Colonic metabolism of dietary grape seed extract: Analytical method development, effect on tight-junction proteins, tissue accumulation, and pan-colonic pharmacokinetics

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Abstract

Procyanidins (PCs) have been extensively investigated for their potential health protective activities, but the prospective bioactivities are limited by their poor bioavailability. The majority of the ingested dose remains unabsorbed and reaches the colon where extensive microbial metabolism occurs. The objectives of these studies are to better understand the roles and activities of PCs in the lower gastrointestinal tract. First, a new high-throughput Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry method was developed to efficiently analyze PCs and an extensive profile of their microbial metabolites. This method is sufficiently sensitive and effective in simultaneously extracting and measuring native PCs and their microbial metabolites in biological samples. Furthermore, administration of grape seed extract increased the expression of gut junction protein occludin and reduced levels of fecal calprotectin, which suggests an improvement of gut barrier integrity and a potential modulation of endotoxemia. Additionally, chronic supplementation of the diet with flavanols did not increase colonic tissue accumulation of PCs or their microbial metabolites over a 12 week feeding study. This was the first long-term study of its kind, and the results indicate that we still do not fully understand the outcome of ingested flavanols in the colon during chronic exposure rather than acute doses. Lastly, new understanding of the microbial metabolism of PCs in the colon has been reached by studying the colon as 4 segments, rather than as a complete unit as previous studies have done. Data show that a gradient is established along the length of the colon for both PCs and their metabolites, with PCs reaching highest concentrations within 3 h after ingestion, while metabolites reach maximum concentrations anywhere form 3-18 h after ingestion. Moreover, data indicate the progressive, step-wise degradation of PCs into small metabolites throughout the length of the colon. Overall, there is greater understanding of the colonic metabolism of dietary PCs derived from GSE and cocoa, the accumulation of these compounds, and their effect on gut permeability. Future work will build off of these novel studies, and will continue to advance the understanding of the health benefits of dietary PCs.

Dedication

To my mother and father for your love, support, understanding, financial assistance, and for encouraging me to continue learning, to follow my love of science, and to pursue my dreams.

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List of abbreviations

Acetonitrile, ACN; methanol, MeOH; catechin, C; cocoa extract, CE; degrees of polymerization, DP; epicatechin, EC; epicatechin gallate, ECG; epigallocatechin, EGC; epigallocatechin gallate, EGCG; gallic acid equivalents, GAE; Institutional Animal Care and Use Committee, IACUC; lower limit of detection, LLOD; lower limit of quantification, LLOQ; signal-to-noise, S/N; percent relative standard deviation, %RSD; multidrug-resistant proteins, MRPs; P-glycoprotein, Pgp; phosphate-buffered saline, PBS; procyanidin, PC; grape seed extract, GSE; Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry, UPLC-MS/MS; electrospray ionization, ESI; tandem quadrupole detector, TQD; multi-reaction monitoring, MRM; endotoxin units, EU; high fat, HF; low fat, LF; limulus amebocyte lysate, LAL; quantitative real-time polymerase chain reaction, qRT-PCR; tight junction protein, TJP; toll-like receptor, TLR; zona occludens, ZO

Attributions

Several colleagues contributed to the writing a research for chapters 3-6 of this dissertation. A brief description of their assistances is included here.

Chapter 3: Simultaneous UPLC-MS/MS Analysis of Native Catechins and Procyanidins and Their Microbial Metabolites in Intestinal Contents and Tissues of Male Wistar

Andrew Neilson, Ph.D (Department of Food Science and Technology, Virginia Tech) is currently a professor at Virginia Tech. Dr. Neilson was a co-author on this paper and assisted with method development.

Chapter 4: Chronic administration of dietary grape seed extract increases colonic expression of gut tight junction protein occludin and reduces fecal calprotectin in a secondary analysis of healthy Wistar Furth rats

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Chapter 5: Dietary supplementation with cocoa flavanols does not alter colon tissue profiles of native flavanols and their microbial metabolites established during habitual dietary exposure in C57BL/6J mice

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Cha**pter 6**: Pan-colonic pharmacokinetics of catechins and procyanidins in male Sprague Dawley rats

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Andrew Neilson, Ph.D, is currently a professor in the Department of Food Science and Technology at Virginia Tech. Dr. Neilson was a co-author on this paper and assisted with the completion of the manuscript.

Chapter 1:
Introduction

Procyanidins (PCs) are dimers, oligomers, and polymers of the flavan-3-ol monomers (+)-catechin and (−)-epicatechin [1-3]. Dietary sources include grapes, apples, cocoa and berries, and PCs are typically concentrated in the skins and the seeds [2, 4]. The majority of ingested PCs reach the colon due to poor absorption in the small intestine [5-8]. Once in the colon, PCs are extensively degraded by colonic microbiota to smaller PCs, flavan-3-ol monomers, valerolactones, and phenylalkyl acids, among other compounds [9, 10]. Concentration gradients of native PCs and their microbial metabolites are likely established along the length of the colon due to progressive metabolism [11, 12]. However, there is little data about the concentration profiles of these compounds in the proximal, mid, and distal regions of the colon.

Furthermore, there is little data about the accumulation of PCs and their microbial metabolites in the tissue of the colon. There have been no studies analyzing if there is a build-up of active compounds in the large intestine after chronic administration of PCs. Previous studies have only shown accumulation after acute doses of PCs [11]. Studying the effects of long-term exposure would have greater implications for the use of PCs as a treatment or preventative measure for disease.

Determining spatial, kinetic, and accumulation profiles of these compounds in the colon would allow for further understanding of PC metabolism and facilitate manipulation of colonic PC delivery and metabolism to improve bioactivity. To determine these profiles, it would be ideal to measure both PCs and their metabolites simultaneously. Current methods using Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) are limiting in that they typically measure only PCs, only metabolites, or very limited numbers of both simultaneously [13-16]. Development of a single, fast, high-throughput method would allow effective and efficient study of these compounds.

The overall goal of these studies is to determine the region of the colon where individual PCs are metabolized, and to determine if there is accumulation of PCs and metabolites in the tissue of the large intestine. Our central hypothesis is that native PCs will be found mainly in the proximal and mid regions of the colon, while microbial metabolites will be found mainly in the

mid and distal regions. In order to achieve our overall objective and test our central hypothesis, the following objectives are proposed:

1) Develop and validate a high-throughput UPLC-MS/MS method that will simultaneously measure both native PCs and their metabolites.
2) Determine the kinetics of delivery and microbial metabolism of GSE monomers and procyanidins in the proximal, mid and distal colon.
3) Determine the impact of chronic exposure of dietary PCs on the accumulation of PCs and metabolites in the tissue of the colon

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Chapter 2:

Literature Review

Phenol Classification

Compounds containing one or more hydroxyl groups attached to an aromatic ring are collectively known as phenols [17, 18]. Phenolic compounds exist naturally in plants. Such compounds are not necessary in plant growth and are not directly involved in primary metabolic processes [18]. Therefore, phenolic compounds are secondary plant metabolites acting to support and contribute to the maintenance of multiple metabolic pathways and can have significance to an organism [1, 17].

There are several classes of phenols including phenolic acids, coumarins, and polyphenols [18]. These classifications vary based on the number of carbons and the number of phenol units in the molecule. Phenolic acids are usually broken into two classes: derivatives of benzoic acid and derivatives of cinnamic acid [2]. Hydroxybenzoic acids are not generally found in abundance except in certain red fruits and onions, and therefore have not been considered to be of any importance [2]. These compounds are often the result is the degradation of larger phenolic compounds [11], and therefore may be more of interest than previously thought. Hydroxycinnamic acids are found more commonly though usually bound with another compound, and are found in large quantities in berries [2].

Polyphenols make up a broad class of compounds found in various plant sources that contain one or more aromatic rings with two or more hydroxyl groups attached to the same ring [1, 3, 4, 18, 19]. One subgroup of polyphenols is the flavonoid group which contains thousands of known structures that can be classified as flavanols, isoflavones, flavonols, flavanones, flavones, and anthocyanins [1-3, 19]. These flavonoid subclasses are considered to be strong phytochemicals and demonstrate a wide range of beneficial properties on both plant and human health [17]. Isoflavones, flavonols, flavanones, flavones, and anthocyanins have a 15 carbon skeleton comprised of three aromatic rings [2, 17, 20]. These compounds are commonly found in plant sources as glycosides or in other complexed structures [3, 20].

Flavan-3-ols

Flavanols (or flavan-3-ols) are identified by their C6-C-3-C6 backbone [1, 3]. In that 3 ring structure, only 2 of the rings are aromatic noting a difference between flavanols and the other 5 flavonoid subgroups described previously. Furthermore, flavanols are not found in glycosylated forms as compared to other flavonoids [2]. The basic monomeric units of flavanols include (+)-catechin (C) and (-)-epicatechin (EC). These two compounds are isomers with regard to the bond positions of the attachment groups on C2 and C3. The bonds for the attachment groups (Aromatic Ring B and hydroxyl group) on these carbons are in the *trans* configuration for C and in the *cis* configuration for EC. The structure for EC is shown in Figure 2.1. Other common monomers are epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG).

Procyanidins (also known as condensed tannins) are oligomers and polymers of C and EC. The degree of polymerization (DP) defines how many monomeric units are in a procyanidin molecule, typically 2-5 units for oligomers, and 6 or more units for polymers [21, 22]. PCs with linkages that occur between C4 of the heterocycle and C6 or C8 of the adjacent monomer are classified as B-type [18, 23-25]. The structures for PCB1 dimer and PCC1 trimer are shown in Figure 2.1. Additionally, A-type PCs have an additional C2-O-C7 or C2-O-C5 linkage [24-27]. Natural dietary sources of PCs include cocoa, tea, peanuts, pine bark, grapes, apples, and berries [2, 4, 28-30]. All sources of PCs contain B-type, but a few sources also contain A-type PCs including cranberries, peanuts, cinnamon, curry, plums, and avocados [4, 26, 31]. Cinnamon and curry have the highest ratio of A-type PCs at 84-90%, followed by cranberries and peanuts at 51-65%, plums at 17-29%, and lastly avocados at <12% total PC content [31, 32].

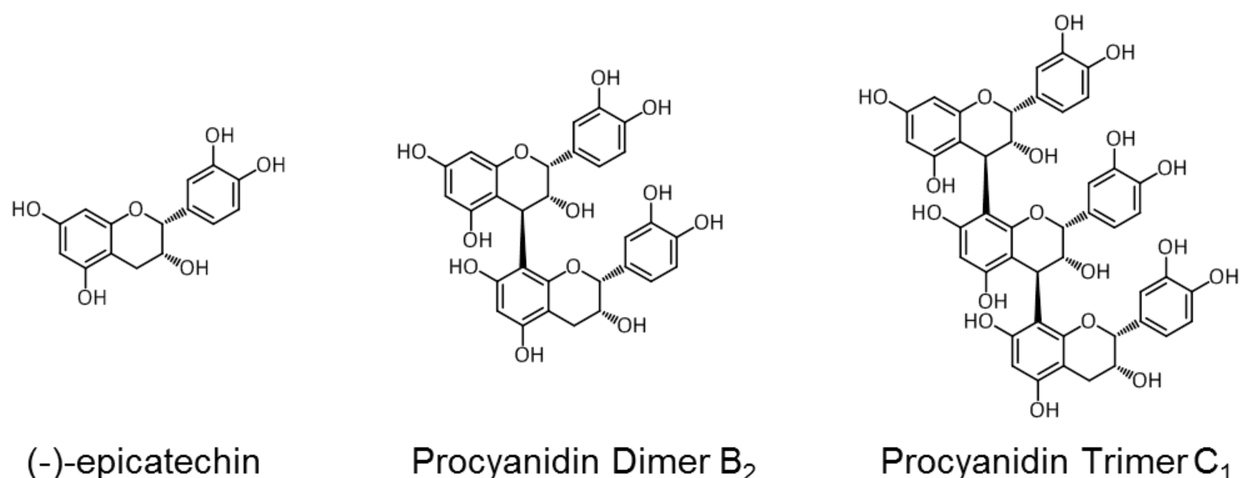


Figure 2.1. *Structures of selected native monomeric flavan-3-ols and B-type procyanidin compounds*

PC content in grapes and cocoa

In grapes (*Vitis vinifera* sp.), monomers and PCs are found in highest concentration in the skin and the seeds [33-36]. Monomers and PCs (including dimers B_1-B_8, dimer gallates, and trimers C_1 and T_2) are found in concentration ranging from 0.06-0.16 mg/g in whole/fresh grapes, 0.14-0.38 mg/g in grape skins, and ranging from 2.30-10.33 mg/g in grape seeds [4, 24, 32, 33, 37]. PC composition in grape skins is 91-99% polymers, while only 77-85% polymers in grape seeds [38]. This results in grape skins having an overall greater average DP than grape seeds [37, 38]. Overall the average DP in grapes is 20.9 ± 0.3 [31]. In grape seeds, the major polyphenol constituent is C, followed in relative abundance by EC, ECG, PCB1, and PCB2 [39]. Contrary to popular belief, PCs have been found to be more concentrated in white grape varieties such as Chardonnay and Riesling rather than red grape varieties such as Merlot [28, 29]. Since PCs tend to lend a purple, red, or brown color to most fruits that contain them, the deep red color found in some grape skins would allow assumption that they contain higher amounts of PCs, but since the majority of grape PCs are found in the seeds the skin color is not an accurate indicator of PC content [29].

One benefit of grape seeds as a source of PCs is their abundance as a commercial byproduct of winemaking [34, 36, 39, 40]. This provides an inexpensive source of PCs that could be utilized in dietary supplementation [36]. To obtain a highly pure source of PCs from grape seeds, the desire compounds must be extracted. Common methods of extraction include solvent extraction using ethyl acetate, methanol, aqueous methanol with organic acid, and a mixture of acetone, water, and organic acid (typically in the ratio of 70:28:2 or 20:29.5:0.5) [41-44]. Another

method of extraction is the use of super-critical fluid carbon dioxide (SFC-CO_2) either with or without solvent (typically ethanol) present [28, 43, 44].

Cocoa has been found to have a higher concentration of PCs, though the profile of native compounds is different than that found in grapes. Lee *et al.* found that cocoa powder has more PCs per serving than red wine, green tea, and black tea, all of which are considered popular sources of PC consumption [45]. Furthermore, research has shown that cocoa tends to have higher concentrations of polymers, including larger PCs with DP of 10 or more [22, 45, 46]. Though most methods only detect PCs up to DP 10, cocoa has been shown to have polymers of 50 units or more [46, 47]. Larger polymeric PCs give cocoa a greater antioxidant capacity than grapes, which may result in overall greater health benefit [45, 46]. PC content of commonly consumed products in the US such as baking chocolate, chocolate sauce, and baking power ranges from 0.91-22.44 mg/g [27, 46, 47]. The amount of PCs in cocoa products has been correlated with the content of nonfat cocoa solids (NFCS) [27, 46, 48, 49]. Products that include higher proportions of NFCS are natural cocoa, chocolate liquor, and dark chocolate, while milk chocolate has one of the lowest total amounts of NFCS [27, 46, 48]. Overall, it has been found that the processing of cocoa has an effect on the PC content of chocolate products including the Dutching, or alkalization, process [27, 48-50]. One study demonstrated this by finding that in natural cocoas PC content is approximately 34.6 mg/g, while it stands at approximately 13.8 mg, 7.8 mg/g, and 3.9 mg/g for lightly, medium, and heavily alkaline processed cocoa powders, respectively [50]. Other studies have shown that the Dutching process can decrease total PC content in cocoa by up to 60% [49, 50]. Milk chocolate products in the United States often use alkalized cocoa which may also account for lower PC content [27]. Health benefits of PCs include prevention or amelioration of cancer, inflammation, obesity, diabetes, cardiovascular disease, and improvement of vascular function [4, 27, 46, 48-51].

Dietary content of PCs

Daily intake of PCs is estimated to be 45-189.7 mg/d for U.S. adults and is distributed within a variety of dietary sources [4, 32, 52, 53]. In the U.K. and Ireland, the intake of total polyphenols is 177-182 mg/day, with 65% of that total coming from anthocyanins and flavonoids [54]. Relative amounts of monomers, dimers, trimer, and larger PCs are 7.1-22%, 11.2-16%, 5-7.8%, and 56-73.9%, respectively [4]. The majority of the estimated total daily intake of PCs is DP > 3 [32]. Most studies found that the major contributors of dietary PCs for adults in the US are

apples, chocolate, and grapes (either fresh or from wine) [4, 32], while another found that sources such as tea, citrus juices and citrus fruits are also major contributors [52]. In fact, consumers of red wine on average have a higher overall daily intake of PCs [32]. These consumption values are based on calculations of PCs content of foods and the USDA Continuing Survey of Food Intake by Individuals. These levels of PC consumption are approximately twice as much as the levels of other flavonoids consumed in the diet on a daily basis [4, 32].

Different factors can influence the amount of dietary PCs consumed on a daily basis. Studies have found that in adults the amount of daily PC intake increases with age [52, 53]. Children also tend to consume greater amounts of PCs than adults [53]. Furthermore, women have a higher average consumption than men [52, 53], though no studies have identified an exact reason for this. Consumption was also higher in alcohol consumers, Caucasians, and vitamin supplement users as compared to their counterparts [52].

An interesting fact pointed out by Vogiatzoglou *et al.* is that the amount of daily intake of PCs for humans is "considerably below the amounts used in most dietary intervention studies" [55]. This presents an interesting consideration that must be taken into account when designing experimental procedures to test the efficacy of PCs as a treatment. While the data is sound there is one aspect that basic dietary consumption values do not take into consideration: bioavailability.

Bioavailability of PCs

While studies have shown that PCs are a relevant dietary compound due to levels of daily consumption and may be reasonably exploitable for their protective properties, it must be noted that they may not have the greatest potential of systemic benefits due to poor bioavailability. Bioavailability (with regard to digestion and absorption) is a contraction of the phrase "biological availability" and is defined as the rate and extent of the absorbance of a substance and reaching systemic circulation [56, 57]. It is a key factor that affects efficacy of a treatment [56]. If a treatment given by oral dosage cannot become systemically available, it cannot be effective. Oral bioavailable of compounds is affected by many factors in the GI tract including solubility, size, digestive enzymes, cellular junctions in intestinal epithelial cells, and cellular transport [56]. First, compounds must be able to survive the harsh, acidic conditions and digestive enzymes of the stomach and pass through to the small intestine [58, 59]. Solubility and absorption can be

affected by factors such as the size of the molecule and its chemical nature (hydrophilic vs. lipophilic) [56]. Absorption is affected by these aspects due to how the molecule is transported across the epithelium of the GI tract, whether it happens via active or passive transport or, in the case of some compounds, not at all. Furthermore, bioavailability is greatly affected by the first hepatic pass after absorption [56]. Liver enzymes will metabolize many compounds before they can reach active sites in the body. Moreover, factors such as age, gender, physiological condition, and consumption of drugs can also affect bioavailability [60]. Understanding the ability and extend of PCs to be absorbed and systemically available is necessary when discussing the consumption of these compounds. Figure 2.2 depicts the general pathways for digestion and absorption.

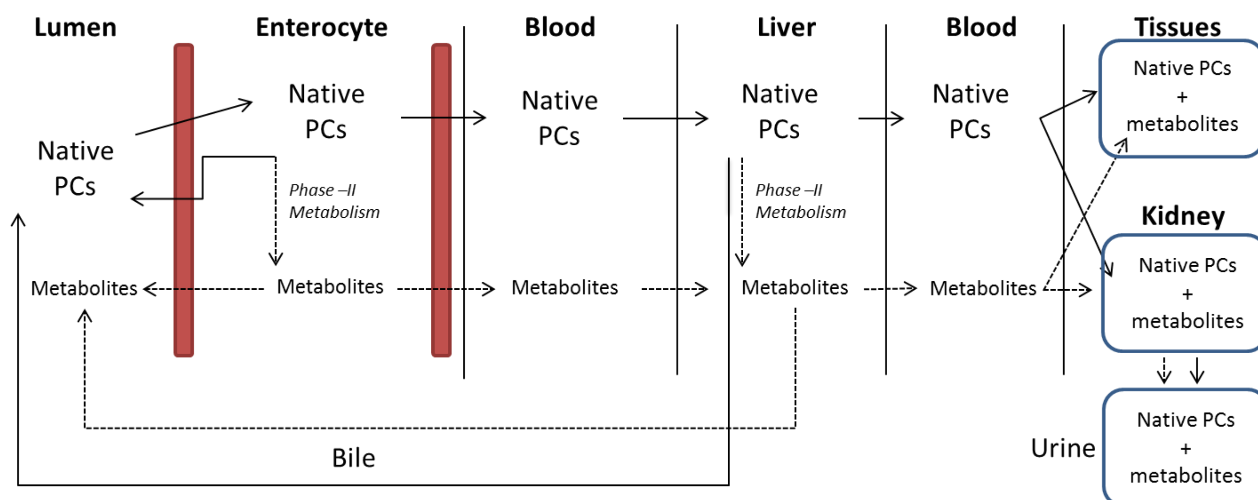


Figure 2.2. *A representation of the path compounds take through digestion and absorption.*

Oral doses of PCs that reach the stomach do not undergo extensive acid hydrolysis [2, 3, 5, 6, 61-63]. Dimers and trimers are stable in acidic conditions and, therefore, in the stomach and to a large extent do not degrade, thusly the majority of dimers and trimers reach the small intestine intact [2, 3, 5, 6, 8, 61, 62]. There is some evidence that PCs consisting of 4 units or more undergo a small amount of hydrolysis in the stomach, but they typically degrade only into trimers or dimers and not completely into individual monomeric units [2, 6, 63]. Monomeric units that reach the small intestine are readily absorbed [6, 7, 61, 63, 64]. C and EC have been shown to undergo Phase II biotransformations primarily in the liver into various *O*-sulfated, *O*-glucuronidated, and *O*-methylated forms [6, 7, 63, 65]. Plasma levels of monomers and their conjugated forms peak at 1-2.5

h after consumption [6, 7, 64]. Studies have shown that EC is more readily absorbed than C, and even if an equal ratio of C and EC are consumed that the amount of C absorbed may be <10% of the amount of EC in the plasma [6, 64]. The extensive absorption of EC results in systemic bioavailability of its native and conjugated forms.

Oligomeric and polymeric PCs have a much lower occurrence of absorption in the small intestine as compared to monomers, specifically EC [2, 5, 7, 8, 62, 63]. Polymers generally cannot be absorbed in their native forms [2]. Typically the degree of absorption decreases as the DP increases for PCs [2, 5, 62, 63]. Dimers absorb more readily than trimers, which absorb more readily than tetramers, and so forth. Doses of PCs with DP 2-10 have shown absorption of only dimers (PCB2 specifically) in very small amounts [66]. Absorption of PCs of 5 units or more is negligible [2, 8]. There are no known transporters for the transfer of PCs across the cell membrane, and therefore PCs must rely on passive transport which is generally unsuccessful [7]. This is potentially due to the hydrophilic nature of larger PCs; the chemical properties of the larger molecules do not allow for passive absorption through the gut wall [2, 7]. Permeability across epithelial cells of polymers with an average DP of 6 have been found to be ~10 times less than C, dimers, and trimers [9]. Studies have shown that the amount of dimers present in plasma are approximately 5-10% of the levels of EC, which puts the amount of dimers absorbed as similar to the amount of C absorbed in the small intestine [7, 19]. Dimers, trimers, and tetramers typically do not undergo Phase II metabolism and are not found in the plasma in conjugated forms [7, 26, 63, 66]. This may result in the conservation of biological activity after absorption [26, 67]. Overall, bioavailability of PCs has been found to be approximately 5-50% of monomers [7, 11, 26, 64, 68, 69]. The wide range of values is most likely due to the variation in administration of the sources of PCs and the ways they were measured.

Metabolite bioavailability

Monomers and PCs can accumulate in tissues system wide. Following oral doses of PCs administered to rodents, compounds that were absorbed in the small intestine have been found accumulated in the digestive tract, liver, lung, pancreas, mammary gland, skin, brain, kidney, uterus, ovaries, and testes [70]. Furthermore, although larger PCs cannot pass through epithelial cells intact, they have a greater ability to adsorb to the surface of epithelial cells than monomers and smaller PCs [9].

In rats, Stoupi *et al.* found that after an oral dose of radio-labeled dimers approximately 40-58% of monomers are excreted in the urine in the first 24 h [71], while Nakamura *et al.* found that 80% of an oral dose of monomers is excreted in 25 h [72]. It should be noted, though, that detection of monomers and PCs in urine has evolved over time. Previously, several studies had found that monomers could be detected in urine, but any PCs with 2 units or more could not be found [63, 66, 72, 73]. These data lead to conclusions that while monomers were absorbed in the small intestine, dimers and larger were not absorbed as readily. More recent studies with more advanced analytical techniques have shown that compounds with larger DPs can be detected in the colon, though in very small amounts [7, 61, 74, 75]. One study in pigs found that the excretion of trimers and dimers was 0.004% and 0.019%, respectively, of the initial dose [61]. This shows that while it is at a very low level, dimers and trimers are absorbed.

Processing and matrix effects

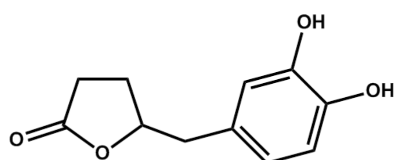
Another consideration for the absorption of monomers and PCs is the possibility of matrix effects [2, 7]. Studies have shown that PCs bound within a food matrix are not available for absorption and that only soluble PCs have significant bioavailability [7]. Diets rich in carbohydrates can significantly decrease the availability of consumed PCs [7]. A study involving different formulations confectionary chocolate products showed that food components such as sucrose and milk may affect the absorption of PCs [76]. This is reasonable due to the chemical nature of PCs to readily bind to fiber, sugar, and protein molecules [67].

General processing methods can affect PCs in food sources. Fermentation processes, such as oxidation that green tea goes through to produce black tea, can alter the profile and availability of PCs [6]. Oxidation causes monomers to react and form different polymeric compounds such as theaflavin which have different chemical properties and activities [6]. Finding an optimum extrusion method to obtain PCs from grape pomace depends on how efficiently the desired compounds are released from the food matrix [29, 77]. Processing conditions such as temperature and shear rate can affect the amount of PCs obtained from grape pomace [29]. Higher temperatures and higher pH during the extrusion process decrease the total PCs recovered [6, 29, 78].

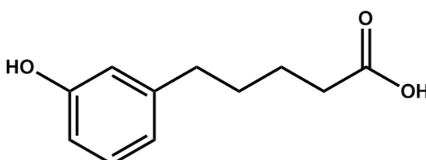
Microbial metabolism of PCs

The result of the poor absorptive characteristics of PCs in the small intestine is that the majority of an oral dose will reach the colon [7, 8]. While this implies that there may be little systemic benefit from PCs, it also indicates that the majority of the benefits may be experienced within the gastrointestinal tract [2], with the major point of exposure as the large intestine. The colon possesses a complex microbial ecology comprised of 10^9-10^{12} cells/g luminal contents [79-81]. The composition of the microbiota found in the colon is comprised primarily of anaerobic bacteria from the *Bacteriodes, Eubacterium, Bifidobacterium, Fusobacterium, Peptostreptococcus* and *Atopobium* groups [79-81]. Research has shown that overall health may be strongly linked to gut health. It has been suggested that colonic microbiota in infants can affect health for the rest of the lifespan due to the effects on the immune system [82]. There have been several types of diseases associated with distortions in gut microbiota: diabetes, inflammatory diseases, neoplastic diseases, and cardiovascular diseases [83]. Administration of PCs has been shown to alter microbial populations in the gut [81, 84]. Choy et al found that administration of GSE to pigs caused a dramatic increase in populations of *Lachnospiraceae, Clostridales, Lactobacillus* and *Ruminococcacceae* [85], while Boto-Ordonez found that giving human subjects doses of red wine increased levels of *Bifidobacterium, Enterococcus* and *Eggerthella lenta* [84]. Furthermore, PCs may repress pathogenic bacteria such as *Clostridium perfringens*, while having no effect on non-pathogenic bacteria [81].

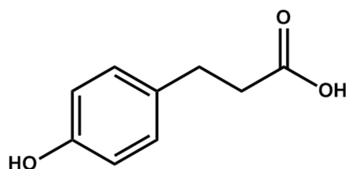
Unabsorbed PCs that reach the colon are metabolized by the colonic microbiota into several smaller constituents such as smaller PCs, monomers, γ-valerolactones, phenolic acids, etc [9, 10, 86]. Predominant microbial metabolites generated from PCs in the colon lumen are 5-(m,p-hydroxyphenyl)-γ-valerolactone, 2-(m,p-hydroxyphenyl) acetic acid, 2-(p-hydroxyphenyl) acetic acid, 2-(m-hydroxyphenyl) acetic acid, 3-phenylpropionic acid, 3-(m-hydroxyphenyl) propionic acid, 3-(p-hydroxyphenyl) propionic acid, 3-(m-hydroxyphenyl) valeric acid, ferulic acid, isoferulic acid, vanillic acid, m-coumaric acid, p-coumaric acid, caffeic acid, gallic acid, and hippuric acid. Structures of a selection of these compounds are shown in Figure 2.3.



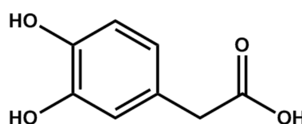
5-(m,p-hydroxyphenyl)-γ-valerolactone



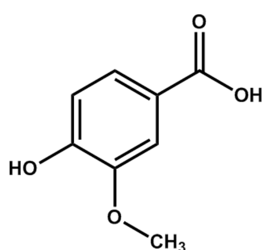
3-(m-hydroxyphenyl) valeric acid



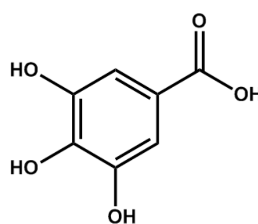
3-(p-hydroxyphenyl) propionic acid



2-(m,p-hydroxyphenyl) acetic acid



Vanillic acid



Gallic acid

Figure 2.3. *Structures of selected microbial metabolites formed from metabolism of monomeric flava-3-ols and procyanidins in the colon*

The amount of degradation may depend on DP [6, 7, 62]. Monomers are metabolized directly into smaller phenolic compounds, while larger compounds must first be broken into smaller DP compounds [10, 65]. Furthermore, larger metabolites are then metabolized into smaller and smaller compounds. Figure 2.4 demonstrates this step-wise degradation process. This may result in slower degradation of larger PCs and the longer persistence of these compounds in the colon as compared to monomers. Though some studies have shown that dimers may be more rapidly metabolized than monomers [87], it is generally believed that larger PCs persist longer in the lower GI tract and therefore impart enhanced benefits. It should be noted that there are no current studies that examine PC oligomers as most studies only measure metabolism of monomers and dimers [11].

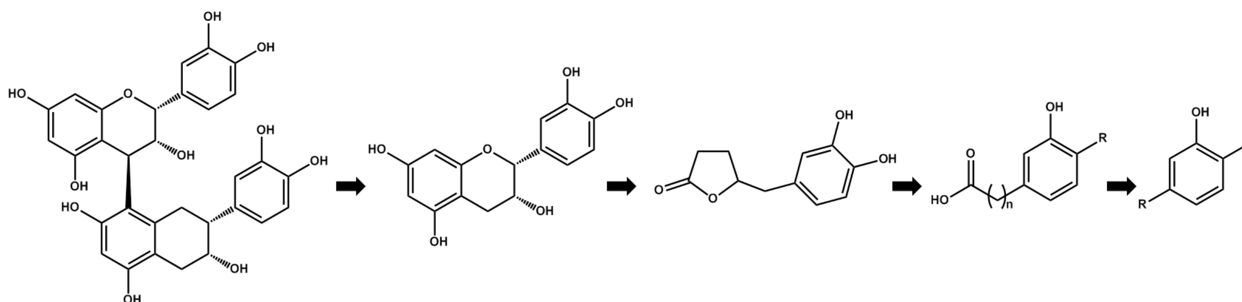


Figure 2.4. *Progressive degradation of PC B2 by microbial metabolism*

Due to the one-way, progressive nature of the GI tract and the extensive metabolism of monomers and PCs, gradients of native PCs and their microbial metabolites are likely established along the length of the colon [11]. Specifically, it is hypothesized that the amount of PCs would start out in large amounts in the more proximal sections of the colon and decrease along the length, while metabolites would start out in small amounts and increase over time. This is demonstrated in Figure 2.5. The effects of dietary PCs likely differ in the proximal and distal colon, concurrent with progressive microbial metabolism [12]. However, there are little data regarding distinct profiles present in the lumen of the proximal, mid, and distal colon.

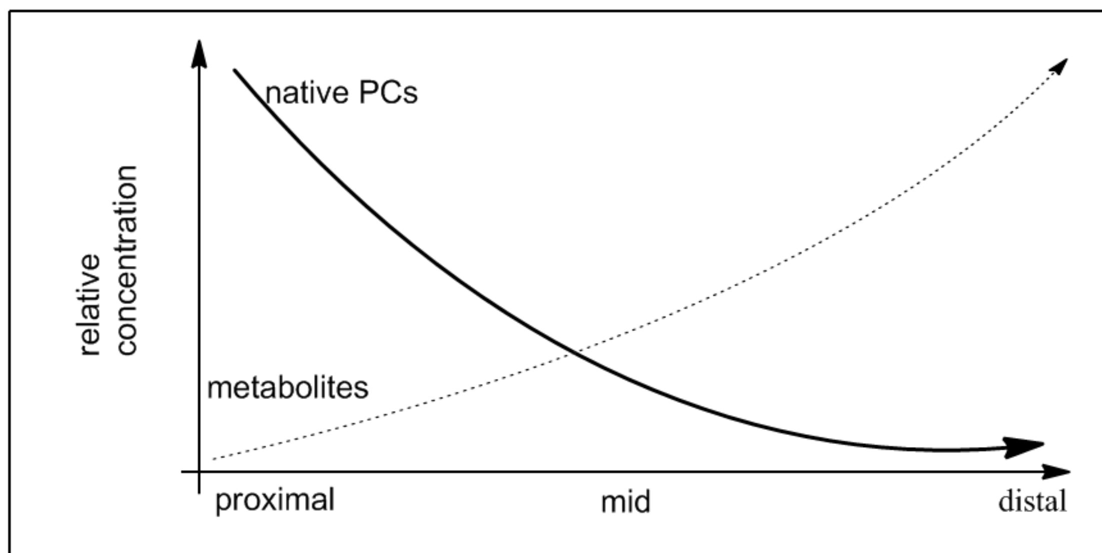


Figure 2.5. *Expected concentration gradients of native PCs and metabolites in specific regions of the colon*

PC Activities

PCs have been shown to have both anti-inflammatory and anti-cancer activities *in vitro* [30, 88-97]. Some studies have shown that PCs can help inhibit the growth of colon cancer cells [88], while others show that PCs can actually induce cell cycle arrest and apoptosis in CaCo-2 cell cultures [89, 90]. A recent study by Cheah *et al.* demonstrated that PCs with DP of 2-6 can enhance the effectiveness of 5-Fluorouracil, a chemotherapeutic drug, on CaCo-2 cells *in vitro* [98]. Due to the majority of a PC dose reaching the colon, these studies have great potential for seeking new treatments and prevention for colon cancer. PCs have also been shown to inhibit the growth of breast and pancreatic cancer cells [96, 97]. The concern that was discussed by both Ramljak and Chung in those previously mentioned studies is the systemic bioavailability of PCs or the lack thereof [96, 97]. The low bioavailability of PCs would not make them an ideal treatment for cancers outside of the GI tract.

Anti-inflammatory properties of PCs have been studied in varying aspects. Schafer *et al.* determined that administration of PCs from pine bark extract can lower both COX-1 and COX-2 levels in human serum [30]. PCs have also been shown to bind NF-kB to prevent inflammation by inhibiting its action [93, 94]. Nunes *et al.* showed the PC induced NF-kB inhibition shows up specifically in the colon, and PCs also inhibits the production of nitric oxide in the gut [94]. Research has also shown that GSE PCs can reduce bronchial inflammation in mouse models of asthma and found decreased levels of Interleukin-4 (IL-4), IL-5, and IL-13 [95]. This study also notes a need to look at the administration of GSE to treat asthma due to low bioavailability.

Another function of PCs found to benefit overall health is the prevention of obesity, insulin tolerance, and impaired glucose tolerance. There have been extensive studies that have shown the benefits of GSE on these factors [99-102]. Hwang *et al.* showed that administration of GSE can lower blood glucose levels [99], while Ohyama *et al.* demonstrated that GSE can prevent weight gain and result in overall smaller fat pads than in a high fat control group [102]. Other studies have linked anti-inflammatory properties of GSE with the potential for the prevention of obesity [100, 101].

Cocoa has also been studied extensively with relation to metabolic disease. PCs extracted from cocoa, like those from GSE, have also been shown to lower blood glucose levels [103-105]. However, the benefits may only be transient and not long-lasting [104]. Cocoa PCs have also been demonstrated to lower serum cholesterol and triglyceride levels, and also contribute to

weight loss [104, 106, 107]. Sanchez *et al.* also demonstrated that administration of soluble cocoa fiber can decrease insulin resistance [106]. Studies have also looked into the differences in effect between cocoa fractions of different DP. Dorenkott *et al.* showed that oligomeric PCs with DP of 2-5 had the largest effect preventing weight gain, fat mass, impaired glucose tolerance, and insulin resistance in a diet induced obesity model [22].

There have been other interesting health benefits provided by PCs previously studied. Doses of EC have been shown to reduce blood pressure in adults [108]. PCs have been demonstrated to have a dose dependent protective capacity for gastric mucosa [109]. Also, doses of GSE PCs have been shown in a double-blind study to reduce leg swelling of sedentary women [110]. Another potential benefit of PCs is the ability to improve healing of previously damaged tissues [111, 112]. PCs have been shown to help repair damage of colon tissue in rats caused by ulcerative colitis [112]. Furthermore, they have been shown to improve kidney damage caused by Type-2 diabetes [111].

Metabolite activities

The microbial metabolites of PCs have certain activities and potential health benefits. Like native PCs, they foster anti-inflammatory and anti-cancer activities [113-115]. Forester *et al.* demonstrated that gallic acid and some of its derivatives have protective effects in the colon to potentially prevent cancer and assist in cancer cell inhibition [114, 115]. Larrosa *et al.* showed that PC metabolites aid in decreasing the expression of cytokines IL-1β, IL-8, and TNF-α [113].

Biology of specific colon regions

The importance of looking at specific segments of the colon rather than as a whole is important due to characteristics of the different regions of the colon, specifically in the occurrence of disease. Various factors may effect whether a person is more likely to develop cancer of the proximal or distal colon. Older people and women tend to be diagnosed with proximal cancer rather than distal, and the opposite is true for younger people and men [116]. Tumor morphology is also different in regions of the colon. Polypoid-type early cancer was found slightly more in the distal colon, while flat-type early cancer had significantly higher occurrence in the proximal colon [117]. Tumors in the proximal colon have been found to be more receptive to chemotherapy treatments than those found in the distal colon [118]. Furthermore,

cancer discovered in the proximal colon is significantly more likely to be discovered in an advanced stage with more severe symptoms [117].

Regional differences are also seen with regard to ulcerative colitis (UC) [119-123]. When patients are first diagnosed with UC, there may be an uneven distribution of lesions [121]. Furthermore, cases of UC in the distal colon can result in inflammation of the proximal colon [122]. Mucin expression in the colon is different depending on region, and can be altered during UC even if there is no histological evidence of UC [123].

Evaluation of bioavailability and activity

As mentioned previously, many studies that attempt to evaluate the bioavailability of PCs and their metabolites do so by using *in vitro* cell culture models of GI cells or colonic microbiota [9, 86, 89-98, 124, 125]. While these studies are well executed and provide excellent basis for proof-of-concept, they are limited in their scope. Serra *et al.* note that while *in vitro* studies are useful, they do not give a comprehensive look at PC activities, specifically static models of the GI tract [5]. Furthermore, inter-individual differences in digestion and especially colonic microbiota present new variables that an *in vitro* model cannot account for [5]. Furthermore, factors such as gender, age, and general health can affect bioavailability, and these factors cannot be taken into account with *in vitro* experiments [60]. This emphasizes the need for *in vivo* studies to evaluate the effectiveness of PCs in the prevention or treatment of disease to allow for a meaningful evaluation of their benefits [5, 60].

UPLC-MS/MS Analysis

The current standard method to identify and quantify both native PCs and their metabolites is by reverse-phase Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS). Current published methods, however, are limited in the scope of analyzing large amounts of complex samples. Most available methods measure a narrow range of compounds and are limited to measuring only native compounds (monomers and PCs) or only microbial metabolites [13, 14, 75, 126-129]. A select number of methods measure both PCs and their metabolites, but those methods are limited to measuring a small number of compounds [15, 74, 130, 131]. These methods provide a more inclusive look at PC metabolism in the gut, but are still severely limited. One reason for this deficiency is due to the diverse chemical nature of PCs and

their metabolites. With varying chemical properties, it is difficult to thoroughly extract all of the compounds desired from biological samples [16, 132]. To achieve a more comprehensive analysis of the metabolism of native PCs by colonic microbiota, it is necessary to develop a UPLC-MS/MS method that can both extract and detect a broader profile of PCs and metabolites simultaneously. This will allow researchers to study a more complete profile of compounds of interest in a more efficient manner.

