

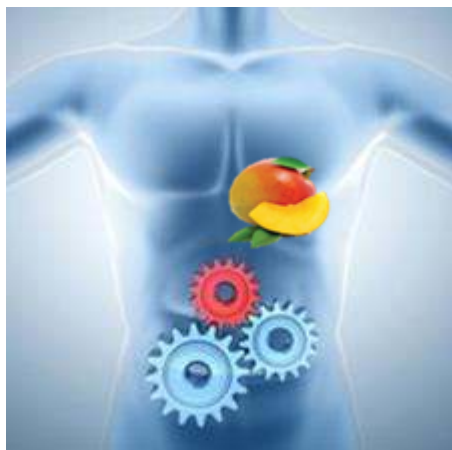


COLLEGE OF AGRICULTURE
AND LIFE SCIENCES

Department of Nutrition and Food Science

Objective I: *In Vitro* Metabolism of Mango Polyphenols and their Anti-inflammatory Activities

Final Report



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Executive Summary:

Objectives: The objectives of this research were to **a)** assess the stability of a mango gallotannin isolate to predict hydrolysis that might occur *in vitro*, which will provide new insight into gallotannin physiological behavior, **b)** investigate the bioaccessibility of mango galloyl derivatives in vitro and in a porcine absorption study, and **c)** assess their anti-inflammatory activities in cell culture.

In summary, both gallic acid and monogalloyl glucose are bioaccessible from different 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. Gallotannins in these in vitro digestion models were found to act as reservoirs of gallic acid which have the potential to release free gallic acid during digestion which can eventually become absorbable gallic acid.

Mango polyphenols metabolize mostly into pyrogallol, which is transformed into pyrogallol-sulfate in the human body. This derivative of mango polyphenols is the more cytotoxic than both its parent compounds and mango extract. Additionally, it exerts significant anti-inflammatory effects in human cells.

Overall, these data show that the generated metabolites from mango gallotannins have potent cancer cytotoxic and anti-inflammatory properties. Mangos have the highest levels in gallotannins among fruits and data show that significant amounts of pyrogallol and gallic acid are generated in the intestines by intestinal bacteria (e.g., *Lactobacillus plantarum*) where they are absorbed into the bloodstream. Consequently, in addition to exerting local anti-inflammatory activities, they exert systemic (in the entire body), overall anti-inflammatory activities that are relevant to cardiovascular disease, arthritis and other chronic diseases.

The performed research resulted in the following research communications:

Abstracts:

RC Barnes, H Kim, NE Deutz, SU Mertens-Talcott, ST Talcott. In Vitro and In Vivo Absorption of Mango Galloyl Derivatives. IFT 2016, Chicago

Manuscripts/peer reviewed publications:

Published

Pyrogallol, an absorbable microbial gallotannins-metabolite and mango polyphenols (*Mangifera Indica* L.) suppress breast cancer ductal carcinoma in situ proliferation in vitro. Nemec MJ, Kim H, Marcianti AB, Barnes RC, Talcott ST, Mertens-Talcott SU. Food Funct. 2016 Sep 14;7(9):3825-33. doi: 10.1039/c6fo00636a. PMID: 27491891

Submission

RC Barnes, H Kim, NE Deutz, SU Mertens-Talcott, ST Talcott. In Vitro Bioaccessibility and In Vivo Absorption of Mango (*Mangifera indica* L.) cv. Ataulfo Galloyl Derivatives, submitted to Food and Function

In preparation

H Kim, Y Minamoto, ME Markel, M Zhu, K Hull, JS Suchodolski, ST Talcott, SU Mertens-Talcott. Anti-inflammatory properties of mango polyphenolic derivatives produced by fecal microbiota in DSS-induced colitis in rats.

Introduction

Our ultimate goal is to identify and demonstrate chemical and biochemical properties of mangos that will allow to better market fresh mangos. This research will also lay vital groundwork towards obtaining legal and marketing claims relating to the composition and health benefits of mangos. We recognize that these foundations are critical to build for a strong working case for these ultimate goals.

