydrolyze to gallic acid under physiological conditions, and are also capable of undergoing hydrolysis by colonic bacteria such as *Lactobacillus plantarum* and *Streptococcus galloylticus* because of their ability to produce tannase, an enzyme capable of hydrolyzing *m*-depside bonds [115, 137]. However, due to the large differences in gut bacteria populations per person it is unlikely each person has similar potential to break down gallotannins. As such there will be a large inter-person variation in the amount of gallic acid that can be produced in the colon.

5.3.4 In Vivo Method Comparison

For additional conformation that the SDS protein precipitation method was more efficient than acetonitrile denaturation and SPE at recovering gallic acid and pyrogallol phase II metabolites were extracted from a pool of plasma at 2 and 8 h, the T_{max} of the respective metabolites (Table 8). Significantly ($p<0.05$) more 4-*O*-methlgallic acid, 4-*O*-methlgallic acid-3-*O*-sulfate, and catechol-*O*-sulfate were recovered using the SDS protein precipitation method with concentrations of 842 ± 39.2, 2,570 ± 90.9, 1,880 ± 4.96 µg/L respectively. A non-significant increase was observed using SDS for methylpyrogallol-*O*-sulfate in comparison to acetonitrile with concentrations of 741 ± 125 and 415 ± 88.3 µg/L, respectively. The remaining concentrations of 4-*O*-

Table 8. Comparison of gallic acid and pyrogallol phase II metabolite plasma concentrations from pooled plasma using SDS protein precipitation method (SDS), acetonitrile protein precipitation (ACN), and solid phase extraction with HLB chemistry (HLB).

	2 h[1]		**8 h**	
	4-*O*-methylgallic acid	**4-*O*-methylgallic acid-3-*O*-sulfate**	**methylpyrogallol-*O*-sulfate**	**catechol-*O*-sulfate**
SDS	842 ± 39.2[a,2]	2570 ± 90.9[a]	741 ± 125[a]	1880 ± 4.96[a]
ACN	336 ± 51.7[b]	509 ± 182[b]	415 ± 88.3[a]	587 ± 99.8[b]
HLB	119 ± 7.06[c]	ND[c]	45 ± 44.5[b]	ND[c]

1. Time points used for pooled plasma samples that were the T_{max} for the respective metabolites.
2. Different letters for each analyte denote a significant ($p<0.05$) difference in concentration based on extraction method with concentration reported as the average ± the standard error of the mean.

methylgallic acid, 4-*O*-methlgallic acid-3-*O*-sulfate, and catechol-*O*-sulfate following extraction with acetonitrile were 336 ± 51.7, 509 ± 182, and 587, ± 99.8 µg/L, respectively. The lowest concentrations of recovered phase II metabolites were observed following extraction with SPE at 119 ± 7.06 and 45 ± 44.5 µg/L for 4-*O*-methlgallic acid and methylpyrogallol-*O*-sulfate. 4-*O*-methlgallic acid-3-*O*-sulfate and catechol-*O*-sulfate were not detected with SPE. Pyrogallol-*O*-sulfate was not detected in any of the extractions from the pooled plasma.

5.4 Conclusions

In conclusion, a novel protein precipitation method utilizing SDS was developed for improved recovery of gallic acid from human plasma. The SDS denaturation protocol was utilized to extract human plasma for gallic acid metabolites generated after an acute feeding of 400 g of fresh mango pulp. Five metabolites, two from gallic acid and three from pyrogallol were characterized and semi-quantified over an 8 h period. The SDS method was proven to be able to extract significantly more gallic acid metabolites from plasma compared to current methods reported in the literature. Plasma extraction of gallic acid and its metabolites with SDS is a simple and rapid method that has potential to be applied in the analysis of other polyphenolic analytes.

CHAPTER VI

DAILY CONSUMPTION OF MANGO CV. ATAULFO FOR 42 DAYS AND ITS INFLUENCE ON THE METABOLISM AND EXCRETION OF GALLIC ACID, GALLOYL GLYCOSIDES, AND GALLOTANNINS

6.1 Introduction

Mangos are one of the most important fruit crops in the world ranking behind only grapes, apples, and bananas in global production [48]. Mango's pulp, peel, and seed is consumed all over the globe and the fruit is widely considered as a good source of many essential nutrients including vitamin C, pro-vitamin A, and dietary fiber. All parts of the mango additionally contain polyphenolics, predominantly galloyl derivatives including free gallic acid, gallic acid glycosides, and gallotannins. Polyphenolic extracts from mango have been reported to have anti-cancer and anti-inflammatory properties in vitro and in animal models [125, 157, 158], and in an obese human subject trial participants that consumed freeze-dried mango pulp for 12 wks had improved fasting blood glucose levels [159]. Recent findings however have demonstrated that polyphenolics undergo extensive biotransformation during digestion potentially negating previous hypotheses that suggested the health promoting properties of polyphenols were due to the un-metabolized parent compounds found in the fruit [16, 112, 160]. Present studies have focused on characterizing metabolites formed during digestion from each individual polyphenolic, gallic acid was previously reported to be absorbed from the small intestine and metabolized in the liver where the endogenous phase II enzymes

catechol-*O*-methyltransferase and sulfotransferases conjugate it to predominantly produce 4-O-methylgallic acid and 4-*O*-methylgallic acid-3-*O*-sulfate [38]. Gallic acid not absorbed will transit to the colon where it is catabolized by host bacteria to produce smaller phenols such as pyrogallol and catechol that are likewise absorbed and metabolized by the host [55, 82]. Monogalloyl glucose was found to have the same metabolic potential as gallic acid, as previously discussed in Chapter IV. Oligomeric galloyl glucoses and gallotannins however are too large to be absorbed intact in the small intestine and must be depolymerized by hydrolytic esterases residing in the gastrointestinal tract or through non-enzymatic hydrolysis to generate bioaccessible gallic acid [71, 107]. Thus, mango pulp is a source of several pro-gallic acid compounds with the potential to produce galloyl and pyrogallol metabolites.

Several studies have proposed the influence of long-term polyphenol intake on reducing inflammatory biomarkers and cancer rates [17, 19, 21, 87]. Current hypotheses propose that the mechanisms behind the health benefits of polyphenols are linked to their concentrations in tissues and plasma [161-163]. Conversely only a limited amount of reports are available that have evaluated plasma concentrations of phenolic metabolites after repetitive dietary intake, and results from these trials can be conflicting depending on the class of polyphenolic consumed. For example, repeated feeding of grape seed extract to rats for 10-days resulted in a 395% increase in gallic acid concentrations in plasma [92]. In contrast, humans that consumed 400 mg of green tea polyphenols daily for 4 weeks had no significant difference in the plasma concentrations of epigallocatechin-gallate but when the dose was increased to 800 mg a 60% increase was

observed [93]. And in the daily consumption of freeze-dried blueberry powder for 30 days with a total of 604 mg of total polyphenols consumed per day no significant changes were observed in their 24 h urinary excretion [164]. As such, the literature suggests that the bioavailability of polyphenolics following repetitive consumption can be different for each class of polyphenolics consumed.

Previously we have observed significant increases in the urinary excretion of pyrogallol-*O*-sulfate and catechol-*O*-sulfate, metabolites sourced from break down of gallic acid in the colon, when 400 g of mango (cv. Keitt) was consumed daily for 10 days [146]. This increase in metabolism was hypothesized to be due to changes in the composition of the subjects' gut microbiota, and recently repetitive mango intake in rats was shown to promote the growth of *Bifidobacteria* bacteria [165-167]. Currently, the metabolic fate of galloyl glycosides and gallotannins in plasma following repetitive consumption is currently unknown. Therefore, the aim of this work was to investigate the effects of daily mango consumption for 42 days on the metabolism and excretion of mango pro-gallic acid compounds in Lean and Obese subjects with increases in bioavailability hypothesized to be due to increased colonic metabolism.

6.2 Materials and Methods

6.2.1 Mango Fruit

Mature, green mangos (cv. Ataulfo) from Mexico were imported through Frontera Produce (Edinburg, TX), shipped to Texas A&M Department of Nutrition and Food Science, and allowed to ripen under ambient conditions. Mangos were deseeded,

pulp removed from the skin, and vacuum sealed in 400 g bags under Good Manufacturing Practices. Mango pulp was frozen within 2 h of processing and stored at -20 ℃ until use.

6.2.2 Chemicals

Standards of ethyl gallate, gallic acid, and pyrogallol were acquired from Sigma Aldrich (St Louis, MO) and 4-*O*-methylgallic acid from Apin Chemicals (Oxfard, UK). Water and methanol containing 0.1% formic acid were acquired from Sigma Aldrich (St Louis, MO) as well as a 10% solution of SDS.

6.2.3 Experimental Design and Sample Processing

Healthy volunteers (n = 32, aged 18 -50) with no medical history of digestive disorders participated in a 42 day mango consumption study divided into three cohorts. The Control cohort (n=11) consumed 400 g of mango pulp only on Days 1 and 42. The remaining two cohorts consumed 400 g mango pulp daily for 42 days and were divided into Lean and Obese groups. The Lean cohort (n=12) had a BMI from 18-25 and the Obese cohort (n= 9) had a BMI > 30. Three days prior to when the study began subjects refrained from consuming foods known to contain gallic acid or pro-gallic acid polyphenolics including mango, grape products, tea, chocolate, and berries. Baseline urine samples were collected prior to mango consumption and post-prandial from 0-3, 3-6, 6-9, 9-12, and 12-24 h and on study Days 1 and 42. The volume of urine output was measured for each collection, and 12 mL acidified with 0.1 mL of 88% formic acid and

passed through a 0.45 µm filter for metabolite analysis by LC-MS. Baseline blood samples were collected prior to mango consumption, and post-prandial at 0.5, 1, 1.5, 2, 3, 4, 6, and 8 h through a blood-draw catheter on Days 1 and 42. Blood samples were centrifuged at 3,000 x g for 5 min, and plasma acidified with 25 µL of formic acid. Plasma samples were stored at -80 °C until processed using the method previously described in Chapter V. Briefly, 50 µL of an internal standard, ethyl gallate, was incubated with 500 µL of acidified plasma and 75 µL of 10% SDS was then added to denature proteins followed by addition of 400 µL of 0.1% formic acid methanol. Plasma was sonicated for 10 min, centrifuged for 5 min at 10,000 x g, and spiked with 50 µL of saturated KCl to remove residual SDS. Samples were held at 4 ºC for 1 h prior to being filtered through a 0.45 µm filter prior to LC-MS analysis. The study protocol was approved by the Institutional Review Board (IRB) of Texas A&M University.

6.2.4 LC-MS Analysis

Metabolites from galloyl derivatives were analyzed on a Thermo Finnigan Surveyor LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI source. Separations were in reversed-phase using a Thermo Finnigan Surveyor HPLC coupled to a Surveyor PDA detector and gradient separations were performed using a Phenomenex Kinetex™ (Bannockburn, Ill) C18 column (150 x 4.6 mm, 2.6 µm) at room temperature. Injections were made into the column by use of a 50 µL sample loop. For separation of urine metabolites mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in methanol run at 0.4 mL/min. A gradient was

run of 0% Phase B for 2 min and changed to 10% Phase B in 4 min, 10 to 15% Phase B in 8.5 min, 15% to 27% Phase B in 11 min, 27% to 90% Phase B in 15 min, 90% was held to 0.5 min before returning to initial conditions. For separation of plasma metabolites mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in methanol run at 0.45 mL/min. A gradient was run of 0% Phase B for 2 min and changed to 10% Phase B in 4 min, 10% Phase B was held to 10 min, 10 to 40% Phase B in 25 min, and 40% to 65% Phase B in 35 min, 65% to 85% Phase B in 41 min, 85% was held to 49 min before returning to initial conditions. The electrospray interface worked in negative ionization mode. Source and capillary temperatures were set at 325°C, source voltage was 4.0 kV, capillary voltage was set at -47 V, and collision energy for MS/MS analysis was set at 35 eV. The instrument operated with sheath gas and auxiliary gas (N_2) flow rates set at 10 units/min and 5 units/min, respectively. The instrument was tuned for 4-*O*-methylgallic acid and metabolites quantified using extracted ion chromatograms from their respective parent compounds as standards.