Furthermore, currently published methods utilize a low UPLC flow rates resulting in longer analysis times [13, 14, 127]. Simply increasing the flow rate will result in a shorter, more efficient analysis while maintaining acceptable peak shape and resolution. This is feasible due to the advanced specifications of the UPLC system that allow the system to run with pressures of up to 15,000 psi, while maintaining optimal chromatographic conditions by using columns with stationary phase comprised of consistently sized small particles (<2um).

Another limitation of existing methods is that they typically do not measure PCs larger than trimers [13-15, 75, 126, 127, 129-131]. Larger PCs are in lower abundance in most natural sources, and are more difficult to extract due to complex chemical properties [16, 132]. Moreover, traditional normal-phase chromatographic methods do not separate larger PCs with sufficient resolution [14, 16]. The development of a more complete extraction process, and advances in UPLC columns should facilitate the resolution of larger PCs in reverse-phase modes.

Conclusion

Currently, studies have shown that PCs exhibit health benefits in the colon as well as in other locations within the body. A flaw with these studies is that they treat the colon as a uniform system. In reality, the human colon is ~2 m long and exhibits distinct biology along the length. Additionally, sequential metabolism in the colon means that proximal and distal colon regions are likely exposed to distinct profiles of active compounds. Further data is required regarding the delivery and efficacy of dietary PCs in different regions of the colon. A novel study will be performed that will view the colon as a series of discrete segments exposed to distinct profiles of native PCs and their bacterial metabolites. This represents a fundamental shift in approach from previous studies, resulting in the first *in vivo* characterization so the delivery and colonic bacterial metabolism of PCs in distinct regions of the colon. This will allow a better understanding of the gradient of PCs and their metabolites along the length of the colon,

and give a broader picture about the delivery of PCs to the colon. The study will consist of an animal study where rats will be fed a chronic dose of GSE over a period of 14 days. After which, the rats will be given one large dose of GSE via gavage, then will be sacrificed at specific intervals. Lumen from the cecum and proximal, mid, and distal colon will be collected, extracted, and analyzed to determine the concentration of PCs and their microbial metabolites. Concentration data will be plotted over time and compared between the regions. Comparisons will also be done between the levels of PCs and metabolites over time. There are three main hypotheses for this study. 1) The concentration of PCs will go from high to low as it progresses from the proximal to the distal colon. 2) Conversely, metabolites will go from low concentration to high as they traverse from the proximal colon to distal. 3) The maximum concentration for the larger PCs (trimers and lager) would be greater in the mid colon compared to that of monomers and dimers due to slower microbial metabolism of the larger compounds.

Another question that needs to be addressed is whether or not there is accumulation of PCs and metabolites in colon tissue. Accumulation of bioactive compounds would result in potential longer term benefits. This will be studied by performing a secondary analysis of murine colon tissue from a previously conducted animal study that was comprised of the 12 wk chronic dietary administration of cocoa extract and cocoa fractions with specific DP. The data from this study will be used to accomplish 2 points: 1) to determine profile of accumulated compounds during chronic exposure, and 2) compare accumulation profiles of PCs and metabolites between fractions of different DP. It is hypothesized that administration of equal amounts of flavanols with distinct qualitative profiles to mice would result in colonic tissue accumulation of distinct qualitative and quantitative profiles of both native flavanols and their microbial metabolites in the colon tissue. Specifically, we hypothesize that 1) Tissue levels of flavanols and metabolites will be increased after administration of cocoa flavanols compared to a low-flavanols control diet, and 2) cocoa extract and cocoa monomers will result in greater levels of monomeric flavanols and metabolites compared to cocoa oligomers and polymers.

To thoroughly analyze these proposed studies, though, development of a new high-throughput UPLC-MS/MS method will make the data collection more efficient. Therefore, the first objective is to develop and validate a method that will simultaneously extract and measure both native PCs and their metabolites to facilitate the analysis of various regions of the colon for profiles of native compounds and their metabolites. Validation of the extraction method and

UPLS-MS/MS analysis will include maximizing the extraction recovery, establishing the repeatability of the instrumental analysis, and determining the lower limits of detection and lower limits of quantification for all compounds of interest. Once a method has been developed, the effectiveness will determined by measuring PCs and metabolites that will be extracted from the issue and intestinal contents from rats administered a chronic aqueous dose of GSE.

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Chapter 3:

Simultaneous UPLC-MS/MS Analysis of Native Catechins and Procyanidins and Their Microbial Metabolites in Intestinal Contents and Tissues of Male Wistar Furth Inbred Rats

Reprinted from Journal of Chromatography B, 953, Katheryn M. Goodrich and Andrew P. Neilson, Simultaneous UPLC-MS/MS Analysis of Native Catechins and Procyanidins and Their Microbial Metabolites in Intestinal Contents and Tissues of Male Wistar Furth Inbred Rats, 63-74, 2014, with permission from Elsevier

Abstract

Procyanidins have been extensively investigated for their potential health protective activities. However, the potential bioactivities of procyanidins are limited by their poor bioavailability. The majority of the ingested dose remains unabsorbed and reaches the colon where extensive microbial metabolism occurs. Most existing analytical methods measure either native compounds (catechins and procyanidins), or their microbial metabolites. The objectives of this study were to develop a high-throughput extraction and UPLC-MS/MS method for simultaneous measurement of both native procyanidins and their metabolites, facilitating high-throughput analysis of native and metabolite profiles in various regions of the colon. The present UPLC-MS/MS method facilitates simultaneous resolution and detection of authentic standards of 14 native catechin monomers and procyanidins, as well as 24 microbial metabolites. Detection and resolution of an additional 3 procyanidin dimers and 10 metabolites for which standards were not available was achieved. Elution and adequate resolution of both native compounds and metabolites were achieved within 10 min. The intraday repeatability for native compounds was between 1.1-16.5%, and the interday repeatability for native compounds was between 2.2-25%. Intraday and interday repeatability for metabolites was between 0.6-24.1% and 1-23.9%, respectively. Observed lower limits of quantification for native compounds were ~9-350 fmol on-column, and for the microbial metabolites were ~0.8-12,000 fmol on-column. Observed lower limits of detection for native compounds were ~4.5-190 fmol on-column, and for metabolites were 0.304-6020 fmol on-column. For native monomers and procyanidins, extraction recoveries ranged from 38-102%. Extraction recoveries for the 9 microbial metabolites tested ranged from 41-95%. Data from tissue analysis of rats gavaged with grape seed extract indicate fairly high accumulation of native compounds, primarily monomers and

dimers, in the cecum and colon. Metabolite data indicate the progressive nature of microbial metabolism as the digesta moves through the lower GI tract. This method facilitates the high-throughput, sensitive, and simultaneous analysis of both native compounds and their microbial metabolites in biological samples and provides a more efficient means of extraction and analysis than previous methods.

Introduction

Procyanidins (PCs) are dimers, oligomers, and polymers of the flavan-3-ol monomers (±)-catechin (C), (−)-epicatechin (EC), and (−)-epicatechin gallate (ECG) [1-3] (**Figure 3.1**). Major dietary sources of PCs include grapes, apples, cocoa and berries[4, 28, 29]. PCs have recently been investigated for their health protective activities. These activities are currently thought to potentially include prevention or amelioration of cancer, inflammation, obesity, diabetes, cardiovascular disease, and improvement of vascular function.

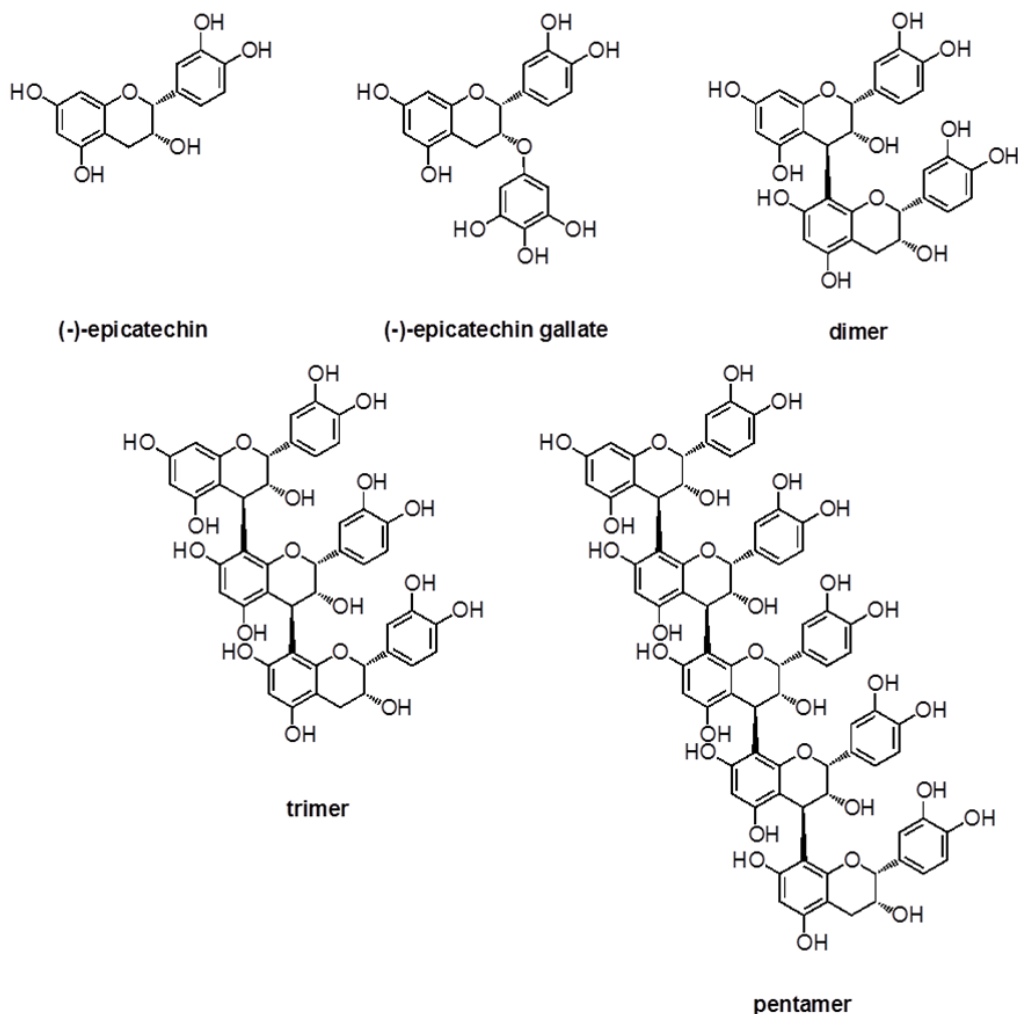

Figure 3.1. *Structures of (-)-epicatechin, (-)-epicatechin gallate, and a representative (-)-epicatechin dimer, trimer, and pentamer*

Bioavailability is a major limiting factor for the potential bioactivity of PCs *in vivo*. The systemic (blood) bioavailability of intact PCs resulting from intestinal absorption is relatively low (0.3-4% of dose)[11, 26, 63, 71, 133, 134]. The majority of the ingested PC dose remains unabsorbed and reaches the colon. The colon harbors an abundant and complex microbial ecology (10^9-10^{12} bacterial cells/g luminal contents) comprised primarily of anaerobes from the *Bacteroidetes* and *Furmicutes* groups[79-81]. Ingested PCs that reach the colon are extensively degraded by colonic microbiota to small PCs, flavan-3-ol monomers, γ-valerolactones, phenylalkyl acids, etc. [9, 11, 74, 87, 135, 136]. Predominant PC metabolites in the colon lumen include hydroxyphenylacetic acids, hydroxyphenylpropionic acids, and hydroxybenzoic acids among other phenolic acid compounds. (**Figure 3.2A**) [9, 10, 86]. Due to progressive bacterial metabolism of the native PCs

present in the proximal colon, gradients of native PCs and their microbial metabolites are likely established along the length of the colon [11, 12] (**Figure 3.2B**). However, previous studies have either examined the colon as a single organ or examine only fecal profiles, and therefore distinct profiles potentially occurring in various regions are not observed.

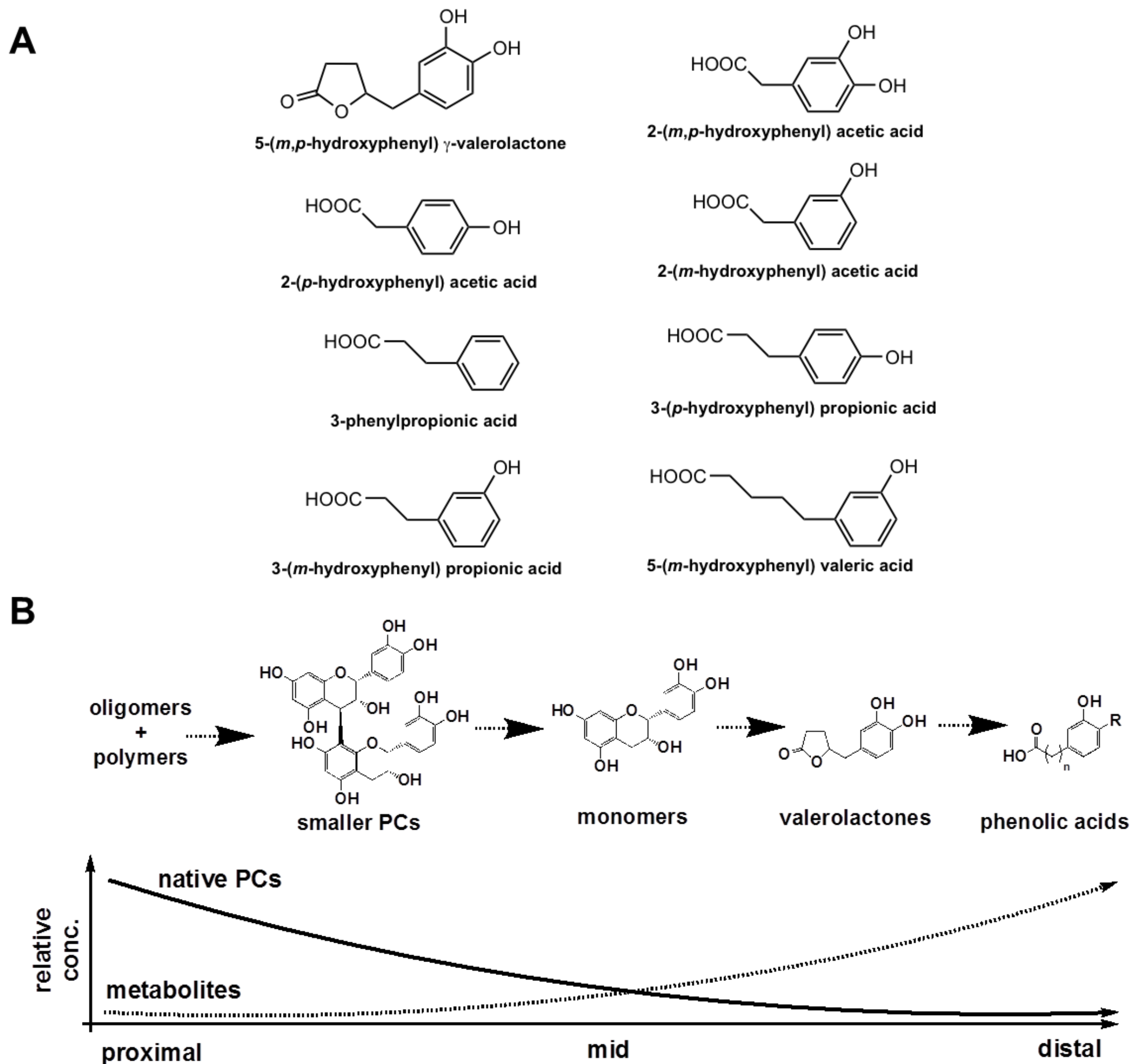

Figure 3.2. *Structures of selected representative metabolites produced by microbial metabolism of native catechins and procyanidins in the colon (A). Schematic demonstrating gradients of native compounds and their microbial metabolites potentially generated throughout the colon due to progressive microbial metabolism (B).*

The standard method to characterize both native PCs and their metabolites is by Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS). However, current published methods impose limitations on experimental design by limiting analytical

throughput. Some methods measure only native compounds (monomers and PCs), while others only measure metabolites[13, 75, 126, 127, 129, 137, 138]. Analysis of PCs and metabolites separately effectively doubles the time and effort required to characterize PC metabolism, significantly lowering analytical throughput. The few existing methods that measure both groups simultaneously only detect a limited number of PCs or metabolites [15, 74, 130, 131]. These methods therefore provide an incomplete view of PC metabolism. To achieve a more inclusive method to fully characterize PC metabolism in the gut, it is necessary to develop an analytical method that can both extract and detect a broader profile of PCs and metabolites together. Furthermore, previous methods have low UPLC flow rates and therefore longer analysis times [13, 127, 137]. By increasing flow rate while maintaining system pressure within UPLC limits (<15,000 psi), a shorter, more efficient analysis can be achieved while maintaining acceptable peak resolution. Another limitation of existing methods is that they do not typically measure PCs larger than trimers [13, 15, 75, 126, 127, 129-131, 137]. However, advances in UPLC columns and improved methodologies should facilitate resolution of larger PCs in reverse-phase modes Moreover, separation and quantification of additional polyphenolic metabolites can be achieved.

The objectives of this study were to develop and validate a high-throughput UPLC-MS/MS method that will simultaneously measure both native PCs and their metabolites and facilitate high-throughput analysis of native and metabolite profiles in various regions of the colon. The effectiveness of this method was determined by measuring PCs and metabolite profiles extracted from intestinal contents and tissue from animals fed a catechin- and PC-rich grape seed extract (GSE).

Materials and methods

Chemicals and Standards. Vitaflavan® GSE was purchased from DRT Nutraceutics (Dax, France). Vitaflavan is a water-soluble extract of *Vitis vinifera* seeds, and manufacturer specifications indicate that it contains 24% w/w monomeric procyanidins, 42% w/w dimeric/trimeric procyanidins, and 10% w/w larger procyanidins (tetramers, pentamers, etc.). LC-MS grade solvents (water, acetonitrile, and formic acid) and ethyl acetate, acetone, and methanol (all ACS grade) were obtained from VWR International (Radnor, PA). Milli-Q water was prepared using a Millipore Milli-Q Gradient system. Ascorbic acid, sodium dodecyl sulfate, phosphoric acid, sodium deoxyxholate, sodium azide, disodium ethylenediaminetetraacetic acid

(EDTA), (+)-catechin hydrate, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, (−)-epigallocatechin gallate, *p*-coumaric acid, *m*-coumaric acid, ferulic acid, isoferulic acid, caffeic acid, protocatechuic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, homovanillic acid, gallic acid, 4-hydroxymandelic acid, pyrogallol, catechol, phenylacetic acid, 2-(3'-hydroxyphenyl) acetic acid, 2-(4'-hydroxyphenyl) acetic acid, 3-(3',4'-dihrdroxyphenyl) acetic acid, 3-phenylpropionic acid, 3-(4'-hydroxyphenyl) propionic acid, 5-phenylvaleric acid, phloretin, hippuric acid, 3-(3'-hydroxyphenyl) propionic acid, 3-(3',4'-dihydroxyphenyl) propionic acid were obtained from Sigma (St. Louis, MO). Procyanidin dimer B_2 and trimer C_1 were obtained from ChromaDex (Irvine, CA). Procyanidin dimers $B_{1,}$ B_5, B_2-gallate, trimer T_2, tetramer A_2, pentamer and hexamer were obtained from Planta Analytica (Danbury, CT).

Animal Treatment and Care. Animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Polytechnic Institute and State University. Sixteen male Wistar Furth inbred rats (6-7 wk old, 120-160 g) were obtained from Harlan Laboratories (Indianapolis, IN) and housed 2/cage on a 12-hour light/dark cycle in a climate controlled facility. Rats were allowed to acclimate to the vivarium environment for 7 d prior to the study. During this period all rats were given food and water *ad libitum*. Rats were fed a nutritionally complete polyphenol-free purified maintenance diet (Harlan Teklad AIN-93M, TD.94048) *ad libitum* for the entirety of the study. Following the acclimation period the rats were randomized to two treatment groups (*n*=8/group). The experimental group was given drinking water containing 0.1% w/v Vitaflavan GSE *ad libitum* to administer an approximate dose of 100 mg/kg/d GSE based on consumption of 10-12 mL water/100 g rat/d. The control group was given standard water without GSE *ad libitum*. Rats were maintained on these treatments for 21 days, during which they were weighed every 3 d and on the final day Following the 21 d treatment period, both groups were provided GSE-free water *ad libitum* for 24 h. The rats were then administered either 1 mL 0.9% saline (control group) or 1.3-1.5 mL of 50 mg/mL GSE in 0.9% saline providing a dose of 250 mg/kg GSE (experimental group) via intragastric gavage. Eight h post-gavage, rats were euthanized by CO_2 asphyxiation followed by bilateral pneumothorax to ensure death. The microbial communities of the colon are known to adjust over time in response to dietary treatments [139, 140]. Polyphenols are also thought to induce alterations to the commensal flora of the colon [141 142] Therefore, the 21 d "wash-in" period was

employed to ensure that the resident colonic microbiota, and their metabolism of catechins and procyanidins, were representative of the situation encountered during habitual dietary exposure to these compounds as opposed to exposure to a single acute dose. Additionally, the higher dose for the acute treatment (250 mg/kg vs. 100 mg/kg/d for habitual exposure) was employed in order to facilitate detection of minor microbial metabolites.

Sample Collection. Following euthanasia, the abdominal cavity was opened ventrally and the intestines were removed by cutting through the rectum and gently removing the intestinal tract from the distal end. The cecum, proximal and distal colon tissues were excised separately and the luminal contents from each region were collected by and immediately snap-frozen in liquid N_2. The proximal and distal colon were defined as equal lengths between the cecum and rectum. Each tissue region was then thoroughly rinsed with pre-chilled 1X phosphate-buffered saline (PBS, VWR) and divided into three equal sections. The outer two sections were collected for polyphenol analysis in 0.5 mL acidified saline (0.1% formic acid v/v in 0.9% NaCL w/v) and immediately snap-frozen in liquid N_2. Samples were stored at −80°C prior to sample preparation.

Sample Preparation. Tissue samples were freeze-dried for > 24 h using a FreeZone 1 L freeze dryer (Labconco, Kansas City, MO). Dry tissues were then pulverized to a fine powder using a Biopulverizer (BioSpec Products, Bartlesville, OK) pre-cooled with liquid N_2. Pulverized samples were stored at −80°C prior to analysis. Luminal contents were freeze dried for > 24 h and reconstituted in 1 mL PBS along with 50 μL 1% (w/v) ascorbic acid. Samples were homogenized by bead beating with zirconium oxide tissue homogenization beads (~100 mg, 0.5-1 mm, Next Advance, Inc., Averill Park, NY) for 10 min (4°C). Homogenates were diluted with 2 mL lysis buffer [0.1% w/v sodium dodecyl sulfate, 0.5% w/v sodium deoxyxholate, 0.02% w/v sodium azide, 5 mM disodium ethylenediaminetetraacetic acid, and 1X Halt protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific Inc., Waltham, MA) in PBS]. Homogenates were further homogenized for 30 s, centrifuged (5820 x g, 10 min, 4°C), and supernatants were snap-frozen in liquid N_2 and stored at −80°C prior to analysis.

Protein Determination. Total protein levels in fecal homogenates were determined using Pierce BCA protein kits (Thermo Fisher S). Samples were diluted 8-fold with PBS and 25 µL diluted samples were assayed in triplicate according to manufacturer's instructions.

Extraction. Pulverized tissue samples were thawed and 25-35 mg was quantitatively weighed for analysis. Zirconium oxide tissue homogenization beads (~100 mg, 0.5-1 mm) were added to each sample along with 50 µL water and a 50 µL 1% (w/v) aqueous ascorbic acid solution. Samples were homogenized in a Bullet Blender (Next Advance) at speed 3 for 1 min at 4°C. Luminal content homogenates were thawed and 500 µL was sampled for analysis. Liquid-liquid extraction was performed for both tissue homogenates and luminal contents homogenates by adding 1 mL ethyl acetate to each sample and blending in a Bullet Blender (5 min, speed 7, 4°C). Samples were then centrifuged (5 min, 17,000 x g, room temperature), and the organic supernatant was collected. The extraction was repeated on the resulting pellet, and the supernatants were pooled for each sample and dried down under a gentle stream of N_2 (35°C). Following liquid-liquid extraction, solid-phase extraction (SPE) was performed on the remaining pellet. An extraction solution (1 mL acetone:water:glacial acetic acid, 70:28:2 v/v/v) was added to each sample along with 100 µL 4% aqueous phosphoric acid. Samples were homogenized in a Bullet Blender (5 min, speed 7, 4°C), centrifuged (5 min, 17,000 x g, room temperature), and the supernatant was collected. The extraction was repeated on the resulting pellet, and the supernatants were pooled for each sample. The pooled extracts were diluted with 12 mL of water. SPE cartridges (Oasis HLB cartridges, 1 cc, 30 mg sorbent, Waters, Milford, MA) were preconditioned with 1 mL MeOH followed by 1 mL water. The diluted samples (2 mL pooled extracts + 12 mL water) were loaded onto the conditioned cartridges using a vacuum manifold. Cartridges were then washed sequentially with 1 mL water followed by 0.5 mL 5% (v/v) aqueous MeOH/0.1 % formic acid (v/v). Cartridges were then eluted sequentially with 2 mL MeOH followed by 2 mL acetone:water:glacial acetic acid (70:28:2) and collected into the tubes containing the dried ethyl acetate extracts from the same sample. Samples were dried under vacuum in a Speed-Vac (45°C) to remove acetone, then frozen on dry ice and freeze-dried >24 h to remove water and acetic acid. Dried samples were resolubilized in 1 mL 0.1% formic acid in water:0.1% formic acid in ACN (95:5 v/v), sonicated in ice water (25 min), and then filtered into

certified LC-MS vials (Waters) using a Samplicity filtration system (0.2 μm PTFE Philic Millex Samplicity filters, Millipore, Billerica, MA). Samples were then analyzed immediately.

UPLC-MS/MS Analysis. UPLC separations were performed on a Waters Acquity H-class separation module equipped with a Waters Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm particle size). The column temperature was set to 40°C, and the samples were maintained at 10°C. The binary mobile phase system was comprised of 0.1% (v/v) aqueous formic acid (phase A) and 0.1% (v/v) formic acid in ACN (phase B). The system flow rate was 0.6 mL/min. Elution was performed based on the following linear gradient: 95% A at 0 min held until 0.5 min, 65% A at 6.5 min, 20% A at 7.5 min held until 8.75 min, 95% A at 8.85 min held until 10.0 min. An injection volume of 10 μL was used for all samples and standards. MS/MS analysis of column effluent was performed by (−)-electrospray ionization (ESI) on a Waters Acquity TQD (triple quadrupole) mass spectrometer equipped with a Z-spray electrospray interface. The ESI capillary voltage was –4.25 kV, and the source and desolvation temperatures were 150°C and 400°C, respectively. The desolvation gas and cone gasses were N_2 at flow rates of 900 L/hr and 75 L/hr, respectively. The MS/MS collision gas was Ar. Data acquisition was carried out with MassLynx software (version 4.1, Waters). MS data collection was set to 10 points/peak with an average peak width of 6 s. The auto-dwell setting was used to automatically calculate dwell time based on an interscan delay time of 0.02 s for each transition. The TQD was operated in quantitation mode, with the mass resolution of the first and second quadrupoles set at 1.0 and 0.75, respectively. The Intellistart function of MassLynx was used to develop and optimize multi-reaction monitoring (MRM) parameters for each compound of interest. Compound solutions were directly infused into the ESI source (0.1 mg/mL in MeOH/0.1% formic acid at a flow rate of 50 μL/min) in combination with a background flow of 50% phase A/50% phase B at 0.6 mL min. Intellistart automatically selected the exact monoisotopic mass of the most abundant daughter ion, optimized the source cone voltage and MS/MS collision energy, and generated a single MRM transition for each compound centered on the exact monoisotopic mass of the parent and daughter ions, with a mass window of 0.2 amu (i.e. exact monoisotopic mass determined by Intellistart ± 0.1). These parameters are listed in **Appendix A Table A1** (native monomers and PCs) and **Appendix A Table A2** (metabolites).

Quantification. All compound peaks were processed and quantified using the TargetLynx function of MassLynx software. Quantification parameters for native compounds and metabolites are shown in **Appendix A Tables A3** and **A4**. Peaks were smoothed using a mean smoothing method with 2-3 smoothing iterations and a smoothing width of 1. Compounds were quantified based on external standard curves of authentic standards in 0.1% formic acid in water:0.1% formic acid in ACN (95:5 v/v); compounds for which authentic standards were not available were quantified based on external standard curves of similar compounds (see **Appendix A Tables A3** and **A4**). Peaks between the lower limits of quantification and detection (LLOQ and LLOD) were tentatively quantified and included in the data, but means containing values in this region are indicated in the data tables (**Appendix A Tables A5-A8**). Peaks below the LLOD were not detected and were therefore included in the data as concentrations of 0.

Determination of Extraction Recovery. The recovery of analytes from the tissue matrix was determined by spiking known quantities of authentic standards into intestinal tissue from control animals. Pulverized tissue samples from control animals were thawed and weighed (30 mg per replicate, n=4).

Zirconium oxide beads (~100 mg, 0.5-1mm) were added to each sample followed by 50 µL of 1% aqueous ascorbic acid (w/v). Samples were then spiked with 25 µL of an aqueous solution containing 40 mg/mL GSE + 4 mg/mL each of 9 representative metabolites (phenylacetic acid; 4-hydroxyphenylacetic acid; 3,4-dihydroxybenzoic acid; 3-phenylpropionic acid; 3-(3'-hydroxyphenyl)propionic acid; 5-phenylvaleric acid; gallic acid; *p*-coumaric acid; hippuric acid). The spiked samples were homogenized in a Bullet Blender at setting 3 for 1 min to evenly distribute the spiked compounds. Samples were then equilibrated at 4°C for 30 min to allow for protein binding and absorption of the spiked compounds into the sample. Samples were then homogenized again and equilibrated at 4°C for an additional 30 min. Samples were then extracted for analysis as described above. In order to determine the expected recovery levels (i.e. 100% of the spike), 25 µL of the spike solution was diluted to 1 mL with 95:5 0.1% formic acid in water:0.1% formic acid in ACN (95:5 v/v), filtered and analyzed as described above. The % recovery for each compound was calculated based on the ratio of the measured amount in the spiked tissue versus the measured amount of each compound in the spiked solution at equivalent dilution.

Determination of Lower Limits of Quantification and Detection. Serial dilutions of native compounds and metabolites were prepared covering a range from 1 μg/mL-10 pg/mL and analyzed via UPLC-MS/MS in triplicate. The LLOD and LLOQ for each compound were determined by averaging the smallest concentration that maintained a signal-to-noise (S/N) ratio of ≥3:1 and ≥10:1, respectively. The LLOD and LLOQ concentrations were converted to fmol-on-column by multiplying by the injection volume. The mol-on-column approach for expressing LLOQ and LLOD was used as opposed to reporting concentrations, as the mol on-column value is independent of the injection volume and more accurately represents true instrument and detector performance.

Determination of Intraday and Interday Reproducibility. The intraday and interday reproducibility of the UPLC-MS/MS method was determined by analyzing separate solutions of native compounds and metabolites (all compounds at 0.01 mg/mL in 1 mL 0.1% formic acid in water:0.1% formic acid in ACN (95:5 v/v) for both metabolites and native compounds). Each solution was injected three times on three consecutive days. For intraday repeatability, the percent relative standard deviation (%RSD) was calculated for raw compound peak areas from the three injections on the first day. For interday repeatability, raw compound peak areas from each day were averaged, and %RSD was calculated using the averages from the three individual days.

Data and Statistical Analysis. Values are expressed as mean ± SEM for n=8 replicates (compound levels in tissue and luminal contents), n=3 replicates (repeatability, and LLOD/LLOQ values), or n=4 replicates (extraction recovery).

Results and Discussion

HPLC Separations and Resolution. The UPLC-MS/MS method facilitated simultaneous resolution and detection of authentic standards of 14 native monomers and PCs and 24 metabolites (**Figure 3.3**). We were also able to detect and resolve 3 PC dimers in the GSE and rat tissues for which standards were not available (likely B_3, B_4, and either B_6, B_7, or B_8) using the MRM function for PC dimer B_2. Additionally, we were able to detect and resolve 10

additional metabolites found in the literature for which standards were not available: [1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol;
3-hydroxyphenylacetic acid; 5-(3’,4’-dihydroxyphenyl)-γ-valerolactone; 5-(3’,4’-dihydroxyphenyl)valeric acid and anther isomer of this compound; 5-(3’-hydroxyphenyl)valeric acid
5-(3’-dihydroxyphenyl)-γ-valerolactone; 5-(4’-dihydroxyphenyl)valeric acid; 5-(4’-dihydroxyphenyl)-γ-valerolactone; 5-hydroxy-5-phenylvaleric acid] using published MRM transitions with cone voltage and collision energy of 30 V and 16 eV, respectively. Therefore, our method is capable of detecting and resolving 51 total compounds:17 native monomers and PCs and 34 metabolites. In addition to resolving authentic standards, resolution was achieved for these compounds extracted from rat intestinal tissue and contents 8 h post-gavage of a dose of GSE (see **Appendix A Figure A1**). This is a dramatic improvement in capacity compared to the few existing methods that attempt to measure native and metabolites simultaneously [74, 87, 127, 136, 143, 144]. The only previously published method that reports measuring > 10 native compounds and >10 metabolites is that of Tabasco *et al.* [145]. This study reported measurement of 13 native compounds and screened for 47 metabolites; however, quantitation is only reported for 3 metabolites. Therefore, it is unknown how many metabolites can actually be quantified from a biological matrix using this method. It should be noted that recent studies have identified numerous dimeric metabolites that are likely early metabolites of procyanidins [136]. Although we did not attempt to profile these metabolites in the present study due to the 8 h period between gavage and collection of tissues, the present method will be adapted to monitor these compounds in future studies of procyanidin metabolism by colonic microbiota.

Elution and adequate resolution of these compounds was achieved within 10 min. This represents an increase in method performance compared to recently published HPLC and UPLC methods that separate metabolites only in 18-21 min [144, 145], natives only in 12.5 min [15], and metabolites + select natives (C, EC, ECG, PC dimers B_1 and B_2 and/or PC trimer C_1) in 12.5-18 min [127, 143, 146]. The method reported by Urpi-Sarda *et al.* [74] also resolves numerous metabolites in 10 min, but resolution of only 2 natives (EC and PC dimer B_2) are reported. Therefore, the present method equals or improves upon the analysis time of current methods, while facilitating simultaneous analysis of the full spectrum of monomers, oligomeric PCs and their metabolites.

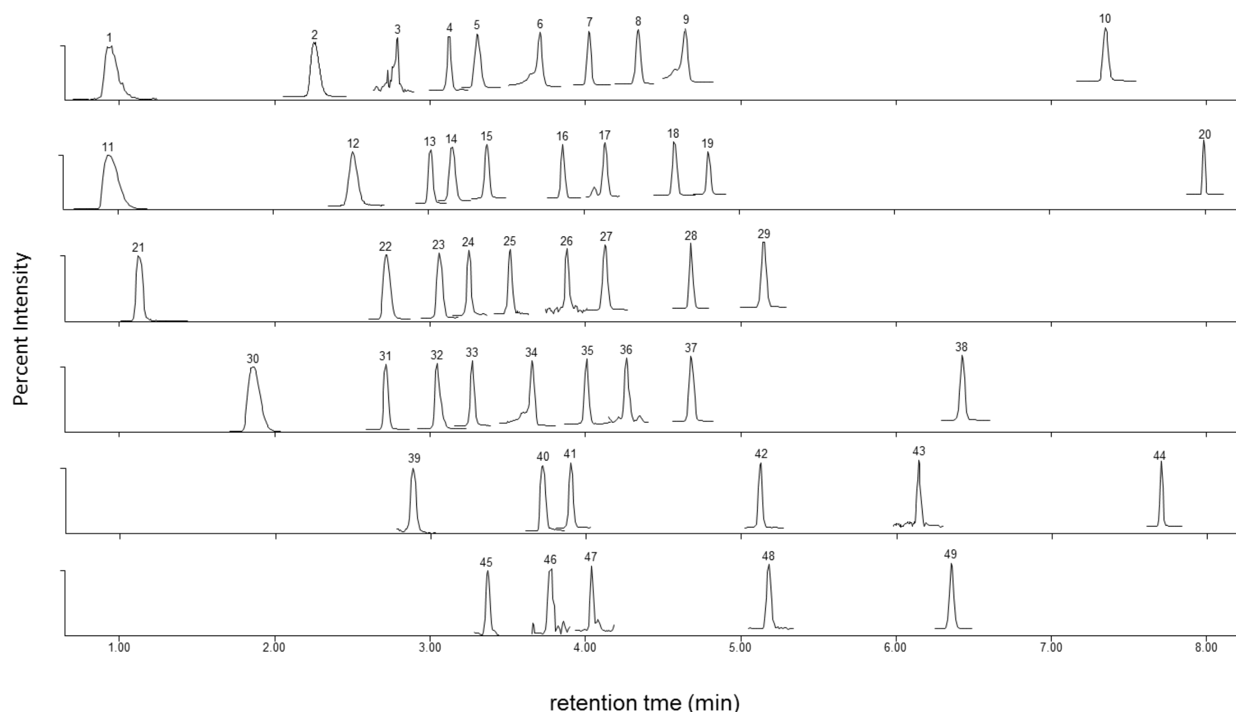

Figure 3.3. *Reconstructed MRM chromatograms showing UPLC separations for native compounds and microbial metabolites. All compounds normalized to equal height. The top four chromatograms represent authentic standards, while the lower two chromatograms were taken from analysis of colon contents following GSE administration. Peak identification: 1) hydroxymandelic acid, 2) 3,4-dihydroxyphenylacetic acid, 3) epigallocatechin, 4) procyanidin trimer T2 5) 3-hydroxybenzoic acid, 6) epigallocatechin gallate, 7) procyanidin dimer B2 gallate, 8) 3-(3'-hydroxyphenyl)propionic acid, 9) epicatechin gallate, 10) phloretin, 11) gallic acid, 12) catechol, 13) catechin, 14) hippuric acid, 15) procyanidin dimer B2, 16) procyanidin trimer C1, 17) procyanidin pentamer, 18) ferulic acid, 19) isoferulic acid, 20) valeric acid, 21) pyrogallol, 22) 4-hydroxybenzoic acid, 23) 3-(3',4'-dihydroxyphenyl)propionic acid, 24) vanillic acid, 25) homovanillic acid, 26) 3-(4'-hydroxyphenyl)propionic acid, 27) p-coumaric acid, 28) procyanidin dimer B5, 29) phenylacetic acid, 30) 3,4-hydroxybenzoic acid, 31) procyanidin dimer B1, 32) 4-hydroxyphenylacetic acid, 33) caffeic acid, 34) epicatechin, 35) cinnamtannin tetramer A2, 36) procyanidin hexamer, 37) m-coumaric acid, 38) 3-phenylpropionic acid, 39) unknown dimer 1, 40) 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)propan-2-ol, 41) 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, 42) 3-(3',4'-dihydroxyphenyl)valeric acid, 43) 5-(4'-hydroxyphenyl)-γ-valerolactone, 44) unknown metabolite 1, 45) unknown dimer 2, 46) 3-hydroxyphenylacetic acid, 47) unknown dimer 3, 48) 5-(3'-hydroxyphenyl)-γ-valerolactone, 49) hydroxyphenylvaleric acid (one of three isomers, see* ***Appendix A Table A2****).*

Repeatability. Intra- and interday repeatability values are shown in **Tables 3.1** and **3.2.** The intraday repeatability for native compounds was between 1.1-16.5%. All compounds except PC trimers, tetramer A_2, and hexamers had interday % RSDs below 8%. The interday repeatability for native compounds was between 2.2-25%, with only PC tetramer A_2 above 11.4% RSD. Intraday repeatability for metabolites was between 0.6-24.1%, with only 5 out of 24 metabolites tested [3-(4'-hydroxyphenyl)propionic acid; catechol; 4-hydroxymandalic acid; pyrogallol; 5-phenylvaleric acid] > 8.8% RSD. Interday repeatability for metabolites was between 1-23.9%, with only 3 of 24 compounds tested [4-hydroxyphenylacetic acid; 3-(4'-

hydroxyphenyl)propionic acid; 5-phenylvaleric acid] > 7.9% RSD. Therefore, both intra- and interday repeatability values for the vast majority of compounds tested were < 10% RSD. These results are generally in agreement with previously reported repeatability data. Urpi-Sarda *et al.* [74] reported interday repeatability values of 3-13.3% RSD for 2 native compounds (EC and PC dimer B2), and values of 0.1-15% RSD for 19 metabolites. Serra *et al.* [147] reported interday repeatability values of 3.5-8.9% RSD for 4 native compounds (C, EC, PC dimer B_2 and a PC trimer). Ortega *et al.* [14] reported interday repeatability values of 0.6-4.7 RSD for 3 native compounds (C, EC, and PC dimer B_2). Gonthier *et al.* [128] previously reported intraday and interday repeatability values of 0.1-14.7% RSD and 1.1-14.5% RSD, respectively, for 15 metabolites. Therefore, the present method generally achieves acceptable repeatability while also measuring a significantly greater suite of native compounds and their microbial metabolites than has previously been achieved.