Background: Mango is a rich source of polyphenolics which have been shown *in vitro* to exert anti-inflammatory and anti-carcinogenic properties. The worldwide popularity of this fruit suggests that it is capable of impacting the health of many. While *in vitro* cell cultures reveal mechanisms for health-promoting properties, it often does not take into consideration the effect of digestion and metabolism on mango phytochemicals. For this reason, it is crucial to investigate the metabolism of mango polyphenols and the anti-inflammatory properties of their bioactive derivatives in a human digestive system.

Polyphenol Metabolism: While several health benefits have been attributed to polyphenols, it is possible that these benefits are limited due to low bioavailability(1), potentially resulting from incomplete dissolution of 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 that is 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(2). The bioavailability, metabolism, and potential health promoting properties of both gallic acid and pentagalloyl glucose have previously been studied(3). 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(4).

Objectives: The objectives of this research were to **a)** assess the stability of a mango gallotannin isolate to predict hydrolysis that might occur *in vitro*, which will provide new insight into gallotannin physiological behavior, **b)** investigate the bioaccessibility of mango galloyl derivatives in vitro and in a porcine absorption study, and **c)** assess their anti-inflammatory activities in cell culture.

Hypotheses: We hypothesize that the differences in the absorption, metabolism, and excretion of gallic acid, monogalloyl glucose, and larger galloyl glycosides could potentially affect the health promoting properties of mangos.

Significance: Currently, the molecular mechanisms of health benefits of mango polyphenols are not well investigated. In order to assess the role of mango polyphenols in health benefits, we are investigating which polyphenols in mango are the most likely to be absorbed. If the health mechanisms of mango polyphenols are clearly understood, it would pave the way towards substantiating health claims on mango consumption.

Study Approach:

Mangos: Fresh mangos (cv. Ataulfo and Keitt) were shipped refrigerated to the Department of Nutrition and Food Science at Texas A&M University. Mangos were ripened under ambient conditions, and when uniform ripeness was achieved the fruit was manually peeled and deseeded. The pulp was cubed, vacuumed sealed and stored at -20°C until use.

In Vitro Bioaccessibility: In vitro digestion conditions for assessing polyphenol bioaccessibility were similar to previously described models with some modifications(5). In triplicate, 10 g of homogenized mango pulp or 10 g of 0.65 mm³ 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 Na₂CO₃ (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 below. 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 described below, solvents evaporated, reconstituted into acidified water acidified, and filtered through a 0.45 µm membrane for HPLC-MS analysis.

Gallotannin In-Vitro Digestion: A gallotannin isolate from Ataulfo was prepared due to Ataulfo's high concentration of gallotannins. A 150 mL portion of the Ataulfo extract was adsorbed to a 6cc sep-pak C18 cartridge and was subsequently washed with a 20% methanol solution to elute non-gallotannin polyphenolics. The compounds remaining on the cartridge were eluted with 100% methanol and evaporated to dryness under vacuum at 45°C. The resulting gallotannin isolate was reconstituted in 15 mL of water acidified with 0.01% HCl and total phenolic content measured in gallic acid equivalents. To assess the stability of gallotannins in conditions similar to the small intestine, the gallotannin isolate was diluted to a final concentration of 280 mg/L GAE in Hank's Balance Salt Solution (HBSS), Hyclone® Thermo-Fisher, and the pH was adjusted to

7.4 with 1.0 M NaOH. A control sample ($t=0$) was immediately taken and the pH re-adjusted to 3.0 ± 0.2 with 1.0 M HCl. The stability of the gallotannin isolate was evaluated following incubation at 37°C and sampled at 0.5, 1, 2, 3, 4, 6, 12, hours, in triplicate. After each sampling, the pH was adjusted to 3.0 ± 0.2 as described and aliquots were centrifuged at 10,000 x g to remove particles and analyzed with LC-MS.

in Vitro Absorption of Gallic Acid and Monogalloyl Glucose: 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. 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 ($> 450 \Omega \text{ cm}^2$) 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.

Absorption of Gallic Acid and Monogalloyl Glucose 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(6). 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. Absolute bioavailability was calculated using:

$$f = \frac{AUC_{\text{oral}} * Dose_{\text{iv}}}{AUC_{\text{iv}} * Dose_{\text{oral}}}$$
 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 \pm standard error of the mean.