6.2.5 Statistical Analyses

Significant differences for metabolites in urine were calculated from the sum of their excretions over 24 h using students t-test in Sigma Plot software (SPSS, Chicago, IL). Significant differences between cohorts were calculated by using ANOVA. Non-compartmental pharmacokinetic analysis was performed by use of the PkSolver Microsoft Excel Add-In [132]. Results are reported at the mean ± standard error of the mean.

6.3 Results and Discussion

The pulp of the Ataulfo mango is known for its relatively high concentrations of gallic acid glycosides and gallotannins compared to other mango varieties. The 400 g of pulp fed to subjects contained a total of 259 mg of galloyl derivatives of which 95.4 mg were non-tannin, including gallic acid (3.64 mg) and monogalloyl glucose (91.7 mg), and the remaining 164 mg were gallotannins ranging in degree of polymerization from 5-10 galloyl groups. Mango pulp contains a majority of pro-gallic acid polyphenolics and they can comprise over 50% of the total phytochemicals present [11].

6.3.1 Urinary Excretion Following 42 Days of Daily Mango Consumption

The urinary excretion of phase II metabolites derived from galloyl derivatives following consumption of 400 g of Ataulfo mango pulp was evaluated in Lean and Obese subjects who consumed mango daily for 42 days and compared to a Control cohort that only consumed mango on Days 1 and 42. Seven gallic acid and pyrogallol based phase II metabolites were tentatively identified in the urine from m/z previously reported [65, 82] (Figure 20) (Table 9).

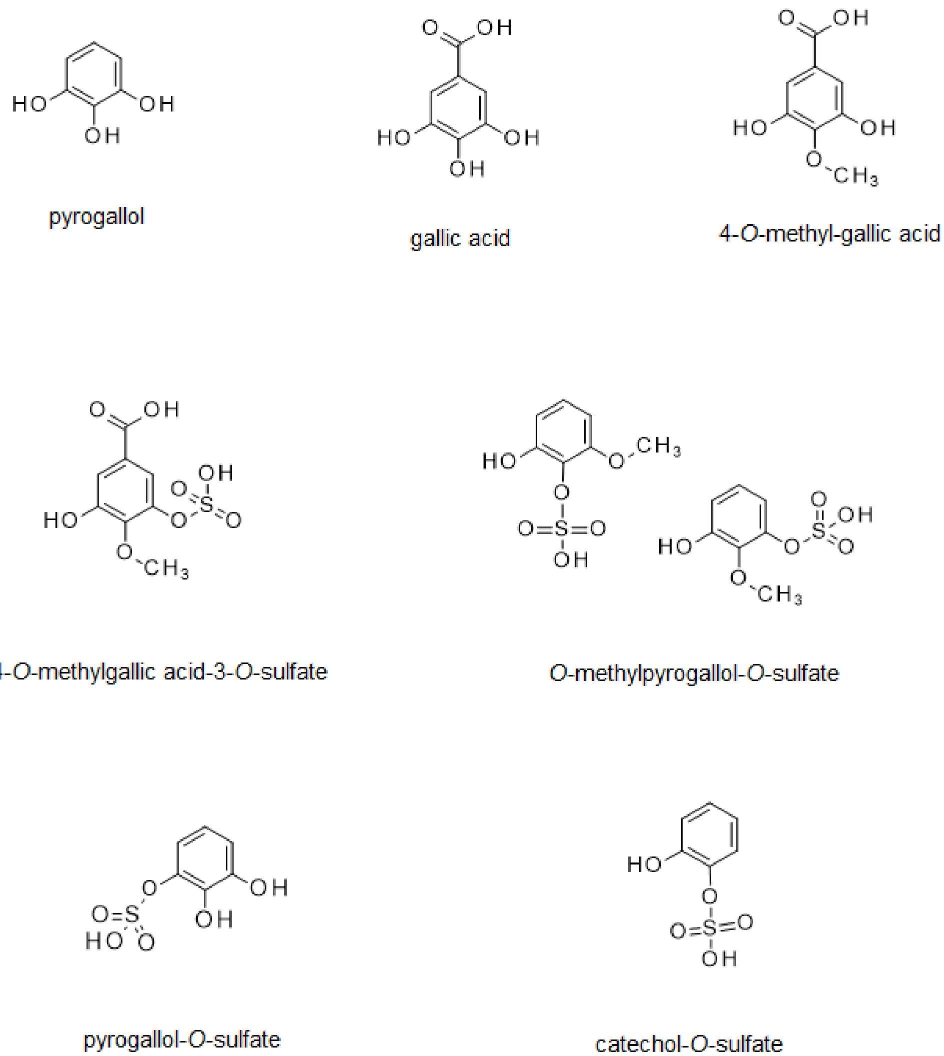

Figure 20. Tentative chemical structures of phase II metabolites following consumption of gallic acid, galloyl glycosides, and gallotannins sourced from 400 g of mango cv. (Ataulfo) pulp.

Table 9. Metabolites characterized and quantified in the plasma and urine of subjects who consumed 400 g of mango cv. Ataulfo pulp daily for 42 days.

Metabolite	Abv	$[M-H]^-$	MS/MS	Urine	Plasma
gallic acid	GA	169	125	Trace[1]	ND[2]
4-*O*-methylgallic acid	MG	183	169, 125	Trace	+[3]
4-*O*-methylgallic acid-3-*O*-sulfate	MGS	263	183, 169	+	+
pyrogallol	PG	125	-	ND	ND
pyrogallol-*O*-sulfate	PGS	205	125	+	+
methylpryogallol-*O*-sulfate	MPGS	219	205, 125	+	+
catechol-*O*-sulfate	CS	189	109	+	+

1. Trace, indicated metabolite was detected but was below the lower limit of quantification, a signal to noise ratio of 5 to 1.
2. ND, metabolite was not detected.
3. **+,** indicates metabolite was quantified.

Five of the seven metabolites, 4-*O*-methylgallic acid-3-*O*-sulfate, pyrogallol-*O*-sulfate, catechol-*O*-sulfate, and two isomers of methylpyrogallol-*O*-sulfate (I and II) were above the lower limit of quantification, a signal to noise ratio of 5 to 1, and were measured in the urine up to 24 h post-prandial. Gallic acid and 4-*O*-methylgallic acid were detected in the urine of subjects however they were below the lower limit of quantification. In the Lean cohort, a significant ($p<0.05$) increase was found in the excretion of all quantifiable metabolites (Figure 21). Specifically, the cumulative 24 h excretion of 4-*O*-methylgallic acid-3-*O*-sulfate increased from 10.6 ± 2.38 to 36.13 ± 5.53 mg, pyrogallol-*O*-sulfate from 14.3 ± 1.50 to 57.8 ± 5.37 mg, methylpyrogallol-*O*-sulfate I from 8.58 ± 1.16 to 40.3 ± 9.51 mg, methylpyrogallol-*O*-sulfate II from 8.40 ± 1.43 to 50.3 ± 8.00, and catechol-*O*-sulfate from 21.7 ± 2.90 to 96.3 ± 12.8 mg between Days 1 and 42 respectively. The same increase in 24 h urinary excretion was observed in the Obese cohort for all quantifiable metabolites except methylpyrogallol-*O*-sulfate I, which was found to have an insignificant increase from 22.1 ± 5.70 to 45.7 ± 11.1. In the Control cohort, no difference in urinary excretion was observed for the galloyl metabolites with the exception of methylpyrogallol-*O*-sulfate I, which was less excreted at Day 42 compared to Day 1 (Figure 21; $p< 0.05$).

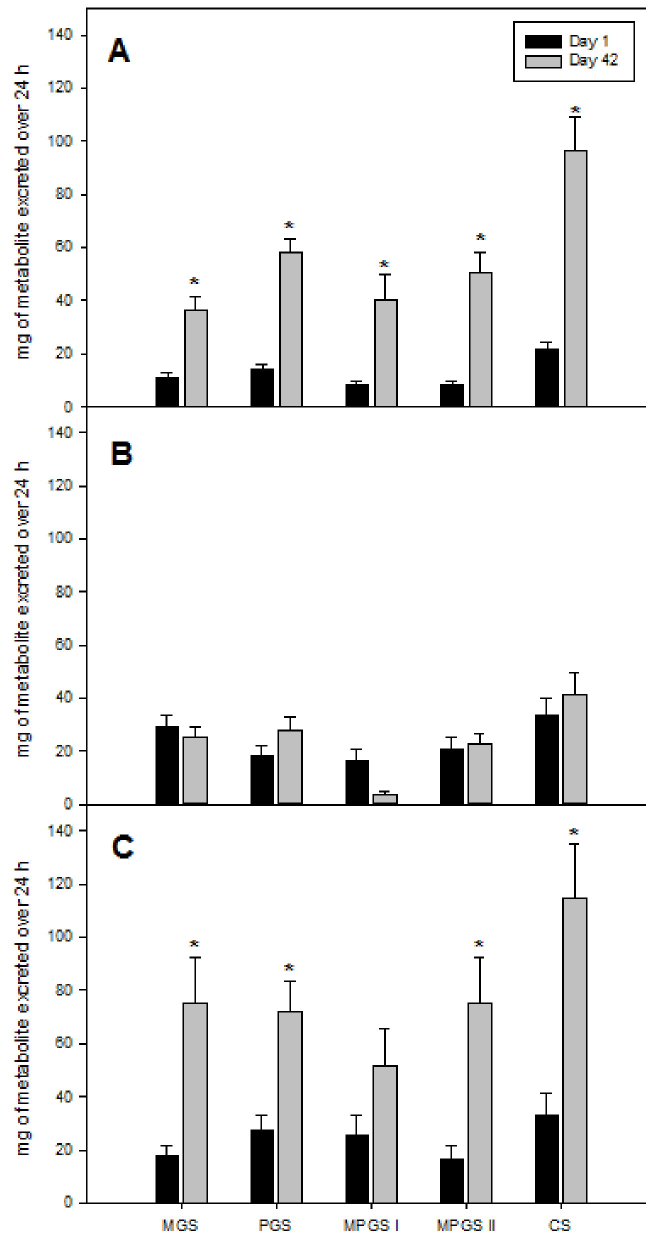

Figure 21. The sum of urinary excretion in mg for the metabolites 4-*O*-methylgallic acid-3-*O*-sulfate (MGS), pyrogallol-*O*-sulfate (PGS), two isomers of methylpyrogallol-*O*-sulfate (MPGS), and catechol-*O*-sulfate (CS) after consumption of 400 g of mango cv. Ataulfo for Days 1 and 42 in the (A) Lean and (C) Obese Cohorts who consumed mango daily for 42 days and the (B) Control Cohort who consumed mango only on Days 1 and 42. A (*) designates a significant difference between Days 1 and 42 for the respective metabolite.