LLOD/LLOQ. The LLOD and LLOQ values are shown in **Tables 3.1** and **3.2**. Observed LLOQs (S/N = 10:1) for native compounds were ~9-350 fmol on-column. Most values fell between 9-70 fmol, but were higher for the larger native compounds (trimer T_2, tetramer A_2, pentamer, and hexamer) which ranged in value from ~135-350 fmol. This is likely due to the poorer peak shape for these compounds. Previously, Urpi-Sarda *et al.* [74] reported LLOQs for EC and dimer B_2 of 51 and 2595 fmol, respectively, for a method that measures these 2 native compounds + 19 metabolites. The present method has a similar LLOQ for EC (~69 fmol) but a vastly improved LLOQ for dimer B_2 (23 fmol). Our reported LLOQs for C, EC and PC dimer B_2 (46, 69 and 23 fmol, respectively) are comparable to those reported by Serra *et al.* [130] for the same compounds (32, 37, and 25 fmol, respectively) and improve upon those reported by Ortega *et al.* [14] (344, 775, and 42 fmol, respectively). Similarly, our LLOQ for PC trimers C_1 and T_2 (42 and ~350 fmol, respectively) are greatly improved compared to that reported by Serra (2450 fmol for an unnamed trimer).

Observed LLOQs for the microbial metabolites were ~0.8-12,000 fmol on-column. The wide range of LLOQs is reflective of the wide range of metabolite compound classes measured in this analysis. Phloretin had the lowest value by far at ~0.8 fmol, while most values were in the range of ~30-750 fmol. Five compounds had LLOQs > 1000 fmol, [3-phenylpropionic acid, 3-(4'-hydroxyphenyl)propionic acid, 5-phenylvaleric acid, and homovanillic acid)] ranging from

1460-12,000. Previously, Urpi-Sarda *et al.* [74] reported LLOQs for ranging from ~40-9,000 fmol on column for these microbial metabolites. The Urpi-Sarda method was more sensitive than the present method for compounds such as caffeic acid (41 vs. 128 fmol, respectively), and less sensitive for compounds such as phenylacetic acid (5,500 vs. 270 fmol, respectively). Overall LLOQs for the two methods are similar, with the present method having the advantage of quantifying more metabolites (24 vs. 19) and also native compounds (17 vs. 2). The only other LC-MS/MS method that reports LLOQs for numerous microbial metabolites was published by Gonthier *et al.* [128], and reported LLOQs of ~5,000-20,000 fmol. It should be noted that Gonthier *et al.* defined LLOQ as S/N=3:1, whereas we defined LLOQ as S/N=10:1.

The observed LLODs (S/N=3:1) for native compounds were ~4.5-190 fmol on-column. The outlier trends for LLOD were similar to those of LLOQ for native compounds: only 4 out of 14 native compounds had LLODs > 50 fmol (generally the larger PCs). These are comparable to or better than previously reported LLODs. Ortega *et al.* [14] reported LLODs of 86, 258 and 29 fmol for C, EC and dimer B_2, respectively. Serra *et al.* [130] reported LLODs of 10, 10, 7.5 and 2,000 fmol for C, EC, dimer B_2 and an unknown trimer, respectively. Finally, Urpi-Sarda *et al.* [74] reported LLODs of 25 and 120 fmol for EC and dimer B_2, respectively.

The observed LLOD values for metabolites were 0.304-6,020 fmol on column. Most compounds ranged from approximately 22-220, with the same outliers as LLOQ. Urpi-Sarda *et al.* [74] found LLODs for metabolites at similar range (4-5,000 fmol) with similar differences as seen in LLOQ.

The present method likely has slightly elevated LLOQ and LLOD values for some compounds due to the reduced MS/MS duty cycle for each individual compound resulting from a significant increase in the number of analytes measured simultaneously. Overall, however, the present method offers similar or improved LLOQs for more compounds than previously reported methods, and significantly improved sensitivity for larger PCs.

The total moles-on-column approach was employed for calculation and presentation of LLOD/LLOQ values. This approach more accurately reflects instrument performance in terms of the true response and sensitivity of the detector and efficiency of the column as opposed to using concentrations (mM, etc.). Moles-on-column calibration is not influenced by injection volume, and therefore it more precisely quantifies the theoretical limitations of the analysis, rather than quantifying the specific case, associated with a particular injection volume. Reporting on-column

performance data provides standardized values that can be easily compared between laboratories and/or methods employing different injection volumes, without laborious data manipulation. This approach also eliminates the artificial improvements in reported LLOD/LLOQ or sensitivity that can be obtained by using a larger injection volume to collect the data. It is also important to note that LLOQ/LLOD for various methods may vary due to the sample matrix in which the compounds were analyzed.

Table 3.1. Method performance parameters for native monomers and procyanidins

Compound	**LLOQ[a] (fmol on column)**		**LLOD[b] (fmol on column)**		**Repeatability (% RSD)**	
	mean	SEM	mean	SEM	Intraday	Interday
(+)-catechin	45.9	11	14.9	1.1	6.0	11.4
(−)-epicatechin	68.9	0.0	28.7	5.7	6.9	10.0
(−)-epicatechin gallate	9.04	0.0	4.52	1.3	1.4	6.1
(−)-epigallocatechin	43.5	11	20.7	6.1	7.9	10.7
(−)-epigallocatechin gallate	9.45	0.73	5.09	0.73	3.7	7.2
procyanidin dimer B_1	13.8	3.5	8.07	0.58	2.4	5.7
procyanidin dimer B_2	23	5.8	10.4	3.5	1.1	6.9
procyanidin dimer B_5	69.2	10	51.9	10	6.2	7.6
procyanidin dimer B_2 gallate	36.5	4.6	20.5	6.8	2.2	6.2
procyanidin trimer C_1	42.3	3.8	34.6	6.7	9.9	9.2
procyanidin trimer T_2	346	0.0	192	38	9.2	2.2
cinnamtannin tetramer A_2	260	87	116	29	16.5	25.0
procyanidin pentamers	139	0.0	46.2	12	5.1	6.9
procyanidin hexamers	135	19	96.3	39	13.7	9.5

[a]lower limit of quantification
[b]lower limit of detection

Table 3.2. Method performance parameters for microbial metabolites

Compound	LLOQ[a] (fmol on column)		LLOD[b] (fmol on column)		Repeatability (% RSD)	
	mean	SEM	mean	SEM	Intraday	Interday
phenylacetic acid	269	24	147	0.0	3.2	4.8
4-hydroxyphenylacetic acid	548	110	416	120	6.5	9.5
3,4-dihydroxyphenylacetic acid	159	20	99.1	20	8.4	5.1
3-hydroxybenzoic acid	724	0.0	193	24	2.2	6.1
4-hydroxybenzoic acid	169	24	60.3	12	8.1	4.3
3,4-dihydroxybenzoic acid	108	22	54.1	11	8.0	4.0
3-phenylpropionic acid	6660	0.0	3110	220	3.7	4.7
3-(3-hydroxyphenyl)propionic acid	40.1	10	24.1	0.0	4.5	4.6
3-(4-hydroxyphenyl)propionic acid	12000	0.0	6020	0.0	10.9	9.7
3-(3,4-dihydroxyphenyl)propionic acid	732	180	220	0.0	5.2	1.0
5-phenylvaleric acid	1120	0.0	374	93	24.1	23.9
gallic acid	98	20	23.5	0.0	8.8	4.8
pyrogallol	211	26	132	26	15.3	1.7
4-hydroxymandalic acid	2040	19	1110	0.0	14.6	7.9
caffeic acid	148	19	74	19	3.1	3.3
m-coumaric acid	122	35	30.5	0.0	1.6	4.2
p-coumaric acid	60.9	0.0	28.4	2.0	2.5	5.7
phloretin	0.729	0.21	0.304	0.061	4.6	3.5
catechol	303	30	151	30	11.2	2.5
hippuric acid	167	0.0	93	19	1.5	2.8
vanillic acid	238	34	119	34	6.7	3.7
homovanillic acid	1460	183	732	183	3.6	5.2
ferulic acid	30.9	10	8.58	3.4	3.3	3.0
isoferulic acid	42.9	8.5	22.3	1.7	0.6	6.5

[a]lower limit of quantification
[b]lower limit of detection

Extraction Recovery. The extraction recoveries for monomers, PCs and select microbial metabolites from tissue homogenates are shown in **Table 3.3**. For native monomers and PCs, the recoveries ranged from 38-102%, with only the PC hexamer having recovery <50%. The recoveries for the predominant GSE constituents (C, EC, ECG, PC dimers and PC trimers) were all >60%, with recoveries for monomers and dimers >70%. These recoveries are similar to those reported by Serra *et al.* [130] from spiked plasma (≥96% for C and EC, 84% for dimer B_2, and 65% for a PC trimer) and are better than those reported by Serra *et al.* [15] for liquid-liquid extraction

from spiked liver (≥77% for C and EC, 65% for dimer B_2). Serra *et al.* [130] were able to achieve recoveries of ≥85% for C, EC and dimer B_2 by liquid-liquid extraction + µSPE from a variety of spiked tissues, but this method did not examine larger PCs nor metabolites [15]. Extraction recoveries for the 9 microbial metabolites tested from tissue homogenates ranged from 41-95%. Four of the 9 compounds had recoveries >90%, with only 5-phenylvaleric acid and hippuric acid having recoveries of <50%. Urpi-Sarda *et al.* [74] reported recoveries of ≥86% for microbial metabolites of monomers and PCs using Waters Oasis MAX and MCX SPE cartridges. However, this method was not suitable for native compounds, as the recovery of PC dimer B_2 by this method was only 10%.

Our initial attempts to perform extraction of all compounds (natives and metabolites) solely using the Oasis HLB SPE columns resulted in generally good recovery of monomers and PCs (25-101% with only EGC having recovery of <54%, data not shown) but poor recovery of metabolites (19-80%, with 6 of the 9 tested compounds having recoveries of ≤50%, data not shown). Therefore, the parallel liquid-liquid + SPE method was developed to improve metabolite recovery. Performing liquid-liquid extraction followed by Waters Oasis HLB SPE, and then combining the two extracts was determined to be more appropriate over HLB alone or other types of solid phase cartridges alone. Waters Oasis Mixed-Mode SPE cartridges were also explored as a possible alternative for extraction of both neutral native and metabolite compounds and weakly acidic metabolites. This technology uses both reverse-phase and ion exchange chemistry for retention and elution. While Mixed-Mode Anion-eXchange (Oasis MAX) cartridges would provide suitable retention for most compounds in the analysis, the required basic (ammonium hydroxide) wash would be unacceptable for native flavonoids due to their known instability above pH 5.5-6 [148, 149]. This appears to be supported by the results of Serra *et al.* who obtained excellent metabolite recoveries but poor recovery of PC dimer B_2 using Oasis MAX and MCX mixed-mode SPE columns [15]. By first extracting the metabolites using ethyl acetate followed by extraction of native compounds via an acidic acetone:water mix that is then passed through Oasis HLP SPE cartridges, we minimized degradation while still achieving acceptable recovery of most analytes. It should be noted that the acetone:water:acetic acid solvent yielded the optimum extraction from the tissue pellet during the SPE phase of the extraction. However, dilution of the acetone:water:acetic acid extract with water (2 mL pooled

extract + 12 mL water) prior to SPE was required in order to achieve optimum loading and retention of the analytes on the SPE cartridges.

Table 3.3. Extraction recovery of compounds spiked into rat colon tissue.

Class	Compound	Recovery[a] (%)	
		mean	SEM
native monomers and procyanidins	(+)-catechin	102	0.85
	(−)-epicatechin	91	0.88
	(−)-epicatechin gallate	83	1.3
	(−)-epigallocatechin	60	1.6
	(−)-epigallocatechin gallate	61	1.1
	procyanidin dimer B_1	89	0.983
	procyanidin dimer B_2	89	0.51
	procyanidin dimer B_5	83	1.5
	procyanidin dimer B_2 gallate	72	1.4
	procyanidin trimer C_1	61	1.9
	procyanidin trimer T_2	64	1.1
	cinnamtannin tetramer A_2	56	2.2
	procyanidin pentamers	54	0.478
	procyanidin hexamers	38	1.9
microbial metabolites	phenylacetic acid	58	2.9
	4-hydroxyphenylacetic acid	94	0.66
	3,4-dihydroxybenzoic acid	92	1.5
	3-phenylpropionic acid	56	2.4
	3-(3-hydroxyphenyl)propionic acid	93	1.0
	5-phenylvaleric acid	41	3.6
	gallic acid	95	2.0
	p-coumaric acid	69	5.9
	hippuric acid	44	0.17

[a]% of spiked compound recovered following extraction (*n*=4)

Intestinal Luminal and Tissue Levels. The measured levels of native compounds and microbial metabolites in the lower GI tract are shown in **Figures 3.4-3.7**. Levels of individual compounds in each region are shown in **Appendix A** (**Tables A5-A8**). The data indicate fairly high accumulation of native compounds, primarily monomers and dimers, in the cecum and colon (**Figure 3.4**). Accumulation of natives was particularly high in the cecum, perhaps due to the high concentration of native compounds and minimal microbial metabolism at this point.

Surprisingly, dimer accumulation levels were relatively high in all three regions of the lower GI tract (**Figure 3.4C**), which could be due to the relatively large % of dimers in GSE. Interestingly, the data also suggest excellent retention of accumulated natives, as significant levels were observed in the cecum even 8 h post-gavage. This is consistent with previous reports of monomer and procyanidin accumulation and retention in the lower GI tract [11, 150].

In the luminal contents, native compounds were elevated in the distal regions (**Figure 3.5**), which is likely indicative that the majority of the gavaged dose had passed through the other regions and resided in the distal colon at 8 h post-gavage. Surprisingly, the highest accumulation of oligomers occurred in the cecum (**Figure 3.4D**) but highest luminal levels were present in the distal colon (**Figure 3.5D**). The actual species and concentrations of catechins, procyanidins and metabolites observed in the colon will depend on the nature and dose of the matrix being consumed.

The metabolite data indicate the progressive nature of microbial metabolism as the bolus moves through the lower GI tract (**Figure 3.7**): valerolactone levels (a marker of early metabolism) were highest in the cecum, while phenolic acids and small aromatics (products of subsequent metabolism), were highest in the distal colon. It should be noted that there were appreciable levels of phenolic acids in the controls, particularly in thee luminal contents. This is to be expected, as phenolic acids such as ferulic acid are found in sources such as grains used to formulate animal diets [151]. The diet used in the present study (Harlan Teklad AIN-93M TD.94048) contained cornstarch and cellulose, both of which could be sources of phenolic acids. However, the presence of valerolactones in the cecum of control mice (**Figure 3.7B**) indicates that there may have been some flavan-3-ols in the background diet, consistent with the observation of small amounts of dimers and oligomers in the controls (**Figures 3.4C-D** and 3.**5C-D**).

In order to identify the potential source of some of the compounds, the study diet was analyzed in triplicate (~30 mg diet analyzed/replicate) following our method. This analysis did not indicate the presence of intact flavonoids such as monomers or PC oligomers. There were detectable levels of six phenolic compounds: 4-hydroxybenzoic acid (0.383 ng/mg diet), 3,4-dihyroxybenzoic acid (0.311 ng/mg diet), p-coumaric acid (4.71 ng/mg diet), ferulic acid (1.31 ng/mg diet), isoferulic acid (0.266 ng/mg diet), and phenylacetic acid (0.233 ng/mg diet). Ferulic acid and p-coumaric acid were not found in any of the control samples, which may suggest

further breakdown of these compounds. The presence of phenolic acids in the diets may explain the presence of phenolic acids in the control animals. The presence of flavonoids, albeit in very small amounts, in the control animals may be explained by the presence of small amount of larger tannins in the diet that are not detectable by the present method but which are metabolized to smaller PCs and valerolactones by gut microbiota

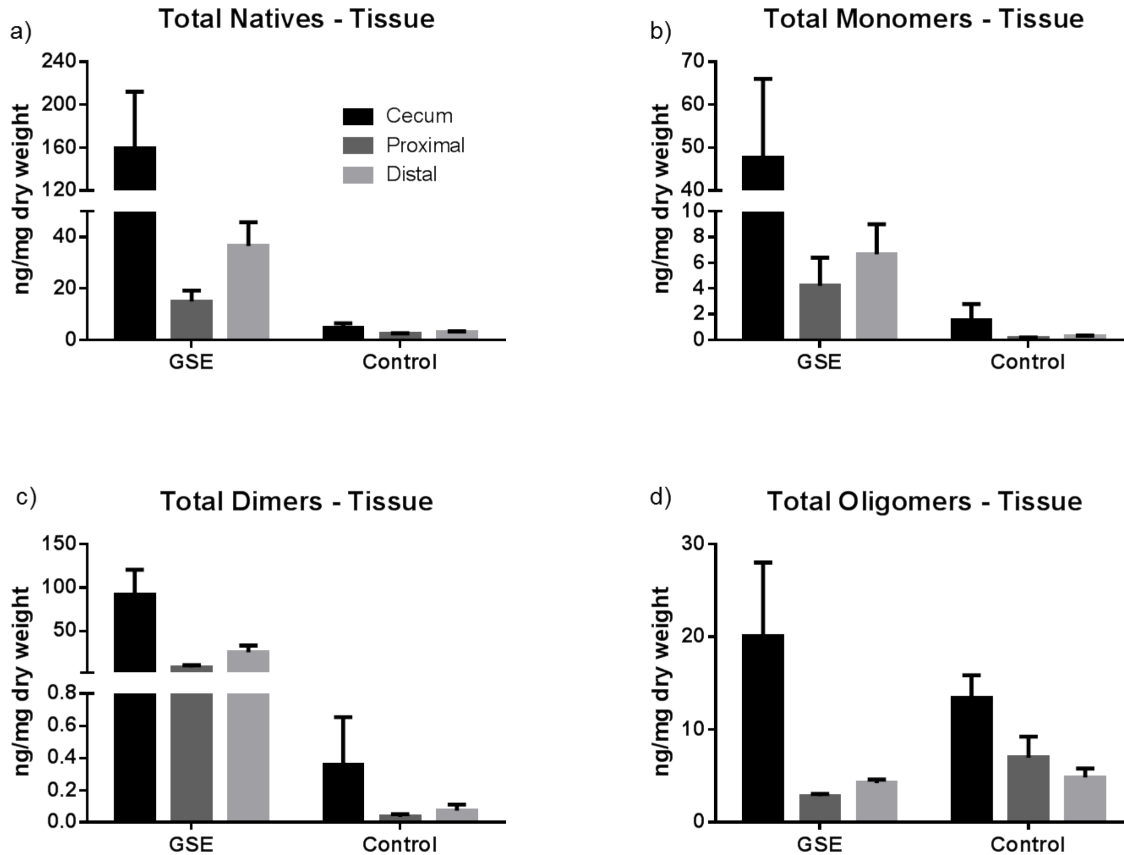

Figure 3.4. *Tissue levels of total native compounds (A), total monomers (B), total dimers (C), and total oligomers (D) in the cecum, proximal colon, and distal colon of rats 8 h post-gavage of GSE. Data are presented as mean ± SEM from n=8 animals.*

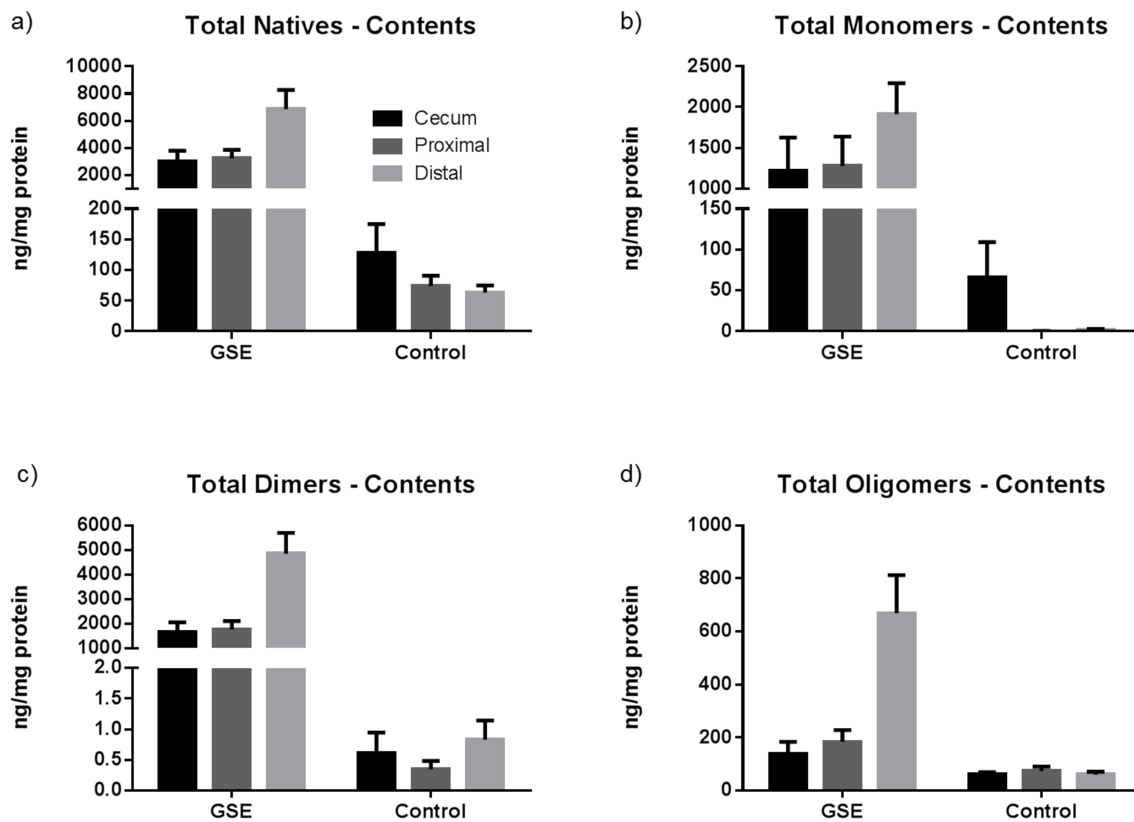

Figure 3.5. *Levels of total native compounds (A), total monomers (B), total dimers (C), and total oligomers (D) in the luminal contents cecum, proximal colon, and distal colon of rats 8 h post-gavage of GSE. Data are presented as mean ± SEM from n=8 animals.*

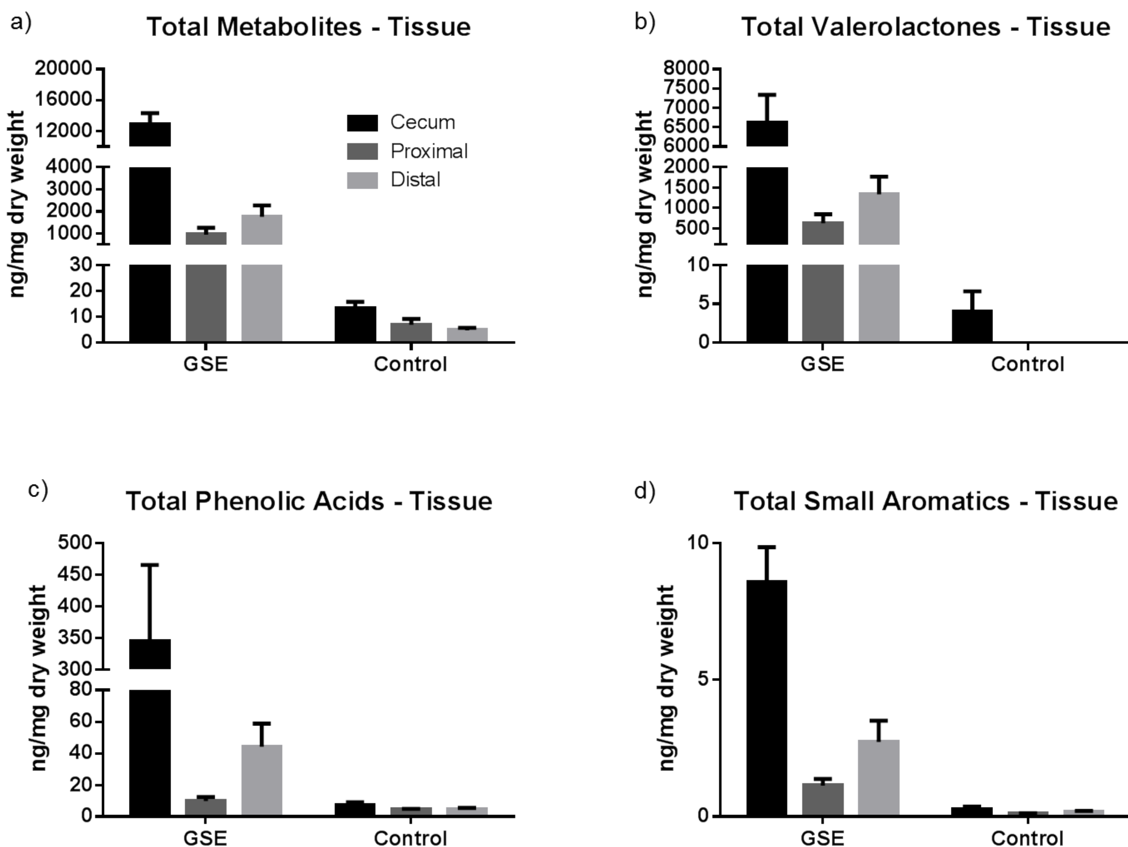

Figure 3.6. *Tissue levels of total metabolites (A), total valerolactones (B), total phenolic acids (C), and total small aromatics (D) in the cecum, proximal colon, and distal colon of rats 8 h post-gavage of GSE. Data are presented as mean ± SEM from n=8 animals. Note this figure includes 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)propan-2-ol (refer to Appendix A for levels of this peak) in the total metabolites, but not in any individual metabolite class as it is not a valerolactone, phenolic acid, not a small aromatic. This accounts for the fact that the sum of B, C, and D is significantly less than A.*

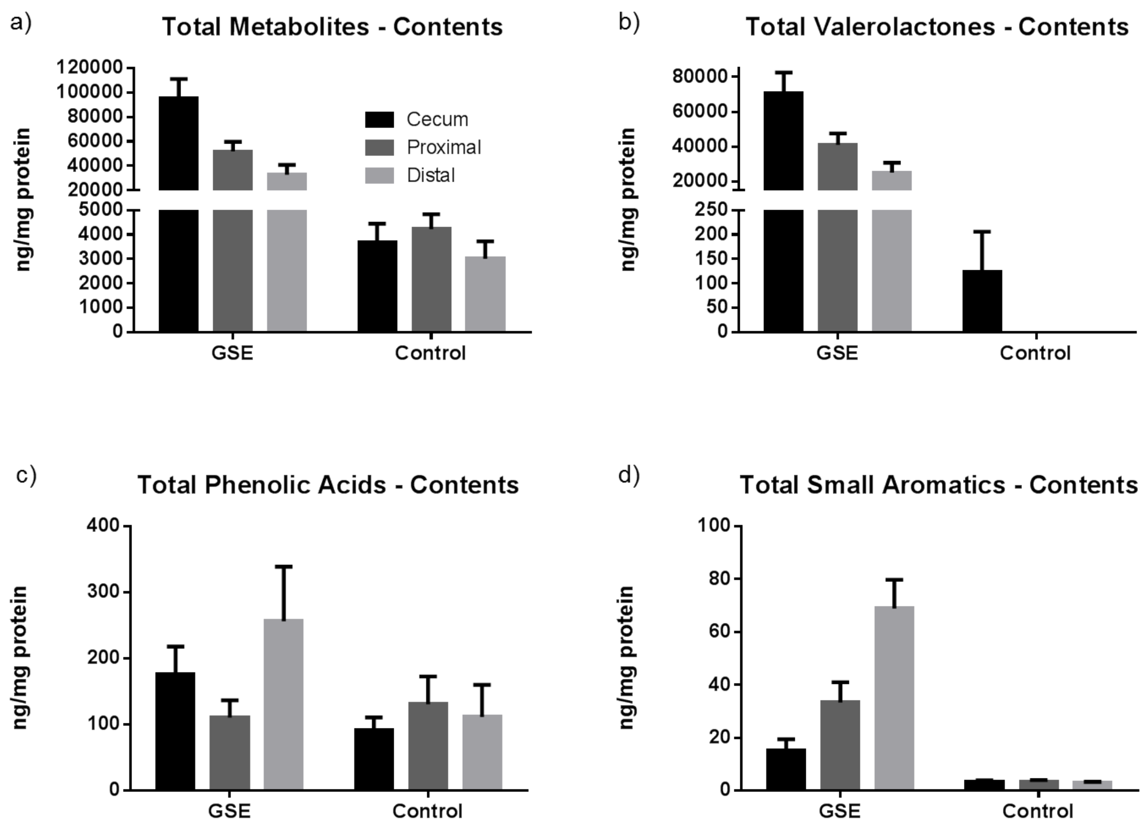

Figure 3.7. *Levels of total metabolites (A), total valerolactones (B), total phenolic acids (C), and total small aromatics (D) in the luminal contents cecum, proximal colon, and distal colon of rats 8 h post-gavage of GSE. Data are presented as mean ± SEM from n=8 animals. Note this figure includes 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)propan-2-ol (refer to Appendix A for levels of this peak) in the total metabolites, but not in any individual metabolite class as it is not a valerolactone, phenolic acid, not a small aromatic. This accounts for the fact that the sum of B, C, and D is lightly less than A.*

Conclusions.

This method provides acceptable extraction and measurement of GSE native compounds and microbial metabolites. The results for extraction recovery are similar to previously reported methods. Although recoveries for certain compounds (e.g. PC hexamer, 5-phenylvaleric acid, and hippuric acid) were lower than other compounds, they were still found to be within the acceptable range for the method and for the wide range of compounds (size and structure) that can be analyzed. Furthermore, the recoveries are acceptable for studies that will compare the differences between treatment and control groups. The run time for the UPLC separation is shorter than previously published methods, which not only allows for the high-throughput of samples, but also for the efficient, simultaneous analysis of both native compounds and

metabolites. The overall analysis has also been shown to be effective in measuring native compounds and microbial metabolites in both the cecum and colon tissue, as well as their luminal contents. Therefore, the present method provides similar performance to existing methods, while increasing the number of compounds that are analyzed and facilitating simultaneous analysis of both native flavan-3-ols and the full suite of their microbial metabolites. This dramatically increases analytical efficiency and throughput, which will facilitate more complete profiling of flavan-3-ol metabolism and permit design of larger experiments. Therefore, we believe that large numbers of both native compounds and their microbial metabolites can be rapidly and simultaneously quantified in complex biological matrices without significantly impairing performance compared to those methods that quantify both classes separately (or those that quantify both classes, but only a few compounds from one of the classes).

We have decreased the per-sample run time of our method compared to existing methods by increasing the flow rate to 0.6 mL/min. While this increased flow rate may result in slightly sub-optimal peak efficiency, the optimum conditions for overall method performance of a UPLC analysis depend on the desired objectives of the analysis. In this case, the objectives of this study were to develop and validate a high-throughput UPLC-MS/MS method that will simultaneously measure both native PCs and their metabolites and facilitate high-throughput analysis of native and metabolite profiles in various regions of the colon. The method we describe has improved performance, for those stated objectives, over existing methods. Specifically, our method quantifies more compounds with acceptable or improved LLOQ/LLOD while also achieving ~25% greater sample throughput compared to the most rapid existing method. Therefore, the use of higher flow rates appropriately facilitates our stated goals without compromising method performance.

One limitation of this method is that Phase-II metabolites (sulfates, glucuronides, and O-methyl derivatives) of the native flavan-3-ols are not measured. The method is focused on monitoring the microbial metabolism of flavan-3-ols, rather than Phase-I and Phase-II metabolism reactions that occur in the gut epithelium, liver, and kidneys. Another concern that must be noted is in regard to the suitability for this method to analyze PC oligomers. Presence of oligomers was found in the control samples for both tissue and contents, although these compounds were not administered to control animals either in the gavaged treatment or the diet.

However, the sensitivity and specificity of MS/MS detection indicate that these compounds are likely present in the control animals due to degradation of larger tannins that are not detectable in the diet due to their complex chemical structure.

This preliminary profiling study is believed to be the first to examine both native compounds and microbial metabolites in various regions of the colon. These preliminary data suggest that various regions of the colon accumulate distinct profiles of potentially bioactive species, which has implications for delivery and efficacy of dietary flavan-3-ols in the colon. Another limitation of the present study is that the animals were sacrificed 8 h post-gavage, which provides only one "snapshot" of microbial metabolism. The levels of all compounds may have been higher in the distal colonic lumen and tissue if this region had been exposed to the digesta for additional time, etc. Therefore, additional pharmacokinetic experiments with animals sacrificed at various time points are required. This method sufficiently improves analytical throughput to a level that facilitates such studies.

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Chapter 4:

Chronic administration of dietary grape seed extract increases colonic expression of gut tight junction protein occludin and reduces fecal calprotectin in a secondary analysis of healthy Wistar Furth rats

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Abstract

Animal studies have demonstrated the potential of grape seed extract to prevent metabolic syndrome, obesity, and type-2 diabetes. Recently, metabolic endotoxemia induced by bacterial endotoxins produced in the colon has emerged as a possible factor in the etiology of metabolic syndrome. Improving colonic barrier function may control endotoxemia by reducing endotoxin uptake. However, the impact of grape seed extract on colonic barrier integrity and endotoxin uptake has not been evaluated. We performed a secondary analysis of samples collected from a chronic GSE feeding study with pharmacokinetic endpoints in order to examine potential modulation of biomarkers of colonic integrity and endotoxin uptake. We hypothesized that a secondary analysis would indicate that chronic GSE administration increases colonic expression of intestinal TJPs and reduces circulating endotoxin levels, even in the absence of an obesity-promoting stimulus. Wistar Furth rats were administered drinking water containing 0.1% grape seed extract for 21 d. Grape seed extract significantly increased expression of gut junction protein occludin in the proximal colon, and reduced fecal levels of the neutrophil protein calprotectin, compared to control. Grape seed extract did not significantly reduce serum or fecal endotoxin levels compared to control, although the variability in serum levels was widely increased by GSE. These data suggest that the improvement of gut barrier integrity, and potential modulation of endotoxemia, warrant investigation as a possible mechanism by which grape seed extract prevents metabolic syndrome and associated diseases. Further investigation of this mechanism in high-fat feeding metabolic syndrome and obesity models is therefore justified.

Introduction

Numerous animal studies have demonstrated the potential of grape seed extract (GSE) to prevent metabolic syndrome, obesity, and type-2 diabetes. GSE reduces weight gain and fat accumulation [152-154], insulin resistance [155, 156], and systemic inflammation [152, 157, 158], while increasing mitochondrial function and fatty acid oxidation [152, 153, 159]. The mechanisms by which GSE acts are not fully understood, but likely include reduction of endotoxin-induced inflammation and stimulation of metabolic gene expression [160].

Recently, metabolic endotoxemia has emerged as a possible factor in the etiology of metabolic syndrome. Circulating endotoxins produced by gut microbiota and absorbed into circulation can induce immune hyperstimulation in skeletal muscle by stimulating toll-like receptors (TLRs), resulting in NF-κB activation, secretion of pro-inflammatory cytokines, and insulin resistance [161, 162]. Endotoxemia is likely regulated by two main factors. First, alterations to gut microbiota can increase endotoxin production in the gut. Animal and human studies have demonstrated that a high fat (HF) diet, obesity and diabetes can alter the composition of the gut microbiota as well as gut endotoxin production [163-170]. Second, elevated gut permeability may increase paracellular endotoxin absorption into circulation through epithelial tight junctions [161, 171, 172]. In mice, altered expression of epithelial tight junction proteins (TJPs) [occludin, claudin isoforms, zona occludens (ZO)-1/2, etc.] and increased permeability are observed in HF feeding and metabolic syndrome, obesity and diabetes models, often preceding clinical disease [169, 170, 173-175].

Reduction of endotoxin production and/or uptake in the gut would represent an ideal mechanistic target for GSE, due to the poor bioavailability and high concentrations of its constituent procyanidins in the gut following consumption [11, 63, 68, 71, 133, 150]. Recently, Song *et al.* [176] demonstrated that GSE reduces small intestinal permeability and increases small intestinal transcription of occludin and ZO-1 genes. This suggests that GSE improves intestinal barrier integrity, with possible implications for endotoxemia and metabolic syndrome. However, the

majority of endotoxin production and uptake likely occurs in the colon, and the impact of GSE on colonic barrier integrity and endotoxin uptake has not been evaluated.

We therefore determined to perform a secondary analysis of fecal, colonic, and blood samples collected form a previous experiment (manuscript in preparation) that was designed to examine the pharmacokinetic behavior of GSE constituents following chronic GSE administration. The purpose of this secondary analysis was to obtain "proof-of-concept" and hypothesis-generating data regarding the potential modulation of colonic integrity and antigen trafficking by chronic GSE administration, thus facilitating justification and design of further experiments to specifically test the impact of GSE on these biomarkers in an obesity model. We hypothesized that a secondary analysis would indicate that chronic GSE administration increases colonic expression of intestinal TJPs and reduces circulating endotoxin levels, even in the absence of an obesity-promoting stimulus.

Materials and Methods

Grape Seed Extract. Vitaflavan™ GSE (>96% polyphenols by weight) containing monomeric catechins, dimeric and trimeric procyanidins, and larger procyanidins (Table 4.1, Figure 4.1) was obtained from DRT Nutraceutics (Dax, France).

Table 4.1. Composition of Vitaflavan™ grape seed extract[ab]

compound	% (w/w)
monomers	**22.0**
catechin	15.3-15.9
epicatechin	4.1-11
epicatechin gallate	2-5.7
dimers + trimers	**32-35**
procyanidin B1	7.7-7.8
procyanidin B2	5.7
procyanidin B3	3.8-4.0
procyanidin B4	1.8
total procyanidins	**>75[b]**
total polyphenols	**>96**

[a]Vitaflavan™ grape seed extract was purchased from DRT Nutraceutics, Dax, France
[b]Composition values based on Vitaflavan™ specification and lot Certificates of Analysis provided by the supplier
[c]Values for total procyanidin content differ based on the analytical method. Procyanidin specifications for Vitaflavan™ are >75% for the gel permeation chromatography method and >65% for the Porter method.

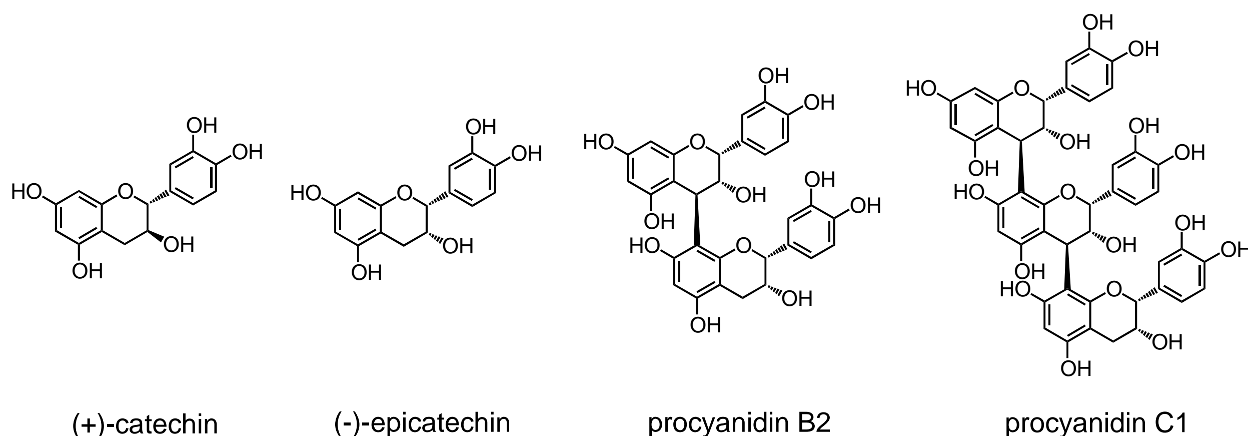

Figure 4.1. *Structures of select predominant flavan-3-ols present in grape seed extract: (+)-catechin, (−)-epicatechin, procyanidin B2, and procyanidin C1.*

Animals. The Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University approved all animal protocols. Male Wistar Furth inbred rats (*n*=16, 6-7 wk old) were obtained from Harlan (Indianapolis, IN) and housed 2/cage on a 12-hour light/dark cycle. Animals were given food and water *ad libitum* throughout the study. The diet was a nutritionally complete polyphenol-free purified diet (Harlan Teklad AIN-93M, TD.94048, Table 2). Animals were acclimated for 7 d prior to the study. Following acclimation, animals were randomized to two groups (*n*=8/group). The experimental group was given drinking water containing 0.1% GSE (~100 mg/kg/d based on ~10-12 mL water/100 g rat/d). This dose of GSE is in the range typically used in pharmacokinetic and obesity prevention studies [152, 153, 177, 178]. The control group was given GSE-free water. Control and GSE-containing water were prepared fresh daily. Animals were treated for 21 d, in order to allow modifications to the gut microbiota, and weighed every 3 d. Following d 21, both groups were provided GSE-free water for 24 h. Animals were then administered either 1 mL 0.9% saline (control group) or ~1.5 mL of 50 mg/mL GSE in 0.9% saline (~250 mg/kg GSE) via intragastric gavage. Animals were euthanized 8 h post-gavage with CO_2 followed by bilateral pneumothorax. As this analysis is a secondary analysis, the model employed for this study (healthy Wistar Furth rats, polyphenol-free rodent chow) is a common model for pharmacokinetic modeling of dietary polyphenols, and the washout and gavage steps were performed in order to observe metabolism of a defined acute GSE dose, which will be reported elsewhere (manuscript in preparation).