LC-MS Analyses: Samples were analyzed on a Thermo Finnigan Surveyor LCQ Deca XP Max MSⁿ 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 KinetexTM (Bannockburn, Ill) C18 column (150 x 4.6 mm, 2.6 μ m) at room temperature. Injections were made into the column by use of a 50 μ L 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 (N₂) 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.

Cytotoxic activities of polyphenolics: To make sure that the used extracts were not be cytotoxic to cells while performing inflammation-related and absorption studies, HT-29 cells were incubated with mango polyphenolics and their metabolites (1-20 mg/L of total polyphenolic equivalents) and their effects on cell growth were determined after 48h using a Resazurin Cell Viability Kit.

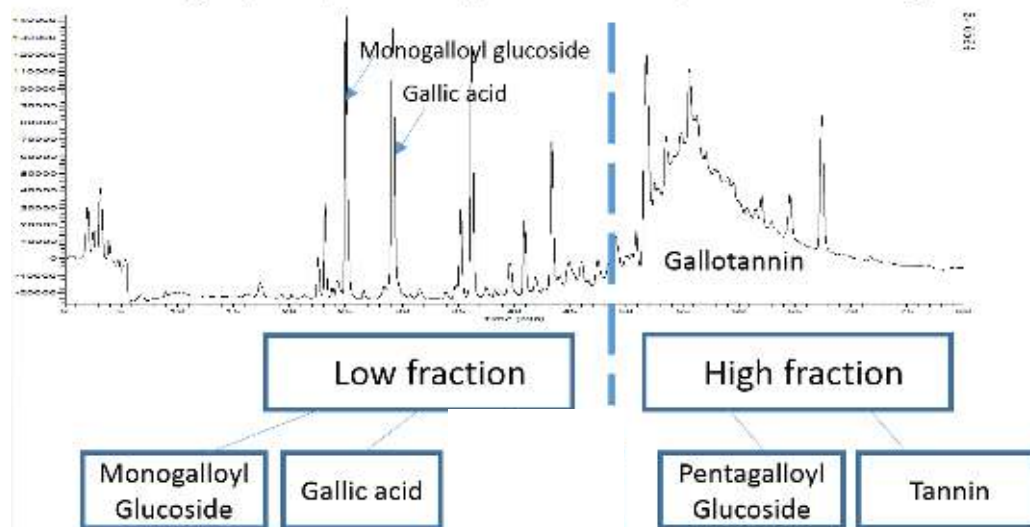
Key inflammation markers were screened by Real-Time PCR (Biomarkers include: IL-1 β , IL-6, and TNF α). CCd-18Co non-cancer human colonocytes were cultured and prepared for experiments as previously described (7). Inflammation were induced with LPS and cells were treated with polyphenolics for 4h.

Statistical Analyses

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 \pm standard error the mean.

Results

Chromatographic profile of phenolic compounds in Mango



Previous reports have documented the high concentrations of gallated phenolics and gallotannins in the pulp of the Ataulfo cultivar compared to other mango varieties.(8) Eight galloyl derivatives were 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. Characterizations were based on UV and mass spectra compared to published values for these compounds.(9) 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 to 117 ± 9.34 mg/kg for nonagalloyl glucose when quantified in equivalents of pentagalloyl glucose

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 1**). 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 $75.1 \pm 6.25\%$, and $61.0 \pm 5.80\%$, 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 $98.0 \pm 4.58\%$ and $85.0 \pm 10.58\%$, respectively, while the concentration of monogalloyl glucose in the mango extract decreased to $81.2 \pm 2.49\%$ of its initial concentration. This

indicates that the mesocarp structure may be critical in stabilizing monogalloyl glucose in both digestive acidic or alkaline conditions.

After 10 h of digestion, a significant loss in monogalloyl glucose was observed and concentrations decreased to $61.6 \pm 7.98\%$, $57.9 \pm 5.23\%$, and $52.1 \pm 8.41\%$ 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 $60.3 \pm 2.41\%$ remained after 10 h (**Figure 2**). Polyphenols are prone to auto-oxidation under duodenal to colonic pH conditions, and this may lead to a progressive formation of oxidized and polymerized compounds in the small and large intestines.(10) 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.