The increase in galloyl metabolism and excretion has been hypothesized to be due to changes in the composition of the subjects' gut microbiota. It has previously been demonstrated that repetitive polyphenolic intake will affect the composition of a hosts' gut microbiota such as an increase in the population sizes of the probiotic bacteria species of *Bifidobacterium* and *Lactobacillus* [168-170]. Corresponding work has shown that changes in the gut microbiota can also result in increases in polyphenol metabolism. Pereira-Caro et al. (2013) observed an increase in the bioavailability, assessed by 24 h urinary excretion, of orange juice polyphenols with an increase from 22% of the initial dose (196 µmol) to 70% in subjects who consumed a *Bifidobacterium longum* probiotic daily for 4 wks [171]. On Day 1 of this study, total urinary excretion of all galloyl metabolites corresponded to 24.3% of the 259 mg of galloyl derivatives consumed in the lean cohort and 45.2% in the obese cohort. Gallotannins are poorly absorbed from the small intestine in comparison to lower molecular weight polyphenolic such as gallic acid, and are hypothesized to transit to the colon and be hydrolyzed by the microbiota to create gallic acid, pyrogallol, and catechol by organisms such as *Lactobacillus plantarum* and *Streptococcus galloylitcus* [115, 172]. We hypothesized that on Day 1 the subjects would not be fully adept at gallotannin hydrolysis, but over time microbiota selectively acclimated to utilize gallotannins as an energy source and produced higher concentrations of absorbable galloyl metabolites. The significant increases in total urinary excretion in the Lean and Obese cohorts from Day 1 to Day 42 support the hypothesis as the 24 h urinary excretion of galloyl derivatives increased to 107 and 130% of the dose of galloyl derivatives fed.

On both Days 1 and 42, pyrogallol-*O*-sulfate, methylpyrogallol-*O*-sulfate I, methylpyrogallol-*O*-sulfate II, and catechol-*O*-sulfate were still being produced and excreted 24 h post-prandial and on Day 42 were found in the baseline urine of subjects in the Lean and Obese cohorts (Figure 22). The prolonged generation of pyrogallol metabolites has previously been reported, and they have been observed to be absorbed and metabolized up to 48 h after a single serving of grape seed or black tea extract [84, 173]. Bowel transit times vary significantly based on factors such as age, race, and diet, and can range in time from 6 to 42 h [174, 175]. Due to the time range that mango could have resided in the colon it is possible that some of the metabolites excreted in the urine of the Lean and Obese on Day 42 were sourced from the mango consumed from a previous days intake. The increase in excretion to 107 and 130% of the 259 mg of galloyl derivatives consumed for the Lean and Obese cohorts, respectively, supports this hypothesis, and ultimately we can't conclude how much of the increase was due to changes in bacteria populations or if it came from the mango consumed from previous dosages.

6.3.2 Plasma Pharmacokinetics Following 42 Days of Daily Mango Consumption

Five phase II galloyl metabolites, 4-*O*-methylgallic acid, 4-*O*-methylgallic acid-3-*O*-sulfate, pyrogallol-*O*-sulfate, methylpyrogallol-*O*-sulfate, and catechol-*O*-sulfate, were characterized and semi-quantified in plasma up to 8 h post-prandial following consumption of Ataulfo mango (Table 9). Extraction of metabolites from plasma was

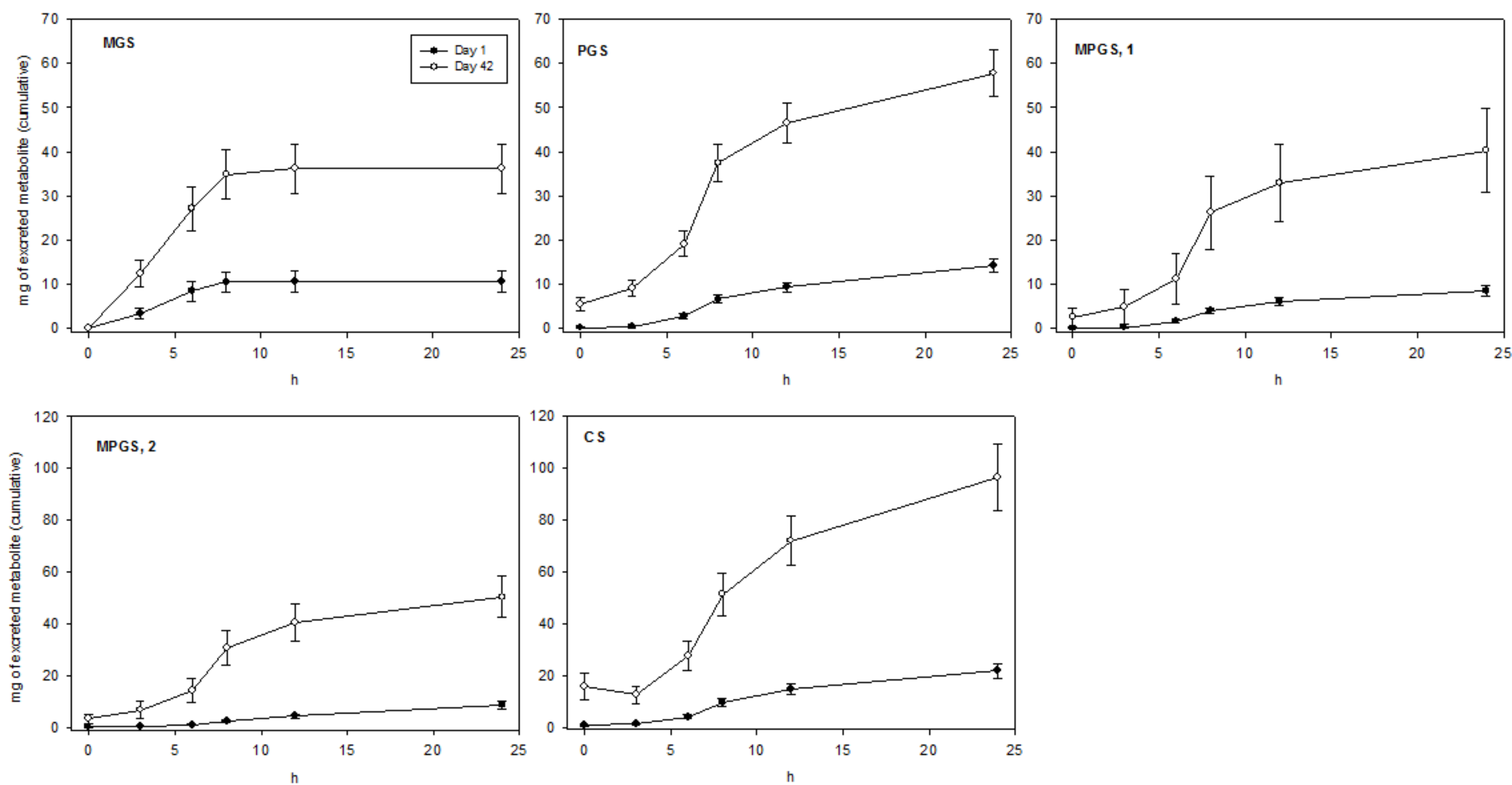

Figure 22. Cumulative excretion in mg of the galloyl metabolites: 4-*O*-methylgallic acid-3-*O*-sulfate (MGS), pyrogallol-*O*-sulfate (PGS), two isomers of methylpyrogallol-*O*-sulfate (MPGS), and catechol-*O*-sulfate (CS) in the Lean Cohort following daily consumption of 400 g of mango cv. Ataulfo for 42 days on Days 1 and 42.

evaluated against an internal standard ethyl gallate whose average recovery was 94.1 ± 0.54% across the three cohorts. In contrast to the higher urinary excretion observed in the Lean and Obese cohorts after 42 days of consuming 400 g of mango no significant increase was observed based on the AUC_{0-8h} for individual plasma metabolites (Table 10). However, a trend of insignificant increases was observed for all individual metabolites in the Lean cohort along with non-significant increases for catechol-*O*-sulfate and 4-*O*-methylgallic acid sulfate in the Obese cohort where high coefficients of variation between subjects ranging from 32 – 118% kept comparisons from being significant. High coefficients of variation are frequently reported for polyphenolic metabolomics investigations, and are hypothesized to be due to differences in gut microbiota compositions per subject [176]. The Control cohort was also found to have no significant differences in the plasma concentrations of gallic acid metabolites on Days 1 and 42. In contrast, the sum of the AUC_{0-8h} for all galloyl metabolites concentrations revealed a significant increase for Lean and Obese cohorts between Days 1 and 42 with total AUC_{0-8h} of 20,400 ± 1,610 to 29,400 ± 2,520 μg/L·h and 13,900 ± 1,010 to 17,400 ± 989 μg/L·h, for the Lean and Obese cohorts respectively (Figure 23). An insignificant decrease was observed for the Control cohort with total AUC_{0-8h} of 24,100 ± 2,670 to 18,010 ± 1,793 μg/L·h. Despite the obese cohort excreting 130% of the initial galloyl derivatives dose on Day 42 when the total AUC_{0-8h} were compared against each other the Lean cohort was significantly higher than the Control and Obese cohorts (ANOVA; $p<0.05$).

Table 10. Area Under the Curve (AUC) from 0 to 8 h of phase II metabolites sourced from gallic acid and galloyl glycosides following daily consumption of mango cv. Ataulfo pulp for 42 days in Lean and Obese Cohorts, and compared against subjects who consumed mango only on days 1 and 42, Control Cohort.

	Control Cohort $AUC_{0\text{-}8h}$ (µg/L · h)		**Lean Cohort $AUC_{0\text{-}8h}$ (µg/L · h)**		**Obese Cohort $AUC_{0\text{-}8h}$ (µg/L · h)**	
Metabolite	**Day 1**	**Day 42**	**Day 1**	**Day 42**	**Day 1**	**Day 42**
4-*O*-methylgallic acid	527 ± 108	515 ± 300	431 ± 233	1,430 ± 760	585 ± 227	504 ± 96.3
4-*O*-methylgallic acid-3-*O*-sulfate	4,440 ± 1,140	5,110 ± 1,450	3,390 ± 487	4,410 ± 728	1,120 ± 299	1,729 ± 457
pyrogallol-*O*-sulfate	5,010 ± 1,580	2,290 ± 317	5,120 ± 1,430	6,830 ± 1,880	1,380 ± 496	1,010 ± 397
methylpyrogallol-*O*-sulfate	4,680 ± 1,380	2,303 ± 298	2,710 ± 562	4,920 ± 1,260	3,550 ± 894	3,440 ± 1,130
catechol-*O*-sulfate	9,420 ± 3,350	7,790 ± 2,200	8,790 ± 1,920	11,800 ± 3,060	7,318 ± 1,980	10,720 ± 1,790

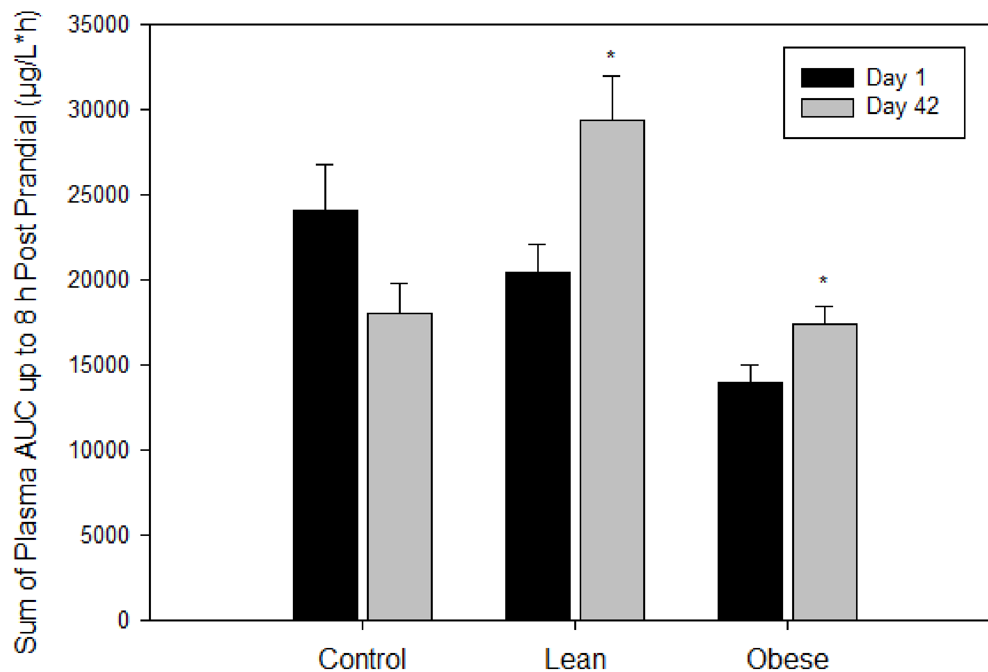

Figure 23. Sum of the area under the curve (AUC) for all galloyl metabolites in the plasma of subjects who consumed mango only on Days 1 and 42 (Control) and those who consumed mango daily for 42 days, on Days 1 and 42 (Lean and Obese). A (*) designates a significant difference between Days 1 and 42 for the respective cohort.