Table 4.2. Composition of the polyphenol-free purified AIN-93M rodent diet[a]

ingredient	g/kg
casein	140.0
L-cysteine	1.8
corn starch	465.692
maltodextrin	155
sucrose	100
soybean oil	40
cellulose	50
mineral mix[b]	35
vitamin mix[c]	10
choline bitartrate	2.5
TBHQ[d]	0.008

[a]Harlan Teklad, Indianapolis, IN (No. 94048)
[b]AIN-93M-MX (Harlan No. 94049)
[c]AIN-93-VX (Harlan No. 94047)
[d]tert-butylhydroquinone (antioxidant)

The cecum, proximal and distal colon were excised. The luminal contents from each region was removed and divided, with half stored in 0.5 mL acidified saline (0.9% NaCl w/v + 0.1% formic acid w/v) and snap-frozen in liquid N_2. Tissue sections were flushed with ice-cold phosphate-buffered saline (PBS); pieces (~5 mm) were collected for RNA analysis in 0.5 mL RNAlater (Qiagen, Valencia, CA) and snap-frozen in liquid N_2. Blood was collected via cardiac puncture, clotted for 30 min, centrifuged (3500 x *g*, 10 min, 4°C), and serum was collected in 1.5 mL with 0.25 mL acidified saline. All samples were stored at −80°C prior to analysis.

Intestinal Junction Protein Expression. Gene expression of intestinal TJPs was performed by quantitative real-time PCR (qRT-PCR) [162]. RNAlater-fixed tissue samples from the cecum, proximal colon and distal colon were thawed on ice, blotted dry, and minced. Tissue was homogenized in 250 μL TRIzol reagent (Ambion, Grand Island, NY) by bead beating (5 min, 4°C) using a Bullet Blender (Next Advance Inc., Averill Park, NY). Homogenates were phase-separated with addition of 125 μL chloroform and centrifuged (12,000 x *g*, 15 min, 4°C). The aqueous layer was removed and RNA was precipitated with 125 μL 70% ethanol. The extract was purified to isolate total RNA using RNEasy Mini kits with RNase-free DNase I digestion following manufacturer's instructions. Purified RNA extracts were stored at −80°C. qRT-PCR

was performed using an ABI PRISM 7900 Sequence Detection System and TaqMan Universal PCR Master Mix was used according to manufacturer's specifications (Applied Biosystems, Inc., Foster City, CA). Primer and probe sets for target genes were pre-validated TaqMan® Gene Expression Assay kits (ABI): ZO-1/TJP-1 (Rn02116071_s1), ZO-2/TJP-2 (Rn01501483_m1), occludin (Rn00580064_m1), claudin-1 (Rn00581740_m1), β-actin (Rn00667869_m1). Relative quantification of target genes was calculated using the $2^{-\Delta CT}$ method, which was validated for each primer/probe set using a 6 point serial standard curve as described previously [179]. Target gene expression was normalized to β-actin mRNA levels.

Preparation of Fecal Homogenates. Luminal contents from the distal colon (referred to hereafter as feces) were freeze dried for 24 h and reconstituted in 1 mL PBS along with 50 µL 1% (w/v) ascorbic acid (Sigma, St. Louis, MO). Samples were then homogenized by bead beating for 10 min (4°C). Homogenates were diluted with 2 mL lysis buffer [0.1% sodium dodecyl sulfate, 0.5% sodium deoxyxholate, 0.02% sodium azide, 5 mM disodium ethylenediaminetetraacetic acid (all from Sigma), and 1X Halt protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific) in PBS]. Homogenates were further homogenized for 30 s, centrifuged (5820 x *g*, 10 min, 4°C), and supernatants were snap-frozen in liquid N_2 and stored at −80°C.

Calprotectin, Endotoxin, and Total Protein Levels. Fecal calprotectin levels were determined using rat calprotectin ELISA kits (Mybiosource.com, San Diego, CA) [180]. Homogenates were diluted 2-fold with PBS and analyzed following manufacturer's instructions. Fecal and serum endotoxin concentrations were determined using PyroGene™ Recombinant Factor C Endotoxin Detection fluorescence assay kits (Lonza Walkersville, Inc., Walkersville, MD) [181]. Limulus Amebocyte Lysate (LAL) assay plates, LAL reagent water, and pyrogen-free tubes (all from Lonza) were employed to minimize exogenous endotoxin contamination. Assays were performed in triplicate on 100 µL undiluted serum or 100 µL homogenate diluted 8-fold with PBS. Fluorescence was quantified using a BioTek Synergy 2 plate reader (Winooski, VT), and data were processed using Gen 5 software (v1.08, BioTek). Total protein levels in fecal homogenates were determined using Pierce BCA protein kits (Thermo Fisher Scientific Inc., Waltham, MA).

Samples were assayed in triplicate according to manufacturer's instructions. Fecal calprotectin and fecal endotoxin levels were normalized with total protein levels in the homogenates.

Statistical Analyses. Statistical analyses for all data were performed using two-tailed unpaired t-tests on GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA) to detect differences between treatments. Significance was defined as $P < \alpha$ (0.05). All data are presented as means ± SEM. The sample size was chosen based on the original objective to observe differences in polyphenol metabolite concentrations. A *post hoc* analysis indicated statistical power of 100% for occludin levels in the proximal colon and 13% for serum endotoxin levels.

Results and Discussion

Weights. Control and GSE groups experienced weights gains of 38 ± 1.4% and 36 ± 1.7%, respectively, from pre-treatment weights [initial weights (means ± SEM) were 199 ± 3 g and 202 ± 2 g for control and GSE groups, respectively]. Weight gains were not statistically significant between control and GSE groups at any time point (Figure 4.2), likely due to the absence of a HF or high-calorie diet needed to induce obesity.

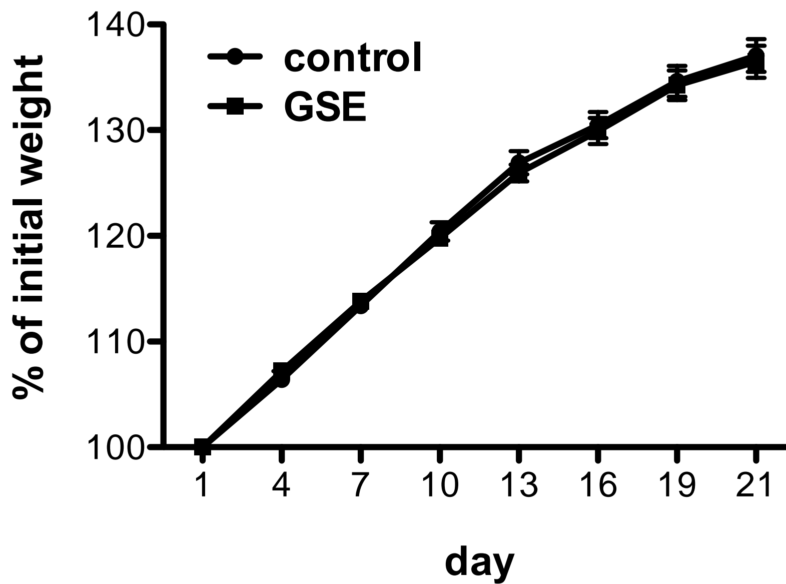

Figure 4.2. *Animal weights over time (expressed as a percentage of initial weight) for control mice (GSE-free) vs. mice administered 100 mg/kg/d GSE in drinking water (n=8/group). Data are expressed as means ± SEM. Initial weights were 199 ± 3 g and 202 ± 2 g for control and GSE groups, respectively. No significant differences between treatments for each day were observed as determined by an unpaired two-tailed t-test with significance defined as P < 0.05.*

Endotoxin. GSE caused a non-significant reduction in serum endotoxin levels (Figure 4.3A). While serum endotoxin levels clustered tightly in the control group [8.19 ± 0.30 endotoxin units (EU)/mL], levels in the GSE group were more variable (7.5 ± 2.1 EU/mL). GSE treatment appeared to alter serum endotoxin concentrations, but the direction of the effect remains unclear. Three animals in the GSE group had extremely low serum endotoxin concentrations (< 2 EU/mL). This trend toward reduced serum endotoxin even when fed a control diet suggests that GSE may potentially inhibit the increase in circulating endotoxin typically observed during HF feeding [167, 169, 170]. To determine whether this trend towards reduced in serum endotoxin levels could be attributed to similar distortions in endotoxin production in the gut, fecal endotoxin levels were determined (Figure 4.3B). The overall clustering pattern of fecal endotoxin levels was similar between groups (clustered at 3.3 ± 0.84 and 4.0 ± 0.95 EU/mg protein in the control and GSE groups, respectively), with a non-significantly higher mean in the GSE group. Therefore, GSE did not appear to significantly modulate endotoxin production in the gut. The different patterns of serum endotoxin levels observed for GSE vs. control, in the absence of differences in the pattern of fecal endotoxin, are provocative and are likely due to other

mechanisms, potentially including altered gut permeability. The variability in observed endotoxin concentrations are likely due to real physiological variations rather than assay variations, as the fluorescence assay employed was highly reproducible (relative standard deviations for triplicate measures of standards, serum samples, and fecal samples were 4.8, 3.8, and 12%, respectively).

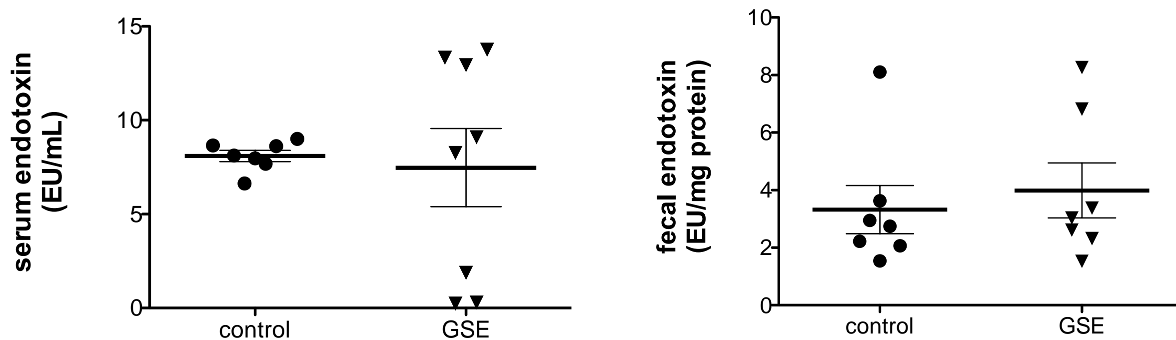

Figure 4.3. *Endotoxin concentrations in serum (**A**) and feces (**B**) of control mice (GSE-free) vs. mice administered 100 mg/kg/d GSE in drinking water (n=8/group). Data are expressed as means ± SEM. Endotoxin is reported as endotoxin units (EU) based on standards provided with the assay (PyroGene™ Recombinant Factor C Endotoxin Detection fluorescence assay kits, Lonza Walkersville, Inc., Walkersville, MD). Serum endotoxin is reported as EU/mL; fecal endotoxin is reported as EU/mg total protein (**B**). No significant differences between treatments in serum or feces were observed as determined by an unpaired two-tailed t-test with significance defined as $P < 0.05$. Note: one serum endotoxin value in the control group, and one fecal endotoxin value in both groups were excluded as an outlier with 99% confidence based on the Q-test.*

Gut Junction Proteins. The expression of TJPs was assessed by qRT-PCR in the cecum as well as the proximal and distal colon (Figure 4.4A-C). In the cecum, GSE significantly lowered the expression of occludin relative to control. No other significant differences were observed in the cecum. In the proximal colon, GSE significantly increased the expression of occludin relative to control. No other significant differences were observed in the proximal colon, although levels of ZO-1/2 were slightly but not significantly elevated in the GSE group. No significant differences were observed in the distal colon, but the same overall trends in expression levels seen in the proximal colon were observed in the distal colon. These data build upon the observations of Song *et al.* [176], who reported that GSE significantly increases transcription of both occludin and

ZO-1 in the small intestine. Notably, we obtained these results in the colon, as opposed to the small intestine, with a dose of only 100 mg/kg/d GSE over 21 d, whereas Song *et al.* employed 250 mg/kg/d over 29 d. More importantly, the increase in select TJP expression suggests that GSE may reduce intestinal permeability in obesity or other disease states, similar to the results of Carrasco-Pozo *et al*. [182] who showed that apple procyanidins (175-350 mg/kg acute dose) prevent indomethacin-induced permeability throughout the gut. However, we did not measure colonic permeability directly. Future studies of the impact of GSE on colonic barrier integrity (including follow-up studies in or laboratory) will require determination of colonic permeability by oral administration of sugar probes (sucrose, lactulose, mannitol, sucralose) followed by analysis of absorption and urinary excretion by HPLC [182], which will more accurately predict permeability/uptake of endotoxin.

The distinct trends in expression observed in the cecum vs. other sections of the colon were unexpected, indicating that distinct regions of the gut may react differently to GSE. This may be due to a variety of factors that vary along the length of the colon, including: distinct polyphenol and metabolite profiles due to progressive microbial metabolism of GSE constituents, differing microbial populations, and distinct tissue architecture and biology [11, 183-185].

While GSE appeared to slightly but not significantly increase the expression of claudin-1 in the cecum and slightly but not significantly lower its expression throughout colon, there are at least 24 known claudins involved in maintaining gut integrity [171]. Due to their key role as determinants of gut permeability [172], further study of the impact of GSE on a variety of claudin isoforms is required.

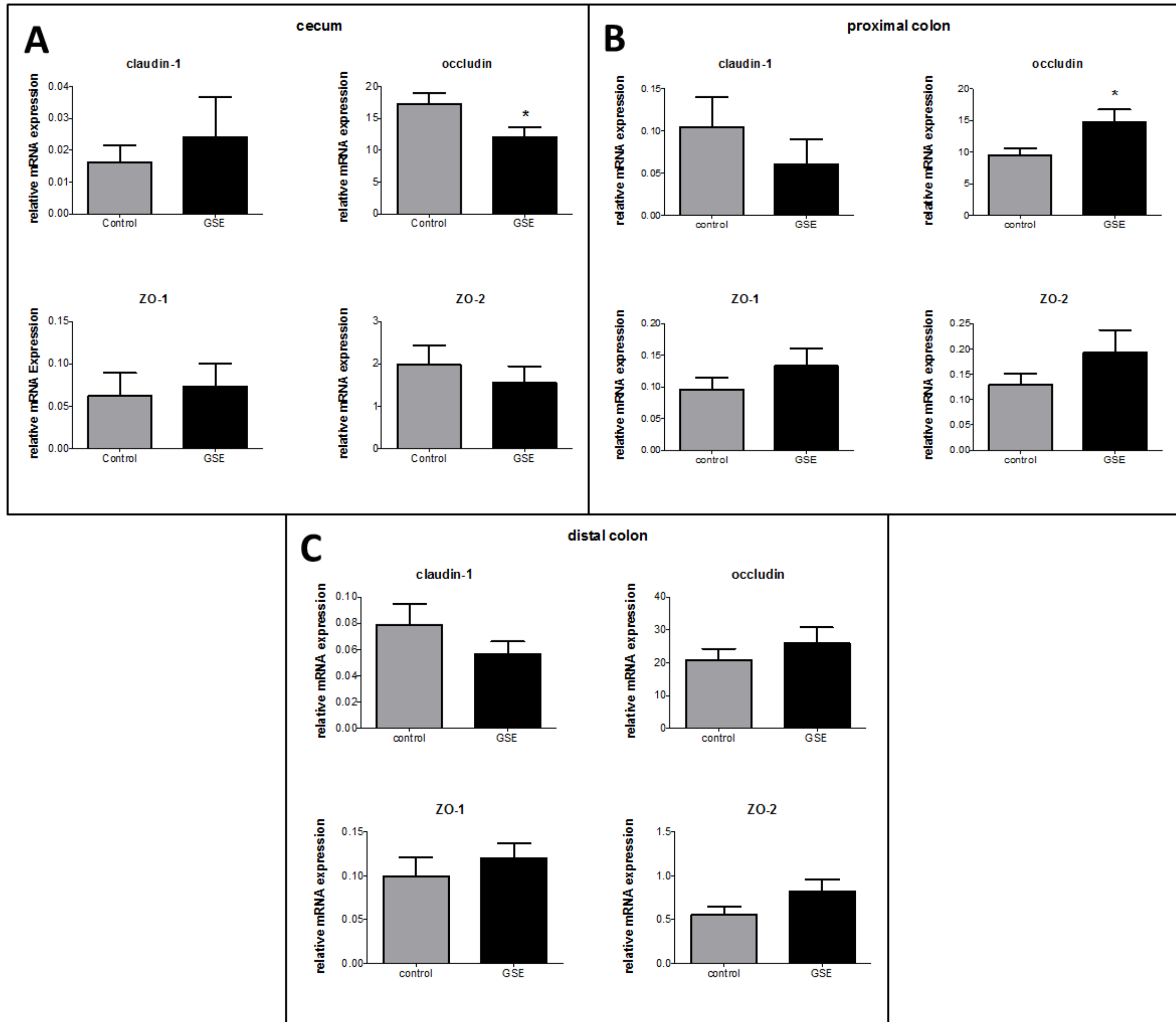

Figure 4.4. *Relative mRNA expression of gut junction proteins in the cecum (**A**), proximal colon (**B**), and distal colon (**C**) of control mice (GSE-free) vs. mice administered 100 mg/kg/d GSE in drinking water (n=8/group). Data are expressed as means ± SEM. mRNA expression was determined by qRT-PCR using TaqMan Universal PCR Master Mix and pre-validated TaqMan® Gene Expression Assay probe set kits for target genes (Applied Biosystems, Inc., Foster City, CA). mRNA levels were normalized to β-actin mRNA. An (*) indicates significantly different from control as determined by an unpaired two-tailed t-test with significance defined as P < 0.05.*

Calprotectin. Finally, fecal calprotectin levels were quantified as a marker of gut neutrophil infiltration (Figure 4.5). GSE significantly reduced fecal calprotectin by greater than 10-fold compared to control (32 ± 9.0 vs. 420 ± 110 pg/mg protein, respectively). This reduction could be due to reduced gut inflammation and subsequent neutrophil recruitment, or reduced gut permeability to neutrophils. Due to the absence of any obesity, inflammation or other gut disease stimulus in the model, we postulate that this difference is attributable to reduced serosal/luminal permeability of the gut to neutrophils and/or calprotectin.

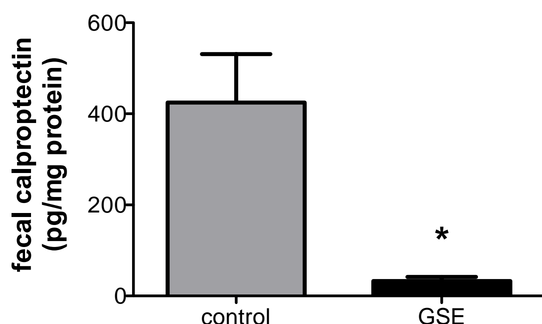

Figure 4.5. *Calprotectin protein levels in feces (the luminal contents of the distal colon) of control mice (GSE-free) vs. mice administered 100 mg/kg/d GSE in drinking water (n=8/group). Data are expressed as means ± SEM. Calprotectin was quantified using rat calprotectin ELISA kits (Mybiosource.com, San Diego, CA). Calprotectin is reported as pg/mg total fecal protein. An (*) indicates significantly different from control as determined by an unpaired two-tailed t-test with significance defined as $P < 0.05$.*

Summary. Based upon these data, we accept our hypothesis that chronic GSE administration can increase colonic expression of intestinal TJPs (particularly occludin) in the absence of an obesity-promoting stimulus, although occludin expression was significantly decreased in the cecum. However, we failed to find evidence that GSE reduces circulating endotoxin levels in the absence of an obesity-promoting stimulus.

Several aspects and limitations of this study warrant mention. First, GSE was removed for 24 h near the end of the study (due to the original study design); this likely muted the observed impact of GSE somewhat. Had this not been performed, observed differences in TJPs, endotoxin, and calprotectin between groups likely would have been even greater. Follow-up studies to confirm these results will maintain GSE exposure throughout the experimental period. Second, this study was conducted without an obesity-promoting stimulus (i.e. HF diet) as this is a secondary analysis of a study originally designed to observe polyphenol metabolism (which

will be reported in a subsequent paper). HF feeding increases gut permeability and serum endotoxin, and would likely result in even greater differences between groups. This is particularly true for the endotoxin data: although the data were inconclusive in this none-obese model, a HF feeding model with elevated fecal and serum endotoxin would likely allow for a negative effect of GSE on endotoxin production or resistance to uptake to be observed. Third, we did not assess gut permeability directly (for example, sugar probe absorption and excretion test). Finally, we employed short-term GSE administration; long-term GSE in obesity models will likely enhance our understanding of the potential impact of GSE on metabolic endotoxemia in at-risk populations.

To our knowledge, this is the first report of the impact of dietary GSE on colonic barrier integrity, fecal calprotectin, gut endotoxin production, and circulating endotoxin levels. Our data suggest that GSE significantly modulates the expression of TJPs in the colon and may also alter luminal-serosal antigen trafficking, particularly for calprotectin. The mechanisms by which these activities occur remain to be investigated, but may include indirect activities of GSE (such as modulation of gut microbial populations and metabolism in the gut lumen) [186, 187] or by direct interaction of adsorbed/absorbed GSE components with the intestinal tissue [125]. Additionally, the active compound(s) in GSE remain to be identified. Procyanidins appear to reduce gut permeability [182], and are therefore likely candidates for active components of GSE. The impact of the degree of polymerization on the activity of monomers/procyanidins warrants future study in order to optimize their impact on TJP expression and permeability.

Based on these preliminary data from this secondary analysis, we hypothesize that the modulation of metabolic endotoxemia through improved gut barrier integrity and reduced permeability may be an additional mechanism by which GSE and other polyphenols prevent metabolic syndrome, in addition to currently known activities (direct inhibition of inflammation, inhibition of lipid digestion, etc.). Further investigation and testing of this hypothesis in a model of impaired gut barrier function and elevated circulating endotoxin is therefore required. To this end, we are currently conducting a follow-up study to examine the long-term dose-dependent effects of GSE on whole-gut permeability (as quantified by oral sugar probe excretion), TJP expression, gut microbiota composition, and fecal/serum endotoxin in a HF feeding metabolic syndrome model in which these parameters are significantly altered from normal values in healthy animals.

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Chapter 5:

Dietary supplementation with cocoa flavanols does not alter colon tissue profiles of native flavanols and their microbial metabolites established during habitual dietary exposure in C57BL/6J mice

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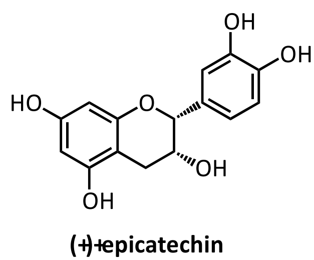
Abstract

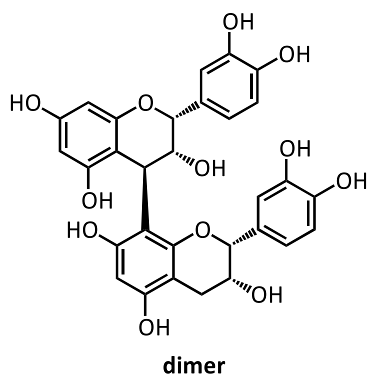
Metabolism of flavanols (catechins, procyanidins) by gut microbiota has been extensively characterized. Comparatively little is known about accumulation of flavanols and their metabolites in the colon tissues, particularly during chronic exposure to low doses. Mice were fed low doses of cocoa flavanols for 12 weeks. Supplementation of the control diet with flavanols did not increase colonic tissue accumulation of flavanols nor microbial metabolites vs. control. The type of cocoa flavanols did not impact colonic tissue accumulation of native flavanols or metabolites. Total phenolic content of the diets indicated that these results are not explained by background levels of undetected phenolics in the control diet. This is the longest known chronic flavanol feeding study to examine colonic tissue accumulation. Vast differences appear to exist between acute high doses and chronic low doses where gut microbiota and epithelium adapt to exposure. Our results indicate that we do not fully understand the fate of flavanols in the colon during chronic exposure.

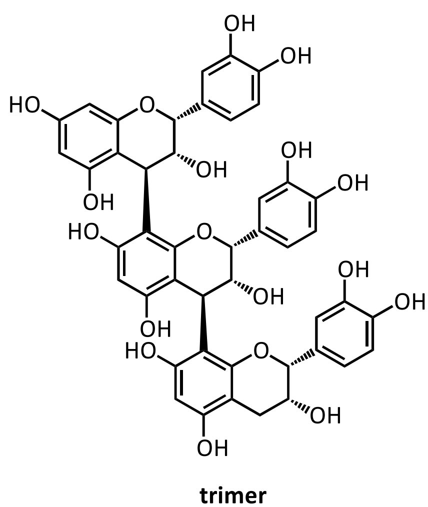
Introduction

Flavanols are a structurally diverse class of compounds comprised of monomeric catechins [(±)-catechin (C), (−)-epicatechin (EC), etc.], and polymers of these monomers known as procyanidins (PCs) (**Figure 5.1**). Cocoa products are one of the most flavanol-rich foods and display a wide range of flavanols[45, 188, 189] with various PC degrees of polymerization (DP).

The overall oral bioavailability of flavanols is generally regarded to be low, particularly for PCs[5, 63, 69, 71]. Therefore, a large portion of an ingested cocoa flavanol dose remains unabsorbed in the small intestine and reaches the colon, where flavanols are extensively degraded by colonic microbiota[9, 11, 71, 74, 135, 190, 191] in the colon lumen. Predominant metabolites in the colon lumen include hydroxyphenylacetic acids, hydroxyphenylpropionic acids, and hydroxybenzoic acids, among other phenolic acid compounds (**Figure 5.2**)[9, 10, 86]. These microbial metabolites are thought to contribute to the observed health-protective activities of ingested flavanols due to their higher relative bioavailability compared to the native flavanols[71, 192], and their demonstrated bioactivities[113, 193].







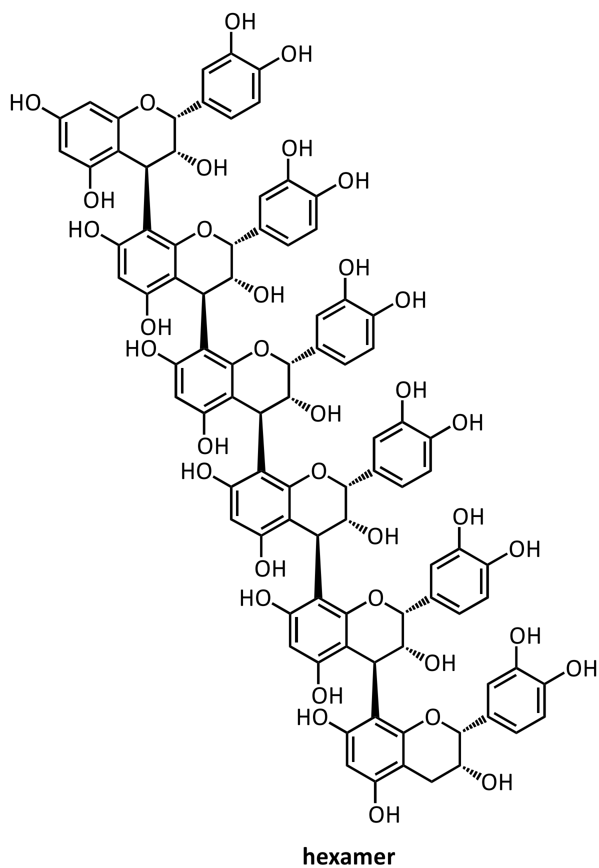


Figure 5.1. *Structures of (−)-epicatechin and a representative (−)-epicatechin dimer, trimer, and hexamer.*

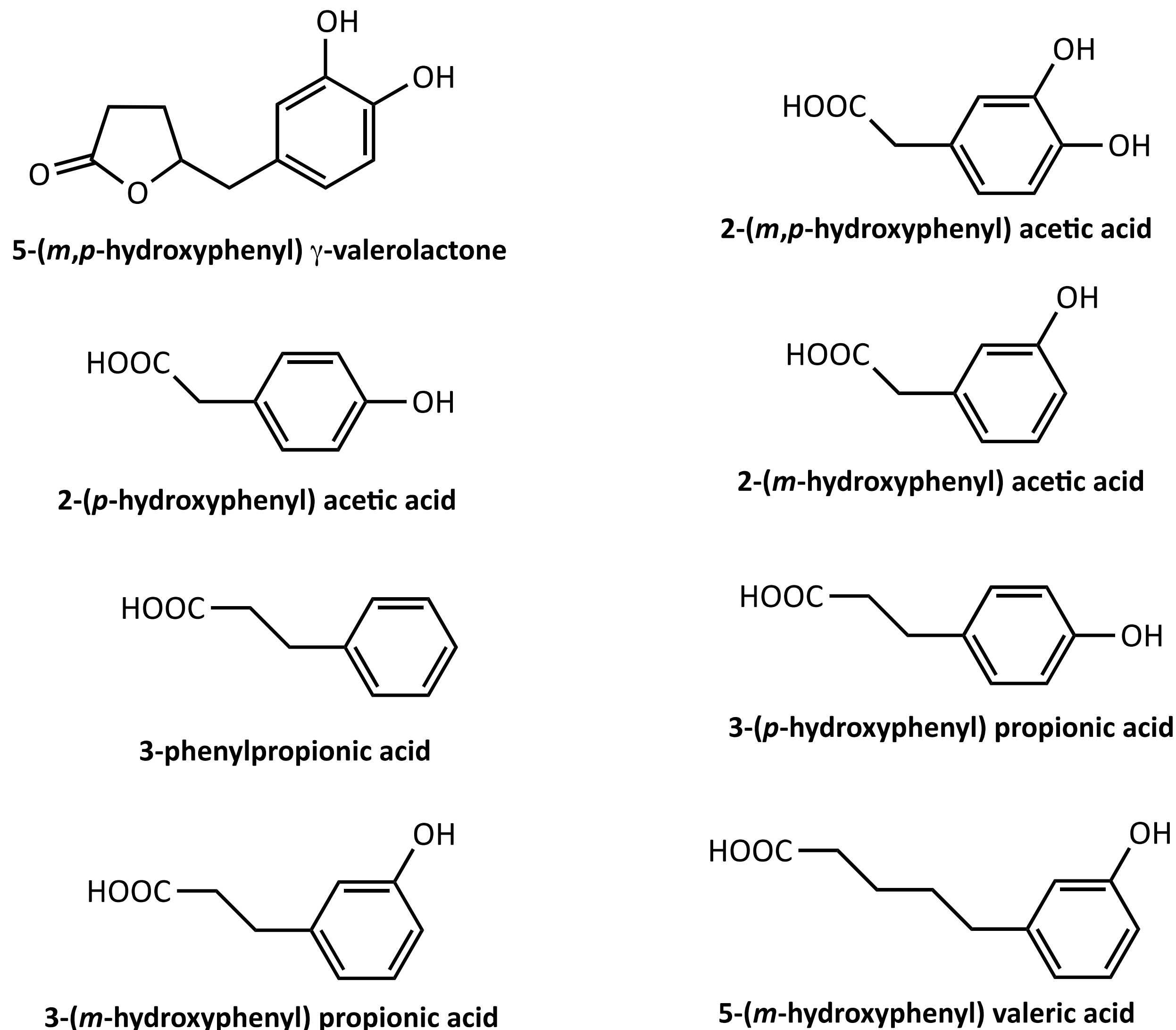


Figure 5.2. *Structures of selected representative metabolites produced by microbial metabolism of native catechins and procyanidins in the colon.*

In addition to systemic activities, native flavanols and their microbial metabolites are thought to act in the colon itself, as both flavanols and their metabolites may be taken up and retained for a period of time in the colonic mucosa. These activities are thought to include anti-inflammatory[113, 124, 193] and anti-carcinogenic[194] activities, improved barrier function[176], reduced permeability to lipopolysaccharide[195, 196], and alterations to the commensal gut flora[197, 198]. Therefore, in addition to determining the bioavailability of flavanols and microbial metabolites, characterizing their local accumulation (i.e. the amount deposited into and retained in the tissue

for a defined period of time) in the colon is a critical step in understanding the health benefits of these compounds.

Despite the critical role that both flavanols and their microbial metabolites appear to play in determining the health benefits of flavanol-rich foods, including activities in the colon itself, comparatively little is known about their accumulation in colonic tissues. Several factors, including the comparative inaccessibility of the colon for sampling and the focus on systemic bioavailability, have contributed to the lack of data on colonic tissue accumulation of these compounds. The majority of studies in animal models have examined availability or accumulation of flavanols and microbial metabolites in the colon following a single acute dose of flavanols[11, 150, 199, 200]; relatively few studies have examined colonic tissue accumulation following habitual dietary exposure[8, 201]. The qualitative and quantitative profiles of accumulated compounds are likely to differ between acute and habitual exposure, as the commensal flora of the colon adjust over time in response to dietary treatments [139, 202], and polyphenols also induce alterations to the commensal flora[141, 142, 197]. Studies of colonic tissue accumulation during habitual dietary exposure are likely more representative of the colonic profile at equilibrium (i.e. when the colonic flora, and their metabolism, have adjusted to flavanol exposure), which are more relevant to health effects of flavanol consumption over extended periods (i.e. human use).

Furthermore, little is known regarding the impact of native dietary flavanol profiles on the resulting colonic tissue accumulation of native flavanols and microbial metabolites. Flavanol monomers and PCs (as well as other classes of flavonoids) appear to be metabolized to a small pool of a few dozen common microbial metabolites[86, 203]. This raises the question of whether the qualitative profile of native flavanols is relevant for modulating the profile of metabolites accumulated in the colon. Very few studies have compared the colonic tissue accumulation of native flavanols and microbial metabolites from distinct dietary profiles of native flavanols[204]; the majority of studies focus on metabolism and accumulation of single compounds or a single flavanol-rich food. Furthermore, the majority of the data on microbial metabolism of flavanols pertains to catechin monomers and small PCs (dimers, trimers, etc.). Very little is known about the metabolism of larger PCs, as these compounds have been studied in matrices that are also rich in smaller compounds (cocoa, apples, GSE, etc.) but have not yet been studied individually. Due to this lack of data, the metabolic fate of large PCs in the gut remains largely unknown.

The objectives of this study were to 1) determine the quantitative and qualitative profile of flavanols and their microbial metabolites accumulated in the colon during habitual exposure to flavanol-rich cocoa extract, and 2) compare profiles accumulated in the colon during habitual exposure to distinct profiles of dietary flavanols (monomer-rich, oligomer-rich, and polymer-rich fractions). We hypothesize that administration of equal amounts of flavanols with distinct qualitative profiles to mice would result in colonic tissue accumulation of distinct qualitative and quantitative profiles of both native flavanols and their microbial metabolites in the colon tissue. Specifically, we hypothesize that 1) administration of cocoa flavanols will increase colonic tissue levels of flavanols and metabolites compared to a low-flavanols control diet, and 2) administration of cocoa oligomers and polymers will results in reduced colonic tissue levels of monomeric flavanols and metabolites compared to cocoa extract or cocoa monomers.

Materials and Methods

Chemicals and Standards. Milli-Q water was prepared using a Millipore Milli-Q Gradient system (Billerica, MA). Ascorbic acid, (+)-catechin hydrate (C), (−)-EC, epigallocatechin [(−)-(EGC)], epicatechin gallate [(−)-ECG], and epigallocatechin gallate [(−)-(EGCG)], *p*-coumaric acid, *m*-coumaric acid, ferulic acid, isoferulic acid, caffeic acid, protocatechuic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, homovanillic acid, gallic acid, 4-hydroxymandelic acid, pyrogallol, catechol, phenylacetic acid, 2-(3'-hydroxyphenyl) acetic acid, 2-(4'-hydroxyphenyl) acetic acid, 3-(3',4'-dihrdroxyphenyl) acetic acid, 3-phenylpropionic acid, 3-(4'-hydroxyphenyl) propionic acid, 5-phenylvaleric acid, phloretin, hippuric acid, 3-(3'-hydroxyphenyl) propionic acid, and 3-(3',4'-dihydroxyphenyl) propionic acid were obtained from Sigma (St. Louis, MO). PC dimer B_2 and trimer C_1 were obtained from ChromaDex (Irvine, CA). Purified PC dimer B_1, dimer B_2-gallate, dimer B_5, trimer T_2, tetramer A_2, and pentamers (DP 5) through decamers (DP 10) were obtained from Planta Analytica (Danbury, CT). Solvents for extraction, fractionation and normal-phase HPLC were ACS grade or better (Fisher, Pittsburg, PA, or VWR, Radnor, PA). Solvents for UPLC-MS/MS were LC-MS grade (VWR).

Cocoa Flavanols. A flavanol-rich cocoa extract (CE) was produced and separated into fractions with distinct flavanol compositions (monomer-, oligomer-, and polymer-rich fractions).

Procedures for extraction, fractionation, and characterization of CE and the fractions were described previously[196]. The levels of total flavanol monomers, dimers, and larger PCs in the CE and cocoa monomer, oligomer, and polymer fractions are presented in **Table 5.1.** The levels of individual compounds are presented in Appendix B (**Appendix B Table B1**). Compared to CE, the monomer-rich fraction is highly enriched for monomers, slightly enriched for dimers, reduced in PCs DP 3-6 and depleted of PCs DP 7-10. The oligomer-rich fraction is highly depleted of monomers, greatly enriched for dimers and PCs DP 3-6, and slightly enriched for DP 7-10. The polymer-rich fraction is highly depleted for monomers, dimers and PCs DP 3-6 and slightly enriched for PCs DP 7-10. It should be noted that these values are from reverse-phase UPLC analysis that only quantified PCs up to DP 10; the composition of larger PCs that make up a large portion of CE and the majority of the polymer fraction (see **Appendix B Figure B1**) are not reflected in the total composition values in **Table 5.1**. The normal-phase HPLC flavanol profiles of CE and cocoa PC fractions are shown in **Appendix B Figure B1**. By comparing relative peak heights and retention times (retention time roughly correlates to degree of polymerization in normal-phase analysis of flavanols) within each chromatogram, the monomer-, oligomer- and polymer-rich fractions were indeed relatively enriched for flavanol monomers, oligomers, and polymers, respectively, compared to CE and each other.

Table 5.1. Levels of native flavanol monomers and procyanidins in cocoa extract and individual cocoa fractions.

Class	**Composition[ab] (mg compound/g dried fraction)**			
	CE[c]	**M[d]**	**O[e]**	**P[f]**
catechin monomers	7.59±0.0806 b	40.2±0.682 c	0.806±0.0129 a	0.0899±0.0146 a
PC dimers	8.48±0.155 b	15.0±0.394 c	24.7±0.267 d	0.347±0.0408 a
PC trimers-hexamers	18.0±0.763 c	10.3±0.422 b	50.4±1.08 d	5.04±0.231 a
PC heptamers-decamers	4.65±0.208 b	0.338±0.153 a	6.83±0.141 b	5.82±1.45 b

[a]Treatments with different letters in the same row are significantly different (*P*<0.05)
[b]Data are reported as mean ± SEM from *n*=3 replicate analyses
[c]Cocoa flavanol extract
[d]Monomer-rich fraction
[e]Oligomer-rich fraction
[f]Polymer-rich fraction

Animals. Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Polytechnic Institute and State University (protocol #13-099-FST). Male C57BL/6J mice (*N*=54, ~25 g) were obtained from Jackson (Bar Harbor, ME).