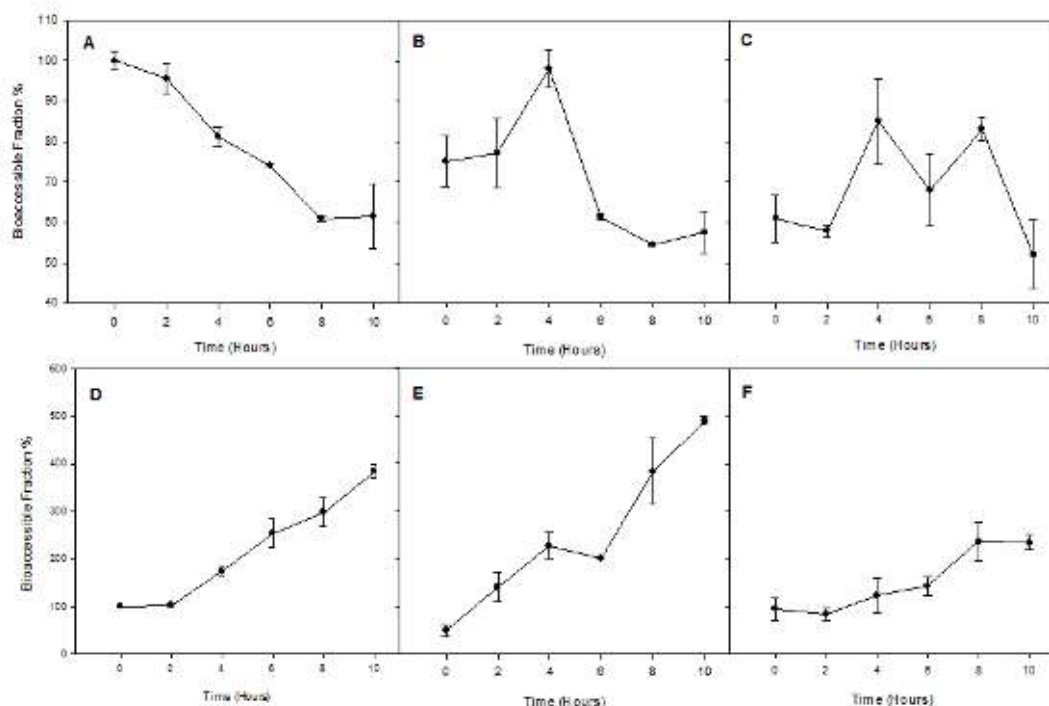


Figure 1. Bioaccessibility of monogalloyl glucose in (A) mango extract, (B) homogenized mango pulp, and (C) cubed mango and free gallic acid in (D) mango extract, (E) homogenized mango pulp, and (F) cubed mango under in vitro digestion conditions at 37 °C, pH 2.0 for 2 h and pH 7.1 for 8 h, with digestive enzymes.

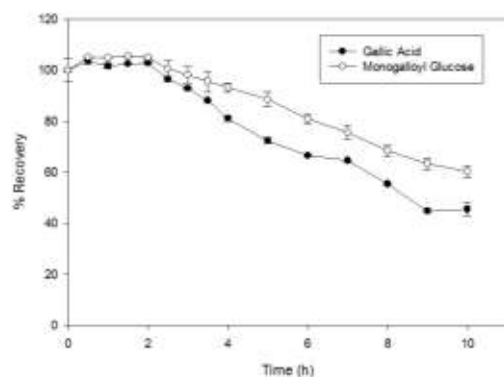


Figure 2. Stability of gallic acid and monogalloyl glucose following in vitro digestion at 37 °C pH 2.0 for 2 h and pH 7.1 for 8 h, with digestive enzymes.