As well when individual metabolites were compared against each other the Lean cohort was significantly higher in the AUC_{0-8h} of 4-*O*-methylgallic acid-3-*O*-sulfate and pyrogallol-*O*-sulfate. Studies are lacking which compare the pharmacokinetics of polyphenolics in different populations though in one study which compared the tissue distribution of catechin in Lean and Obese rats the Lean rats had higher adipose tissue concentrations of catechin and its phase II metabolites methylcatechin-glucuronide [177]. Thus, while significant differences were not found for individual metabolite plasma concentrations in the Lean and Obese cohorts, a significant increase was

observed for the sum of all metabolites with the Lean cohort having a significantly higher total $AUC_{0\text{-}8h}$ on Day 42 compared to the other cohorts.

Similar observations were made when a grape and blueberry polyphenolic extract fed at a dose of 3.5 mg gallic acid / kg body weight to rats for 15 days produced no significant difference for gallic acid concentrations in plasma [178]. Likewise, it was reported that daily administration of grape seed extract dosed at 3.1 mg of gallic acid / kg of body weight to rats had no significant effect on the concentration of gallic acid metabolites in brain, liver, or heart tissues [177]. The lack of meaningful accumulation of gallic acid on its metabolites in blood observed both in this study and in others is likely the result of their rapid metabolism and excretion. Our prior work in a porcine model as discussed in Chapter IV found the half-life of gallic acid was 6 min, considerably shorter than other polyphenolics such as epigallocatechin gallate

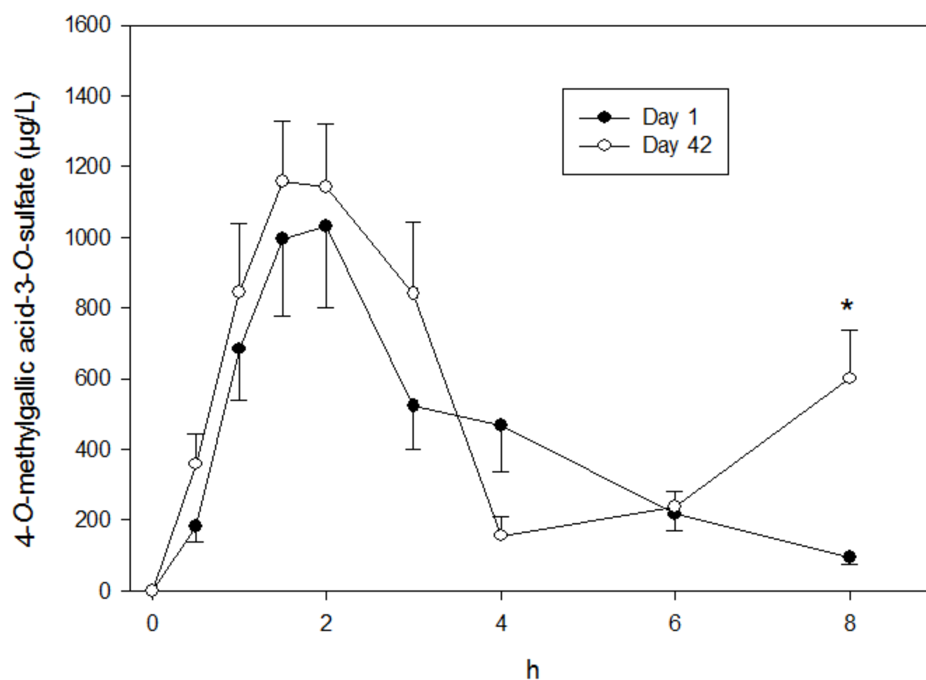

Figure 24. Plasma concentrations of 4-*O*-methylgallic acid-3-*O*-sulfate in human plasma on Days 1 and 42 following consumption of 400 g of mango (cv. Ataulfo) pulp in the Lean Cohort who consumed mango daily for 42 days. A (*) designates a significant difference between Days 1 and 42.

which was found in have a half-life of 51 min in rats indicating a rapid metabolic turn-over of free gallic acid and mono-galloylglucose present in mango [143]. In contrast, when 50, 100, 150 mg quercetin supplements were administered daily for two weeks a significant difference in plasma concentrations was observed across all three dosing regiments up to 24 h post prandial. It is possible that the 8 h post-prandial blood collection used in this study was not long enough to observe the significant changes found in the 24 h urinary excretion. As an example, in the Lean cohort 4-*O*-methylgallic acid-3-*O*-sulfate was found to have a significantly higher concentration at 8 h on Day 42 when compared to Day 1 (Figure 24). Ultimately, Del Rio et al. (2012) concluded that

the characterization and quantification of polyphenol metabolites in blood is an inadequate measurement of bioavailability and rather the cumulative urinary excretion is likely more accurate for mass balance calculations since any absorbed polyphenols will ultimately be excreted [179]. Observations from this study support this hypothesis as plasma concentrations from 8 h post-prandial did not experience the same increase as observed in urine in the same time range.

It is unknown exactly how rapid excretion of these metabolites affects the potential health benefits from consumption of gallic acid glycosides and gallotannins. Though studies which have investigated the bioefficacy of habitual consumption of pro-gallic acid and pro-pyrogallol foods such as strawberries, raspberries, grapes, and tea have reported benefits such as reduction in pro-inflammatory biomarkers, however the exact mechanisms remain unknown [180-182]. However, results from this study and others suggest that galloyl metabolites are rapidly eliminated from the body indicating they will likely have short incubation times in tissues. For galloyl derivatives it is possible that their health benefits are not tied to their systemic concentrations, but through their influence on cell signaling mechanisms [183]. In order to fully understand mechanisms of action future work needs to be performed with polyphenol phase II metabolites at the sites of action or investigate if their bioactivity is linked to regulation of our systemic anti-oxidant and anti-inflammatory pathways.

6.4 Conclusions

The urinary excretion and plasma concentrations of phase II metabolites sourced from mango gallic acid, galloyl glycosides, and gallotannins was assessed in lean and obese subjects who consumed mango daily for 42 days, and compared against subjects who consumed mango only on Days 1 and 42. In the Lean and Obese cohorts, significant increases were found in the cumulative 24 h urinary excretion of 4-*O*-methylgallic acid-3-*O*-sulfate, methylpyrogallol-*O*-sulfate, pyrogallol-*O*-sulfate, and catechol-*O*-sulfate and were hypothesized to be due to adaptations in the subjects gut microflora. No significant differences were found in the individual plasma concentrations of these metabolites up to 8 h post-prandial. It was hypothesized that the galloyl and pyrogallol metabolites are rapidly excreted and will not accumulate in plasma. Studies investigating the mechanisms of action for galloyl phase II metabolites need to be performed to see if their bioefficacy is limited to a specific site of action or through regulation of cell signaling pathways.

CHAPTER VII

SUMMARY AND CONCLUSIONS

The pulp of Ataulfo, Tommy Atkins, Keitt, Haden, and Kent mango cultivars were investigated for their galloyl derivative content. Ataulfo had the highest among all cultivars with 21.6 ± 4.53 mg/kg of gallic acid, 189 ± 2.09 mg/kg of ester-monogalloyl glucose, and 3,040 ± 962 mg/kg of gallotannins while Kent had the lowest with 41.3 ± 0.68 of gallic acid, 41.3 ± 2.6 mg/kg of ester-monogalloyl glucose, 127 ± 30 mg/kg and of gallotannins. Additionally, the enzymatic hydrolysis of gallotannins with tannase was evaluated and the intermediates characterized, and when pentagalloyl glucose was hydrolyzed two tetragalloyl glucoses, six trigalloyl glucoses, and five digalloyl glucoses were characterized each hypothesized to have unique stereochemistry and as a result a unique bioavailability. Galloyl glycosides and other gallated species are found in many foods stuffs and have the potential to affect the diet of a large percent of the global population.

The polyphenol composition of mango pulp is comprised primarily of galloyl derivatives. In this study, the galloyl derivatives monogalloyl glucose and gallic acid were found to be bioaccessible in both homogenized and cubed mango pulp and have the potential to be absorbed and metabolized. A significant amount of gallotannins remained bound within the food matrix for both homogenized and cubed mango suggesting limited bioaccessibility in the small intestine and will transit to the colon. For the first time gallic acid and monogalloyl glucose were shown to be transported across Caco-2

monolayers at a similar rate. In a porcine model, similar amounts of phase II galloyl metabolites were found for gallic acid and monogalloyl glucose following oral administration, and this may suggest that the differences in composition of non-tannin galloyl derivatives between mango cultivars is not a major factor impacting the physiological production of gallic acid metabolites.

In conclusion, a novel protein precipitation method utilizing SDS was developed for improved recovery of gallic acid from human plasma. The SDS denaturation protocol was utilized to extract human plasma for gallic acid metabolites generated after an acute feeding of 400 g of fresh mango pulp. Five metabolites, two from gallic acid and three from pyrogallol were characterized and semi-quantified over an 8 h period. The SDS method was proven to be able to extract significantly more gallic acid metabolites from plasma compared to current methods reported in the literature. Plasma extraction of gallic acid and its metabolites with SDS is a simple and rapid method that has the potential to be applied in the analysis of other polyphenolic analytes.

The urinary excretion and plasma concentrations of phase II metabolites sourced from mango gallic acid, galloyl glycosides, and gallotannins was assessed in lean and obese subjects who consumed mango daily for 42 days, and compared against subjects who consumed mango only on Days 1 and 42. In the Lean and Obese cohorts, significant increases were found in the cumulative 24 h urinary excretion of 4-*O*-methylgallic acid-3-*O*-sulfate, methylpyrogallol-*O*-sulfate, pyrogallol-*O*-sulfate, and catechol-*O*-sulfate and were hypothesized to be due to adaptations in the subjects gut microflora. No significant differences were found in the individual plasma concentrations of these

metabolites up to 8 h post-prandial. It was hypothesized that the galloyl and pyrogallol metabolites are rapidly excreted and will not accumulate in plasma. Studies investigating the mechanisms of action for galloyl phase II metabolites need to be performed to see if their bioefficacy is limited to a specific site of action or through regulation of cell signaling pathways.

REFERENCES

[1] Singh, Y. N., Traditional medicine in Fiji: Some herbal folk cures used by Fiji Indians. *Journal of Ethnopharmacology* 1986, *15*, 57-88.

[2] Sairam, K., Hemalatha, S., Kumar, A., Srinivasan, T. Ganesh, J., Shankar, M., & Venkataraman, S., Evaluation of anti-diarrhoeal activity in seed extracts of Mangifera indica. *Journal of Ethnopharmacology* 2003, *84*, 11-15.

[3] Tharanathan, R. N., Yashoda, H. M., Prabha, T. N., Mango (Mangifera indica L.), "The King of Fruits"—An Overview. *Food Reviews International* 2006, *22*, 95-123.