Following arrival, mice were maintained on standard rodent chow for 2 wk under standard conditions (3 mice/cage, 12 h light/dark cycle, 30-70% relative humidity, 20-26°C) to acclimatize to the vivarium. To reduce coprophagy, bedding was changed twice per wk throughout the study. Mice were provided food and water *ad libitum* unless otherwise specified.

Diets. Mice were assigned to one of five diet/treatment combinations (n=9/group). Animals were fed a basal high-fat diet (60% kcal from fat) from the diet-induced obesity model series (D12492, Research Diets, Inc., New Brunswick, NJ). This a semi-purified diet with minimal polyphenol or phenolic background level that is practically attainable; the composition of this basal diet was reported previously[196] and is presented in **Appendix B Table B4**. The diet was provided alone (control) or supplemented with CE or one of the three cocoa fractions. The diets containing CE or cocoa fractions were formulated (262.53 mg/kg diet) to provide doses of 25 mg/kg body weight (BW)/d based on an estimated daily food consumption of 95.23 g food/kg BW/d for male C57BL/6J mice from previous studies in our lab (data not shown). Diets were maintained at −20 °C throughout the study to prevent degradation of lipids or cocoa compounds. In order to maintain freshness and prevent degradation of fat or cocoa compounds, food was replaced twice per wk for all cages. Animals were maintained on diets for 12 wk. Food intake for each cage was measured twice per wk by weighing food provided and weighing leftover food prior to replacing with fresh food. Food intake did not differ between treatment groups (data not shown)[196].

Animal Procedures. Animal body weights (weekly), body composition (weeks 1, 5, 8, 11), glucose tolerance (week 10) and insulin tolerance (week 11) were measured. These results have been described previously[196].

Euthanasia and Necropsy. At the end of week 12, mice were fasted overnight and euthanized by CO_2 (following AVMA Guidelines on Euthanasia) followed by bilateral pneumothorax. Following euthanasia, the abdominal cavity was opened ventrally and the intestines were removed by cutting through the rectum and gently removing the intestinal tract from the distal end. The colon (between the cecum and rectum) was excised and thoroughly flushed with pre-chilled 1X phosphate-buffered saline (PBS, VWR) and immediately snap-frozen in liquid N_2. Samples were stored at −80°C prior to sample preparation.

Extraction of Flavanols and Microbial Metabolites. Sample preparation and extraction was performed using our previously published method with modifications[201]. Tissue samples were freeze-dried for > 24 h using a FreeZone 1 L freeze dryer (Labconco, Kansas City, MO). Zirconium oxide tissue homogenization beads (~100 mg, 0.5-1 mm, Next Advance, Averill Park, NY) were added to each sample (25-35 mg dry tissue) along with 50 µL Milli-Q water and 50 µL of a 1% (w/v) aqueous ascorbic acid solution. Samples were homogenized in a Bullet Blender (Next Advance, speed 7, 1 min, 4°C). Liquid-liquid extraction was performed by adding 1 mL ethyl acetate and blending in a Bullet Blender (5 min, speed 9, 4°C, Next Advance). Samples were centrifuged (5 min, 17,000 x *g*, room temperature), and the organic supernatant was collected. The extraction was repeated on the resulting pellet, and the supernatants were pooled for each sample and dried under a gentle stream of N_2 (35°C). Following liquid-liquid extraction, solid-phase extraction (SPE) was performed on the remaining pellet. An extraction solution (1 mL acetone:water:glacial acetic acid, 70:28:2 v/v/v) was added to each sample along with 100 µL 4% aqueous phosphoric acid. Samples were homogenized in a Bullet Blender (5 min, speed 9, 4°C), centrifuged (5 min, 17,000 x *g*, room temperature), and the supernatant was collected. The extraction was repeated and the supernatants were pooled for each sample. The pooled extracts were diluted with 12 mL water. Oasis HLB SPE cartridges (1 cc, 30 mg sorbent, Waters, Milford, MA) were preconditioned with 1 mL methanol (MeOH) followed by 1 mL water. The diluted samples (2 mL pooled extracts + 12 mL water) were loaded onto the cartridges using a vacuum manifold. Cartridges were washed sequentially with 1 mL acidified water (0.1% formic acid, v/v) followed by 0.5 mL 5% (v/v) aqueous MeOH containing 0.1% formic acid (v/v). Cartridges were eluted sequentially with 2 mL acidified MeOH (0.1% formic acid, v/v) followed by 2 mL acetone:water:glacial acetic acid (70:28:2) and collected into the tubes containing the dried ethyl acetate extracts from the same sample. Samples were dried under vacuum in a Speed-Vac (45°C) to remove acetone and MeOH, then frozen on dry ice and freeze-dried > 24 h to remove water and acetic acid. Dried samples were resolubilized in 1 mL 0.1% formic acid in water:0.1% formic acid in acetonitrile (ACN) (95:5 v/v), sonicated in ice water (25 min), and then filtered into certified LC-MS vials (Waters) using a Samplicity filtration system (0.2 µm PTFE Philic Millex Samplicity filters, Millipore). Filtered samples were

analyzed immediately. The extraction recovery of native flavanols and metabolites by this method was validated in our previous method paper[201].

For extraction of the diet, 3 g samples of each diet were weighed in triplicate and defatted using hexane. Samples were vortexed with 20 mL hexane, sonicated on ice (10 min), centrifuged (5 min, 3500 x *g*, room temperature), the supernatant was discarded, and the procedure was repeated. An extraction solution (10 mL acetone:water:glacial acetic acid, 70:28:2 v/v/v) was added to each sample. Samples were then vortexed for 30 seconds then sonicated on ice for 10 min. Samples were centrifuged (5 min, 3500 x *g*, room temperature) and the supernatant was collected. The extraction was repeated. The supernatants were pooled for each sample and 2 mL was diluted with 12 mL water. Oasis HLB SPE cartridges (1 cc, 30 mg sorbent, Waters) were preconditioned as described above. The diluted samples (2 mL pooled extracts + 12 mL water) were loaded onto the cartridges using a vacuum manifold. Cartridges were washed and eluted as described above into tubes containing the dried ethyl acetate extracts from the same sample. Samples were dried under vacuum in a Speed-Vac (45°C) to remove acetone and methanol, then frozen on dry ice and freeze-dried > 24 h to remove water and acetic acid. Dried samples were resolubilized, sonicated, and filtered into LC-MS vials as described above. Filtered samples were analyzed immediately.

UPLC-MS/MS Analysis of Flavanols and Microbial Metabolites. UPLC-MS/MS analysis was performed using our previously published method with modifications[201]. Details of the methodology are found in **Appendix B**.

Folin-Ciocalteu Assay. To extract phenolics from the diet, samples of each diet were weighed in triplicate defatted twice, and extracted twice as described above. For the Folin assay, 200 µL of each sample was combined with 2.5 mL 0.2 N Folin-Ciocalteau reagent. Then, 2.0 mL saturated aqueous Na_2CO_3 was added to each sample, and samples were incubated at room temperature for 2 h. The absorbance was then read at 765 nm. The standard curve was prepared using gallic acid, and total phenolics were calculated as mg gallic acid equivalents (GAE)/g of material

Data and Statistical Analysis. Outliers were identified and removed using Dixon's Q-test at the α=0.05 level if necessary. Data were analyzed by one-way ANOVA to determine overall

significance of treatment. If significance was detected, Tukey's HSD post-hoc test was then performed to compare all treatment means. Significance was defined as $P<0.05$.

Results and Discussion

Dietary Levels of Native Flavanols. Levels of native cocoa catechins and PCs in the diet are presented in **Appendix B Figure B2.** UPLC-MS/MS analysis of the diets showed that the profile of native flavanols in the diet matched those of the pure extracts. As seen in **Appendix B Figure B2A**, the amount of total native flavanols (catechins and PCs) in the control diet is negligible, including an undetectable level of oligomers. This demonstrates that there is essentially no background contribution of catechins or PCs (that can be measured by our UPLC-MS/MS method, i.e. DP 1-10) in the basal control diet, and that the only source of catechins and PCs in each diet is the CE or the cocoa fraction added to the basal diet. **Appendix B Figure B2B-D** show that the monomer- and oligomer-supplemented diets were enriched with monomeric and oligomeric flavanols, respectively, at levels that are significantly higher than those in the other fractions. Overall, the total amount of native flavanols that can be measured by our UPLC-MS/MS method (DP 1-10) found in the polymer diet were lower than in the diets supplemented with CE, monomers or oligomers the other fractions, as seen in **Appendix B Figure B2A**. This is due to the fact that the larger PC compounds (DP > 10) that make up a large portion of the polymer fraction (see **Appendix B Figure B1**) are not resolved nor detected with this method as previously shown. Therefore the measured levels are not reflective of all compounds present in the polymer fraction, but rather those that can be accurately measured using the present UPLC-MS/MS method. These data demonstrate that the vast majority of native flavanols in the diet are from the cocoa fractions and the basal diet itself.

Dietary Levels of Metabolites. Background levels of metabolite compounds (compounds that we identified as microbial metabolites of catechins and PCs but which were present in the diets before being administrated to mice) present in the diets are shown in **Appendix B Figure B3**. There was a significant concentration of these compounds in the basal control diet (1.13 ± 0.0273 mg/g, **Appendix B Figure B3A**); supplementation of the basal diet with CE or cocoa fractions did not significantly alter the levels present in the diet (1.23 ± 0.0402 - 1.71 ± 0.292 mg/g). The majority of the metabolites present in the diets are compounds other than the

phenylalkyl and benzoic acid derivatives (**Appendix B Figure B3D**), such as ferulic acid, etc. These compounds are common in plant materials[151], and are likely introduced from plant-based ingredients in the semi-purified diet (**Appendix B Table B4**). There were somewhat higher (but not statistically different) levels of phenylalkyl and benzoic acid derivatives in the monomer-supplemented diet (**Appendix B Figure B4B-C**). This is potentially due to fractionation, which may cause phenylalkyl and benzoic acids occurring in cocoa to elute with the monomer fraction during SPE. It should be noted that we have previously observed appreciable levels of these metabolites in intestines of rats fed a semi-purified basal control diet[201]; these compounds appear to be unavoidable background components in plant-based rodent diets. Overall, these data show that there is no significant contribution of metabolite compounds by the CE or cocoa fractions above levels present in the basal diet alone and that levels of metabolites present in the diets are similar across treatment groups.

Based on analysis of the diets, differences in the levels of native flavanols (catechins and PCs) and their microbial metabolites in the colon of mice fed these diets would be expected to be due solely to the composition of the CE or fraction with which each diet was supplemented.

Colon Tissue Levels of Native Flavanols. The levels of native flavanols detected in mouse colons are shown in **Figure 5.3**. Supplementation of the basal control diet with flavanols did not increase the colonic tissue accumulation of these compounds compared to the basal control diet alone. While tissue accumulation levels of native flavanols were extremely low, the chromatograms shown in **Figure 5.4A** demonstrate that the peaks are large, accurately measurable peaks, as opposed to very small peaks and/or baseline noise. Furthermore, quantified peaks were found to be above the signal-to-noise ratio limit of 10:1 for lower limit of quantification. These data suggest that there was very little tissue accumulation of native flavanols in the colon during the 12 wk feeding period, but tissue accumulation was measurable. Our data differ from the results of previous studies of colonic tissue accumulation that have used much higher doses (125-1000 mg/kg) and found significant colonic tissue accumulation of flavanols[8, 11, 201]. This finding could be due to the comparatively low dose (25 mg/kg) or the 12 h fast before euthanasia. Both of those factors contribute to the accumulation and retention of these native flavanols in colonic tissues. However, the 12 h fast alone would not result in significant depletion of ingested native flavanols and their metabolites, partly due to the gastrointestinal

transit time required to reach the colon [a portion of food ingested in the hours prior to fasting would still be in the lower GI tract at 12 h, as reflected by the fact that ~½ of the mouse colons contained fecal matter at the time of sacrifice in the present study (data not shown)]. Furthermore, acute studies have shown appreciable levels of native flavanols and their metabolites in the colon at from 8-18 h post-administration of flavanols[11, 150, 201], albeit with much higher doses administered.

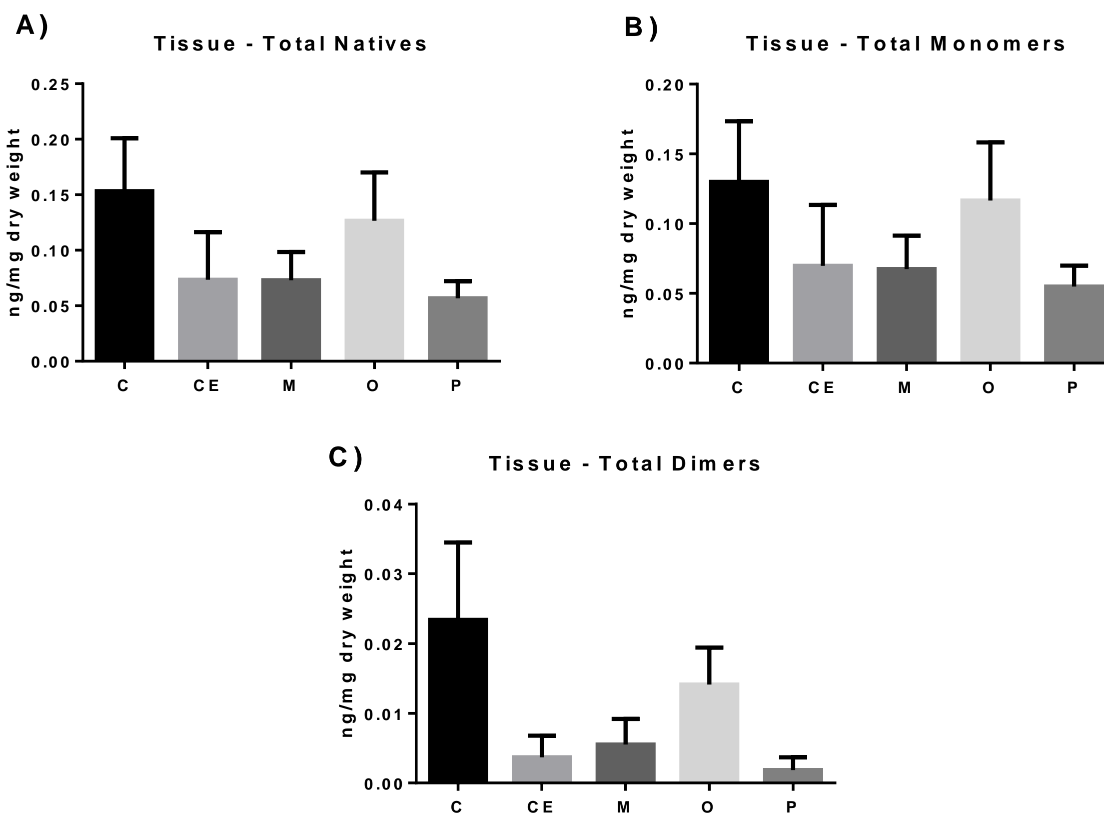


Figure 5.3. *Tissue levels of total native compounds (A), total monomers (B), and total dimers (C) in mouse colon tissue following 12-week exposure to the experimental diets. Mice were fasted 12 h prior to euthanasia and collection of colon tissue. Data are presented as mean ± SEM from n=9 animals. No significant differences were detected between treatments for total native compounds, total monomers, or total dimers (one-way ANOVA with Tukey's HSD post-hoc test, significance defined as P<0.05). Treatments: mice fed basal control diet (C), mice fed control diet supplemented with cocoa extract (CE), mice fed control diet supplemented with cocoa monomers (M), mice fed control diet supplemented with cocoa oligomers (O), and mice fed control diet supplemented with cocoa polymers (P).*

The finding that chronic feeding of flavanols did not elevate levels of intact flavanols in the colon was completely unexpected, due to the fact that the basal diet had roughly 10^5- to 10^6-

fold lower levels of native flavanols than any of the cocoa-supplemented diets (**Appendix B Figure B2A**). One possible explanation for this is that the 12 wk feeding of the cocoa-supplemented diet may have stimulated the gut microbiota in these animals to adapt and become extremely efficient at metabolizing native flavanols; the gut microbiota in animals fed the basal control diet would have had little stimulus to increase their metabolism of the native flavanols. The net effect of 12 wk feeding of flavanols would be to accumulate less of the native flavanols in the colon, despite higher dietary levels. Another possibility is that chronic exposure to flavanols could have unregulated detoxification pathways in the gut epithelium (Phase-I/II enzymes and apical efflux transporters such as Multidrug-resistant proteins (MRPs) and P-glycoprotein (Pgp)][205, 206]. Similar to the first possibility, the gut epithelium in animals fed a semi-purified (and largely polyphenol-free) diet would have had little stimulus to increase their metabolism of the native flavanols. Phase-I/II metabolites were not analyzed in the present study due to the sheer number of compounds measured. Provocative data have shown that chronic dosing of grape flavanols for 10 d upregulates both Phase-II metabolism of catechins and depolymerization of procyanidins (likely by the gut microbiota) from an acute dose compared to an acute dose without prior chronic dosing, although this study reported only blood levels[207]. This suggests that both mechanisms may be responsible for our data. Future studies could employ various strategies to quantify Phase-I/II metabolites of flavanols[76]. For example, parallel analyses could be performed on the same tissue, with one sample first incubated in β-glucuronidase/sulfatase prior to extraction and the other sample extracted without enzymatic deconjugation, with the difference in measured native flavanols between the two assays representing the extent of glucuronidation and sulfation. Assessment of *O*-methylated metabolites is more challenging, as enzymatic deconjugation is not feasible and *O*-methyl derivatives must be measured directly, doubling or tripling the number of compounds to monitor.

Interestingly, no PCs larger than trimers (DP 3) were detected above the lower limit of detection (LLOD) in the colon tissue, despite the fact that these larger compounds made up significant portions of the oligomer- and polymer-supplemented diets (**Table 5.1**, **Appendix B Figure B1, Figure B2D**) and were easily detectable using our UPLC-MS/MS method. Therefore, it appears that larger PCs larger are particularly impacted by alterations to colonic metabolism occurring during chronic flavanol exposure.

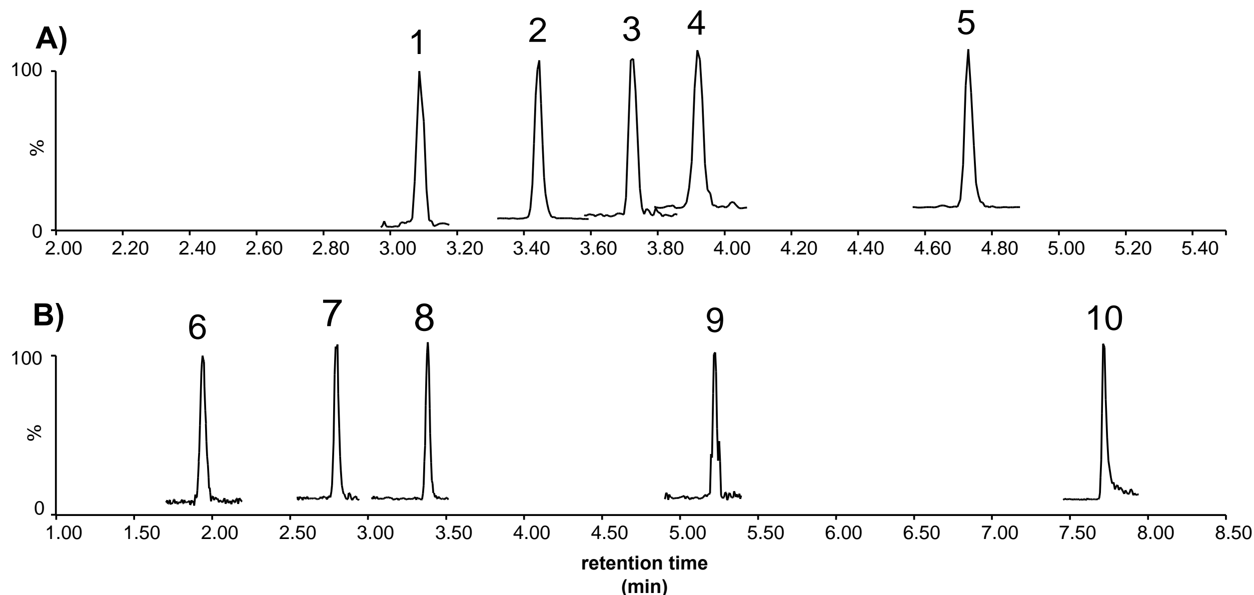


Figure 5.4. *Representative UPLC-MS/MS multi-reaction monitoring (MRM) chromatograms of detected native flavanols (A) and microbial metabolites (B) in the colon tissue of a mouse fed a basal diet supplemented with cocoa oligomers for 12 weeks. All chromatograms are normalized to equal height (100% relative intensity). All chromatograms are from the same animal. Compound identities: 1) catechin 2) PC B_2 3) (−)-epicatechin 4) PC C_1 5) PC B_5 6) 3,4-dihydroxybenzoic acid 7) 4-hydroxybenzoic acid 8) 3-hydroxybenzoic acid 9) phenylacetic acid 10) unknown metabolite [an isomer of 5-(3',4'-dihydroxyphenyl)valeric acid].*

Colon Tissue Levels of Metabolites. The levels of microbial metabolites detected in mouse colons are shown in **Figure 5.5**. Similar to the native flavanols, the UPLC-MS/MS peaks for the predominant metabolites (**Figure 5.4B**) were sizeable and not merely "noise", confirming the accuracy and reliability of these data. Mean tissue accumulation levels of microbial metabolites were notably greater than accumulation of native flavanols (13.9-15.2 vs. 0.0568-0.153 ng/mg tissue, respectively). However, there were no statistically significant differences in the accumulated levels of total metabolites, or in any sub-class of metabolites (**Figure 5.5B-D**), between treatments. Considering only the four treatments supplemented with CE or cocoa fractions, the data suggest that no matter the source or size (DP) of flavanols, they are all metabolized into similar qualitative and quantitative profiles of compounds during chronic exposure. The findings that the levels of microbial metabolites were not elevated in the flavanol-supplemented diets compared to control were quite surprising, given the differences in dietary levels of flavanols between the diets (**Appendix B Figure B2**). Our hypothesis that extensive microbial metabolism of native flavanols potentially led to their low tissue levels (**Figure 5.3**) would suggest that extremely high levels of the resulting metabolites should be found in the colon of flavanol-fed mice compared to basal control; clearly this was not the case. Therefore,

the reason for the similarity in colonic levels of native flavanols and microbial metabolites between basal control and flavanol-fed mice remains unclear.

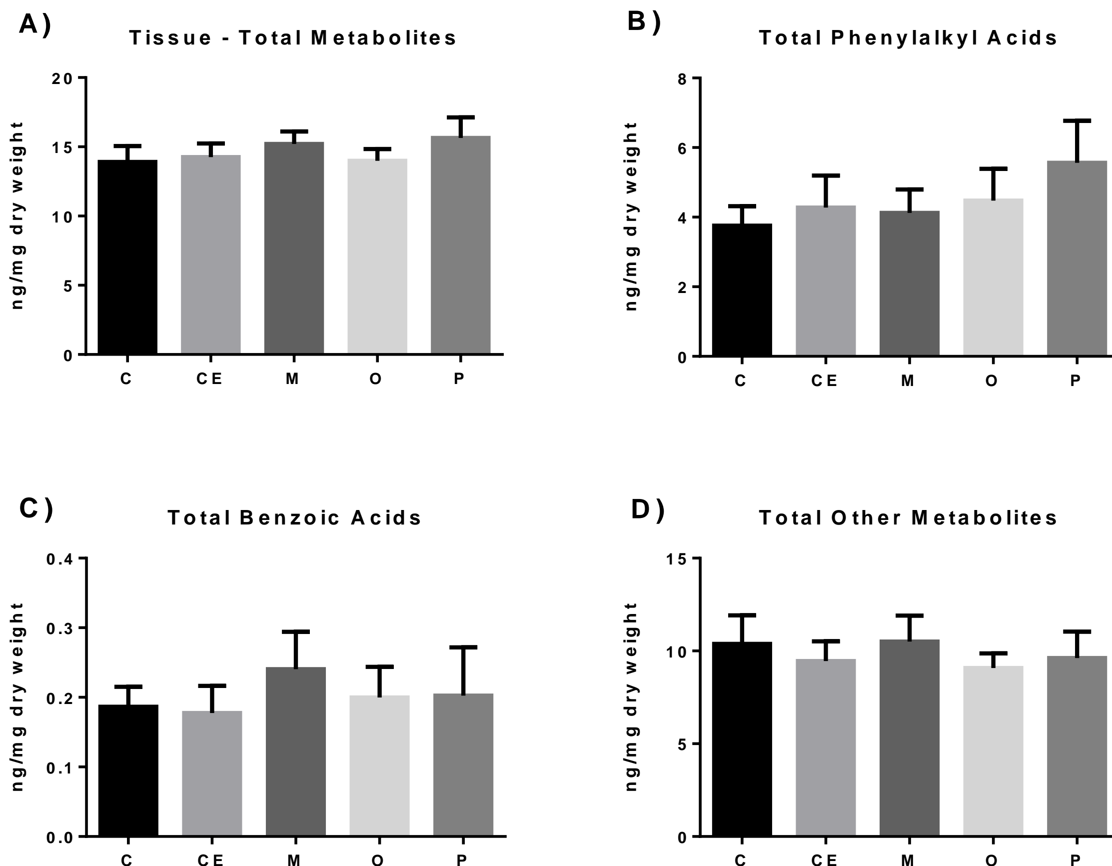


Figure 5.5. *Tissue levels of total metabolites (A), total phenylalkyl (phenylacetic, phenylpropionic, and phenylvaleric) acid derivatives, (B), total benzoic acid derivatives (C), and total other metabolites (D) in mouse colon tissue following 12-week exposure to the experimental diets. Mice were fasted 12 h prior to euthanasia and collection of colon tissue. Data are presented as mean ± SEM from n=9 animals. No significant differences were detected between treatments for total native compounds, total monomers, or total dimers (one-way ANOVA with Tukey's HSD post-hoc test, significance defined as P<0.05). Treatments: mice fed basal control diet (C), mice fed control diet supplemented with cocoa extract (CE), mice fed control diet supplemented with cocoa monomers (M), mice fed control diet supplemented with cocoa oligomers (O), and mice fed control diet.*

Folin-Ciocalteu Assay. The surprising finding that total levels of accumulated metabolites were similar for the basal control diet and diets supplemented with cocoa flavanols suggested the possibility that the basal diet contained other phenolics that were not detected by the UPLC-MS/MS assay. UPLC-MS/MS was specific for common catechin monomers and B-type PCs; the

presence of other phenolics in the diets that could be metabolized to the few dozen common metabolites could explain the data in **Figure 5.5**. Therefore, the Folin-Ciocalteu assay was performed on the diets to determine the total concentration of phenolics that could have been metabolized to the common metabolites measured by UPLC-MS/MS. As shown in **Appendix B Figure B4**, the concentrations of total phenolics in all diets supplemented with CE or the cocoa fractions were significantly higher than that of the basal control diet. These data are in agreement with UPLC-MS/MS data showing that supplementation of the basal control diet with cocoa flavanols significantly increased the phenolic content of the diet, and the available substrates for microbial metabolism. However, the magnitude of the differences in levels of phenolic compounds between basal control vs. supplemented diets differed greatly for the two assays (~2.2-fold for the Folin assay vs. ~10^5- to 10^6-fold for the UPLC-MS/MS assay). It should be noted that the Folin assay more accurately represents the total antioxidant activity, including non-phenolic constituents[208]). Nevertheless, both measures confirm significantly greater levels of phenolics available for microbial metabolism in flavanol-supplemented diets compared to control, but these differences are not reflected in tissue accumulation of microbial metabolites in the colon tissue (**Figure 5.5**). Therefore, similarities in colon metabolite levels between control and flavanol-supplemented mice are not due to large levels of undetected phenolics in the background diet but rather due to colonic metabolism and clearance of native flavanols in flavanol-supplemented mice. Lignins are a potential source of the same metabolites that are produced by metabolism of flavanols, but which may not have been accounted for by the Folin assay. Lignins are found in virtually all plant materials used for animal feeds[209], as well as cacao beans[210]. However, lignins in the diets used for the present study would likely be almost exclusively from the basal control diet, as lignin would not be appreciably extracted from the cocoa (efficient lignin extraction generally requires high heat, whereas our extractions were performed at room temperature)[211-213]. These compounds were not measured by our specific UPLC-MS/MS method, and therefore would not be accounted for in our analysis of native flavanols. It is unknown if the levels of lignins present in the basal control diet contributed to the measured Folin values. Lignins have Folin activity[214, 215], but it is inversely proportion to lignin DP[216]. Therefore, large lignins would have contributed little to observed Folin values. Anaerobic microbial metabolism of lignins results in formation of several phenolic metabolites also formed by metabolism of flavanols[217], including phenylacetic acid, benzoic acid, 3-phenylpropionic acid,

acid, vanillic acid, ferulic acid, caffeic acid, and catechol. Therefore, the presence of high levels of large lignins in the basal control diet could partly explain why this diet produced similar levels of phenolic metabolites compared to flavanol-supplemented diets (assuming that supplementation with flavanols did not significantly increase the level of precursor compounds above that present in the basal diet).

One limitation of the present study is that the mice were fed a high-fat, low carbohydrate diet (60% and 20% kcal from fat and carbohydrates, respectively, **Appendix B Table B4**)[196]. It is unknown how the high-fat diet impacted gut microbiota composition and subsequent metabolism and tissue accumulation of flavanols. Future work is required to examine the colon tissue accumulation during low-fat (~10% kcal from fat) feeding). However, the present data are relevant, as there is interest in using cocoa flavanols to combat obesity and type-2 diabetes[195, 196], which typically occur in the context of a high-fat diet.

To the best of our knowledge, the present study represents the longest known chronic flavanol feeding study for which colonic tissue accumulation data are reported. Although colonic tissue accumulation levels may be transient (compounds present in the tissue may be metabolized inside cells and/or effluxed into circulation or back into the lumen over time), these levels represent the typical profile of flavanols and their metabolites to which the colon tissue is exposed during chronic flavanol intake.

Our findings, and those of Wang *et al*.[207], highlight the pronounced differences observed between single acute doses in animals not previously exposed to flavanols[11] and chronic low doses where the gut microbiota and epithelium adapt to continuous exposure. The results from the present study indicate that we do not yet fully understand the fate of ingested flavanols in the colon during chronic dietary exposure to these compounds. There have been comparatively few studies that characterize the fate of flavanols during long-term chronic exposure to these compounds. Our data demonstrate that these factors (time and dose) warrant further consideration in future studies comparing various doses and various exposure times. The rationale for these studies is that chronic, low-dose exposure to flavanols is more relevant to human health than high-dose, acute exposure in animals not previously exposed to flavanols.

Additionally, due to the unexpected findings of this study, little is still known regarding the metabolism of larger PCs. We hoped that feeding oligomeric and polymeric PCs would provide data regarding their metabolism. It is known that PC dimers can be metabolized by

microbiota to dimeric ring-opening intermediates without depolymerization[191]. This key discovery suggests that larger PCs may also be incrementally metabolized from the ends inward, and are not necessarily depolymerized prior to ring-opening and other reactions. However, it is still unknown how the dimeric intermediates are further metabolized. Potential metabolic fates of larger PCs include formation of similar intermediates by ring opening (trimers → trimeric intermediates, tetramers → tetrameric intermediates, etc.), depolymerization to smaller PCs and monomers (this has been shown to be minimal in the stomach and small intestine, but occurrence in the colon remains unknown) prior to further metabolism[207]; alternatively, these larger PCs compounds may remain largely intact and unmetabolized.

Therefore, studies are warranted to compare colonic metabolism and tissue accumulation of flavanols, as well as blood pharmacokinetics[207], during acute vs. chronic exposure. Such studies should compare the quantitative and qualitative profiles of native flavanols and their microbial metabolites in the colon at relevant time points of dietary flavanol exposure. Relevant time points for such studies would likely be following a single acute dose without prior exposure, 1 week chronic exposure, 1 month chronic exposure, and so forth. Such studies are necessary in order to generate data relevant to human use, where flavanols are typically ingested throughout the lifespan from foods as well as dietary supplements. Additionally, the correlation between colonic tissue accumulation profiles and colonic activity resulting from distinct dietary flavanol profiles warrants further consideration. We have previously shown that distinct flavanol profiles result in distinct biological activities, but the impact of small vs. large PCs on colon health remains poorly understood. This study suggests that differences in colon tissue activities would not be predicted by differences in accumulation profiles.

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Chapter 6:

Pan-colonic pharmacokinetics of catechins and procyanidins in male Sprague Dawley rats

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Pan-colonic pharmacokinetics of catechins and procyanidins in male Sprague Dawley rats,
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Abstract

Poor absorption and bioavailability of procyanidins from the upper gastrointestinal tract results in the majority of the dose reaching the colon. During colonic transit, progressive microbial metabolism likely produces gradients of procyanidins and microbial metabolites along the length of the colon, suggesting that proximal and distal regions are exposed to different profiles of procyanidins and metabolites. However, previous studies have largely treated the colon as a single organ or looked at fecal profiles, and differences in the profiles of native and metabolite compounds between regions have not been observed. The metabolism kinetics of procyanidins larger than trimers, and formation of metabolites, in the colon has not been well characterized. Therefore, the objective of this study was to determine the kinetics of delivery and microbial metabolism of monomeric, dimeric and oligomeric procyanidins in the cecum and proximal, mid, and distal colon. Sprague Dawley rats were gavaged grape seed extract and sacrificed over 18 h. Analysis of luminal contents showed distinct native and metabolite profiles for each region. Procyanidins had maximum concentrations at approximately 3 h post gavage for all sections. Metabolites reached maximum concentrations from 3-18 h post gavage. The appearance of metabolites was highly dependent on species: larger metabolites were found at earlier times in the more proximal segments, and smaller metabolites found at later times in more distal regions. This study allowed for the observation of regional in the lower gastrointestinal tract, giving insight into the distribution and delivery of procyanidins and their microbial metabolites throughout the colon.

Introduction

Procyanidins (PCs) are dimers, oligomers, and polymers of the flavan-3-ol monomers (±)-catechin (C), (−)-epicatechin (EC), and (−)-epicatechin gallate (ECG) [1-3] (**Figure 6.1A**). Major dietary sources of PCs include grapes, apples, cocoa and berries [4, 28, 29]. There is significant interest in the potential therapeutic and health protective activities of PCs. These activities are currently thought to include prevention or amelioration of cancer, inflammation, obesity, diabetes [22], cardiovascular disease, and improvement of vascular function.

Bioavailability and target tissue delivery are major limiting factors for the potential bioactivity of PCs *in vivo*. The systemic (blood) bioavailability of intact PCs resulting from intestinal absorption is relatively low (0.3-4% of dose), particularly for trimers and larger species [11, 26, 63, 71, 133, 134]. Previous research has shown that bioavailability decreases as the degree of polymerization increases, with monomers being the most readily bioavailable species and bioavailability drops to close to zero for trimers and larger [11, 26, 63, 134]. Therefore, the majority of the ingested PC dose remains unabsorbed and reaches the colon. The colon harbors an abundant and complex microbial ecology (10^9-10^{12} bacterial cells/g luminal contents) comprised primarily of anaerobes from the *Bacteroidetes* and *Furmicutes* groups [79-81]. Ingested PCs that reach the colon are extensively degraded by colonic microbiota to small PCs, flavan-3-ol monomers, γ-valerolactones, phenylalkyl acids, etc. (**Figure 6.1B**) [9, 11, 74, 87, 135, 136]. Predominant PC metabolites in the colon lumen include hydroxyphenylacetic acids, hydroxyphenylpropionic acids, and hydroxybenzoic acids among other phenolic acid compounds [9, 10, 86].

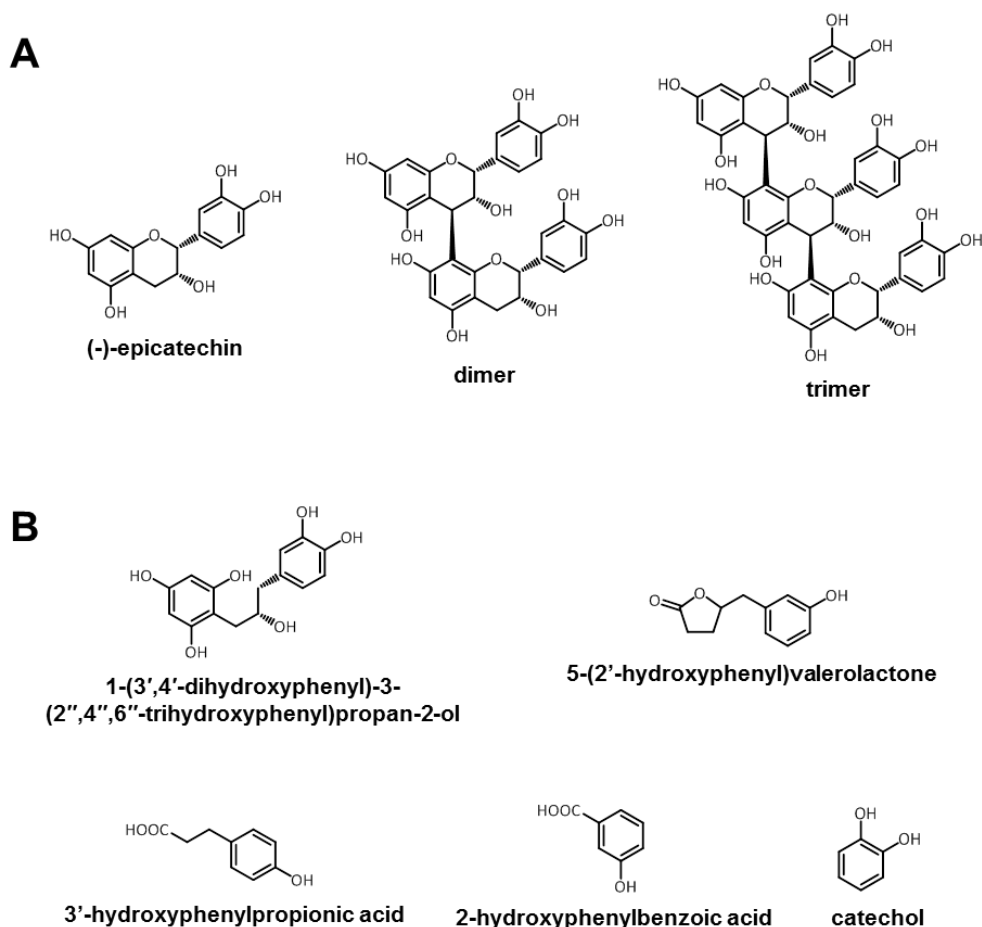

Figure 6.1. *Structures of select native procyanidins (A) and select microbial metabolites (B).*

Due to progressive bacterial metabolism of the native PCs present in the proximal colon, gradients of native PCs and their microbial metabolites are likely established along the length of the colon [11, 12]. Additionally, sequential metabolism in the colon means that proximal and distal colon regions are likely exposed to distinct profiles of active compounds. However, previous studies have either examined the colon as a single organ or examined only fecal profiles, and therefore distinct profiles potentially occurring in various regions are not observed. Further data are required regarding the delivery and efficacy of dietary PCs in different regions of the colon. There is need for an innovative experimental approach that views the colon as a series of discrete segments exposed to distinct profiles of native PCs and their bacterial metabolites. This represents a fundamental shift in approach from previous studies, resulting in the first *in vivo* characterization of the delivery and colonic bacterial metabolism of PCs in distinct regions of the colon.

Recently, we performed a pilot study to develop a high-throughput UPLC-MS/MS method for analyzing PCs and their bacterial metabolites [218], as similar methods for analyzing these compounds were inefficient and limited in both the quantity and the diversity of compounds analyzed [13, 126, 143]. The method that was developed is capable of simultaneously detecting 17 native catechin monomers and PCs, as well as 34 microbial metabolites [218], an improvement upon previous methods that only measured 5-21 native PCs and their metabolites [74, 219-221]. We employed this method to measure PC and metabolite profiles in cecal, proximal colonic and distal colonic tissue and luminal contents in animals fed grape seed extract (GSE)[218]. This analysis showed that there are differences in PC and metabolite levels in separate regions of the lower GI tract. This study was limited, though, by the fact that all animals were sacrificed at one time point post-gavage (8h). While this "snapshot" of data gave some insight to the profiles of the compounds of interest, it was determined that a further experiment was needed to show the profiles over a time by collecting samples at intermittent time points throughout the study.