In contrast 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 $51.5 \pm 8.12\%$ and $88.5 \pm 1.03\%$ of the initial amount of gallotannins remained in the fruit matrix for homogenized mango pulp and cubed mango, respectively (**Figure 3**~~Error! Reference source not found.~~). The amount of gallotannins remaining in the cubed mango matrix was significantly ($p < 0.05$) higher compared to homogenized mango pulp 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.(11) 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.(12)

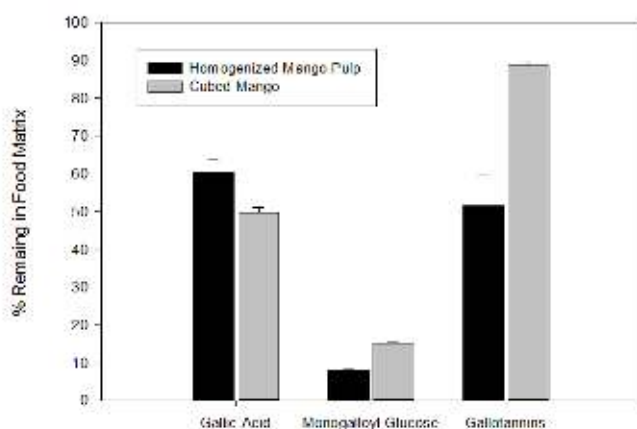


Figure 3. Gallic acid, monogalloyl glucose, and gallotannins bound to cellular matrices of homogenized mango pulp and cubed mango following in vitro digestion at 37 °C pH 2.0 for 2 h and pH 7.1 for 8 h with digestive enzymes.

Free gallic acid significantly ($p < 0.05$) increased after 10 h of the in vitro digestion by $384 \pm 14.9\%$, $490 \pm 9.68\%$ and $233 \pm 15.8\%$ for mango extract, homogenized mango pulp, and cubed mango, respectively (**Figure 1**). 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 $45.4 \pm 2.51\%$ of its initial concentration over 10 h (**Figure 2**). 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.(13) 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(14), and it may be beneficial to choose a food matrix that retains gallotannins throughout upper intestinal digestion to support its generation in the colon.

Gallotannin In-Vitro Digestion

The molecular weight distribution of gallotannins following incubation in cell culture and intestinal conditions, pH 7.4 and 37°C, was evaluated to determine the extent of gallotannin hydrolysis and to estimate the subsequent amount of liberated gallic acid that could potentially be included in the biologically available portion of mango polyphenolics. Prior to incubation, over 63% of the gallotannin isolate (Figure 4) was comprised of compounds with molecular weights corresponding to 8GG, 9GG, 10GG, 11GG, and 12GG, respectively.

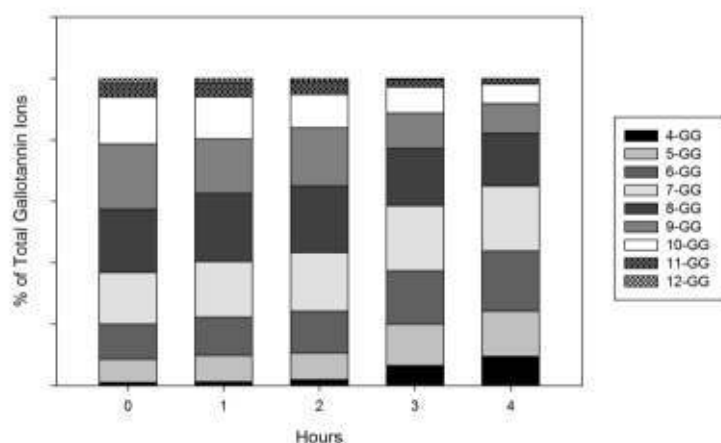


Figure 4. Hydrolysis of Gallotannins in Physiological pH conditions.

Over a four-hour period, the equilibrium shifted to a predominance of gallotannins with molecular weights corresponding to 4GG, 5GG, 6GG and 7GG. The increase in lower molecular weight gallotannins was due to the hydrolysis of larger gallotannin molecules that generated free gallic acid that can be absorbed or be a substrate for the microbiome. The degradation of the gallotannins used in the present study produced 23 mg/L of potentially more bioavailable free gallic acid in four hours.

In Vivo and in Vitro Absorption of Gallic Acid and Monogalloyl Glucose

For the first time the transport of monogalloyl glucose was evaluated in a Caco-2 monolayer model and compared to gallic acid. 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(15). 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 1**). 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.(16)

Table 1. Transport of 50 µg/mL gallic acid and monogalloyl glucose 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

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

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(17), 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 2**).