[4] Yi, W., Fischer, J., Krewer, G., Akoh, C. C., Phenolic Compounds from Blueberries Can Inhibit Colon Cancer Cell Proliferation and Induce Apoptosis. *Journal of Agricultural and Food Chemistry* 2005, *53*, 7320-7329.

[5] Kim, H., Banerjee, N., Ivanov, I., Talcott, S., Mertens-Talcott, S., Comparison of anti-inflammatory mechanisms of mango (Mangifera indica L.) and pomegranate (Punica granatum L.) in DSS-induced colitis in rats (372.8). *The FASEB Journal* 2014, *28*.

[6] Makare, N., Bodhankar, S., Rangari, V., Immunomodulatory activity of alcoholic extract of Mangifera indica L. in mice. *Journal of Ethnopharmacology* 2001, *78*, 133-137.

[7] Fantini, M., Benvenuto, M., Masuelli, L., Frajese, G. Tresoldi, I., Modesti, A., & Bei, R., In Vitro and in Vivo Antitumoral Effects of Combinations of Polyphenols, or

Polyphenols and Anticancer Drugs: Perspectives on Cancer Treatment. *International Journal of Molecular Sciences* 2015, *16*, 9236-9282.

[8] Tomás-Barberán, F. A., Andrés-Lacueva, C., Polyphenols and Health: Current State and Progress. *Journal of Agricultural and Food Chemistry* 2012, *60*, 8773-8775.

[9] Berardini, N., Carle, R., Schieber, A., Characterization of gallotannins and benzophenone derivatives from mango (Mangifera indica L. cv. 'Tommy Atkins') peels, pulp and kernels by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Communications in Mass Spectrometry* 2004, *18*, 2208-2216.

[10] Berardini, N., Fezer, R., Conrad, J., Beifuss, U. Carle, R., & Schieber, A., Screening of Mango (Mangifera indica L.) Cultivars for Their Contents of Flavonol O- and Xanthone C-Glycosides, Anthocyanins, and Pectin. *Journal of Agricultural and Food Chemistry* 2005, *53*, 1563-1570.

[11] Krenek, K. A., Barnes, R. C., Talcott, S. T., Phytochemical Composition and Effects of Commercial Enzymes on the Hydrolysis of Gallic Acid Glycosides in Mango (Mangifera indica L. cv. 'Keitt') Pulp. *Journal of Agricultural and Food Chemistry* 2014, *62*, 9515-9521.

[12] Pierson, J. T., Monteith, G. R., Roberts-Thomson, S. J., Dietzgen, R. G. Gidley, M. J., & Shaw, P. N., Phytochemical extraction, characterisation and comparative distribution across four mango (Mangifera indica L.) fruit varieties. *Food Chemistry* 2014, *149*, 253-263.

[13] Ribeiro, S. M. R., Barbosa, L. C. A., Queiroz, J. H., Knödler, M., Schieber, A., Phenolic compounds and antioxidant capacity of Brazilian mango (Mangifera indica L.) varieties. *Food Chemistry* 2008, *110*, 620-626.

[14] Noratto, G. D., Bertoldi, M. C., Krenek, K., Talcott, S. T., Stringheta, P. C., & Mertens-Talcott, S. U., Anticarcinogenic Effects of Polyphenolics from Mango (Mangifera indica) Varieties. *Journal of Agricultural and Food Chemistry* 2010, *58*, 4104-4112.

[15] Percival, S. S., Talcott, S. T., Chin, S. T., Mallak, A. C., Lounds-Singleton, A., & Pettit-Moore, J., Neoplastic Transformation of BALB/3T3 Cells and Cell Cycle of HL-60 Cells are Inhibited by Mango (Mangifera indica L.) Juice and Mango Juice Extracts. *The Journal of Nutrition* 2006, *136*, 1300-1304.

[16] Kawada, M., Ohno, Y., Ri, Y., Ikoma, T., Yuugetu, H., Asai, T., Watanabe, M., Yasuda, N., Akao, S., Takemura, G., Minatoguchi, S., Gotoh, K., Fujiwara, H., Fukuda, K., Anti-tumor effect of gallic acid on LL-2 lung cancer cells transplanted in mice. *Anti-Cancer Drugs* 2001, *12*, 847-852.

[17] Bradbury, K. E., Appleby, P. N., Key, T. J., Fruit, vegetable, and fiber intake in relation to cancer risk: findings from the European Prospective Investigation into Cancer and Nutrition (EPIC). *The American Journal of Clinical Nutrition* 2014, *100*, 394S-398S.

[18] Dauchet, L., Amouyel, P., Hercberg, S., Dallongeville, J., Fruit and Vegetable Consumption and Risk of Coronary Heart Disease: A Meta-Analysis of Cohort Studies. *The Journal of Nutrition* 2006, *136*, 2588-2593.

[19] Hu, D., Huang, J., Wang, Y., Zhang, D., Qu, Y., Fruits and Vegetables Consumption and Risk of Stroke: A Meta-Analysis of Prospective Cohort Studies. *Stroke* 2014, *45*, 1613-1619.

[20] Wang, Q., Chen, Y., Wang, X., Gong, G., Li, G., & Li, C., Consumption of fruit, but not vegetables, may reduce risk of gastric cancer: Results from a meta-analysis of cohort studies. *European Journal of Cancer* 2014, *50*, 1498-1509.

[21] Wang, X., Ouyang, Y., Liu, J., Zhu, M., Zhao, G., Bao, W., & Hu, F. B., Fruit and vegetable consumption and mortality from all causes, cardiovascular disease, and cancer: systematic review and dose-response meta-analysis of prospective cohort studies. *The BMJ* 2014, *349,* 4490.

[22] Yang, Y., Zhang, D., Feng, N., Chen, G., Liu, J., Chen, G., & Zhu, Y., Increased Intake of Vegetables, But Not Fruit, Reduces Risk for Hepatocellular Carcinoma: A Meta-analysis. *Gastroenterology* 2014, *147*, 1031-1042.

[23] Willett, W. C., Sacks, F., Trichopoulou, A., Drescher, G., Ferro-Luzzi, A., Helsing, E., & Trichopoulos, D., Mediterranean diet pyramid: a cultural model for healthy eating. *The American Journal of Clinical Nutrition* 1995, *61*, 1402S-1406S.

[24] State-specific trends in fruit and vegetable consumption among adults --- United States, 2000-2009. *MMWR. Morbidity And Mortality Weekly Report* 2010, *59*, 1125-1130.

[25] Michels Blanck, H., Gillespie, C., Kimmons, J. E., Seymour, J. D., Serdula, M. K., Trends in Fruit and Vegetable Consumption Among U.S. Men and Women, 1994–2005. *Preventing Chronic Disease* 2008, *5*, A35.

[26] Crino, M., Sacks, G., Vandevijvere, S., Swinburn, B., Neal, B., The Influence on Population Weight Gain and Obesity of the Macronutrient Composition and Energy Density of the Food Supply. *Curr Obes Rep* 2015, *4*, 1-10.

[27] Dietz, W. H., The Response of the US Centers for Disease Control and Prevention to the Obesity Epidemic. *Annual Review of Public Health* 2015, *36*, 575-596.

[28] Produce for Better Health Foundation. State of the Plate, 2015 Study on America's Consumption of Fruit and Vegetables. *Produce for Better Health Foundation* 2015. http://www.PBHFoundation.org

[29] Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L., Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition* 2004, *79*, 727-747.

[30] Naczk, M., Shahidi, F., Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis* 2006, *41*, 1523-1542.

[31] Talapatra, S., Talapatra, B., *Chemistry of Plant Natural Products*, Springer Berlin Heidelberg 2015, pp. 625-678.

[32] Bravo, L., Polyphenols: Chemistry, Dietary Sources, Metabolism, and Nutritional Significance. *Nutrition Reviews* 1998, *56*, 317-333.

[33] Tsao, R., Chemistry and Biochemistry of Dietary Polyphenols. *Nutrients* 2010, *2*, 1231-1246.

[34] Niemetz, R., Gross, G. G., Enzymology of gallotannin and ellagitannin biosynthesis. *Phytochemistry* 2005, *66*, 2001-2011.

[35] Hofmann, A. S., Gross, G. G., Biosynthesis of gallotannins: Formation of polygalloylglucoses by enzymatic acylation of 1,2,3,4,6-penta-O-galloylglucose. *Archives of Biochemistry and Biophysics* 1990, *283*, 530-532.

[36] Dixon, R. A., Achnine, L., Kota, P., Liu, C.-J.*, et al.*, The phenylpropanoid pathway and plant defence—a genomics perspective. *Molecular Plant Pathology* 2002, *3*, 371-390.

[37] Soto-Vaca, A., Gutierrez, A., Losso, J. N., Xu, Z., Finley, J. W., Evolution of Phenolic Compounds from Color and Flavor Problems to Health Benefits. *Journal of Agricultural and Food Chemistry* 2012, *60*, 6658-6677.

[38] Pimpão, R. C., Dew, T., Figueira, M. E., McDougall, G. J.*, et al.*, Urinary metabolite profiling identifies novel colonic metabolites and conjugates of phenolics in healthy volunteers. *Molecular Nutrition & Food Research* 2014, *58*, 1414-1425.

[39] Serrano, J., Puupponen-Pimiä, R., Dauer, A., Aura, A.-M., Saura-Calixto, F., Tannins: Current knowledge of food sources, intake, bioavailability and biological effects. *Molecular Nutrition & Food Research* 2009, *53*, S310-S329.

[40] Hager, T. J., Howard, L. R., Liyanage, R., Lay, J. O., Prior, R. L., Ellagitannin Composition of Blackberry As Determined by HPLC-ESI-MS and MALDI-TOF-MS. *Journal of Agricultural and Food Chemistry* 2008, *56*, 661-669.

[41] Lu, M.-J., Chu, S.-C., Yan, L., Chen, C., Effect of tannase treatment on protein–tannin aggregation and sensory attributes of green tea infusion. *LWT-Food Science and Technology* 2009, *42*, 338-342.

[42] Leopoldini, M., Marino, T., Russo, N., Toscano, M., Antioxidant Properties of Phenolic Compounds: H-Atom versus Electron Transfer Mechanism. *The Journal of Physical Chemistry A* 2004, *108*, 4916-4922.

[43] Sosulski, F., Krygier, K., Hogge, L., Free, esterified, and insoluble-bound phenolic acids. 3. Composition of phenolic acids in cereal and potato flours. *Journal of Agricultural and Food Chemistry* 1982, *30*, 337-340.

[44] Williamson, G., Manach, C., Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *The American Journal of Clinical Nutrition* 2005, *81*, 243S-255S.

[45] Siebert, K. J., Effects of Protein−Polyphenol Interactions on Beverage Haze, Stabilization, and Analysis. *Journal of Agricultural and Food Chemistry* 1999, *47*, 353-362.

[46] Chung, K.-T., Wei, C.-I., Johnson, M. G., Are tannins a double-edged sword in biology and health? *Trends in Food Science & Technology* 1998, *9*, 168-175.

[47] Litz, R. E., *Compendium of Transgenic Crop Plants*, John Wiley & Sons, Ltd 2009.

[48] Food and Agriculture Organization of the United Nations, *FAOSTAT Database*, 2014. http://faostat.fao.org/

[49] Hirschberg, J., Carotenoid biosynthesis in flowering plants. *Current Opinion in Plant Biology* 2001, *4*, 210-218.

[50] Namitha, K. K., Negi, P. S., Chemistry and Biotechnology of Carotenoids. *Critical Reviews in Food Science and Nutrition* 2010, *50*, 728-760.