The objective of this study was therefore to determine the kinetics (concentration as a function of time) of both delivery and subsequent microbial metabolism of native GSE catechin monomers and PCs in the lumen of the cecum and proximal, mid, and distal colon. Our working hypotheses were threefold. First, we hypothesized that the maximal concentrations for native compounds would be highest in the proximal colon due to progressive microbial metabolism of these compounds in the proximal ⟶ distal direction. Second, we hypothesized that the maximal concentrations of microbial metabolites would be highest in the distal colon for the same reason. Finally, we hypothesize that the maximal concentrations for larger PCs (trimers and lager) would be greater in the mid colon compared to that of monomers and dimers due to slower microbial metabolism of the larger compounds. To achieve this objective, we performed an animal experiment to study the kinetics of delivery and PC metabolism in the proximal, mid and distal colon.

Materials and Methods

Chemicals and Standards. Vitaflavan® GSE was purchased from DRT Nutraceutics (Dax, France). Vitaflavan is a water-soluble extract of white *Vitis vinifera* seeds. Manufacturer specifications indicate that Vitaflavan® contains >96% polyphenols by weight, and >75% catechin monomers and procyanidins by weight. Specifically, Vitaflavan® contains 24% w/w

monomeric procyanidins, 42% w/w dimeric/trimeric procyanidins, and 10% w/w larger procyanidins (tetramers, pentamers, etc.). LC-MS grade solvents (water, acetonitrile, and formic acid) and ACS grade solvents (ethyl acetate, acetone, and methanol) were obtained from VWR International (Radnor, PA). Milli-Q water was prepared using a Millipore Milli-Q Gradient system (Billerica, MA). Ascorbic acid, phosphoric acid, (+)-catechin hydrate, (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin gallate (EGCG), *p*-coumaric acid, ferulic acid, isoferulic acid, caffeic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, homovanillic acid, gallic acid, pyrogallol, catechol, phenylacetic acid, 2-(4'-hydroxyphenyl) acetic acid, 3-(3',4'-dihrdroxyphenyl) acetic acid, 5-phenylvaleric acid, hippuric acid, 3-(3'-hydroxyphenyl) propionic acid, 3-(3',4'-dihydroxyphenyl) propionic acid were obtained from Sigma (St. Louis, MO). Procyanidin dimer B_2 and trimer C_1 were obtained from ChromaDex (Irvine, CA). Procyanidin dimers $B_{1,}$ B_5, B_2-gallate, trimer T_2, and tetramer A_2 were obtained from Planta Analytica (Danbury, CT).

Animal Treatment and Care. Animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Polytechnic Institute and State University. Fifty male Sprague Dawley rats (5 wk old) were obtained from Harlan Laboratories (Indianapolis, IN) and housed 2/cage on a 12-hour light/dark cycle in a climate controlled facility. Rats were allowed to acclimate to the vivarium environment for 14 d prior to the study. During this period all rats were given a standard rodent chow (2018 Teklad Global 18% Protein Rodent Diet, Harlan Teklad, Indianapolis, IN) and water *ad libitum*. Following the acclimation period the rats were randomized and given a nutritionally complete polyphenol-free growth diet (AIN-93G, TD.94048, Harlan Teklad) supplemented with 0.1% w/w Vitaflavan® to provide ~100 mg/kg bodyweight/d (based on estimated food intake) *ad libitum* for 14 d. Gut microbial communities of the colon are known to adjust over time in response to dietary treatments [139, 140], and polyphenols are also thought to induce alterations to the commensal flora of the colon [141, 142]. Therefore, this 14 d "wash-in" period was employed to ensure that the resident colonic microbiota, and their metabolism of catechins and procyanidins, were representative of the situation encountered during habitual dietary exposure to these compounds as opposed to exposure to a single acute dose [222]. On day 15, rats were switched to standard (GSE-free) chow *ad libitum* for 48 h and then fasted for 12 h. This was done to flush the GSE

polyphenols from the digestive tract to obtain a consistent baseline. On day 18, the rats were administered a solution of Vitaflavan® in saline providing a dose of 250 mg/kg GSE via intragastric gavage [the higher dose for the acute treatment (250 mg/kg vs. 100 mg/kg/d for habitual exposure) was employed in order to facilitate detection of minor microbial metabolites]. Food (standard, GSE-free chow) was restored *ad libitum* immediately following gavage. Rats were euthanized in groups of *n*=8 per time point at 0, 3, 6, 9, 12, and 18 h post-gavage (rats euthanized at 0 h post-gavage were not provided with food, as they were euthanized immediately). Euthanasia was performed by CO_2 asphyxiation according to AVMA guidelines followed by bilateral pneumothorax to ensure death.

Sample Collection. Following euthanasia, the abdominal cavity was opened ventrally and the intestines were removed by cutting through the rectum, gently removing the intestinal tract from the distal end, and then sequentially excising the cecum, proximal, mid, and distal colon tissues. The luminal contents from each region were collected by manually removing feces from tissue. Contents were placed in 0.5 mL acidified saline (0.1% formic acid v/v in 0.9% NaCL w/v) and immediately snap-frozen in liquid N_2. We chose to focus on luminal contents (feces), as opposed to tissue in order simplify the analysis by excluding Phase-II metabolites generated by the epithelium. Samples were stored at −80°C prior to analysis.

Sample Preparation. Luminal contents were freeze dried for >24 h until dry. Approximately 25-35 mg of the dried sample was reconstituted in 1 mL 0.1% formic acid in H_2O with 0.2% (w/v) ascorbic acid. Samples were homogenized by bead beating with zirconium oxide tissue homogenization beads (~100 mg, 0.5-1 mm, Next Advance, Inc., Averill Park, NY) for 10 min (4°C). Homogenized samples were divided into two tubes, reserving 300 µL for protein analysis and the rest for polyphenol analysis. Samples were again stored at −80°C prior to further analysis.

Protein Determination. Total protein levels in fecal homogenates were determined using Pierce BCA protein kits (Thermo Fisher, Waltham, MA). Samples were diluted 5, 12.5, or 50-fold with PBS and 25 µL diluted samples were assayed in triplicate according to manufacturer's instructions.

Extraction. Extraction of native compounds and metabolites was performed based on our published method [218]. Previously homogenized luminal contents were thawed and 300 μL was sampled for analysis. Liquid-liquid extraction was performed on the homogenates by adding 100 μL 1% aqueous (w/v) ascorbic acid and 1 mL ethyl acetate to each sample and vortexing for 10 s. Zirconium oxide beads were added to each sample and were homogenized in a Bullet Blender (5 min, speed 9, 4°C). Samples were then centrifuged (5 min, 17000 x g, RT), and the organic supernatant was collected. The extraction was performed again on the resulting pellet, and the supernatants were pooled for each sample. Ethyl acetate supernatants were dried under a gentle stream of N_2 (30°C). Solid-phase extraction was then performed on the remaining pellet. To each sample, 1 mL of an extraction solution (acetone:water:glacial acetic acid, 70:28:2 v/v/v) was added along with 100 μL 4% aqueous phosphoric acid. Samples were blended in a Bullet Blender (5 min, speed 9, 4°C), centrifuged (5 min, 17,000 x g, RT), and the supernatant was collected. The extraction was repeated on the resulting pellet without the addition of the phosphoric acid, and the supernatants were pooled for each sample. Water (13 mL) was added to the pooled supernatants and samples were mixed well. SPE cartridges (Oasis HLB cartridges, 1 cc, 30 mg sorbent, Waters, Milford, MA) were preconditioned using 1 mL MeOH followed by 1 mL water. A vacuum manifold was used to load the diluted samples onto the conditioned cartridges. The SPE cartridges were then washed with 1 mL acidified water followed by 0.5 mL 5% (v/v) aqueous MeOH in 0.1 % formic acid (v/v). Cartridges were then eluted with 2 mL acetone:water:glacial acetic acid (70:28:2) followed by 2 mL acidified MeOH. Eluents were collected into the tubes containing the dried ethyl acetate extracts obtained from liquid-liquid extraction from the same sample. Samples were dried under a steady stream of N_2 (30°C) to remove the MeOH and the acetone, frozen and freeze-dried for >12 h to remove water and acetic acid. To each dried sample, 1 mL 0.1% formic acid in water: 0.1% formic acid in ACN (95:5 v/v) was added for resolubilization. Samples were then sonicated in ice water (20 min), then filtered into certified LC-MS vials (Waters) using a Samplicity filtration system (0.2 μm PTFE Philic Millex Samplicity filters, Millipore). Samples were then analyzed immediately.

UPLC-MS/MS Analysis. Measurement of native compounds and metabolites was performed based on our published UPLC-MS/MS method [218]. UPLC separations were performed on a

Waters Acquity H-class separation module with a binary mobile phase system was comprised of 0.1% (v/v) aqueous formic acid (phase A) and 0.1% (v/v) formic acid in ACN (phase B). Elution was performed based on the following linear gradient: 95% A at 0 min held until 0.5 min, 65% A at 6.5 min, 20% A at 7.5 min held until 8.75 min, 95% A at 8.85 min held until 10.0 min. The system flow rate was 0.6 mL/min, and the samples were maintained at 10°C. Separations were performed with a Waters Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm particle size) maintained at 40°C. An injection volume of 10 μL was used for all samples and standards. MS/MS analysis of column effluent was performed by (−)-electrospray ionization (ESI) on a Waters Acquity TQD (tandem quadrupole detector) mass spectrometer equipped with a Z-spray electrospray interface. The ESI capillary voltage was –4.25 kV, and the desolvation gas and cone gasses were N_2 at flow rates of 900 L/hr and 75 L/hr, respectively. The source and desolvation temperatures were 150°C and 400°C, respectively. The MS/MS collision gas was Ar. Data acquisition was carried out with MassLynx software (version 4.1, Waters). Multi-reaction monitoring (MRM) parameters are listed in **Appendix C Table C1** (native monomers and PCs) and **Appendix C Table C2** (metabolites).

Quantification. The TargetLynx function of MassLynx software was used to process and quantify all compound peaks based on external standard curves prepared from authentic standards as described previously [218]. A mean smoothing method was used with 2-3 smoothing iterations and a smoothing width of 1. Quantification was based on external standard curves of authentic standards. Compounds for which authentic standards were not available were quantified based on external standard curves of similar compounds. For ease of interpretation, individual compound values were added together within classes of compounds for figure presentation.

Data and Statistical Analysis. Outliers were identified and removed using Dixon's Q-test at the $\alpha=0.05$ level if necessary. Data were analyzed by separate 1-way ANOVA analyses to determine 1) overall significance of the effect of sampling time on compound levels in each colonic section, and 2) overall significance of location (colonic section) on compound levels within each time point. If significance was detected, Tukey's HSD post-hoc test was then performed to compare all pairs of means. Significance was defined as $P<0.05$.

Results and Discussion

The levels of native PCs and microbial metabolites as measured from the luminal contents of the lower GI tract are shown in **Figures 6.2 and 6.3**, respectively. The figures show the concentrations of compounds measured in each segment over 0-18 h post gavage. These data demonstrate how the native compounds move throughout the colon and are sequentially metabolized by the microbiota. Maximal levels of total native compounds peaked between 3 and 6 h post gavage in all regions of the lower GI tract: cecum, proximal, mid, and distal colon (**Figure 6.2A**).

The maximal levels of native compounds in the cecum were found to be lower overall than in the other segments. This is most likely due to the early time points that were selected for this experiment (0 and 3 h). The maximum concentration of native compounds in the cecum presumably occurred between 0 and 3 h post gavage, due to rapid transit through the stomach and small intestine. In future studies, additional early time points (1 h, 2 h, etc.) will be considered to more accurately capture these values.

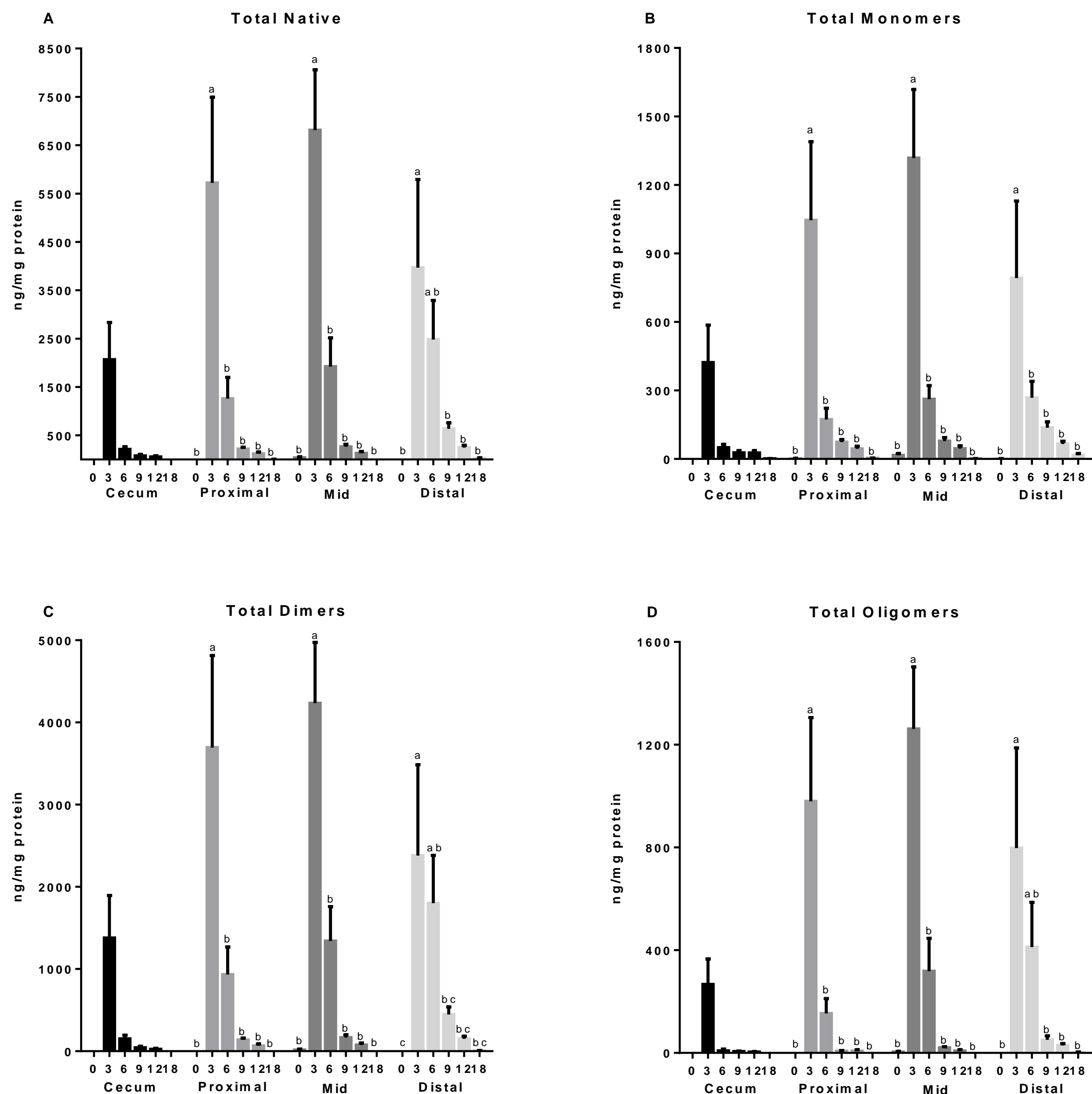

Figure 6.2. *Levels of total native compounds (A), total monomers (B), total dimers (C), and total oligomers (D) in the luminal contents cecum, proximal colon, mid colon, and distal colon of rats post-gavage of GSE. Data are presented as mean ± SEM from n = 8 animals. Values on the x-axis are times post-gavage (h). Times within the same region with distinct superscripts have significantly different concentrations (P<0.05).*

Overall, the maximal level of native compounds present in the distal colon never reached the amount present in the more proximal regions due to microbial degradation of the natives. Most of the degradation (i.e. loss of natives) occurred by 6 h with the majority of the native compounds no longer present in all sections after 12 h post gavage. A previous study had shown a similar decrease in concentration during the same time period [11]. This demonstrates that the exposure of high levels of native compounds to the lower GI tract is relatively short, suggesting

that the optimum colonic bioactivities of these native compounds may also be limited to less than 12 h post-consumption. Therefore, frequent consumption of flavanols (multiple times a day) appears to be necessary to maintain relatively high colonic levels of native compounds. **Table 6.1** shows the AUC (approximating total exposure) data for each class of PC in each segment. Total concentrations of PCs in each colonic segment (proximal, mid, and distal) are all close in magnitude over 18 h (3700-6800 ng/mg protein at 3 h and ~1300-2500 ng/mg protein at 6 h), but the levels present in the cecum are much lower (~2100 ng/mg protein at 3 h and ~210 ng/mg protein at 6 h). This is possibly due to the lack of earlier time points as mentioned previously.

Table 6.1. Maximum concentration (C_{MAX}), time of maximum concentration (t_{MAX}), and total area under the curve (AUC) for native PCs.

		Region			
Compound	**Parameter**	**Cecum**	**Proximal Colon**	**Mid colon**	**Distal Colon**
Total natives	C_{MAX} (ng/mg protein)	-	5720 ± 1770	6810 ± 1250	3970 ± 1820
	t_{MAX} (h)	-	3	3	3
	AUC (ng*h/mg)	7360	22100	27600	22500
Total monomers	C_{MAX} (ng/mg protein)	-	1050 ± 343	1320 ± 300	793 ± 338
	t_{MAX} (h)	-	3	3	3
	AUC (ng*h/mg)	1640	4090	5210	3940
Total dimers	C_{MAX} (ng/mg protein)	-	3695 ± 1119	4234 ± 739	2380 ± 1110
	t_{MAX} (h)	-	3	3	3
	AUC (ng*h/mg)	4870	14600	17600	14700
Total oligomers	C_{MAX} (ng/mg protein)	-	980 ± 326	1260 ± 241	797 ± 390
	t_{MAX} (h)	-	3	3	3
	AUC (ng*h/mg)	858	3450	4840	3920

[a]Parameters were calculated as follows: Values for C_{MAX} and t_{MAX} were selected for each region from the largest average value(s). Multiple time points were chosen for C_{MAX} and presented as a range for values not significant different from the greatest values, with the selected time points given as a range of t_{MAX}. No value was selected for C_{MAX} or t_{MAX} if there was no statistically significant difference between concentrations of any time points. C_{MAX} SEM values determined from averaging multiple data points from each time point. Values for t_{MAX} are presented as ranges with no SEM due to the values being qualitative and not averaged. AUC is presented as the cumulative sum of data across all time points, with no SEM due to the fact that AUCs were determined from aggregate and not from individual AUC values. It is important to note that each animal was sampled at only one time point, and therefore these are not "true" pharmacokinetic values, but rather estimates from the available data.

In the distal colon, the concentration of monomers peaked at 3 h and was statistically lower at 6 h, with small (non-significant) decreases from 6-18 h. PC dimer and oligomer levels also peaked at 3 h in the distal colon. However, for dimers and oligomers, the concentrations present in the distal colon at 3 and 6 h were not statistically different. This suggests that degradation of the larger PCs is slower than for monomers, and therefore dimers and oligomers persist in the colon for a longer period of time than monomers. The slower metabolism of dimers and oligomers compared to monomers could be due to a variety of factors, slower kinetics of the initial steps required for metabolism of larger compounds [191], and lower abundance of bacteria possessing these activities. However, *in vitro* fecal culture experiments have suggested that dimers are metabolized more rapidly than monomers [190]. Therefore, the reason for the distinct degradation kinetics of monomers vs. larger PCs *in vivo* remains unknown. These data suggest that the potential benefits of PCs in the colon may be enhanced, or persist longer, for dimers and oligomers compared to the monomers, which are mostly degraded by 6 h post gavage. **Figure 6.2D** shows the levels of oligomers present in each section. Additionally, the data on oligomers are novel, as previous studies only examined colonic degradation of dimers and monomers [11].

Figure 3 shows the concentration of microbial metabolites in each section over 18 h post gavage. In contrast to the native species, maximum total metabolite concentrations are in each segment were observed anywhere from 3-18 h post gavage (**Figure 6.3A**). The overall average (total metabolites) is affected by the differences between the types of metabolites. Larger metabolites such as 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol and those in the valerolactone group (**Figures 6.3B** and **6.3C**) had highest concentrations in the cecum and proximal colon at earlier time points (3-6 h post gavage). These larger compounds are the first metabolites produced in the colon, and are further degraded during transit through the colon. The presence of 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol (**Figure 6.3B**) is gone by 18 h in all sections, which demonstrates that this is an intermediate metabolite that is produced early and then continues to be degraded throughout the colon, and is completely eliminated at 18 h. Therefore, these larger, early metabolites are more likely to exert transitory activities compared to other classes of metabolites.

The smaller metabolites (**Figures 6.3D-6.3F**) reached their maximum concentrations at later time points (6-18 h). For phenyl alkyl acids (**Figure 6.3D**), the next class produced after valerolactones, maximum concentrations were observed at ~12 h for all segments, demonstrating

that these metabolites appear after extensive degradation of PCs and larger metabolites. The AUCs (**Table 6.2**) for phenyl alkyl acids were higher in the cecum and proximal colon than in the mid and distal colon (~110,000-120,000 ng*h/mg protein and 57,000-64,000 ng*h/mg protein, respectively), showing that these metabolites are further degraded by the microbiota in the colon.

The maxima for benzoic acid derivatives (**Figure 6.3E**), produced after phenylalkyl acids, ranged from 6-18 h, with the later maxima occurring in the more distal regions. Total amount of benzoic acid derivatives as shown by AUCs are at a maximum in the middle portion of the segments analyzed (proximal and mid colon), indicating that these metabolites are the product of degradation of larger metabolites, yet still undergo further degradation. Lastly, all other metabolites had the greatest AUCs in the last two segments of the colon. These compounds are the smallest ones measured, and it is logical that they show up latest and have the greatest concentration after extensive degradation.

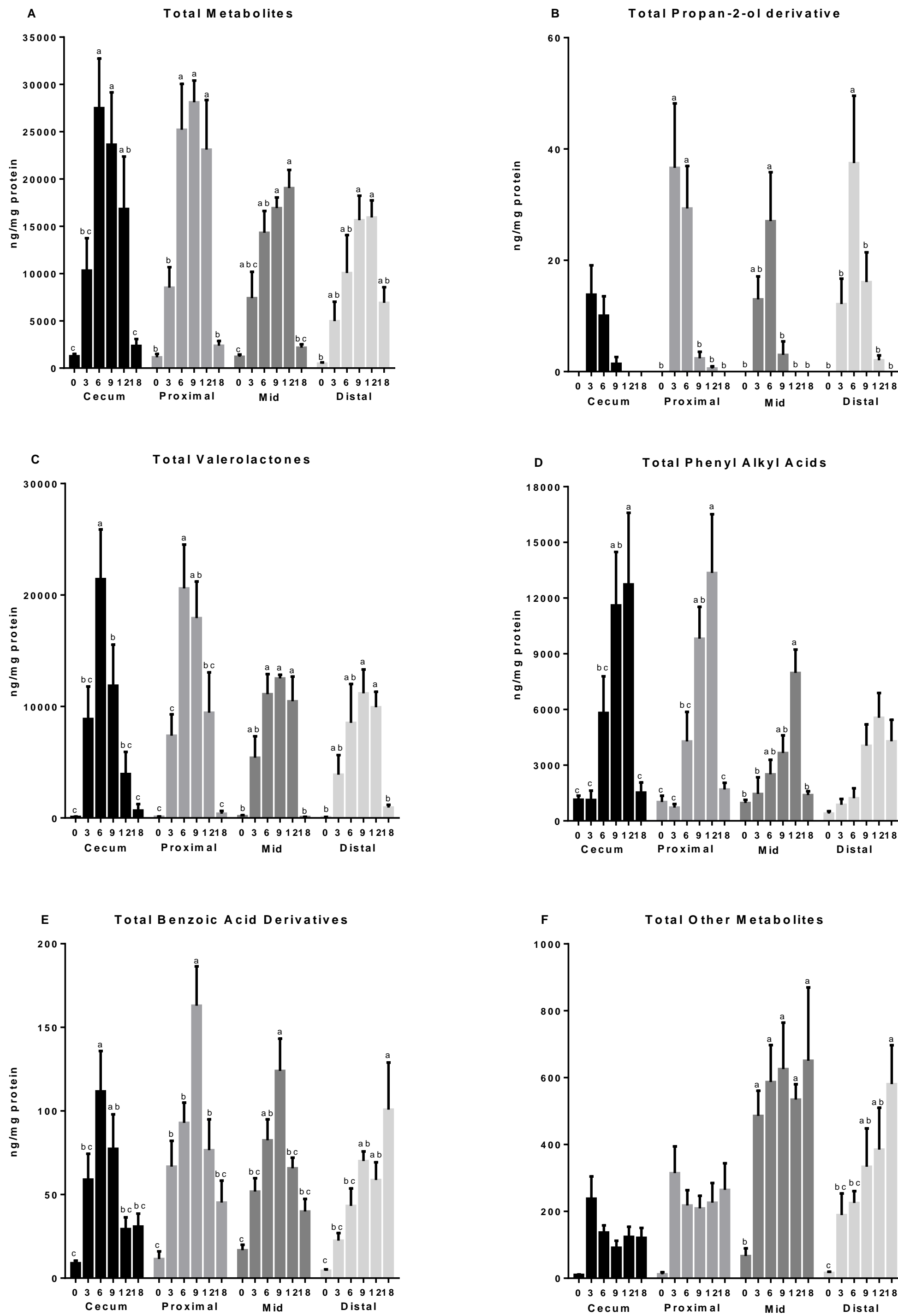

Figure 6.3. *Levels of total metabolites (A), 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)propan-2-ol (B), total valerolactones (C), total phenylalkyl acid (D), and total benzoic acid derivatives (E), and total other metabolites (F), in the luminal contents cecum, proximal colon, mid colon, and distal colon of rats post-gavage of GSE. Data are presented as mean ± SEM from n = 8 animals. Values on the x-axis are times post-gavage (h). Times within the same region with distinct superscripts have significantly different concentrations (P<0.05).*

Table 6.2. Maximum concentration (C_{MAX}), time of maximum concentration (t_{MAX}), and total area under the curve (AUC) for metabolites.

Compound	Parameter[a]	Region Cecum	Proximal Colon	Mid colon	Distal Colon
Total metabolites	C_{MAX} (ng/mg protein)	16800 ± 5540 – 27500 ± 5230	23100 ± 5230 – 28100 ± 2300	7400 ± 2780 – 19000 ± 1920	4970 ± 2060 – 15900 ± 1810
	t_{MAX} (h)	6-12	6-12	3-12	3-18
	AUC (ng*h/mg)	269000	298000	210000	182000
Total P2ol[b]	C_{MAX} (ng/mg protein)	-	29 ± 8 – 37 ± 12	13 ± 4 – 27 ± 9	37 ± 12
	t_{MAX} (h)	-	3-6	3-6	6
	AUC (ng*h/mg)	76	207	129	206
Total valerolactones	C_{MAX} (ng/mg protein)	21400 ± 4450	17900 ± 3280 – 20600 ± 3950	5400 ± 1940 – 12500 ± 329	3880 ± 1770 – 11200 ± 2150
	t_{MAX} (h)	6	6-9	3-12	3-12
	AUC (ng*h/mg)	146000	181000	135000	118000
Total phenylalkyl acids	C_{MAX} (ng/mg protein)	11600 ± 2880 – 12700 ± 3860	9810 ± 1710 – 13400 ± 3160	2510 ± 779 – 7970 ± 1260	-
	t_{MAX} (h)	9-12	9-12	6-12	-
	AUC (ng*h/mg)	119000	111000	64300	56800
Total benzoic acids	C_{MAX} (ng/mg protein)	59 ± 15 – 112 ± 24	163 ± 24	82 ± 13 – 124 ± 19	101 ± 28
	t_{MAX} (h)	3-12	9	6-9	18
	AUC (ng*h/mg)	982	1460	1220	980
Total other metabolites	C_{MAX} (ng/mg protein)	-	-	485 ± 75 – 651 ± 219	334 ± 114 – 581 ± 116
	t_{MAX} (h)	-	-	3-18	9-18
	AUC (ng*h/mg)	2340	4050	9550	5740

[a]Parameters were calculated as follows: Values for C_{MAX} and t_{MAX} were selected for each region from the largest average value(s). Multiple time points were chosen for C_{MAX} and presented as a range for values not significant different from the greatest values, with the selected time points given as a range of t_{MAX}. No value was selected for C_{MAX} or t_{MAX} if there was no statistically significant difference between concentrations of any time points. C_{MAX} SEM values determined from averaging multiple data points from each time point. Values for t_{MAX} are presented as ranges with no SEM due to the values being qualitative and not averaged. AUC is presented as the cumulative sum of data across all time points, with no SEM due to the fact that AUCs were determined from aggregate and not from individual AUC values. It is important to note that each animal was sampled at only one time point, and therefore these are not "true" pharmacokinetic values, but rather estimates from the available data.
[b]P2ol: 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol

These data present several novel findings on several fronts. First, to the best of our knowledge, the levels of these microbial metabolites in the colon have not been previously reported as a function of time or in distinct regions of the colon. Prior work has focused on fecal levels or the colon as a whole. In the present work, the colon has been separated into 4 distinct sections that were individually analyzed. Second, these data show that there are distinct characteristics of each region, rather than treating the colon as one unit. This allows for the observation of differences not previously seen that allows each region to be distinguished from one another. This information can potentially assist in novel delivery systems for PCs to different regions of the colon. Degradation of native PCs, and formation of all metabolites, occurs in all regions of the lower GI tract. Furthermore, all classes of microbial metabolites appear to be generated in each regions of the lower GI tract. This suggests that the cecum, proximal colon, mid colon and distal colon all possess resident microbiota capable of completely metabolizing native PCs to the smallest observed metabolites (benzoic acid derivatives). Metabolism therefore appears to largely be controlled by time as opposed to the distinct populations of microbiota in each region of the lower GI tract. The delivery kinetics of native compounds and their microbial metabolites in the colon are distinctly different: high levels of natives are present early and are only transiently present, while metabolites are consistently present over longer periods of time. These differences have significant implications for the impacts of these compounds on the physiology and biochemistry of the colon. Using the knowledge of the kinetics of each class of compounds, various strategies (slow-release delivery, alteration of colonic microbiota profiles and metabolic activities by pre- or probiotics, oral delivery of pre-formed metabolites, etc.) could be employed in order to take advantage of the potential bioactivities of both native and metabolite compounds in the colon. Third, we have shown that the degradation kinetics of PCs may differ *in vivo* compared to *in vitro* experiments. This may suggest a potential limitation of *in vitro* experiments for predicting degradation rates. Finally, to the best of our knowledge, this is the first study in which the kinetics of PC degradation in the colon have been characterized following "preconditioning" with chronic exposure to PCs. We, and others, have used this approach previously for single time point studies [218, 223, 224], but not for kinetic studies. Therefore, the present results are more relevant for translation to humans as they represent degradation of Pcs and formation of metabolites during

chronic PC exposure, as opposed to exposure to a single acute dose in animals that have never been exposed to these compounds previously.

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Chapter 7:
Conclusions and Future Work

The UPLC-MS/MS analysis developed provides acceptable extraction and quantification of native PCs found in GSE and their microbial metabolites and the results for the extraction recovery are similar to previously reported methods. Furthermore, the results for recovery are sufficient for studies that will compare the differences between control groups and treatment groups. The analysis allows for both the high-throughput of samples and for the efficient, simultaneous analysis of both PCs and their metabolites and is also effective is measuring compounds of interest in both tissue and luminal contents from the cecum and colon. This more efficient method will allow more complete profiling of flavan-3-ol metabolism as well as allow for larger experiments to be efficiently analyzed.

One limitation is that this method does not include the analysis of Phase-II metabolites (sulfates, glucuronides, and O-methyl derivatives). This method is focused on quantifying the microbial metabolism of flavan-3-ols in the colon, rather than Phase-I and Phase-II metabolism reactions that occur in the gut epithelium, liver, and kidneys. Future work can look into these secondary metabolites to see if they are present and quantifiable in the colon tissue and luminal contents, as well as potentially in plasma and urine.

With regard to TJPs, it has been demonstrated that the chronic administration of GSE can increase the expression of TJPs (specifically occludin) in the absence of an obesity-promoting stimulus. We failed to find, though, any evidence that the chronic administration of GSE reduces circulating endotoxin levels in the absence of an obesity-promoting stimulus. There are several limitations in this study that should be noted including the removal of GSE from the diet for 1 d, the lack of obesity-promoting stimuli, and the relatively short treatment period. It is worth mentioning, though, that the TJP and endotoxin analyses were performed as a secondary analysis on samples from another study. Therefore, the limitations are not due to poor planning or experimental design, but due to the analyses taking place as an afterthought to a primary experiment. Future studies are required to investigate this hypothesis by including experiments that include a model of impaired gut barrier function and elevated circulating endotoxin (i.e. obesity promoting stimulus).

The study presented in Chapter 5 is thought to be the longest known chronic PC feeding study for which colonic tissue accumulation data are reported. Although colonic tissue accumulation levels may be transient, these levels represent the typical profile of flavanols and their metabolites to which the colon tissue is exposed during chronic flavanol intake. Our findings highlight the differences observed between chronic low doses and a single acute dose. The data from this analysis indicate that we do not have a complete understanding of the fate of flavanols during long-term dietary administration. The data show that the factors of time and dose merit further consideration in continuing studies. Future studies will compare the effects of various doses and various lengths of exposure. The justification for these studies is that chronic, low-dose exposure to flavanols is more relevant to human health as a preventative treatment option than an acute, high-dosage in animals not previously exposed to flavanols.

Finally, the levels of these microbial metabolites in the colon reported as a function of time or in distinct regions of the colon is novel. Contrary to prior work where the colon has been treated as one unit, these data show individually analyzed data from four distinct sections. The results show that there are distinct characteristics of each region which had not been previously seen. This information can potentially assist in novel delivery systems for PCs to different regions of the colon. Degradation of native PCs, and formation of all metabolites, occurs in all regions of the lower GI tract suggesting that the cecum, proximal colon, mid colon and distal colon all possess resident microbiota capable of completely metabolizing native PCs. Therefore it appears that metabolism is more controlled by time rather than by distinct microbial populations in each region. The delivery kinetics, and therefore the impact, of native compounds and their microbial metabolites in the colon are distinctly different: high levels of natives are present early and are only transiently present, while metabolites are consistently present over longer periods of time. Furthermore, we have shown that there is a difference in degradation kinetics *in vivo* as compared to *in vitro* suggesting a potential limitation of in vitro experiments for predicting degradation rates. One limitation of this pseudo-pharmacokinetic study is that we were unable to have strong statistical conclusions from the AUC data due to samples coming from different animals. The nature of the study makes it impossible to analyze the data from a single animal, so it may be necessary to alter the method of sampling. Performing a similar experiment but taking blood samples periodically is one option, though as stated several times previously, the low absorption of larger PCs would present an issue.

Overall, advances have been made understanding the colonic metabolism of dietary PCs derived from GSE and cocoa. There is greater knowledge about the gradients of PCs and their metabolites along the length of the colon from novel studies that look at distinct segments of the colon rather than treating it as a whole unit. Furthermore, there is new insight on the accumulation of dietary PCs in the colon tissue after chronic exposure and changes that occur in the presence of TJP. All of this was possible due to a novel analysis method using UPLC-MS/MS. Future work will build off of these novel studies, and will continue to advance the understanding of the health benefits of dietary PCs.