Table 2. Pharmacokinetic parameters for gallic acid (GA) and monogalloyl glucose (MGG) following oral and IV administration in a 10 h pilot porcine absorption study.

	Oral			IV			
	C _{max}	T _{max}	AUC _{oral}	AUC _{IV}	Half-life	V _d	Bioavailability _{abs} ¹
	(mg/L)	(h)	(mg/L·h)	(mg/L·h)	(h)	(L)	(%)
GA	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
MGG	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.

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 $19.3 \pm 4.40\%$ and $0.28 \pm 0.20\%$, 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.(18) 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. 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.(19, 20)

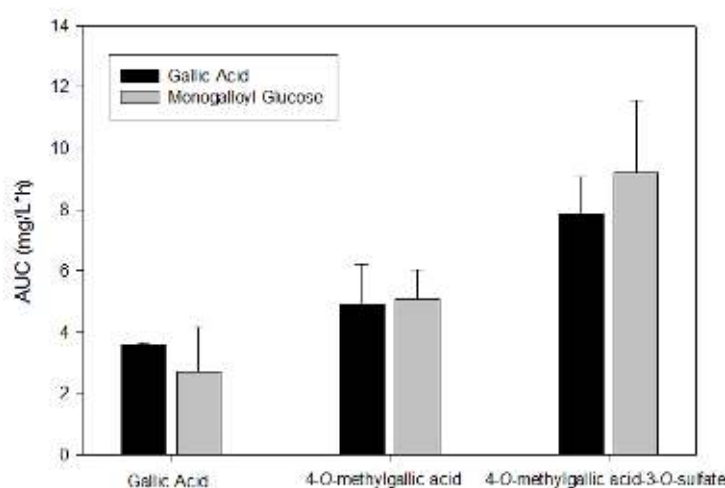


Figure 5. Area under the curve (AUC) for the metabolites gallic acid, 4-O-methylgallic acid, and 4-O-methylgallic acid-3-O-sulfate after oral administration of gallic acid and monogalloyl glucose determined in a porcine model.

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 5**). 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 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.

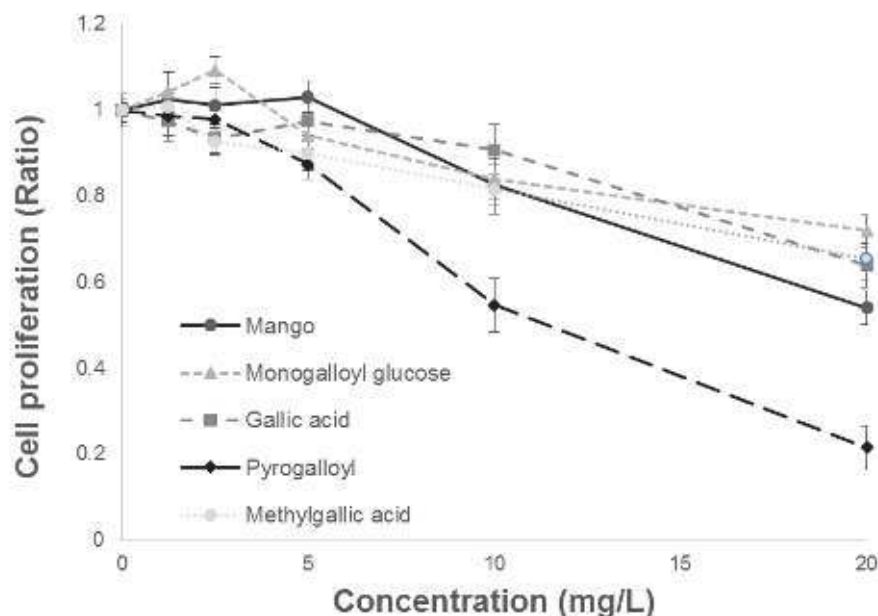


Figure 6. Effects of mango polyphenols and their metabolites on cell proliferation

After incubating mango polyphenols with pig cecum microbiota, we determined that pyrogallol is the major metabolite. We treated HT-29 cells with mango polyphenols, including monogalloyl glucose and gallic acid, as well as the major metabolites pyrogallol and 4-methyl gallic acid. In human colon cancer cells, pyrogallol had more anti-proliferative activity than parent compounds (**Figure 6**).