[51] Ornelas-Paz, J. d. J., Yahia, E. M., Gardea-Bejar, A., Identification and Quantification of Xanthophyll Esters, Carotenes, and Tocopherols in the Fruit of Seven Mexican Mango Cultivars by Liquid Chromatography−Atmospheric Pressure Chemical Ionization−Time-of-Flight Mass Spectrometry [LC-(APcI+)-MS]. *Journal of Agricultural and Food Chemistry* 2007, *55*, 6628-6635.

[52] Mercadante, A. Z., Rodriguez-Amaya, D. B., Britton, G., HPLC and Mass Spectrometric Analysis of Carotenoids from Mango. *Journal of Agricultural and Food Chemistry* 1997, *45*, 120-123.

[53] Ornelas-Paz, J. d. J., Yahia, E. M., Gardea, A. A., Changes in external and internal color during postharvest ripening of 'Manila' and 'Ataulfo' mango fruit and relationship with carotenoid content determined by liquid chromatography–APcI+-time-of-flight mass spectrometry. *Postharvest Biology and Technology* 2008, *50*, 145-152.

[54] Saleh, N. A. M., El–Ansari, M. A. I., POLYPHENOLICS OF TWENTY LOCAL VARIETIES OF MANGIFERA INDICA. *Planta Med* 1975, *28*, 124-130.

[55] Pimpão, R. C., Ventura, M. R., Ferreira, R. B., Williamson, G., Santos, C. N., Phenolic sulfates as new and highly abundant metabolites in human plasma after ingestion of a mixed berry fruit purée. *British Journal of Nutrition* 2015, *113*, 454-463.

[56] Motilva, M.-J., Serra, A., Rubió, L., Nutrikinetic studies of food bioactive compounds: from in vitro to in vivo approaches. *International Journal of Food Sciences and Nutrition* 2015, *66*, S41-S52.

[57] Kirita, M., Tanaka, Y., Tagashira, M., Kanda, T., Maeda-Yamamoto, M., Cloning and characterization of a novel O-methyltransferase from Flammulina velutipes that

catalyzes methylation of pyrocatechol and pyrogallol structures in polyphenols. *Bioscience, Biotechnology, and Biochemistry* 2015, 1-8.

[58] Jiamboonsri, P., Pithayanukul, P., Bavovada, R., Gao, S., Hu, M., A validated liquid chromatography–tandem mass spectrometry method for the determination of methyl gallate and pentagalloyl glucopyranose: Application to pharmacokinetic studies. *Journal of Chromatography B* 2015, *986–987*, 12-17.

[59] Wang, X., Li, W., Ma, X., Chu, Y., *et al.*, Simultaneous determination of caffeic acid and its major pharmacologically active metabolites in rat plasma by LC-MS/MS and its application in pharmacokinetic study. *Biomedical Chromatography* 2014, 552-559.

[60] Qiao, J., Kong, X., Kong, A., Han, M., Pharmacokinetics and Biotransformation of Tea Polyphenols. *Current Drug Metabolism* 2014, *15*, 30-36.

[61] Margalef, M., Pons, Z., Muguerza Marquinez, B., Arola-Arnal, A., A rapid method to determine the colonic microbial metabolites derived from grape flavanols in rat plasma by liquid chromatography-tandem mass spectrometry. *Journal of Agricultural and Food Chemistry* 2014, *62*, 7698-7706.

[62] Hodgson, A. B., Randell, R. K., Mahabir-Jagessar-T, K., Lotito, S., *et al.*, Acute Effects of Green Tea Extract Intake on Exogenous and Endogenous Metabolites in Human Plasma. *Journal of Agricultural and Food Chemistry* 2014, *62*, 1198-1208.

[63] Lan, W., Bian, L., Zhao, X., Jia, P., *et al.*, Liquid Chromatography/Quadrupole Time-of-Flight Mass Spectrometry for Identification of In Vitro and In Vivo Metabolites of Bornyl Gallate in Rats. *Journal of Analytical Methods in Chemistry* 2013, *2013*, 10.

[64] Borges, G., Lean, M. E. J., Roberts, S. A., Crozier, A., Bioavailability of dietary (poly)phenols: a study with ileostomists to discriminate between absorption in small and large intestine. *Food & Function* 2013, *4*, 754-762.

[65] Yan, Z., Chen, Y., Li, T., Zhang, J., Yang, X., Identification of metabolites of Si-Ni-San, a traditional Chinese medicine formula, in rat plasma and urine using liquid chromatography/diode array detection/triple–quadrupole spectrometry. *Journal of Chromatography B* 2012, *885–886*, 73-82.

[66] Stalmach, A., Edwards, C. A., Wightman, J. D., Crozier, A., Identification of (Poly)phenolic Compounds in Concord Grape Juice and Their Metabolites in Human Plasma and Urine after Juice Consumption. *Journal of Agricultural and Food Chemistry* 2011, *59*, 9512-9522.

[67] Borges, G., Mullen, W., Mullan, A., Lean, M. E. J.*, et al.*, Bioavailability of multiple components following acute ingestion of a polyphenol-rich juice drink. *Molecular Nutrition & Food Research* 2010, *54*, S268-S277.

[68] Bazoti, F. N., Gikas, E., Tsarbopoulos, A., Simultaneous quantification of oleuropein and its metabolites in rat plasma by liquid chromatography electrospray ionization tandem mass spectrometry. *Biomedical Chromatography* 2010, *24*, 506-515.

[69] Green, R. J., Murphy, A. S., Schulz, B., Watkins, B. A., Ferruzzi, M. G., Common tea formulations modulate in vitro digestive recovery of green tea catechins. *Molecular Nutrition & Food Research* 2007, *51*, 1152-1162.

[70] Jakobek, L., Interactions of polyphenols with carbohydrates, lipids and proteins. *Food Chemistry* 2015, *175*, 556-567.

[71] Krook, M. A., Hagerman, A. E., Stability of polyphenols epigallocatechin gallate and pentagalloyl glucose in a simulated digestive system. *Food Research International* 2012, *49*, 112-116.

[72] Hollman, P. C. H., Bijsman, M. N. C. P., van Gameren, Y., Cnossen, E. P. J., *et al.*, The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Radical Research* 1999, *31*, 569-573.

[73] Rubio, L., Macia, A., Motilva, M.-J., Impact of Various Factors on Pharmacokinetics of Bioactive Polyphenols: An Overview. *Current Drug Metabolism* 2014, *15*, 62-76.

[74] Konishi, Y., Kobayashi, S., Shimizu, M., Transepithelial Transport of p-Coumaric Acid and Gallic Acid in Caco-2 Cell Monolayers. *Bioscience, Biotechnology, and Biochemistry* 2003, *67*, 2317-2324.

[75] Halestrap, A. P., *Comprehensive Physiology*, John Wiley & Sons, Inc. 2011.

[76] Johnston, K., Sharp, P., Clifford, M., Morgan, L., Dietary polyphenols decrease glucose uptake by human intestinal Caco-2 cells. *FEBS Letters* 2005, *579*, 1653-1657.

[77] Peters, C. M., Green, R. J., Janle, E. M., Ferruzzi, M. G., Formulation with ascorbic acid and sucrose modulates catechin bioavailability from green tea. *Food Research International* 2010, *43*, 95-102.

[78] Xu, C., Li, C.-T., Kong, A.-N., Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res* 2005, *28*, 249-268.

[79] Manach, C., Williamson, G., Morand, C., Scalbert, A., Rémésy, C., Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *American Journal of Clinical Nutrition* 2005, *81*, 230S-242S.

[80] Gleichenhagen, M., Schieber, A., Current challenges in polyphenol analytical chemistry. *Current Opinion in Food Science* 2016, *7*, 43-49.

[81] Booth, A. N., Masri, M. S., Robbins, D. J., Emerson, O., *et al.*, The metabolic fate of gallic acid and related compounds. *Journal of Biological Chemistry* 1959, *234*, 3014-3016.

[82] Yasuda, T., Inaba, A., Ohmori, M., Endo, T., *et al.*, Urinary Metabolites of Gallic Acid in Rats and Their Radical-Scavenging Effects on 1,1-Diphenyl-2-picrylhydrazyl Radical. *Journal of Natural Products* 2000, *63*, 1444-1446.

[83] Li, L., Zhang, J., Shaik, A., Nhkata, K., *et al.*, Abstract 2871: Pharmacokinetics studies of anticancer gallotannin penta-O-galloyl-beta-D-glucose (PGG) in mice. *Cancer Research* 2010, *70*, 2871.

[84] van der Pijl, P. C., Foltz, M., Glube, N. D., Peters, S., Duchateau, G. S. M. J. E., Pharmacokinetics of black tea-derived phenolic acids in plasma. *Journal of Functional Foods* 2015, *17*, 667-675.

[85] O'Donovan, L., Brooker, J. D., Effect of hydrolysable and condensed tannins on growth, morphology and metabolism of Streptococcus gallolyticus (S. caprinus) and Streptococcus bovis. *Microbiology* 2001, *147*, 1025-1033.

[86] Díaz-Rubio, M. E., Pérez-Jiménez, J., Martínez-Bartolomé, M., Álvarez, I., Saura-Calixto, F., Regular Consumption of an Antioxidant-rich Juice Improves Oxidative

Status and Causes Metabolome Changes in Healthy Adults. *Plant Foods Hum Nutr* 2015, *70*, 9-14.

[87] Tresserra-Rimbau, A., Rimm, E. B., Medina-Remón, A., Martínez-González, M. A.*, et al.*, Inverse association between habitual polyphenol intake and incidence of cardiovascular events in the PREDIMED study. *Nutrition, Metabolism and Cardiovascular Diseases* 2014, *24*, 639-647.

[88] Cowan, T. E., Palmnäs, M. S. A., Yang, J., Bomhof, M. R.*, et al.*, Chronic coffee consumption in the diet-induced obese rat: impact on gut microbiota and serum metabolomics. *The Journal of Nutritional Biochemistry* 2014, *25*, 489-495.

[89] Assunção, M., Santos-Marques, M. J., Carvalho, F., Lukoyanov, N. V., Andrade, J. P., Chronic green tea consumption prevents age-related changes in rat hippocampal formation. *Neurobiology of Aging* 2011, *32*, 707-717.

[90] Geleijnse, J. M., Habitual coffee consumption and blood pressure: An epidemiological perspective. *Vascular Health and Risk Management* 2008, *4*, 963-970.

[91] Yang, Y., Lu, F., Wu, J., Wu, C., Chang, C., THe protective effect of habitual tea consumption on hypertension. *Archives of Internal Medicine* 2004, *164*, 1534-1540.

[92] Ferruzzi, M. G., Lobo, J. K., Janle, E. M., Cooper, B.*, et al.*, Bioavailability of gallic acid and catechins from grape seed polyphenol extract is improved by repeated dosing in rats: implications for treatment in Alzheimer's disease. *Journal Of Alzheimer's Disease: JAD* 2009, *18*, 113-124.

[93] Chow, H.-H. S., Cai, Y., Hakim, I. A., Crowell, J. A.*, et al.*, Pharmacokinetics and Safety of Green Tea Polyphenols after Multiple-Dose Administration of

Epigallocatechin Gallate and Polyphenon E in Healthy Individuals. *Clinical Cancer Research* 2003, *9*, 3312-3319.

[94] Kimble, L., Mathison, B., McKay, D., Chen, C. O.*, et al.*, Chronic Consumption of High Polyphenol Content Cranberry Beverage Improves Phenolic Acid and Flavonoid Bioavailability in Healthy Overweight Humans. *The FASEB Journal* 2015, *29*.

[95] Barnes, R. C., Krenek, K. A., Meibohm, B., Mertens-Talcott, S. U., Talcott, S. T., Urinary Metabolites from Mango (Mangifera indica L. cv. Keitt) Galloyl Derivatives and In Vitro Hydrolysis of Gallotannins in Physiological Conditions. *Molecular Nutrition & Food Research* 2015, *60*, 542-550.