Appendix A: Supplementary Data for Chapter 3

Table A1. MS/MS settings for MRM detection of native monomers and procyanidins

Compounds	t_R[a] (min)	MW (g mol^{-1})	[M – H]$^-$ (m/z)[b]	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
procyanidin dimer B_1	2.68	578.136	577.136	289.105	38	24
(−)-epigallocatechin	2.76	306.038	305.038	124.977	40	22
unknown dimer 1[c]	2.92	578.136	577.136	425.102	36	16
(+)-catechin	2.99	290.028	289.028	245.057	36	14
procyanidin trimer T_2	3.09	866.218	865.218	289.102	36	48
unknown dimer 2[c]	3.29	578.136	577.136	425.102	36	16
procyanidin dimer B_2	3.34	578.136	577.136	425.102	36	16
(−)-epicatechin	3.63	290.092	289.092	245.056	42	12
(−)-epigallocatechin gallate	3.67	458.038	457.038	168.982	34	16
procyanidin trimer C_1	3.82	866.218	865.218	287.085	46	32
cinnamtannin tetramer A_2	3.97	1154.808	576.404	125.020	26	34
procyanidin dimer B_2 gallate	3.99	730.164	729.164	407.129	42	32
unknown dimer 3[c]	4.07	578.136	577.136	425.102	36	16
procyanidin pentamer	4.10	1442.820	720.410	125.022	26	44
procyanidin hexamer	4.23	1731.038	864.519	125.020	32	56
(−)-epicatechin gallate	4.60	442.076	441.076	168.968	38	18

[a]retention time
[b]m/z values represent monoisotopic masses detected by IntelliStart
[c]likely procyanidin dimers B_3, B_4, and either B_6, B_7 or B_8

Table A2. MS/MS settings for MRM detection of microbial metabolites

Compounds	t_R[a] (min)	MW (g mol^{-1})	$[M – H]^-$ (m/z)[b]	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
4-hydroxymandelic acid	0.88	167.958	166.958	122.968	30	10
gallic acid	0.93	169.962	168.962	124.953	30	12
pyrogallol	1.13	125.864	124.864	78.974	40	14
3,4-dihydroxybenzoic acid	1.83	153.948	152.948	108.987	28	14
3,4-dihydroxyphenylacetic acid	2.23	167.968	166.968	122.973	18	10
catechol	2.49	109.860	108.860	90.954	38	16
4-hydroxybenzoic acid	2.69	137.882	136.882	92.966	28	12
4-hydroxyphenylacetic acid	3.03	151.948	150.948	107.064	22	6
3-(3,4-dihydroxyphenyl) propionic acid	3.04	181.968	180.968	108.984	30	14
hippuric acid	3.13	178.972	177.972	133.984	30	12
vanillic acid	3.25	167.962	166.962	152.002	28	14
3-hydroxybenzoic acid	3.28	137.874	136.874	92.963	30	12
caffeic acid	3.27	179.968	178.968	134.977	34	16
homovanillic acid	3.51	181.968	180.968	136.985	24	8
1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	3.76	292.100	291.100	247.630	30	16
3-hydroxyphenylacetic acid	3.77	151.948	150.948	107.020	30	16
3-(4-hydroxyphenyl)propionic acid	3.88	165.952	164.952	121.014	30	12
5-(3’,4’-dihydroxyphenyl)-γ-valerolactone	3.94	208.066	207.066	163.010	30	16
p-coumaric acid	4.09	163.952	162.952	119.010	28	14
3-(3-hydroxyphenyl)propionic acid	4.31	165.958	164.958	105.982	32	20
ferulic acid	4.56	193.972	192.972	133.969	32	16
m-coumaric acid	4.65	163.948	162.948	119.005	30	14
isoferulic acid	4.77	193.972	192.972	177.978	32	12

Table A2. continued

Compounds	t_R[a] (min)	MW (g mol^{-1})	[M – H]$^{-}$ (m/z)[b]	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
phenylacetic acid	5.13	135.942	134.942	90.956	18	6
5-(3',4'-dihydroxyphenyl)valeric acid	5.16	210.082	209.082	191.090	30	16
5-(3'-hydroxyphenyl)valeric acid[c]; 5-(4'-dihydroxyphenyl)valeric acid[c]; 5-hydroxy-5-phenylvaleric acid[c]	5.20 5.90 6.37	194.087 194.087 194.087	193.087 193.087 193.087	175.050 175.050 175.050	30 30 30	16 16 16
5-(3'-dihydroxyphenyl)-γ-valerolactone[d]; 5-(4'-dihydroxyphenyl)-γ-valerolactone[d]	5.22 6.19	192.071 192.071	191.071 191.071	147.090 147.090	30 30	16 16
3-phenylpropionic acid	6.40	149.982	148.982	105.016	32	10
phloretin	7.32	274.080	273.080	166.981	40	16
unknown metabolite[e]	7.72	210.082	209.082	97.120	30	16
5-phenylvaleric acid	7.97	178.032	177.032	159.086	36	14

[a]retention time
[b]m/z values represent monoisotopic masses detected by IntelliStart
[c]isomers cannot be distinguished by retention time or MRM due to lack of authentic standards
[d]isomers cannot be distinguished by retention time or MRM due to lack of authentic standards
[e]isomer of 5-(3',4'-dihydroxyphenyl)valeric acid

Table A3. Calibration curve parameters for native monomers and procyanidins

Compounds	Slope	Intercept	R^2	Concentration Range (µg/mL)	
				Minimum	Maximum
(+)-catechin	1.119×10^7	9.07	0.992	0.0004	6
(−)-epicatechin	7.449×10^6	14.17	0.991	0.0002	6
(−)-epicatechin gallate	4.426×10^7	22.71	0.960	0.0002	6
(−)-epigallocatechin	9.188×10^3	-0.17	0.9581	0.6	6
(−)-epigallocatechin gallate	2.603×10^7	-4.49	0.996	0.0006	6
procyanidin dimer B_1[b]	1.099×10^7	-3.53	0.997	0.001	8
	3.104×10^6	70062.10	0.967	8	80
procyanidin dimer B_2[ab]	9.391×10^6	-6.81	0.997	0.001	8
	2.595×10^6	53461.50	0.945	6	10
procyanidin dimer B_5	3.320×10^6	0.53	0.997	0.00008	8
procyanidin dimer B_2 gallate	5.894×10^6	-3.23	0.995	0.00008	8
procyanidin trimer C_1	2.711×10^6	-1.12	0.996	0.012	18
procyanidin trimer T_2	7.398×10^5	-11.09	0.999	0.04	18
cinnamtannin tetramer A_2	4.747×10^5	-14.64	0.997	0.04	18
procyanidin pentamers	9.561×10^5	-30.81	0.996	0.02	18
procyanidin hexamers	4.031×10^5	-13.17	0.990	0.04	18

[a]calibration curve used to quantify unknown dimers 1-3

[b]two calibration curves covering different ranges were employed for dimers B_1 and B_2

Table A4. Calibration curve parameters for microbial metabolites

Compounds	Slope	Intercept	R^2	Concentration Range (µg/mL)	
				Minimum	Maximum
phenylacetic acid	$3.358x10^6$	-6.25	0.999	0.008	18
4-hydroxyphenylacetic acid[a]	$2.108x10^6$	67.49	0.966	0.02	4
3,4-dihydroxyphenylacetic acid	$1.504x10^7$	84.54	0.991	0.004	18
3-hydroxybenzoic acid	$2.620x10^7$	340.91	0.973	0.004	18
4-hydroxybenzoic acid	$3.337x10^7$	234.69	0.990	0.004	18
3,4-dihydroxybenzoic acid	$3.435x10^7$	79.48	0.994	0.002	18
3-phenylpropionic acid	$6.745x10^5$	-74.14	0.997	0.2	18
3-(3-hydroxyphenyl)propionic acid	$7.226x10^6$	13.47	0.981	0.0012	18
3-(3,4-dihydroxyphenyl)propionic acid	$3.872x10^6$	18.34	0.991	0.008	18
5-phenylvaleric acid[b]	$3.930x10^5$	33.68	0.939	0.01	500
gallic acid	$2.473x10^7$	33.86	0.997	0.0012	18
pyrogallol	$2.642x10^6$	13.12	0.986	0.002	18
m-coumaric acid	$5.434x10^7$	32.26	0.992	0.0012	4
p-coumaric acid	$4.816x10^6$	205.20	0.977	0.002	18
phloretin	$9.159x10^7$	20.11	0.954	0.00002	18
catechol	$1.337x10^6$	10.23	0.974	0.002	18
hippuric acid	$1.618x10^7$	86.67	0.983	0.002	18
ferulic acid	$2.163x10^7$	15.28	0.991	0.0008	18
isoferulic acid	$6.603x10^6$	-26.41	0.994	0.002	18

[a]calibration curve used to quantify 3-hydroxyphenylacetic acid

[b]calibration curve used to quantify 5-hydroxy-5-phenylvaleric acid, 5-(3’-hydroxyphenyl)valeric acid, 5-(4’-dihydroxyphenyl)valeric acid, 5-(3’,4’-dihydroxyphenyl)valeric acid, 5-(3’-dihydroxyphenyl)-γ-valerolactone, 5-(4’-dihydroxyphenyl)-γ-valerolactone, 5-(3’,4’-dihydroxyphenyl)-γ-valerolactone, 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol, and unknown metabolite 1

Key for Tables S5-S8	
Number of values included in the average that are greater than the LLOD, yet lower than the LLOQ	
	0
(yellow)	1-2
(green)	3-4
(grey)	≥5

Table A5. Concentrations of native compounds in intestinal tissue (mean ± SEM from *n*=8 replicates)[a]

Compound	Control						GSE					
	Cecum		Proximal Colon		Distal Colon		Cecum		Proximal Colon		Distal Colon	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
(+)-catechin	0.813	0.666	0.0900	0.0439	0.149	0.0533	7.92	2.33	1.09	0.558	3.39	1.14
(−)-epicatechin	0.732	0.547	0.0646	0.0298	0.111	0.0450	6.88	1.84	0.742	0.311	1.07	0.356
(−)-epicatechin gallate	0.0179	0.0179	ND	ND	ND	ND	3.52	1.72	0.00618	0.00301	0.0421	0.0203
(−)-epigallocatechin	ND	ND	ND	ND	ND	ND	29.2	13.6	2.36	1.56	2.16	2.16
(−)-epigallocatechin gallate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
procyanidin dimer B_1	0.0873	0.0799	0.0110	0.00556	0.0337	0.0177	41.1	11.9	4.32	1.46	13.8	4.23
procyanidin dimer B_2	0.0858	0.0698	0.00646	0.00646	0.0297	0.0168	17.2	5.61	2.25	0.803	7.52	2.30
procyanidin dimer B_5	0.0478	0.0450	ND	ND	ND	ND	9.19	2.99	0.508	0.152	1.98	0.651
procyanidin dimer B_2 gallate	ND	ND	ND	ND	0.00419	0.00419	13.773	7.23	0.0174	0.00946	0.0823	0.0354
Unknown Dimer 1[b]	0.0748	0.0639	0.0111	0.00718	0.00554	0.00554	5.01	1.28	0.456	0.152	0.884	0.199
Unknown Dimer 2[b]	0.0457	0.0308	ND	ND	ND	ND	3.94	1.23	0.348	0.111	1.00	0.262
Unknown Dimer 3[b]	0.0148	0.0105	0.00591	0.00591	ND	ND	1.36	0.441	0.0575	0.0280	0.361	0.129
procyanidin trimer C_1	ND	ND	ND	ND	ND	ND	5.44	2.58	0.0281	0.0187	0.209	0.136
procyanidin trimer T_2	0.0856	0.0856	ND	ND	ND	ND	8.46	4.10	0.225	0.150	0.729	0.260
cinnamtannin tetramer A_2	0.803	0.253	0.614	0.217	0.748	0.193	2.44	1.16	0.947	0.137	1.09	0.0338
procyanidin pentamers	1.02	0.155	0.788	0.204	0.914	0.153	1.22	0.143	0.806	0.176	1.07	0.0122
procyanidin hexamers	0.980	0.230	0.771	0.200	1.106	0.0147	2.49	0.700	0.810	0.178	1.12	0.0189

[a]all means and SEMs are in units of ng/mg protein
[b]likely PC dimers B_3, B_4, and either B_6, B_6, B_7

Table A6. Concentrations of native compounds in intestinal contents (mean ± SEM from *n*=8 replicates)[a]

Compound	Control						GSE					
	Cecum		Proximal Colon		Distal Colon		Cecum		Proximal Colon		Distal Colon	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
(+)-catechin	1.40	0.582	0.136	0.136	1.50	0.757	470	181	220	67.7	397	72.7
(−)-epicatechin	0.483	0.341	0.0143	0.0143	0.332	0.202	355	122	164	36.5	205	34.8
(−)-epicatechin gallate	ND	ND	ND	ND	ND	ND	35.4	17.8	40.8	9.92	109	27.4
(−)-epigallocatechin	63.7	42.8	ND	ND	ND	ND	360.	137	856	303	1203	293
(−)-epigallocatechin gallate	ND	ND	ND	ND	0.0239	0.0239	0.166	0.0520	0.571	0.0932	1.29	0.310
procyanidin dimer B_1	0.357	0.262	0.271	0.137	0.232	0.108	777	133	841	153	2290	408
procyanidin dimer B_2	0.0720	0.0720	0.0780	0.0780	0.404	0.171	396	111	454	108	1280	227
procyanidin dimer B_5	0.0887	0.0887	ND	ND	0.154	0.154	242	94.0	229	56.6	597	120
procyanidin dimer B_2 gallate	0.0922	0.0922	ND	ND	ND	ND	35.8	14.2	71.0	19.1	252	51.2
Unknown Dimer 1[b]	ND	ND	ND	ND	0.0414	0.0414	62.8	18.9	62.6	11.4	149	20.8
Unknown Dimer 2[b]	ND	ND	ND	ND	ND	ND	86.7	23.7	68.2	10.8	164	26.8
Unknown Dimer 3[b]	ND	ND	ND	ND	ND	ND	41.8	22.0	31.3	8.81	121	29.9
procyanidin trimer C_1	ND	ND	ND	ND	ND	ND	24.8	8.51	45.8	14.0	193	41.4
procyanidin trimer T_2	ND	ND	ND	ND	ND	ND	41.6	16.1	68.9	29.5	321	70.6
cinnamtannin tetramer A_2	23.9	4.35	24.0	9.30	19.9	3.99	45.5	22.1	33.1	8.50	108	36.0
procyanidin pentamers	16.2	4.03	30.1	8.67	23.3	2.95	9.90	2.85	14.7	2.67	8.07	1.57
procyanidin hexamers	22.3	4.63	19.4	7.64	17.4	5.46	16.9	2.3	21.1	4.65	39.9	7.85

[a]all means and SEMs are in units of ng/mg protein
[b]likely PC dimers B_3, B_4, and either B_6, B_6, B_7

Table A7. Concentrations of microbial metabolites in intestinal tissue (mean ± SEM from *n*=8 replicates)[a]

Compound	Control						GSE					
	Cecum		Proximal Colon		Distal Colon		Cecum		Proximal Colon		Distal Colon	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
4-hydroxymandelic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
gallic acid	0.116	0.0985	0.00467	0.00467	0.00614	0.00454	4.94	0.908	0.90	0.240	1.82	0.396
pyrogallol	ND	ND	ND	ND	ND	ND	3.07	0.835	0.0910	0.0813	0.760	0.443
3,4-dihydroxybenzoic acid	0.0952	0.0105	0.0514	0.0362	0.0887	0.0774	1.02	0.173	0.0856	0.0325	0.211	0.0682
3,4-dihydroxyphenylacetic acid	ND	ND	ND	ND	ND	ND	4.88	1.27	0.120	0.0427	0.135	0.0565
catechol	ND	ND	ND	ND	ND	ND	0.290	0.0969	ND	ND	ND	ND
4-hydroxybenzoic acid	0.468	0.261	0.00347	0.00347	0.0209	0.0106	0.839	0.151	0.0594	0.0390	0.204	0.0882
4-hydroxyphenylacetic acid	0.307	0.223	0.281	0.225	1.42	0.676	3.26	1.00	0.000211	0.000211	0.177	0.132
3-(3,4-dihydroxyphenyl) propionic acid	ND	ND	ND	ND	ND	ND	2.69	1.07	0.0813	0.0418	0.101	0.0415
hippuric acid	ND	ND	ND	ND	0.0338	0.0338	0.0261	0.0261	ND	ND	ND	ND
vanillic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3-hydroxybenzoic acid	0.0797	0.0547	ND	ND	ND	ND	0.622	0.183	ND	ND	ND	ND
caffeic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
homovanillic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	ND	ND	ND	ND	ND	ND	67.3	21.0	4.90	1.91	21.3	6.74
3-hydroxyphenylacetic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.0337	0.0337
3-(4-hydroxyphenyl)propionic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5-(3’,4’-dihydroxyphenyl)-γ-valerolactone	3.95	2.68	ND	ND	ND	ND	5910	662	602	208	1290	414
p-coumaric acid	ND	ND	ND	ND	ND	ND	0.0114	0.00816	ND	ND	ND	ND
3-(3-hydroxyphenyl)propionic acid	0.0701	0.0241	0.0390	0.0328	0.100	0.0674	47.8	14.9	2.36	0.768	5.94	1.91
ferulic acid	ND	ND	ND	ND	ND	ND	0.00740	0.00437	0.0000341	0.0000341	0.0181	0.0181
m-coumaric acid	0.00195	0.00173	ND	ND	ND	ND	0.0964	0.0156	0.00623	0.00397	0.000302	0.000302
isoferulic acid	0.159	0.0119	0.0982	0.0254	0.134	0.00106	0.133	0.00438	0.132	0.00169	0.138	0.00555
phenylacetic acid	2.29	0.542	1.02	0.138	0.816	0.360	9.19	1.49	0.783	0.160	0.381	0.0918
5-(3’,4’-dihydroxyphenyl)valeric acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5-(3’-hydroxyphenyl)valeric acid[b]; 5-(4’-dihydroxyphenyl)valeric acid[b]; 5-hydroxy-5-phenylvaleric acid[b]	1.09	0.681	2.41	2.35	0.00665	0.00665	5830	803	308	87.3	352	82.2
5-(3’-dihydroxyphenyl)-γ-valerolactone[c];	ND	ND	ND	ND	ND	ND	434	84.2	17.2	5.42	30.5	11.8
5-(4’-dihydroxyphenyl)-γ-valerolactone[c]	ND	ND	ND	ND	ND	ND	271.5	50.8	10.9	5.32	13.1	4.71
3-phenylpropionic acid	4.13	1.36	2.95	0.765	2.22	0.786	15.6	1.35	3.24	0.770	3.50	0.523
phloretin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
unknown metabolite[d]	0.640	0.294	0.112	0.0944	0.00632	0.00632	1.08	0.332	0.114	0.0784	0.325	0.325
5-phenylvaleric acid	ND	ND	ND	ND	ND	ND	259	112	3.21	1.41	33.7	12.7

[a]all means and SEMs are in units of ng/mg protein
[b]isomers cannot be distinguished by retention time or MRM due to lack of authentic standards
[c]isomers cannot be distinguished by retention time or MRM due to lack of authentic standards
[d]isomer of 5-(3’,4’-dihydroxyphenyl)valeric acid

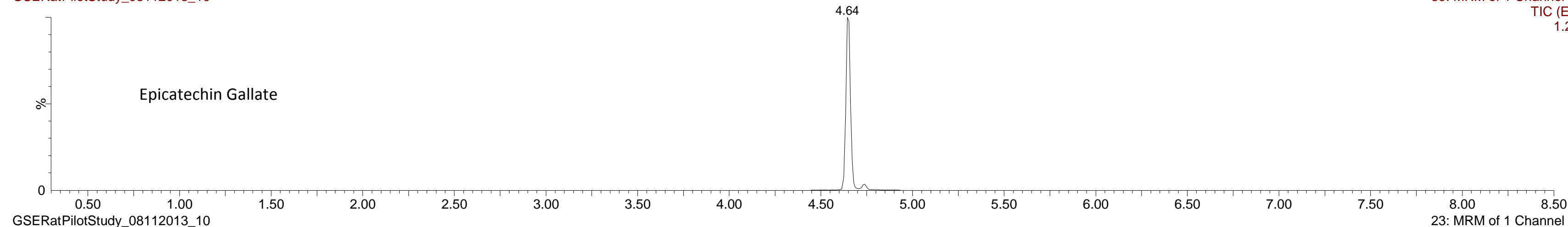
Table A8. Concentrations of microbial metabolites in intestinal contents (mean ± SEM from *n*=8 replicates)[a]

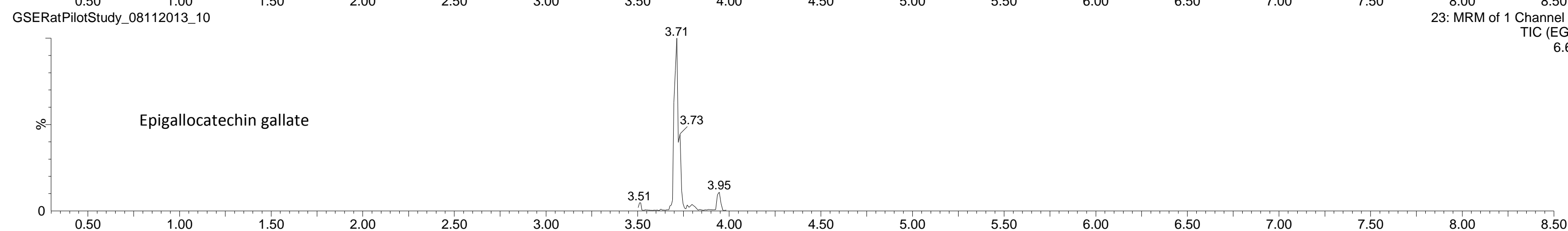
Compound	Control						GSE					
	Cecum		Proximal Colon		Distal Colon		Cecum		Proximal Colon		Distal Colon	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
4-hydroxymandelic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
gallic acid	0.0591	0.0591	ND	ND	ND	ND	11.1	4.11	25.3	4.00	57.2	10.1
pyrogallol	ND	ND	ND	ND	ND	ND	1.85	1.43	6.34	5.48	10.7	4.68
3,4-dihydroxybenzoic acid	ND	ND	ND	ND	ND	ND	2.88	0.723	1.22	0.344	3.35	0.821
3,4-dihydroxyphenylacetic acid	ND	ND	ND	ND	ND	ND	1.22	0.733	1.26	0.792	2.53	1.08
catechol	ND	ND	ND	ND	ND	ND	0.0960	0.0629	ND	ND	ND	ND
4-hydroxybenzoic acid	ND	ND	ND	ND	1.39	1.14	0.221	0.221	ND	ND	0.118	0.0991
4-hydroxyphenylacetic acid	0.877	0.877	9.01	8.95	32.5	31.0	ND	ND	ND	ND	ND	ND
3-(3,4-dihydroxyphenyl) propionic acid	ND	ND	ND	ND	ND	ND	0.131	0.0867	0.251	0.251	0.712	0.470
hippuric acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
vanillic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3-hydroxybenzoic acid	ND	ND	ND	ND	ND	ND	0.0619	0.0619	ND	ND	ND	ND
caffeic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
homovanillic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	ND	ND	ND	ND	ND	ND	812	216	1400	188	1230	100
3-hydroxyphenylacetic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.192	0.146
3-(4-hydroxyphenyl)propionic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5-(3’,4’-dihydroxyphenyl)-γ-valerolactone	124	82.2	ND	ND	ND	ND	67600	11200	40200	6510	24500	5340
p-coumaric acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3-(3-hydroxyphenyl)propionic acid	ND	ND	0.0341	0.0341	2.22	1.69	44.6	14.9	18.3	4.18	17.3	4.02
ferulic acid	ND	ND	ND	ND	0.0706	0.0706	0.0396	0.0307	ND	ND	0.0435	0.0323
m-coumaric acid	0.226	0.115	0.0501	0.0340	0.0115	0.0115	0.409	0.309	0.0273	0.0178	0.0660	0.0537
isoferulic acid	3.137	0.414	3.38	0.642	2.90	0.349	1.64	0.251	1.73	0.336	0.945	0.129
phenylacetic acid	19.1	2.31	28.7	6.55	26.5	6.36	11.5	3.03	6.50	0.663	3.81	0.868
5-(3’,4’-dihydroxyphenyl)valeric acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5-(3’-hydroxyphenyl)valeric acid[b]; 5-(4’-dihydroxyphenyl)valeric acid[b]; 5-hydroxy-5-phenylvaleric acid[b]	21.2	21.2	ND	ND	ND	ND	21600	5060	6910	1750	4960	2190
5-(3’-dihydroxyphenyl)-γ-valerolactone[c];	ND	ND	ND	ND	ND	ND	2160	629	622	130	465	190
5-(4’-dihydroxyphenyl)-γ-valerolactone[c]	ND	ND	ND	ND	ND	ND	727	270	204	81.2	222	137
3-phenylpropionic acid	71.5	18.8	93.1	33.7	48.9	17.1	57.2	13.0	41.5	12.9	23.9	5.47
phloretin	ND	ND	ND	ND	ND	ND	0.00687	0.00687	0.00539	0.00502	0.0753	0.0357
unknown metabolite[d]	3450	669	4090	586	2900	697	2010	521	2290	362	1240	262
5-phenylvaleric acid	ND	ND	ND	ND	ND	ND	58.7	45.5	41.6	19.9	205	75.5

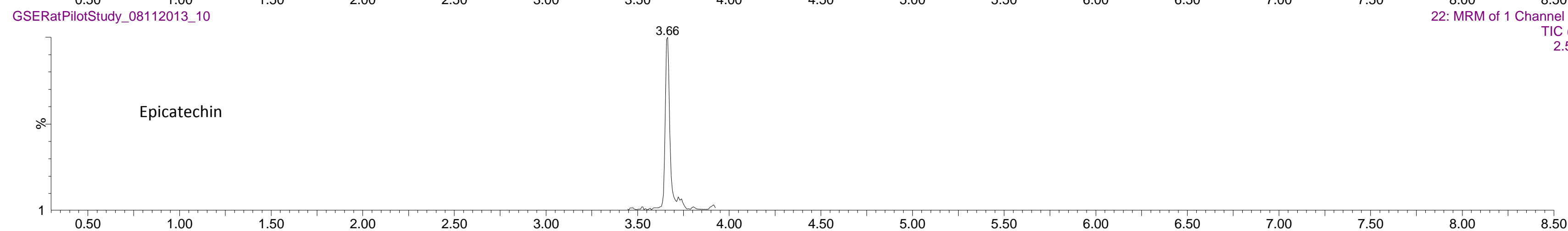
[a]all means and SEMs are in units of ng/mg protein
[b]isomers cannot be distinguished by retention time or MRM due to lack of authentic standards
[c]isomers cannot be distinguished by retention time or MRM due to lack of authentic standards
[d]isomer of 5-(3’,4’-dihydroxyphenyl)valeric acid

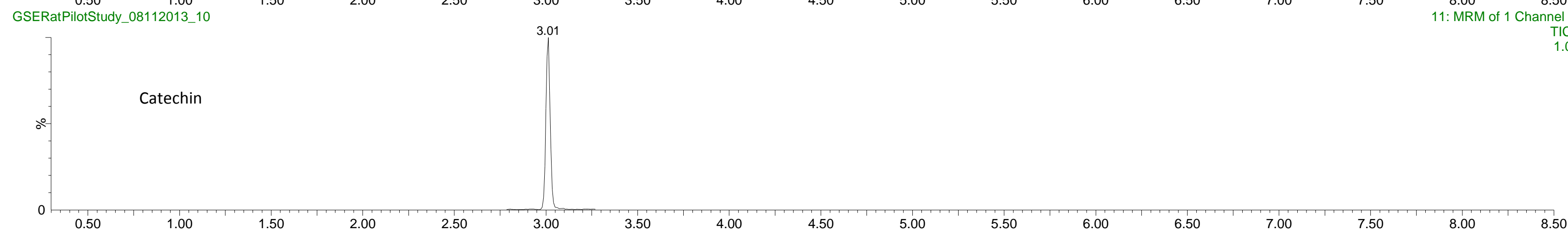
Figure A1. MRM chromatograms of detected native catechins and procyanidins and their microbial metabolites in the luminal contents of the distal colon of a male inbred Wistar-Furth rat, 8 h post-gavage of Vitaflavan GSE (250 mg/kg body weight). See materials and methods section for UPLC-MS/MS conditions and parameters. All compounds normalized to equal height.

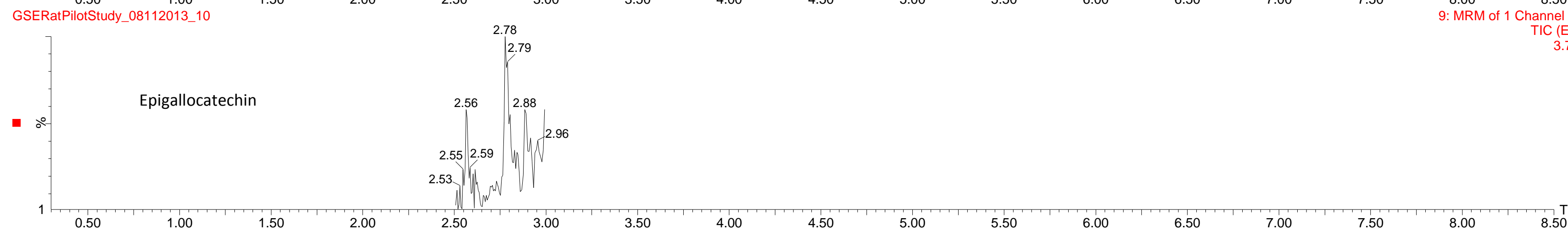
911 Distal Contents











911 Distal Contents

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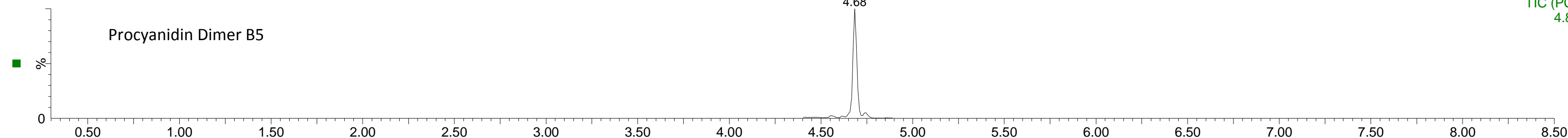
37: MRM of 1 Channel ES-
TIC (PCB5)
4.89e5



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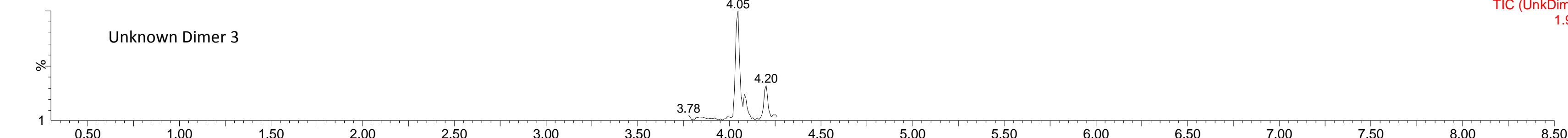
31: MRM of 1 Channel ES-
TIC (UnkDimer3)
1.99e5



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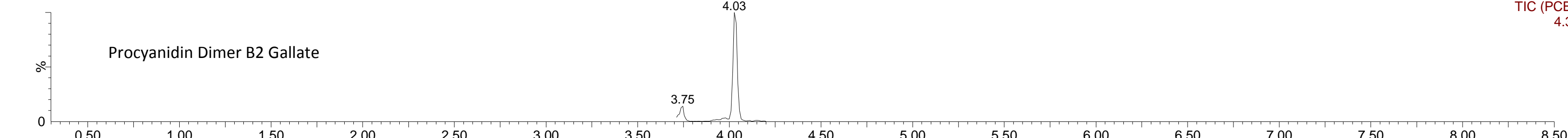
30: MRM of 1 Channel ES-
TIC (PCB2G)
4.34e5



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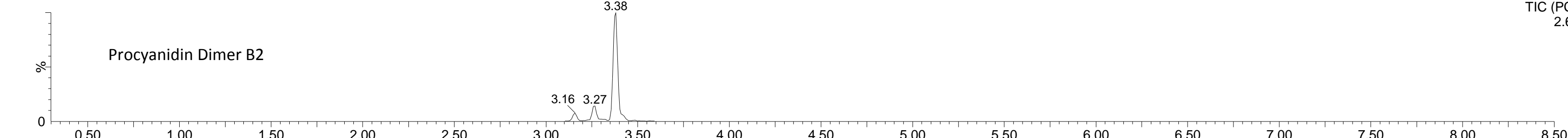
20: MRM of 1 Channel ES-
TIC (PCB2)
2.67e6



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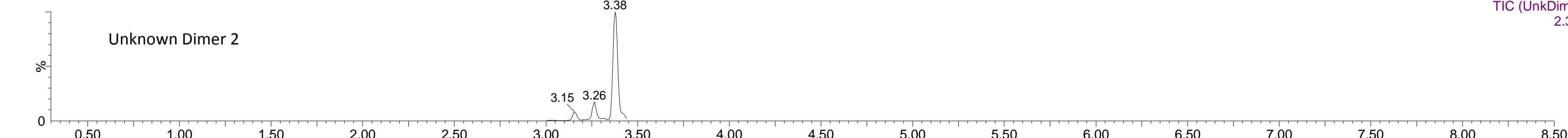
16: MRM of 1 Channel ES-
TIC (UnkDimer2)
2.38e6



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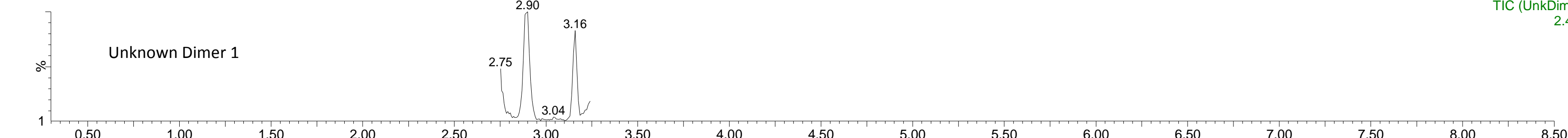
10: MRM of 1 Channel ES-
TIC (UnkDimer1)
2.43e5



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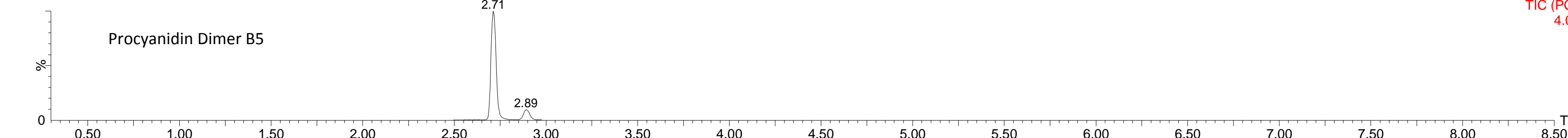

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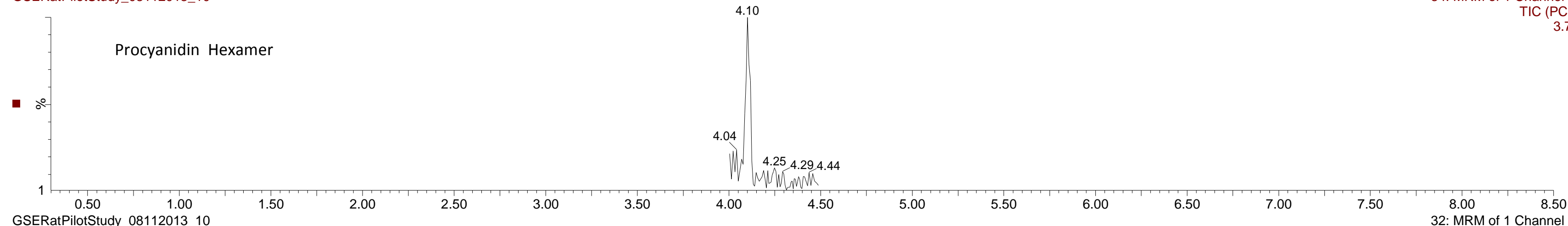

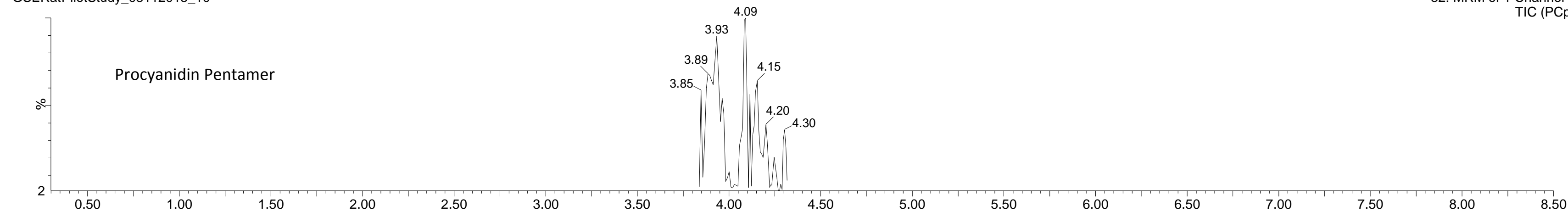

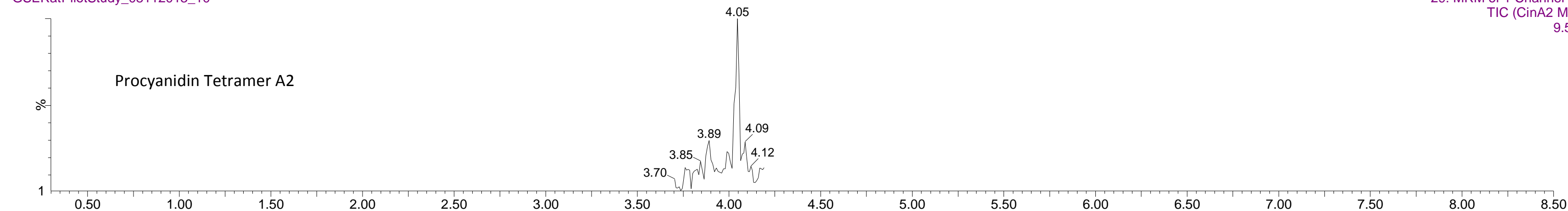

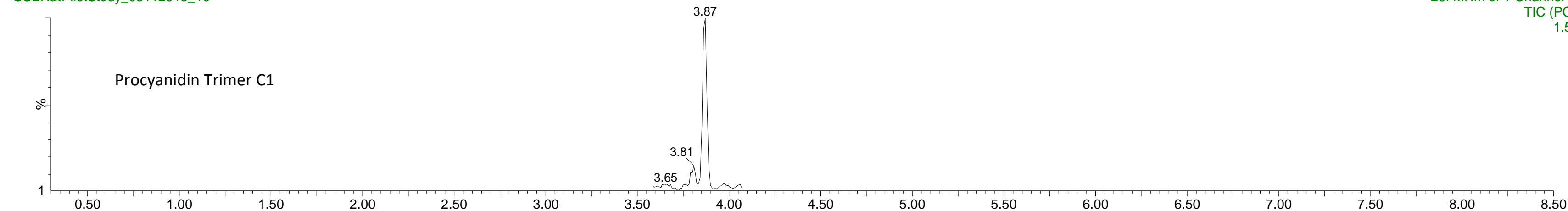

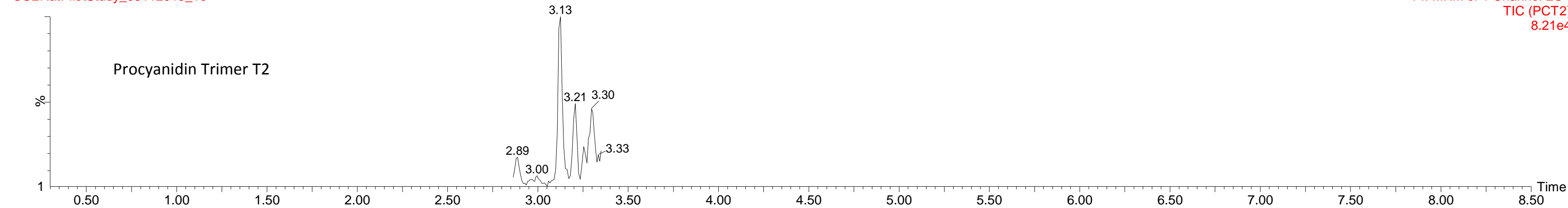

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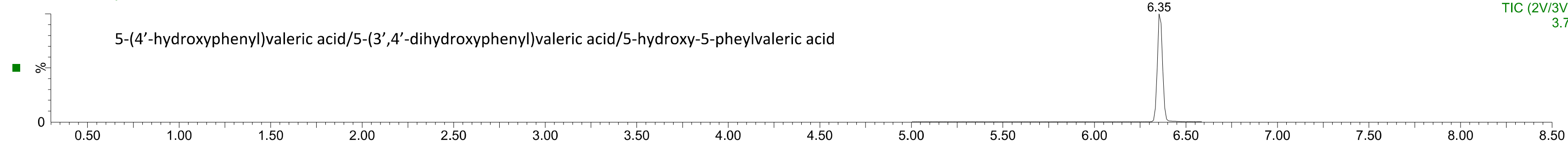

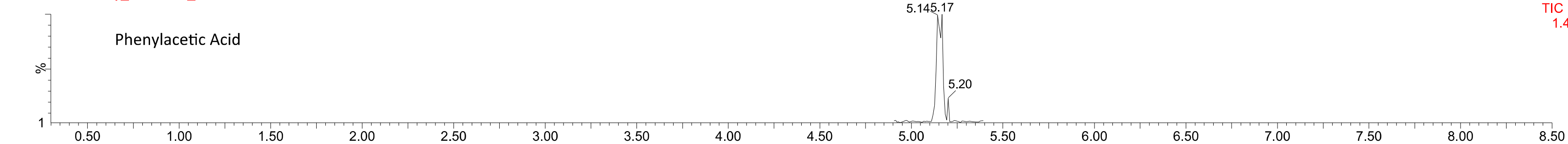

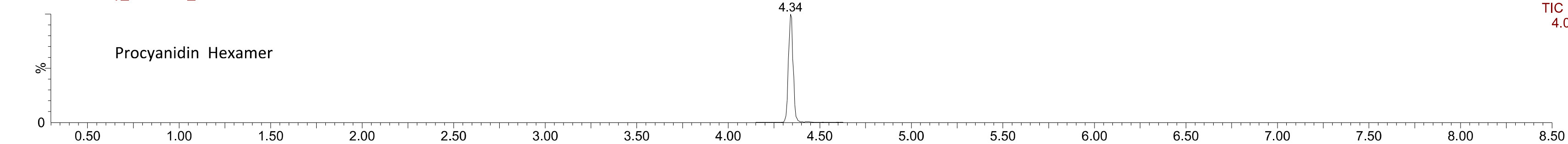

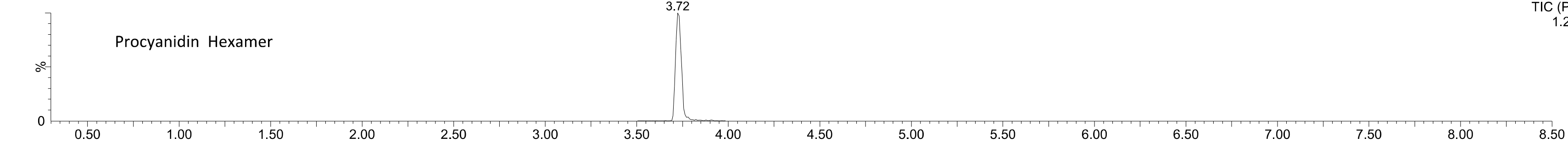

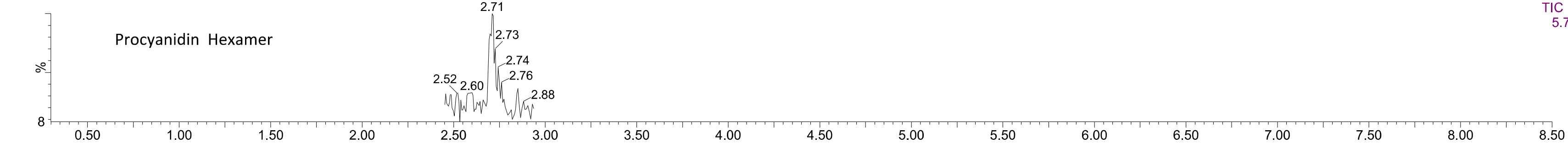

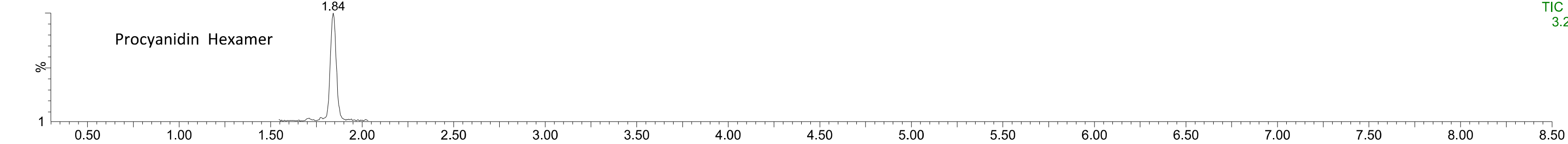

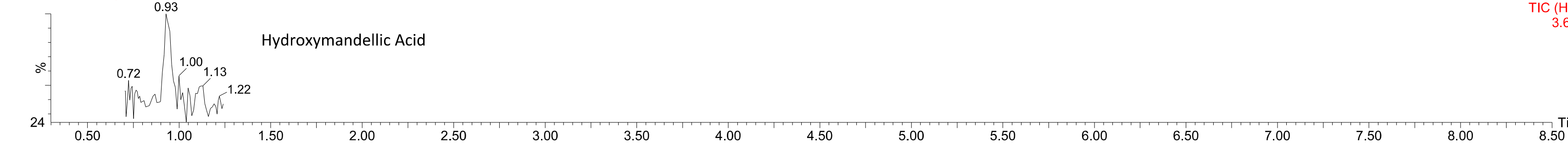

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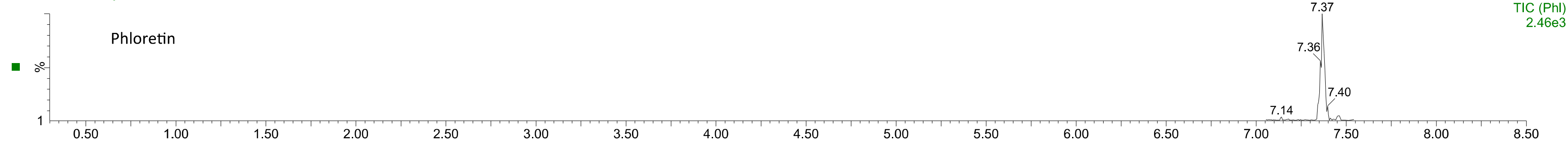