The IC₅₀ of Monogalloyl glucose, gallic acid, and 4-methyl gallic acid were 31.95, 30.39, and 30.85, respectively. These concentrations were higher than the IC₅₀ of mango extract (IC₅₀ of mango extract: 22.75). This may be due to synergistic effects of compounds in mango polyphenols. Interestingly, the major metabolite pyrogallol had the lowest IC₅₀ (IC₅₀ of pyrogallol: 12.78). This suggests that the metabolism of mango polyphenols increases cytotoxicity.

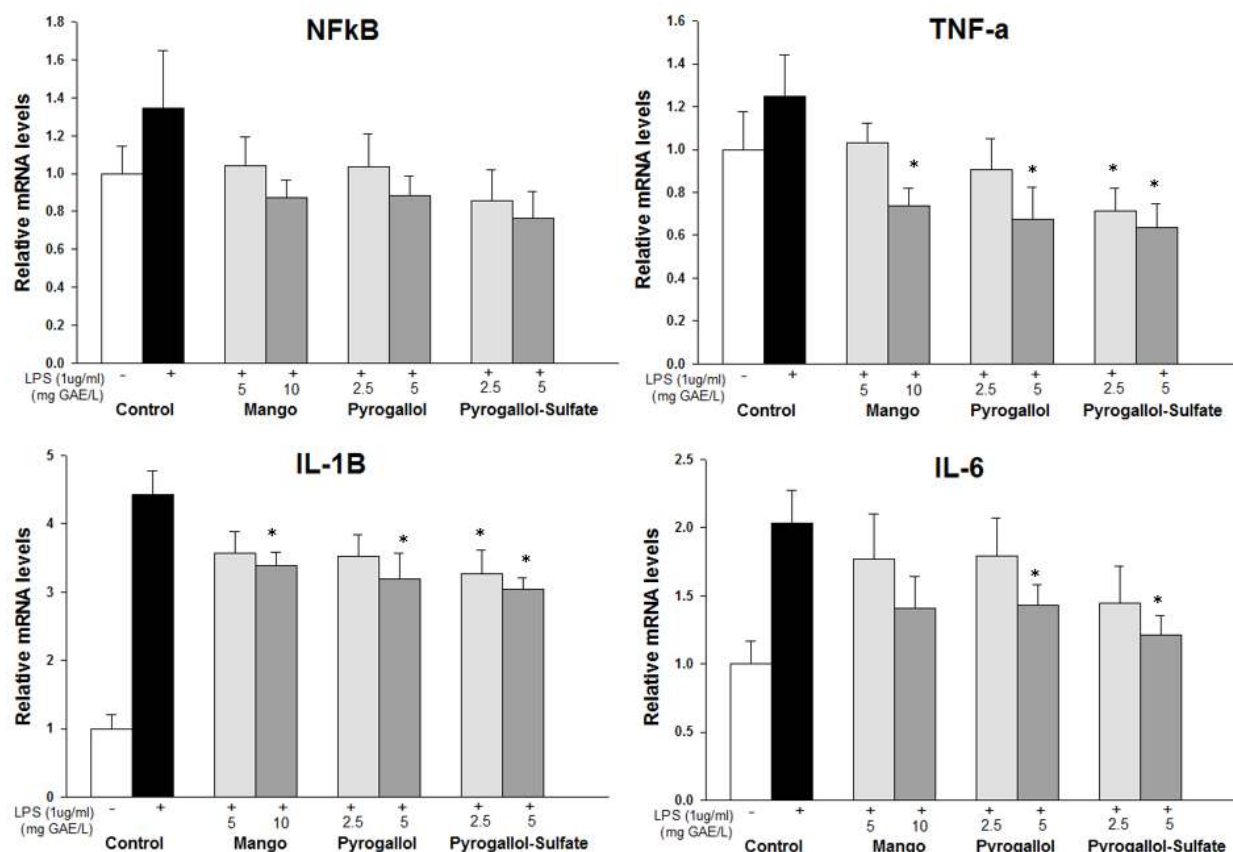


Figure 7. Effects of tannins and their metabolites on the mRNA levels of pro-inflammatory cytokines in LPS-treated CCD-18 cells