[96] Rothwell, J. A., Urpi-Sarda, M., Boto-Ordoñez, M., Llorach, R.*, et al.*, Systematic analysis of the polyphenol metabolome using the Phenol-Explorer database. *Molecular Nutrition & Food Research* 2015, 203-211.

[97] Gasperotti, M., Masuero, D., Guella, G., Mattivi, F., Vrhovsek, U., Development of a targeted method for twenty-three metabolites related to polyphenol gut microbial metabolism in biological samples, using SPE and UHPLC-ESI-MS/MS. *Talanta* 2014, *128*, 221-230.

[98] de Ferrars, R. M., Czank, C., Saha, S., Needs, P. W.*, et al.*, Methods for Isolating, Identifying, and Quantifying Anthocyanin Metabolites in Clinical Samples. *Analytical Chemistry* 2014, *86*, 10052-10058.

[99] Ma, F., Gong, X., Zhou, X., Zhao, Y., Li, M., An UHPLC–MS/MS method for simultaneous quantification of gallic acid and protocatechuic acid in rat plasma after oral

administration of Polygonum capitatum extract and its application to pharmacokinetics. *Journal of Ethnopharmacology* 2015, *162*, 377-383.

[100] Sun, Z., Zhao, L., Zuo, L., Qi, C., *et al.*, A UHPLC–MS/MS method for simultaneous determination of six flavonoids, gallic acid and 5,8-dihydroxy-1,4-naphthoquinone in rat plasma and its application to a pharmacokinetic study of Cortex Juglandis Mandshuricae extract. *Journal of Chromatography B* 2014, *958*, 55-62.

[101] Seeram, N. P., Lee, R., Heber, D., Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (Punica granatum L.) juice. *Clinica Chimica Acta* 2004, *348*, 63-68.

[102] Xiao, J., Kai, G., A Review of Dietary Polyphenol-Plasma Protein Interactions: Characterization, Influence on the Bioactivity, and Structure-Affinity Relationship. *Critical Reviews in Food Science and Nutrition* 2012, *52*, 85-101.

[103] Luo, F., Fu, Y., Xiang, Y., Yan, S., *et al.*, Identification and quantification of gallotannins in mango (Mangifera indica L.) kernel and peel and their antiproliferative activities. *Journal of Functional Foods* 2014, *8*, 282-291.

[104] Schieber, A., Ullrich, W., Carle, R., Characterization of polyphenols in mango puree concentrate by HPLC with diode array and mass spectrometric detection. *Innovative Food Science & Emerging Technologies* 2000, *1*, 161-166.

[105] Jiamboonsri, P., Pithayanukul, P., Bavovada, R., Leanpolchareanchai, J., *et al.*, Factors Influencing Oral Bioavailability of Thai Mango Seed Kernel Extract and Its Key Phenolic Principles. *Molecules* 2015, *20*, 21254-21273.

[106] Hollman, P., De Vries, J., van Leeuwen, S. D., Mengelers, M., Katan, M. B., Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *The American journal of clinical nutrition* 1995, *62*, 1276-1282.

[107] Rodríguez, H., Rivas, B. d. l., Gómez-Cordovés, C., Muñoz, R., Degradation of tannic acid by cell-free extracts of Lactobacillus plantarum. *Food Chemistry* 2008, *107*, 664-670.

[108] Newsome, A. G., Li, Y., van Breemen, R. B., Improved Quantification of Free and Ester-Bound Gallic Acid in Foods and Beverages by UHPLC-MS/MS. *Journal of Agricultural and Food Chemistry* 2016, *64*, 1326-1334.

[109] Belmares, R., Contreras-Esquivel, J. C., Rodríguez-Herrera, R., Coronel, A. R. r., Aguilar, C. N., Microbial production of tannase: an enzyme with potential use in food industry. *LWT - Food Science and Technology* 2004, *37*, 857-864.

[110] Aguilar, C. N., Rodríguez, R., Gutiérrez-Sánchez, G., Augur, C., *et al.*, Microbial tannases: advances and perspectives. *Applied Microbiology and Biotechnology* 2007, *76*, 47-59.

[111] Yao, J., Guo, G. S., Ren, G. H., Liu, Y. H., Production, characterization and applications of tannase. *Journal of Molecular Catalysis B: Enzymatic* 2014, *101*, 137-147.

[112] Kaur, M., Velmurugan, B., Rajamanickam, S., Agarwal, R., Agarwal, C., Gallic Acid, an Active Constituent of Grape Seed Extract, Exhibits Anti-proliferative, Pro-apoptotic and Anti-tumorigenic Effects Against Prostate Carcinoma Xenograft Growth in Nude Mice. *Pharm Res* 2009, *26*, 2133-2140.

[113] Wu, M., Wang, Q., McKinstry, W. J., Ren, B., Characterization of a tannin acyl hydrolase from Streptomyces sviceus with substrate preference for digalloyl ester bonds. *Applied Microbiology and Biotechnology* 2015, *99*, 2663-2672.

[114] Cruz, R., de Lima, J. S., Fonseca, J. C., dos Santos Fernandes, M. J., *et al.*, Diversity of filamentous fungi of area from Brazilian Caatinga and high-level tannase production using mango (Mangifera indica L.) and surinam cherry (Eugenia uniflora L.) leaves under SSF. *Advances in Microbiology* 2013, *3*, 52.

[115] López de Felipe, F., de las Rivas, B., Muñoz, R., Bioactive compounds produced by gut microbial tannase: implications for colorectal cancer development. *Frontiers in Microbiology* 2014, *5*, 684.

[116] Hagerman, A. E., *Tannin Handbook* 2011. http://www.users.miamioh.edu/hagermae/

[117] Singleton, V. L., Rossi, J. A., Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture* 1965, *16*, 144-158.

[118] Sáyago-Ayerdi, S. G., Moreno-Hernández, C. L., Montalvo-González, E., García-Magaña, M. L., *et al.*, Mexican ‘Ataulfo’ mango (Mangifera indica L) as a source of hydrolyzable tannins. Analysis by MALDI-TOF/TOF MS. *Food Research International* 2013, *51*, 188-194.

[119] Manthey, J. A., Perkins-Veazie, P., Influences of Harvest Date and Location on the Levels of β-Carotene, Ascorbic Acid, Total Phenols, the in Vitro Antioxidant

Capacity, and Phenolic Profiles of Five Commercial Varieties of Mango (Mangifera indica L.). *Journal of Agricultural and Food Chemistry* 2009, *57*, 10825-10830.

[120] Battestin, V., Macedo, G. A., De Freitas, V. A. P., Hydrolysis of epigallocatechin gallate using a tannase from Paecilomyces variotii. *Food Chemistry* 2008, *108*, 228-233.

[121] Chang, F.-S., Chen, P.-C., Chen, R. L. C., Lu, F.-M., Cheng, T.-J., Real-time assay of immobilized tannase with a stopped-flow conductometric device. *Bioelectrochemistry* 2006, *69*, 113-116.

[122] Grundhöfer, P., Niemetz, R., Schilling, G., Gross, G. G., Biosynthesis and subcellular distribution of hydrolyzable tannins. *Phytochemistry* 2001, *57*, 915-927.

[123] Ren, B., Wu, M., Wang, Q., Peng, X., *et al.*, Crystal Structure of Tannase from Lactobacillus plantarum. *Journal of Molecular Biology* 2013, *425*, 2737-2751.

[124] Garrido, G., González, D., Lemus, Y., García, D., *et al.*, In vivo and in vitro anti-inflammatory activity of Mangifera indica L. extract (VIMANG®). *Pharmacological Research* 2004, *50*, 143-149.

[125] Wilkinson, A. S., Monteith, G. R., Shaw, P. N., Lin, C.-N., *et al.*, Effects of the Mango Components Mangiferin and Quercetin and the Putative Mangiferin Metabolite Norathyriol on the Transactivation of Peroxisome Proliferator-Activated Receptor Isoforms. *Journal of Agricultural and Food Chemistry* 2008, *56*, 3037-3042.

[126] Kamiloglu, S., Capanoglu, E., Bilen, F. D., Gonzales, G. B., *et al.*, Bioaccessibility of Polyphenols from Plant-Processing Byproducts of Black Carrot (Daucus carota L.). *Journal of Agricultural and Food Chemistry* 2015, *64*, 2450-2458.

[127] Rodríguez, H., de las Rivas, B., Gómez-Cordovés, C., Muñoz, R., Characterization of tannase activity in cell-free extracts of Lactobacillus plantarum CECT 748T. *International Journal of Food Microbiology* 2008, *121*, 92-98.

[128] Bouayed, J., Deußer, H., Hoffmann, L., Bohn, T., Bioaccessible and dialysable polyphenols in selected apple varieties following in vitro digestion vs. their native patterns. *Food Chemistry* 2012, *131*, 1466-1472.

[129] Kaulmann, A., André, C. M., Schneider, Y.-J., Hoffmann, L., Bohn, T., Carotenoid and polyphenol bioaccessibility and cellular uptake from plum and cabbage varieties. *Food Chemistry* 2016, *197, Part A*, 325-332.

[130] Hidalgo, I. J., Borchardt, R. T., Transport of a large neutral amino acid (phenylalanine) in a human intestinal epithelial cell line: Caco-2. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1990, *1028*, 25-30.

[131] van der Pijl, P. C., Kies, A. K., Ten Have, G. A. M., Duchateau, G. S. M. J. E., Deutz, N. E. P., Pharmacokinetics of proline-rich tripeptides in the pig. *Peptides* 2008, *29*, 2196-2202.

[132] Zhang, Y., Huo, M., Zhou, J., Xie, S., PKSolver: An add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. *Computer Methods and Programs in Biomedicine* 2010, *99*, 306-314.

[133] Friedman, M., Jürgens, H. S., Effect of pH on the Stability of Plant Phenolic Compounds. *Journal of Agricultural and Food Chemistry* 2000, *48*, 2101-2110.

[134] Low, D. Y., D'Arcy, B., Gidley, M. J., Mastication effects on carotenoid bioaccessibility from mango fruit tissue. *Food Research International* 2015, *67*, 238-246.

[135] Englyst, H. N., Hay, S., Macfarlane, G. T., Polysaccharide breakdown by mixed populations of human faecal bacteria. *FEMS Microbiology Letters* 1987, *45*, 163-171.

[136] Goel, G., Kumar, A., Beniwal, V., Raghav, M.*, et al.*, Degradation of tannic acid and purification and characterization of tannase from Enterococcus faecalis. *International Biodeterioration & Biodegradation* 2011, *65*, 1061-1065.

[137] Jimenez, N., Reveron, I., Esteban-Torres, M., Lopez de Felipe, F.*, et al.*, Genetic and biochemical approaches towards unravelling the degradation of gallotannins by Streptococcus gallolyticus. *Microbial cell factories* 2014, *13*, 154.

[138] Han, Y. H., Kim, S. Z., Kim, S. H., Park, W. H., Apoptosis in pyrogallol-treated Calu-6 cells is correlated with the changes of intracellular GSH levels rather than ROS levels. *Lung Cancer* 2008, *59*, 301-314.

[139] Yang, C.-J., Wang, C.-S., Hung, J.-Y., Huang, H.-W.*, et al.*, Pyrogallol induces G2-M arrest in human lung cancer cells and inhibits tumor growth in an animal model. *Lung Cancer* 2009, *66*, 162-168.

[140] Indahl, S. R., Scheline, R. R., Decarboxylation of 4-hydroxycinnamic acids by Bacillus strains isolated from rat intestine. *Applied Microbiology* 1968, *16*, 667.