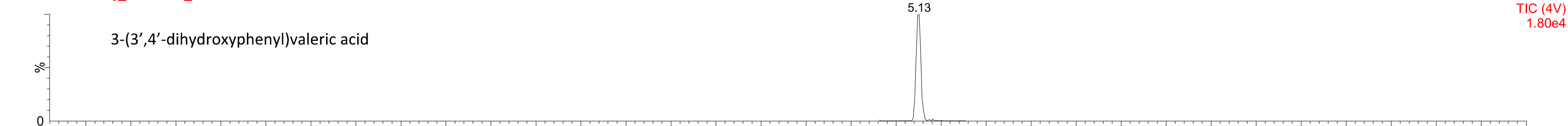

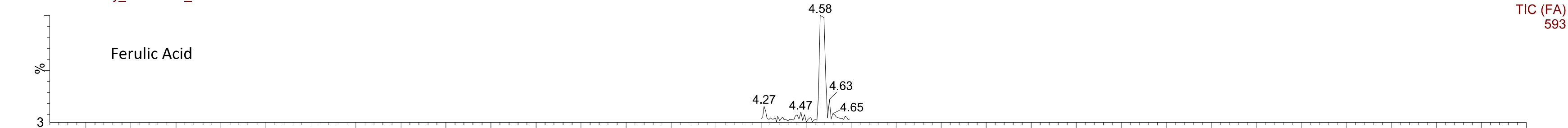

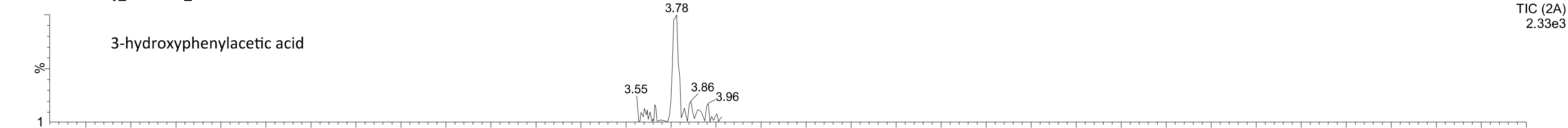

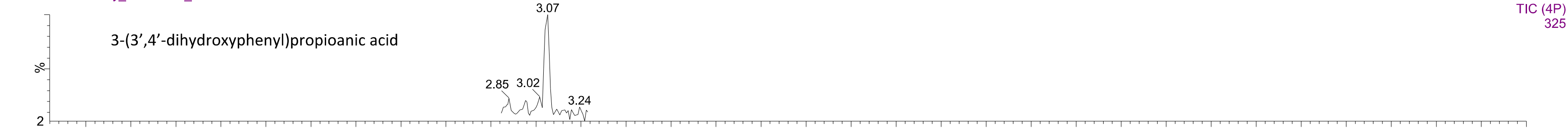

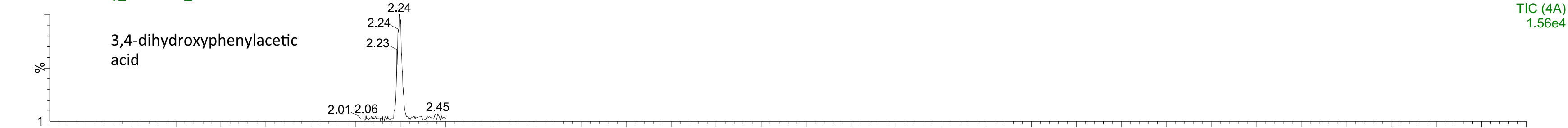

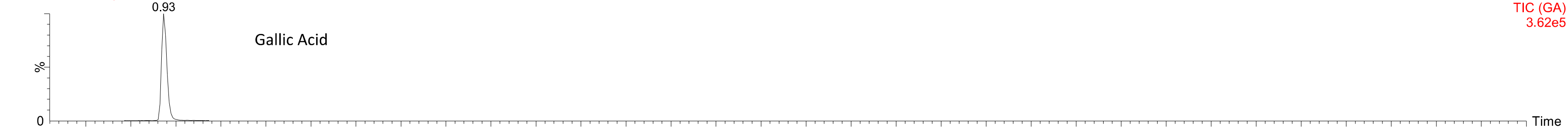

911 Distal Contents

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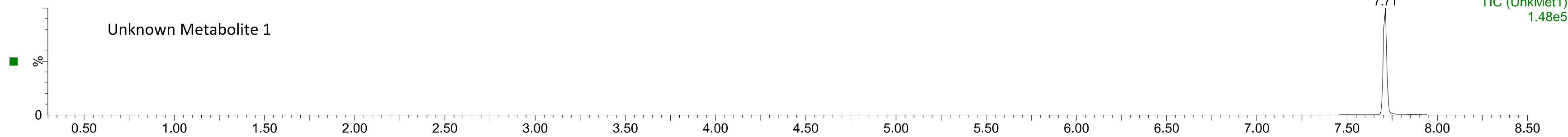
47: MRM of 1 Channel ES-
TIC (UnkMet1)
1.48e5



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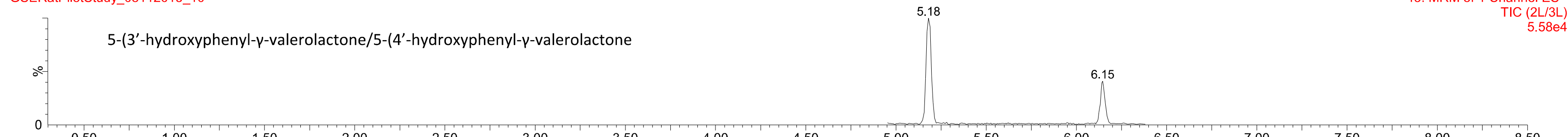
43: MRM of 1 Channel ES-
TIC (2L/3L)
5.58e4



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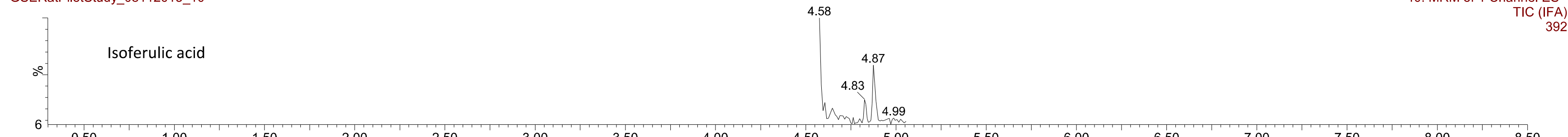
40: MRM of 1 Channel ES-
TIC (IFA)
392



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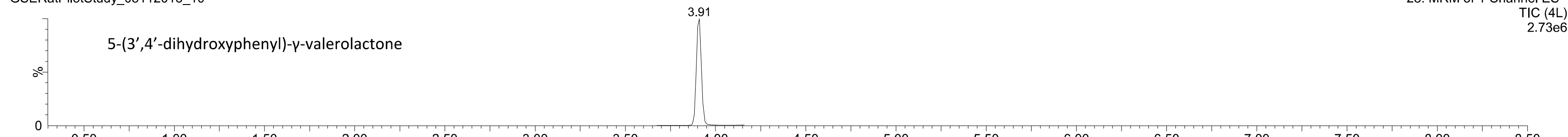
28: MRM of 1 Channel ES-
TIC (4L)
2.73e6



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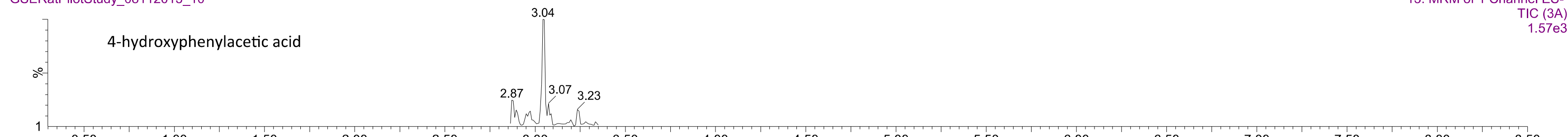
13: MRM of 1 Channel ES-
TIC (3A)
1.57e3



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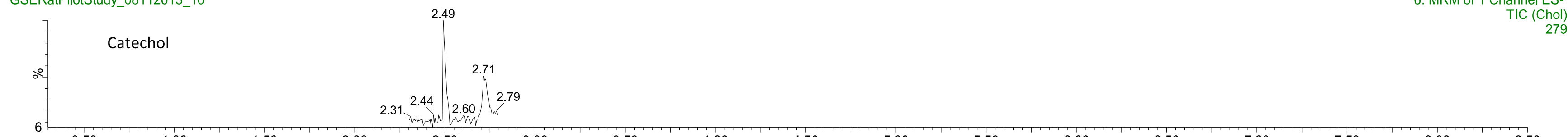
6: MRM of 1 Channel ES-
TIC (Chol)
279



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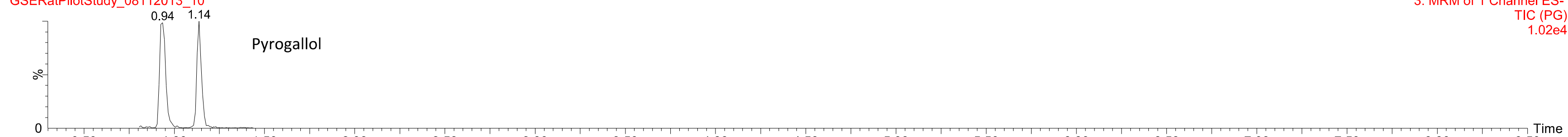
3: MRM of 1 Channel ES-
TIC (PG)
1.02e4



Appendix B: Supporting Information for Chapter 5

Table B1. Levels of Native Monomers and Procyanidins in Cocoa Extract and Individual Cocoa Fractions.

Compound	**Composition[ab] (mg compound/g dried fraction)**			
	CE[d]	**M[e]**	**O[f]**	**P[g]**
(±)-catechin[c]	1.67±0.0183 b	9.52±0.180 c	0.119±0.00183 a	0.0129±0.00328 a
(−)-epicatechin	5.90±0.0635 b	30.6±0.530 c	0.667±0.0152 a	0.0726±0.00914 a
(−)-epigallocatechin	0.0151±0.000498 b	0.0237±0.00322 b	0.0199±0.00102 b	0.0132±0.00440 a
procyanidin dimer B_1	0.356±0.0126 b	0.476±0.0103 c	1.12±0.00765 d	0.0135±0.00100 a
procyanidin dimer B_2	4.88±0.149 b	6.12±0.125 c	15.4±0.201 d	0.250 ±0.00346 a
procyanidin dimer B_5	3.10±0.0298 b	7.52±0.246 c	7.32±0.206 c	0.0838±0.0419 a
unknown procyanidin dimer 1[h]	0.0360±0.00645 b	0.0474±0.0109 b	0.114±0.00897 c	0 a
unknown procyanidin dimer 2[h]	0.116±0.00989 b	0.157±0.0140 b	0.433±0.0222 c	0 a
unknown procyanidin dimer 3[h]	0 a	0.678±0.0118 c	0.311±0.156 b	0 a
procyanidin trimer C_1	8.12±0.197 c	6.31±0.276 b	26.3±0.427 d	0.932±0.0330 a
cinnamtannin tetramer A_2	3.52±0.296 c	2.29±0.290 b	10.6±0.209 d	0.534±0.0493 a
procyanidin pentamers	3.69±0.261 b	1.73±0.0943a	9.77±0.438 c	1.40±0.0732 a
procyanidin hexamers	2.70±0.164 b	0 a	3.65±0.193 c	2.18±0.216 b
procyanidin heptamers	2.14±0.116 b	0 a	1.93±0.187 b	2.72±0.255 c
procyanidin octamers	0.736±0.0757 c	0.196±0.0122 b	1.88±0.0598 d	0a
procyanidin nonamers	1.78±0.292 b	0.142±0.142 a	3.03±0.186 c	1.87±0.123 b
procyanidin decamers	0 a	0 a	0 a	3.68±1.228 a
Total monomers	7.59±0.0806 b	40.2±0.682 c	0.806±0.0129 a	0.0899±0.0146 a
Total dimers	8.48±0.155 b	15.0±0.394 c	24.7±0.267 d	0.347±0.0408 a
Total procyanidins DP 3-6	18.0±0.763 c	10.3±0.422 b	50.4±1.08 d	5.04±0.231 a
Total procyanidins DP 7-10	4.65±0.208 b	0.338±0.153 a	6.83±0.141 b	5.82±1.45 b

[a]Data reported are from Dorenkott *et al.*[196]
[b]Data are reported as mean ± SEM from n=3 replicate analyses. Treatments with different letters for the same compound (or sum of compounds) are significantly different ($P<0.05$)
[c]Note that (+)-C was used as then analytical standard; results are reported for total (±)-C
[d]Cocoa extract
[e]Monomer-rich fraction
[f]Oligomer-rich fraction
[g]Polymer-rich fraction
[g]Likely procyanidin dimers B_3, B_4, and either B_6, B_7 or B_8

UPLC-MS/MS Methodology. UPLC separations were performed on a Waters Acquity H-class separation module equipped with a Waters Acquity UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm particle size). The column temperature was set to 40°C, and samples were maintained at 10°C. The binary mobile phase system was comprised of 0.1% (v/v) aqueous formic acid (phase A) and 0.1% (v/v) formic acid in ACN (phase B). The system flow rate was 0.6 mL/min. Elution was performed based on the following linear gradient: 95% A at 0 min held until 0.5 min, 65% A at 6.5 min, 20% A at 7.5 min held until 8.75 min, 95% A at 8.85 min held until 10.0 min. An injection volume of 25 μL was used for all samples and standards. MS/MS analysis of column effluent was performed by (−)-electrospray ionization (ESI) on a Waters Acquity TQD (triple quadrupole) mass spectrometer equipped with a Z-spray electrospray interface. The ESI capillary voltage was –4.25 kV, and the source and desolvation temperatures were 150°C and 400°C, respectively. The desolvation gas and cone gasses were N_2 at flow rates of 900 L/hr and 75 L/hr, respectively. The MS/MS collision gas was Ar. Data acquisition was carried out with MassLynx software (version 4.1, Waters). MS data collection was set to 10 points/peak with an average peak width of 6 s. The auto-dwell setting was used to automatically calculate dwell time based on an interscan delay time of 0.02 s for each transition. The TQD was operated in quantitation mode, with the mass resolution of the first and second quadrupoles set at 1.0 and 0.75, respectively. The Intellistart function of MassLynx was used to develop and optimize multi-reaction monitoring (MRM) parameters for each compound of interest. Compound solutions were directly infused into the ESI source (0.1 mg/mL in MeOH/0.1% formic acid at a flow rate of 50 μL/min) in combination with a background flow of 50% phase A/50% phase B at 0.6 mL min. Intellistart automatically selected the exact monoisotopic mass of the most abundant daughter ion, optimized the source cone voltage and MS/MS collision energy, and generated a single MRM transition for each compound centered on the exact monoisotopic mass of the parent and daughter ions, with a mass window of 0.2 amu (i.e. exact monoisotopic mass determined by Intellistart ± 0.1). These parameters are listed in Supplementary Table S2 (native monomers and PCs) and Supplementary Table S3 (metabolites). All compound peaks were processed and quantified using the TargetLynx function of MassLynx software. Peaks were smoothed using a mean smoothing method with 2-3 smoothing iterations and a smoothing width of 1. Compounds were quantified based on external standard curves of authentic standards in 0.1% formic acid in water:0.1% formic acid in ACN (95:5 v/v); compounds for which authentic standards were not available were quantified based on external standard curves of similar compounds. Peaks between the lower limits of quantification and detection (LLOQ and LLOD, previously described [201]) were tentatively quantified and included in the data. Peaks below the LLOD were not detected and were therefore included in the data as concentrations of 0.

Table B2. MS/MS Settings for MRM Detection of Native Monomers and Procyanidins

Compounds	t_R[a] (min)	MW (g mol^{-1})	$[M-H]^-$ (m/z)[b]	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
procyanidin dimer B_1	2.68	578.136	577.136	289.105	38	24
(−)-epigallocatechin	2.76	306.038	305.038	124.977	40	22
unknown dimer 1[c]	2.92	578.136	577.136	425.102	36	16
(+)-catechin	2.99	290.028	289.028	245.057	36	14
procyanidin trimer T_2	3.09	866.218	865.218	289.102	36	48
unknown dimer 2[c]	3.29	578.136	577.136	425.102	36	16
procyanidin dimer B_2	3.34	578.136	577.136	425.102	36	16
(−)-epicatechin	3.63	290.092	289.092	245.056	42	12
(−)-epigallocatechin gallate	3.67	458.038	457.038	168.982	34	16
procyanidin trimer C_1	3.82	866.218	865.218	287.085	46	32
cinnamtannin tetramer A_2	3.97	1154.808	576.404	125.020	26	34
procyanidin dimer B_2 gallate	3.99	730.164	729.164	407.129	42	32
procyanidin octamers	4.04	2307.17	1152.58	125.17	48	68
unknown dimer 3[c]	4.07	578.136	577.136	425.102	36	16
procyanidin pentamers	4.10	1442.820	720.410	125.022	26	44
procyanidin hexamers	4.23	1731.038	864.519	125.020	32	56
procyanidin nonamers	4.33	2586.36	864.12	125.17	28	46
procyanidin heptamers	4.41	2018.80	1008.40	125.17	36	56
(−)-epicatechin gallate	4.60	442.076	441.076	168.968	38	18
procyanidin decamers	4.60	2883.55	960.18	125.17	30	52
procyanidin dimer B_5	4.64	578.136	577.136	289.107	30	26

[a]Retention time

[b]m/z values represent monoisotopic masses detected by Intellistart; all MRMs used singly-charged parent ions except for cinnamtannin tetramer A_2, pentamers, hexamers, heptamers, octamers, which were doubly-charged ($[M-2H]^{2-}$), and nonamers and decamers, which were triply-charged ($[M-3H]^{3-}$)

[c]Likely procyanidin dimers B_3, B_4, and either B_6, B_7 or B_8

Table B3. MS/MS Settings for MRM Detection of Microbial Metabolites

Compounds	t_R[a] (min)	MW (g mol^{-1})	$[M – H]^-$ (m/z)[b]	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
4-hydroxymandelic acid	0.88	167.958	166.958	122.968	30	10
gallic acid	0.93	169.962	168.962	124.953	30	12
pyrogallol	1.13	125.864	124.864	78.974	40	14
3,4-dihydroxybenzoic acid	1.83	153.948	152.948	108.987	28	14
3,4-dihydroxyphenylacetic acid	2.23	167.968	166.968	122.973	18	10
catechol	2.49	109.860	108.860	90.954	38	16
4-hydroxybenzoic acid	2.69	137.882	136.882	92.966	28	12
4-hydroxyphenylacetic acid	3.03	151.948	150.948	107.064	22	6
3-(3,4-dihydroxyphenyl)propionic acid	3.04	181.968	180.968	108.984	30	14
hippuric acid	3.13	178.972	177.972	133.984	30	12
vanillic acid	3.25	167.962	166.962	152.002	28	14
3-hydroxybenzoic acid	3.28	137.874	136.874	92.963	30	12
caffeic acid	3.27	179.968	178.968	134.977	34	16
homovanillic acid	3.51	181.968	180.968	136.985	24	8
1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	3.76	292.100	291.100	247.630	30	16
3-hydroxyphenylacetic acid	3.77	151.948	150.948	107.020	30	16
3-(4-hydroxyphenyl)propionic acid	3.88	165.952	164.952	121.014	30	12
5-(3’,4’-dihydroxyphenyl)-γ-valerolactone	3.94	208.066	207.066	163.010	30	16
p-coumaric acid	4.09	163.952	162.952	119.010	28	14
3-(3-hydroxyphenyl)propionic acid	4.31	165.958	164.958	105.982	32	20
ferulic acid	4.56	193.972	192.972	133.969	32	16
m-coumaric acid	4.65	163.948	162.948	119.005	30	14
isoferulic acid	4.77	193.972	192.972	177.978	32	12
phenylacetic acid	5.13	135.942	134.942	90.956	18	6
5-(3’,4’-dihydroxyphenyl)valeric acid	5.16	210.082	209.082	191.090	30	16
5-(3’-hydroxyphenyl)valeric acid[c];	5.20	194.087	193.087	175.050	30	16
5-(4’-dihydroxyphenyl)valeric acid[c];	5.90	194.087	193.087	175.050	30	16
5-hydroxy-5-phenylvaleric acid[c]	6.37	194.087	193.087	175.050	30	16
5-(3’-dihydroxyphenyl)-γ-valerolactone[d];	5.22	192.071	191.071	147.090	30	16
5-(4’-dihydroxyphenyl)-γ-valerolactone[d]	6.19	192.071	191.071	147.090	30	16
3-phenylpropionic acid	6.40	149.982	148.982	105.016	32	10
phloretin	7.32	274.080	273.080	166.981	40	16
unknown metabolite[e]	7.72	210.082	209.082	97.120	30	16
5-phenylvaleric acid	7.97	178.032	177.032	159.086	36	14

[a]Retention time
[b]m/z values represent monoisotopic masses detected by Intellistart; all MRMs used singly-charged parent ions
[c]Isomers cannot be distinguished by retention time or MRM due to lack of authentic standards
[d]Isomers cannot be distinguished by retention time or MRM due to lack of authentic standards
[e]Isomer of 5-(3’,4’-dihydroxyphenyl)valeric acid

Table B4. Composition of the Basal Mouse Diet[ab] from the Research Diets Diet-Induced Obesity (DIO) Model Series.

Component	%	
	(w/w)	(kcal)
protein	26.2	20.0
carbohydrate	26.3	20.0
fat	34.0	60.0
total		100.0
kcal/g	5.2	
Ingredient	**g**	**kcal**
casein, 80 mesh	200.0	800.0
L-cystine	3.0	12.0
corn starch	0.0	0.0
maltodextrin 10	125.0	500.0
sucrose	68.8	275.2
cellulose, BW200	50.0	0.0
soybean oil	25.0	225.0
lard[c]	245.0	2205.0
mineral mix s10026	10.0	0.0
dicalcium phosphate	13.0	0.0
calcium carbonate	5.5	0.0
potassium citrate, 1 H20	16.5	0.0
vitamin mix v10001	10.0	40.0
choline bitartrate	2.0	0.0
FD&C yellow dye #5	0.0	0.0
FD&C blue dye #1	0.1	0.0
total	773.9	4057.0

[a]Research Diets D12492

[b]Cocoa extract or the monomer-, oligomer-, or polymer-rich fractions were added to the diet at 262.53 mg/Kg

[c]Lard contributed 72 mg cholesterol/100 g lard. The cholesterol content of the diet was 279.6 mg/kg

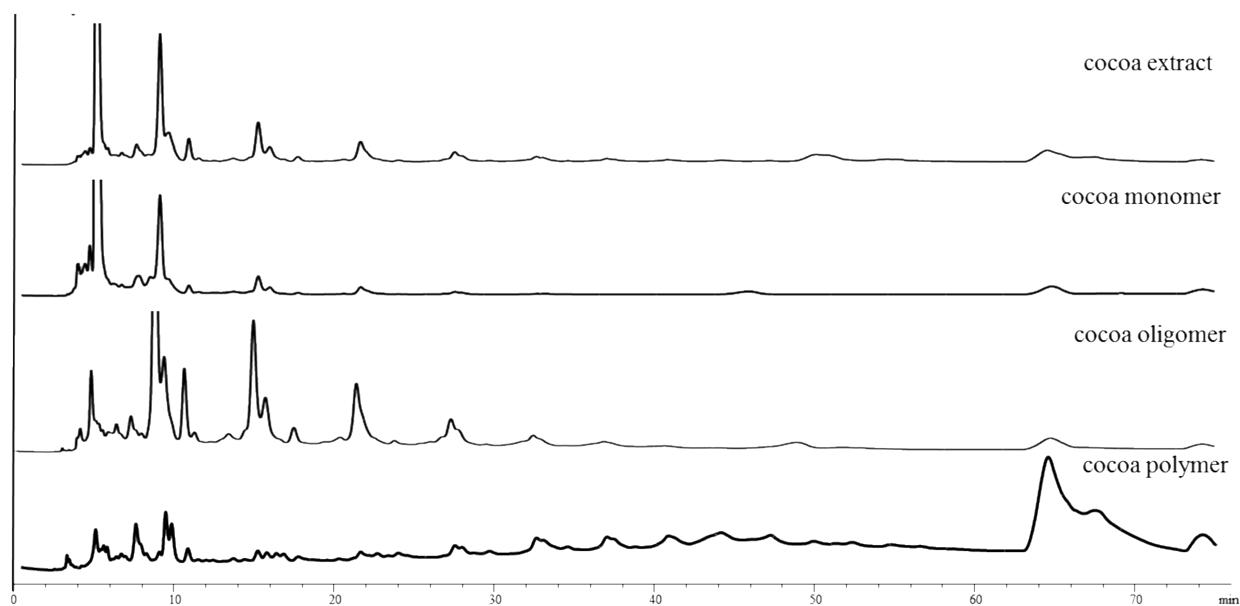


Figure B1. Fluorescence profiles of normal-phase HPLC chromatograms of the cocoa extract, monomer, oligomer, and polymer fractions used in the mouse diets. Note that the scale is different for each chromatogram (chromatograms were scaled/cropped to emphasize relative peak heights within each trace). The scale for each chromatogram in fluorescence units (LU) is as follows: CE, 230 LU; monomer-rich fraction, 160 LU; oligomer-rich fraction, 150 LU; polymer-rich fraction, 40 LU. It should be noted fluorescence chromatograms do not accurately represent relative PC composition, as relative response factors for fluorescence detection decrease rapidly with increasing DP[47]. This chromatogram was originally published in our previous paper[196].

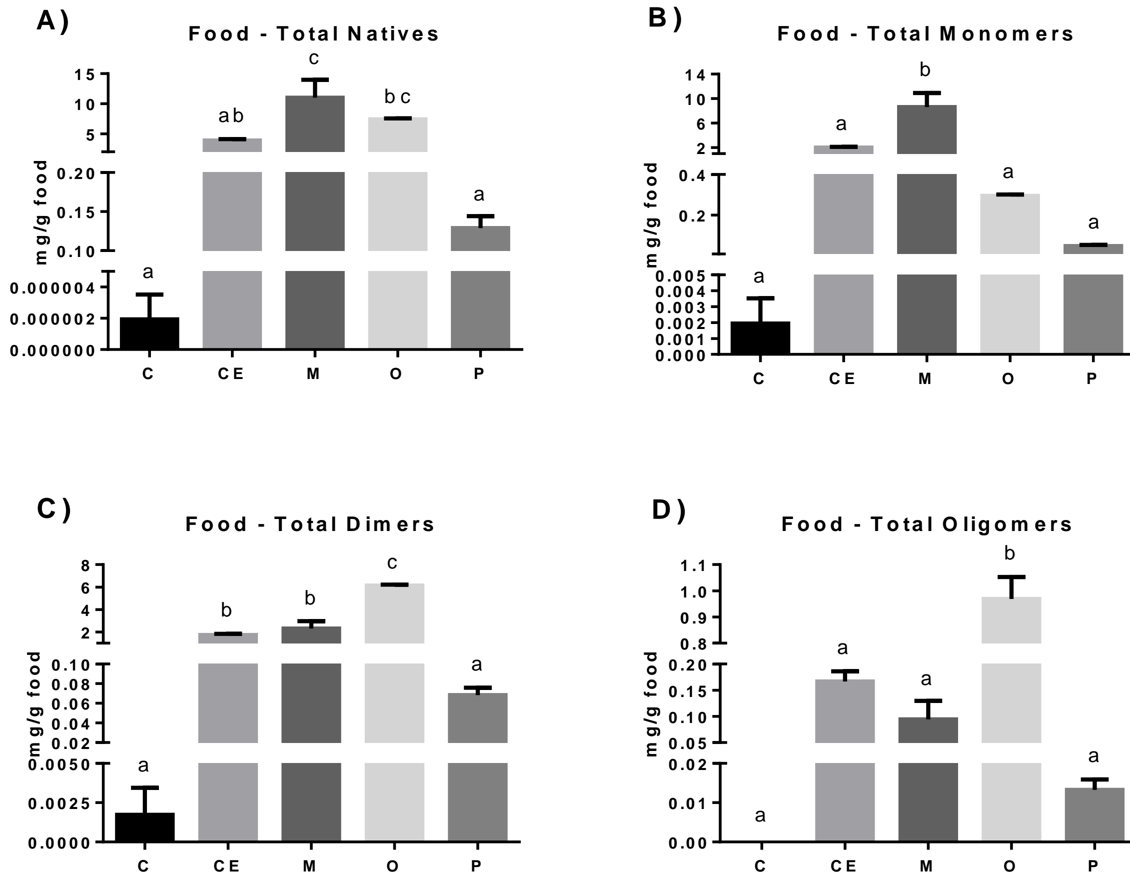


Figure B2. Levels of total native compounds (A), total monomers (B), total dimers (C), and total oligomers (D) in the formulated experimental diets fed to mice. Data are presented as mean ± SEM from n=3 replicate analyses. Bars with different letters are significantly different (one-way ANOVA with Tukey's HSD post-hoc test, significance defined as $P<0.05$). Treatments: basal control diet (C), control diet supplemented with cocoa extract (CE), control diet supplemented with cocoa monomers (M), control diet supplemented with cocoa oligomers (O), and control diet supplemented with cocoa polymers (P).

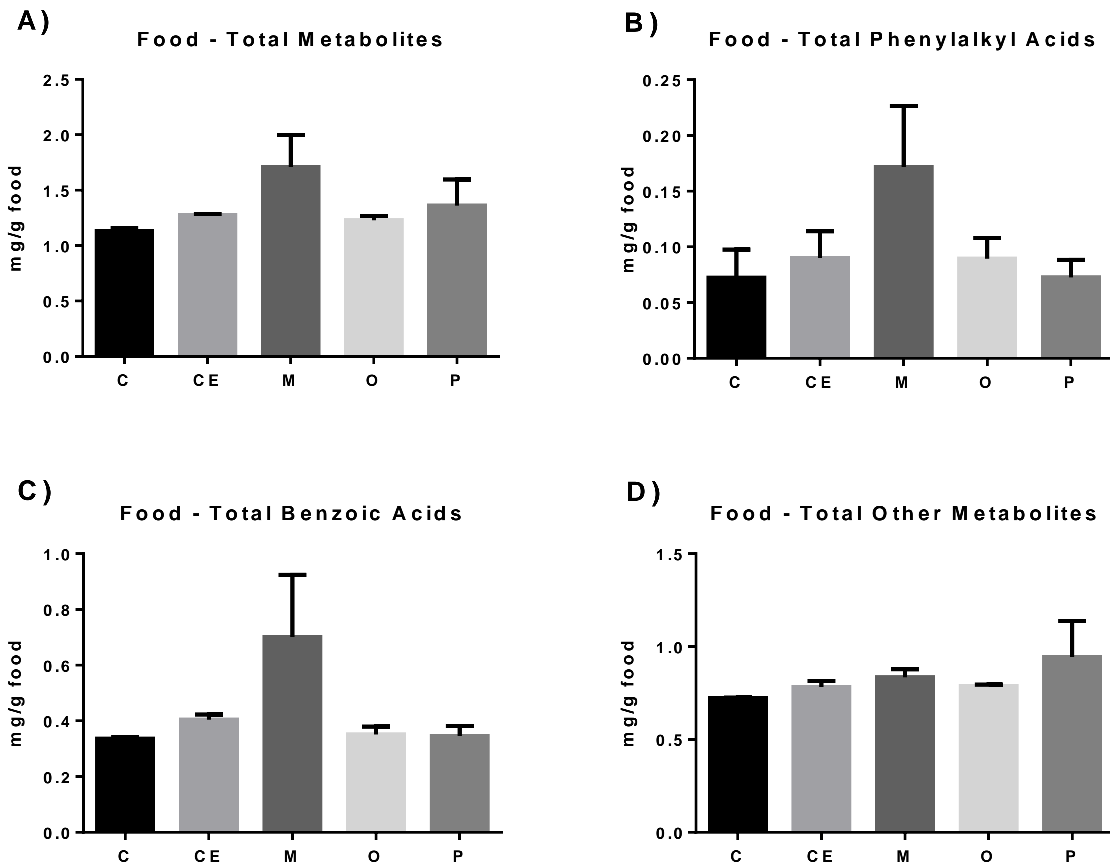


Figure B3. Levels of total metabolites (A), total phenylalkyl (phenylacetic, phenylpropionic, and phenylvaleric) acid derivatives, (B), total benzoic acid derivatives (C), and total other metabolites (D) in the formulated experimental diets fed to mice. Data are presented as mean ± SEM from n=3 replicate analyses. Bars with different letters are significantly different (one-way ANOVA with Tukey's HSD post-hoc test, significance defined as P<0.05). Treatments: basal control diet (C), control diet supplemented with cocoa extract (CE), control diet supplemented with cocoa monomers (M), control diet supplemented with cocoa oligomers (O), and control diet supplemented with cocoa polymers (P).

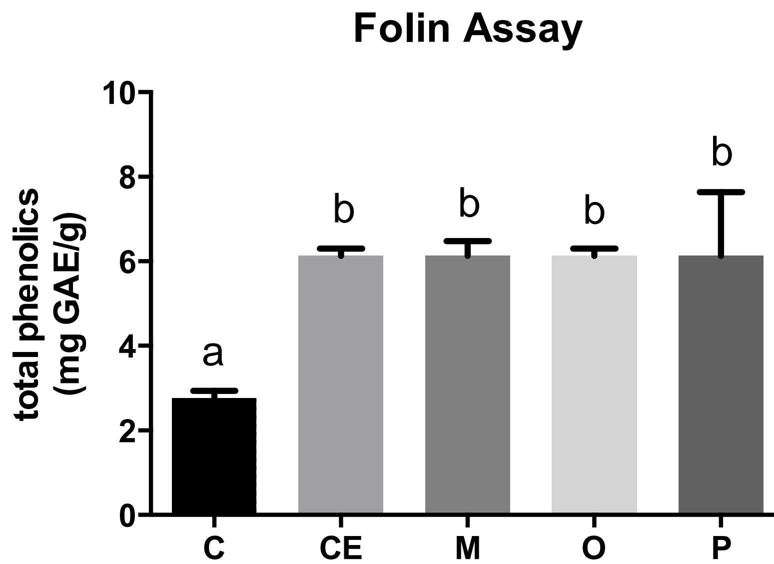


Figure B4. Total polyphenols content [expressed as mg gallic acid equivalents (GAE)/g of material] of diets as quantified by the Folin−Ciocalteu assay. Values are presented as the mean ± SEM from n=3 replicate analyses. Bars with different letters are significantly different (one-way ANOVA with Tukey's HSD post-hoc test, significance defined as P<0.05). Treatments: basal control diet (C), control diet supplemented with cocoa extract (CE), control diet supplemented with cocoa monomers (M), control diet supplemented with cocoa oligomers (O), and control diet supplemented with cocoa polymers (P).

Appendix C: Supporting Information for Chapter 6

Table C1. MS/MS Settings for MRM Detection of Native Monomers and Procyanidins

Compounds	t_R[a] (min)	MW (g mol^{-1})	$[M – H]^-$ (m/z)[b]	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
procyanidin dimer B_1	2.68	578.136	577.136	289.105	38	24
(−)-epigallocatechin	2.76	306.038	305.038	124.977	40	22
unknown dimer 1[c]	2.92	578.136	577.136	425.102	36	16
(+)-catechin	2.99	290.028	289.028	245.057	36	14
procyanidin trimer T_2	3.09	866.218	865.218	289.102	36	48
unknown dimer 2[c]	3.29	578.136	577.136	425.102	36	16
procyanidin dimer B_2	3.34	578.136	577.136	425.102	36	16
(−)-epicatechin	3.63	290.092	289.092	245.056	42	12
(−)-epigallocatechin gallate	3.67	458.038	457.038	168.982	34	16
procyanidin trimer C_1	3.82	866.218	865.218	287.085	46	32
cinnamtannin tetramer A_2	3.97	1154.808	576.404	125.020	26	34
procyanidin dimer B_2 gallate	3.99	730.164	729.164	407.129	42	32
procyanidin octamers	4.04	2307.17	1152.58	125.17	48	68
unknown dimer 3[c]	4.07	578.136	577.136	425.102	36	16
procyanidin pentamers	4.10	1442.820	720.410	125.022	26	44
procyanidin hexamers	4.23	1731.038	864.519	125.020	32	56
procyanidin nonamers	4.33	2586.36	864.12	125.17	28	46

[a]Retention time
[b]m/z values represent monoisotopic masses detected by Intellistart; all MRMs used singly-charged parent ions except for cinnamtannin tetramer A_2, pentamers, hexamers, heptamers, octamers, which were doubly-charged ($[M – 2H]^{2-}$), and nonamers and decamers, which were triply-charged ($[M – 3H]^{3-}$)
[c]Likely procyanidin dimers B_3, B_4, and either B_6, B_7 or B_8

Table C2. MS/MS Settings for MRM Detection of Microbial Metabolites

Compounds	t_R [a] (min)	MW (g mol^{-1})	$[M - H]^-$ (m/z) [b]	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
4-hydroxymandelic acid	0.88	167.958	166.958	122.968	30	10
gallic acid	0.93	169.962	168.962	124.953	30	12
pyrogallol	1.13	125.864	124.864	78.974	40	14
3,4-dihydroxybenzoic acid	1.83	153.948	152.948	108.987	28	14
3,4-dihydroxyphenylacetic acid	2.23	167.968	166.968	122.973	18	10
catechol	2.49	109.860	108.860	90.954	38	16
4-hydroxybenzoic acid	2.69	137.882	136.882	92.966	28	12
4-hydroxyphenylacetic acid	3.03	151.948	150.948	107.064	22	6
3-(3,4-dihydroxyphenyl)propionic acid	3.04	181.968	180.968	108.984	30	14
hippuric acid	3.13	178.972	177.972	133.984	30	12
vanillic acid	3.25	167.962	166.962	152.002	28	14
3-hydroxybenzoic acid	3.28	137.874	136.874	92.963	30	12
caffeic acid	3.27	179.968	178.968	134.977	34	16
homovanillic acid	3.51	181.968	180.968	136.985	24	8
1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol	3.76	292.100	291.100	247.630	30	16
3-hydroxyphenylacetic acid	3.77	151.948	150.948	107.020	30	16
3-(4-hydroxyphenyl)propionic acid	3.88	165.952	164.952	121.014	30	12
5-(3’,4’-dihydroxyphenyl)-γ-valerolactone	3.94	208.066	207.066	163.010	30	16
p-coumaric acid	4.09	163.952	162.952	119.010	28	14
3-(3-hydroxyphenyl)propionic acid	4.31	165.958	164.958	105.982	32	20
ferulic acid	4.56	193.972	192.972	133.969	32	16
m-coumaric acid	4.65	163.948	162.948	119.005	30	14
isoferulic acid	4.77	193.972	192.972	177.978	32	12
phenylacetic acid	5.13	135.942	134.942	90.956	18	6
5-(3’,4’-dihydroxyphenyl)valeric acid	5.16	210.082	209.082	191.090	30	16
5-(3’-hydroxyphenyl)valeric acid[c];	5.20	194.087	193.087	175.050	30	16
5-(4’-dihydroxyphenyl)valeric acid[c];	5.90	194.087	193.087	175.050	30	16
5-hydroxy-5-phenylvaleric acid[c]	6.37	194.087	193.087	175.050	30	16
5-(3’-dihydroxyphenyl)-γ-valerolactone[d];	5.22	192.071	191.071	147.090	30	16
5-(4’-dihydroxyphenyl)-γ-valerolactone[d]	6.19	192.071	191.071	147.090	30	16
3-phenylpropionic acid	6.40	149.982	148.982	105.016	32	10
phloretin	7.32	274.080	273.080	166.981	40	16
unknown metabolite[e]	7.72	210.082	209.082	97.120	30	16
5-phenylvaleric acid	7.97	178.032	177.032	159.086	36	14

[a]Retention time

[b]m/z values represent monoisotopic masses detected by Intellistart; all MRMs used singly-charged parent ions

[c]Isomers cannot be distinguished by retention time or MRM due to lack of authentic standards

[d]Isomers cannot be distinguished by retention time or MRM due to lack of authentic standards

[e]Isomer of 5-(3’,4’-dihydroxyphenyl)valeric acid