In our human pilot study, we determined that most pyrogallol in human plasma is in the form of pyrogallol-sulfate. We determined from the previous cell proliferation assay that only pyrogallol exerted a significant, positive effect on HT-29 cells. As a result, we only tested the effects of mango extract, pyrogallol, and pyrogallol-sulfate on inflamed CCD-18 human epithelial cells. PG-O-sulfate was as effective as PG in decreasing TNF- α , IL-1 β , and IL-6 mRNA levels in LPS-treated CCD-18 fibroblasts (**Figure 7**). This shows that the major metabolite of mango polyphenols does exert anti-inflammatory effects.

References:

1. 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.
2. Kamiloglu, S.; Capanoglu, E.; Bilen, F. D.; Gonzales, G. B.; Grootaert, C.; Van de Wiele, T.; Van Camp, J., Bioaccessibility of Polyphenols from Plant-Processing Byproducts of Black Carrot (*Daucus carota* L.). *Journal of Agricultural and Food Chemistry* **2015**.
3. Ferruzzi, M. G.; Lobo, J. K.; Janle, E. M.; Cooper, B.; Simon, J. E.; Wu, Q.-L.; Welch, C.; Ho, L.; Weaver, C.; Pasinetti, G. M., 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.

4. 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.
5. 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.
6. Ten Have, G. A.; van der Pijl, P. C.; Kies, A. K.; Deutz, N. E., Enhanced Lacto-Tri-Peptide Bio-Availability by Co-Ingestion of Macronutrients. *PloS one* **2015**, *10*, e0130638.
7. Noratto, G. D.; Angel-Morales, G.; Talcott, S. T.; Mertens-Talcott, S. U., Polyphenolics from acai (*Euterpe oleracea* Mart.) and red muscadine grape (*Vitis rotundifolia*) protect human umbilical vascular Endothelial cells (HUVEC) from glucose- and lipopolysaccharide (LPS)-induced inflammation and target microRNA-126. *Journal of agricultural and food chemistry* **2011**, *59*, 7999-8012.
8. Sáyago-Ayerdi, S. G.; Moreno-Hernández, C. L.; Montalvo-González, E.; García-Magaña, M. L.; Mata-Montes de Oca, M.; Torres, J. L.; Pérez-Jiménez, J., 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.
9. 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.
10. 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.
11. Englyst, H. N.; Hay, S.; Macfarlane, G. T., Polysaccharide breakdown by mixed populations of human faecal bacteria. *FEMS Microbiology Letters* **1987**, *45*, 163-171.
12. Goel, G.; Kumar, A.; Beniwal, V.; Raghav, M.; Puniya, A. K.; Singh, K., Degradation of tannic acid and purification and characterization of tannase from *Enterococcus faecalis*. *International Biodeterioration & Biodegradation* **2011**, *65*, 1061-1065.
13. Jimenez, N.; Reveron, I.; Esteban-Torres, M.; Lopez de Felipe, F.; de Las Rivas, B.; Munoz, R., Genetic and biochemical approaches towards unravelling the degradation of gallotannins by *Streptococcus gallolyticus*. *Microbial cell factories* **2014**, *13*, 154.
14. 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.
15. Hollman, P. C. H.; Bijlsman, M. N. C. P.; van Gameren, Y.; Cnossen, E. P. J.; de Vries, J. H. M.; Katan, M. B., The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Radical Research* **1999**, *31*, 569-573.
16. 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.
17. 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.
18. 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.
19. 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.
20. Pimpão, R. C.; Dew, T.; Figueira, M. E.; McDougall, G. J.; Stewart, D.; Ferreira, R. B.; Santos, C. N.; Williamson, G., Urinary metabolite profiling identifies novel colonic metabolites and conjugates of phenolics in healthy volunteers. *Molecular Nutrition & Food Research* **2014**, *58*, 1414-1425.