[141] Scheline, R. R., Decarboxylation and demethylation of some phenolic benzoic acid derivatives by rat caecal contents. *Journal of Pharmacy and Pharmacology* 1966, *18*, 664-669.

[142] Gao, R., Yuan, Z., Zhao, Z., Gao, X., Mechanism of pyrogallol autoxidation and determination of superoxide dismutase enzyme activity. *Bioelectrochemistry and Bioenergetics* 1998, *45*, 41-45.

[143] Chen, L., Lee, M.-J., Li, H., Yang, C. S., Absorption, Distribution, and Elimination of Tea Polyphenols in Rats. *Drug Metabolism and Disposition* 1997, *25*, 1045-1050.

[144] Shahrzad, S., Aoyagi, K., Winter, A., Koyama, A., Bitsch, I., Pharmacokinetics of Gallic Acid and Its Relative Bioavailability from Tea in Healthy Humans. *The Journal of Nutrition* 2001, *131*, 1207-1210.

[145] Shahrzad, S., Bitsch, I., Determination of gallic acid and its metabolites in human plasma and urine by high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications* 1998, *705*, 87-95.

[146] Barnes, R. C., Krenek, K. A., Meibohm, B., Mertens-Talcott, S. U., Talcott, S. T., Urinary metabolites from mango (Mangifera indica L. cv. Keitt) galloyl derivatives and in vitro hydrolysis of gallotannins in physiological conditions. *Molecular Nutrition & Food Research* 2016, *60*, 542-550.

[147] Wang, D., Williams, B. A., Ferruzzi, M. G., D'Arcy, B. R., Microbial metabolites, but not other phenolics derived from grape seed phenolic extract, are transported through differentiated Caco-2 cell monolayers. *Food Chemistry* 2013, *138*, 1564-1573.

[148] Xiao, J., Mao, F., Yang, F., Zhao, Y., *et al.*, Interaction of dietary polyphenols with bovine milk proteins: Molecular structure–affinity relationship and influencing bioactivity aspects. *Molecular Nutrition & Food Research* 2011, *55*, 1637-1645.

[149] Hagerman, A. E., Butler, L. G., Protein precipitation method for the quantitative determination of tannins. *Journal of Agricultural and Food Chemistry* 1978, *26*, 809-812.

[150] Hartzfeld, P. W., Forkner, R., Hunter, M. D., Hagerman, A. E., Determination of Hydrolyzable Tannins (Gallotannins and Ellagitannins) after Reaction with Potassium Iodate. *Journal of Agricultural and Food Chemistry* 2002, *50*, 1785-1790.

[151] Laemmli, U. K., Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* 1970, *227*, 680-685.

[152] Bhuyan, A. K., On the mechanism of SDS-induced protein denaturation. *Biopolymers* 2010, *93*, 186-199.

[153] Department of Health and Human Services, Food and Drug Administration, Guidance for Industry on Bioanalytical Method Validation. *Fed. Regist.* 2001, *66*, 28526.

[154] Marklund, S., Marklund, G., Involvement of the Superoxide Anion Radical in the Autoxidation of Pyrogallol and a Convenient Assay for Superoxide Dismutase. *European Journal of Biochemistry* 1974, *47*, 469-474.

[155] Antharavally, B. S., Mallia, K. A., Rosenblatt, M. M., Salunkhe, A. M.*, et al.*, Efficient removal of detergents from proteins and peptides in a spin column format. *Analytical Biochemistry* 2011, *416*, 39-44.

[156] Gasperotti, M., Masuero, D., Guella, G., Mattivi, F., Vrhovsek, U., Development of a targeted method for twenty-three metabolites related to polyphenol gut microbial

metabolism in biological samples, using SPE and UHPLC–ESI-MS/MS. *Talanta* 2014, *128*, 221-230.

[157] Ajila, C. M., Naidu, K. A., Bhat, S. G., Rao, U. J. S. P., Bioactive compounds and antioxidant potential of mango peel extract. *Food Chemistry* 2007, *105*, 982-988.

[158] Kim, H., Banerjee, N., Barnes, R. C., Pfent, C. M., *et al.*, Mango polyphenolics reduce inflammation in intestinal colitis—involvement of the miR-126/PI3K/AKT/mTOR axis in vitro and in vivo. *Molecular Carcinogenesis* 2016.

[159] Evans, S. F., Meister, M., Mahmood, M., Eldoumi, H., *et al.*, Mango Supplementation Improves Blood Glucose in Obese Individuals. *Nutrition and Metabolic Insights* 2014, *7*, 77-84.

[160] Zhang, J., Li, L., Kim, S.-H., Hagerman, A., Lü, J., Anti-Cancer, Anti-Diabetic and Other Pharmacologic and Biological Activities of Penta-Galloyl-Glucose. *Pharm Res* 2009, *26*, 2066-2080.

[161] Ali, F., Ismail, A., Kersten, S., Molecular mechanisms underlying the potential antiobesity-related diseases effect of cocoa polyphenols. *Molecular Nutrition & Food Research* 2014, *58*, 33-48.

[162] Koutsos, A., Tuohy, K. M., Lovegrove, J. A., Apples and Cardiovascular Health—Is the Gut Microbiota a Core Consideration? *Nutrients* 2015, *7*, 3959-3998.

[163] Fraga, C. G., Oteiza, P., S8-4 - Flavanols and vascular health: molecular mechanisms to build evidence-based recommendations. *Free Radical Biology and Medicine* 2014, *75, Supplement 1*, S12.

[164] Feliciano, R. P., Istas, G., Heiss, C., Rodriguez-Mateos, A., Plasma and Urinary Phenolic Profiles after Acute and Repetitive Intake of Wild Blueberry. *Molecules* 2016, *21*, 1120.

[165] Patel, R., Maru, G., Polymeric black tea polyphenols induce phase II enzymes via Nrf2 in mouse liver and lungs. *Free Radical Biology and Medicine* 2008, *44*, 1897-1911.

[166] Lambert, J. D., Sang, S., Lu, A. Y., Yang, C. S., Metabolism of dietary polyphenols and possible interactions with drugs. *Current drug metabolism* 2007, *8*, 499-507.

[167] Ojo, B., El-Rassi, G. D., Payton, M. E., Perkins-Veazie, P., *et al.*, Mango Supplementation Modulates Gut Microbial Dysbiosis and Short-Chain Fatty Acid Production Independent of Body Weight Reduction in C57BL/6 Mice Fed a High-Fat Diet. *The Journal of Nutrition* 2016, *146*, 1483-1491.

[168] Moreno-Indias, I., Sanchez-Alcoholado, L., Perez-Martinez, P., Andres-Lacueva, C., *et al.*, Red wine polyphenols modulate fecal microbiota and reduce markers of the metabolic syndrome in obese patients. *Food & Function* 2016, *7*, 1775-1787.

[169] Duque, A. L. R. F., Monteiro, M., Adorno, M. A. T., Sakamoto, I. K., Sivieri, K., An exploratory study on the influence of orange juice on gut microbiota using a dynamic colonic model. *Food Research International* 2016, *84*, 160-169.

[170] Vendrame, S., Guglielmetti, S., Riso, P., Arioli, S., *et al.*, Six-Week Consumption of a Wild Blueberry Powder Drink Increases Bifidobacteria in the Human Gut. *Journal of Agricultural and Food Chemistry* 2011, *59*, 12815-12820.

[171] Pereira-Caro, G., Oliver, C. M., Weerakkody, R., Singh, T.*, et al.*, Chronic administration of a microencapsulated probiotic enhances the bioavailability of orange juice flavanones in humans. *Free Radical Biology and Medicine* 2015, *84*, 206-214.

[172] Ayed, L., Hamdi, M., Culture conditions of tannase production by Lactobacillus plantarum. *Biotechnology Letters* 2002, *24*, 1763-1765.

[173] Margalef, M., Pons, Z., Bravo, F. I., Muguerza, B., Arola-Arnal, A., Plasma kinetics and microbial biotransformation of grape seed flavanols in rats. *Journal of Functional Foods* 2015, *12*, 478-488.

[174] Evans, R., Kamm, M., Hinton, J., Lennard-Jones, J., The normal range and a simple diagram for recording whole gut transit time. *International journal of colorectal disease* 1992, *7*, 15-17.

[175] Kim, E. R., Rhee, P.-L., How to interpret a functional or motility test-colon transit study 2012, *18*, 94-99.

[176] Lee, M.-J., Maliakal, P., Chen, L., Meng, X.*, et al.*, Pharmacokinetics of tea catechins after ingestion of green tea and (−)-epigallocatechin-3-gallate by humans formation of different metabolites and individual variability. *Cancer Epidemiology Biomarkers & Prevention* 2002, *11*, 1025-1032.

[177] Margalef, M., Pons, Z., Iglesias-Carres, L., Bravo, F. I.*, et al.*, Lack of Tissue Accumulation of Grape Seed Flavanols after Daily Long-Term Administration in Healthy and Cafeteria-Diet Obese Rats. *Journal of Agricultural and Food Chemistry* 2015, *63*, 9996-10003.

[178] Dudonné, S., Dal-Pan, A., Dubé, P., Varin, T. V.*, et al.*, Potentiation of the bioavailability of blueberry phenolic compounds by co-ingested grape phenolic compounds in mice, revealed by targeted metabolomic profiling in plasma and feces. *Food & Function* 2016, *7*, 3421-3430.

[179] Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M.*, et al.*, Dietary (Poly)phenolics in Human Health: Structures, Bioavailability, and Evidence of Protective Effects Against Chronic Diseases. *Antioxidants & Redox Signaling* 2012, *18*, 1818-1892.

[180] Zern, T. L., Wood, R. J., Greene, C., West, K. L.*, et al.*, Grape polyphenols exert a cardioprotective effect in pre- and postmenopausal women by lowering plasma lipids and reducing oxidative stress. *J Nutr* 2005, *135*, 1911-1917.

[181] Ellis, C. L., Edirisinghe, I., Kappagoda, T., Burton-Freeman, B., Attenuation of Meal-Induced Inflammatory and Thrombotic Responses in Overweight Men and Women After 6-Week Daily Strawberry (Fragaria) Intake— A Randomized Placebo-Controlled Trial. *Journal of Atherosclerosis and Thrombosis* 2011, *18*, 318-327.

[182] Steptoe, A., Gibson, E. L., Vuononvirta, R., Hamer, M., Wardle, J., Rycroft, J., Marin, J., Erusalminsky, J., The effects of chronic tea intake on platelet activation and inflammation: A double-blind placebo controlled trial. *Atherosclerosis* 2007, *193*, 277-282.

[183] Vauzour, D., Rodriguez-Mateos, A., Corona, G., Oruna-Concha, M. J., Spencer, J. P. E., Polyphenols and Human Health: Prevention of Disease and Mechanisms of Action. *Nutrients* 2010, *2*, 1106-1131.

APPENDIX

Plasma Extraction of Gallic Acid

Additional methods to extract the standards gallic acid, 4-*O*-methylgallic acid, and ethyl gallate from plasma were evaluated besides the three described in Chapter V. The solvent ethyl acetate was investigated for its ability to partition polyphenolics from plasma. Extraction with ethyl acetate led to poor recovery of standards from plasma likely due to the aforementioned affinities between galloyl phenolics and plasma proteins. Trichloroacetic acid was additionally tested as a potential extraction aide. It was able to denature plasma proteins, however, it too strongly affected the ionization of the ESI source and led to poor ionization of analytes. Lastly, a cationic surfactant, cetyl trimethylammonium bromide (CTAB), was also investigated, however, CTAB lacked the ability to precipitate plasma proteins in acidic pH conditions, and was judged to be not applicable for extraction of polyphenol metabolites. The three methods outlined here produced poor recovery of gallic acid and where not further investigated for the extraction of galloyl metabolites following consumption of mango.