Biotransformation of Polymethoxyflavones by Gut Microbiome and Molecular Characterization of Polymethoxyflavones by Surface Enhanced Raman Spectroscopy

Changchu Ma
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BIOTRANSFORMATION OF POLYMETHOXYFLAVONES BY GUT MICROBIOME AND MOLECULAR CHARACTERIZATION OF POLYMETHOXYFLAVONES BY SURFACE ENHANCED RAMAN SPECTROSCOPY

A Dissertation Presented

By

CHANGCHU MA

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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September 2015

The Department of Food Science
BIOTRANSFORMATION OF POLYMETHOXYFLAVONES BY GUT MICROBIOME AND MOLECULAR CHARACTERIZATION OF POLYMETHOXYFLAVONES BY SURFACE ENHANCED RAMAN SPECTROSCOPY

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DEDICATION

To my father and mother,

Any of my families and friends,

Without whom none of my success would be possible
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ABSTRACT

BIOTRANSFORMATION OF POLYMETHOXYFLAVONES BY GUT MICROBIOME AND MOLECULAR CHARACTERIZATION OF POLYMETHOXYFLAVONES BY SURFACE ENHANCED RAMAN SPECTROSCOPY

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Polymethoxyflavones (PMFs), a unique class of flavonoids found in citrus fruits has shown beneficial bioactivities. Biological fate of PMFs in the gastrointestinal tract is critical for their bioactivities. PMFs can be biotransformed to produce various metabolites with different bioactivities, and PMFs can also interact with the food matrix, which in turn affects their bioactivities. Therefore, we investigated the biotransformation of two PMFs, namely nobiletin (NBT) and 5-hydroxynobiletin (5HN) by the gut microbiome. Mice were fed with NBT or 5HN, and small intestinal contents were collected and subjected to anaerobic fermentation by gut microbiome isolated from mice and human volunteers. HPLC analysis demonstrated that the gut microbiome largely deconjugated glucuronide and sulfate conjugates of NBT, 5HN and their metabolites and caused demethylation at certain positions of flavonoid. Moreover, the colonic microbial metabolites showed much stronger anti-carcinogenic effect than those found in the small intestine. The second part of this dissertation focuses on characterizing different PMFs, and their interaction with dietary protein by surface enhanced Raman spectroscopy.
(SERS). 3’-hydroxynobiletin (3HN), 4’-hydroxynobiletin (4HN), and 5-hydroxynobiletin (5HN) exhibited significantly different SERS behaviors after binding with silver dendrites. 5HN had the highest peak intensity, while 3HN had the lowest peak intensity. The HPLC analysis revealed that 36.13 ±1.06% of 5HN, 18.40 ± 3.31% of 4HN and 9.66 ± 0.94% of 3HN were bound to silver dendrites. We speculated that different positions of hydroxylation of PMFs were critical for different binding affinities. Furthermore, the molecular interaction of NBT and 5, 3’, 4’- trihydroxynobiletin (THN), and native and thermal denatured kappa casein, were characterized by SERS and fluorescence spectroscopy. Both SERS and fluorescence quenching studies showed that increased binding of NBT to thermal denatured casein than native casein. However, binding affinity of THN to thermal denatured casein decreased. Our results suggested that NBT interacted with kappa casein through hydrophobic interaction. THN interacted with kappa casein through hydrogen bonding and hydrophobic interaction, and hydrogen binding was the driving force. This dissertation demonstrates the significance of the microbial biotransformation of PMFs in the colon, and the feasibility of SERS to characterize PMFs and their interactions with protein.
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CHAPTER 1
INTRODUCTION

Flavonoids have long been believed to display health-promoting effects on human health. Abundant in fruits and vegetables, more than 4000 flavonoid structures have been identified to date.\(^1\) So far, there are more than 20 polymethoxylated flavonoids being isolated and identified from different tissues of citrus plants, including hydroxylated polymethoxyflavones, polymethoxyflavones, polymethoxyflavanones, and polymethoxylchalcone.\(^2\) Different varieties of citrus species contain different types and contents of polymethoxylated flavonoids. Almost exclusively found in citrus peels, polymethoxyflavones (PMFs), a unique class of flavonoid, has been of particular interest as they have been demonstrated to ameliorate human health problems by revealing a broad spectrum of biological activities including anti-inflammatory, anti-carcinogenic, anti-atherogenic, antioxidant, and antimicrobial properties.\(^3\)–\(^16\)

The beneficial biological properties of dietary flavonoids are believed to correlate to its absorption, distribution, metabolism and excretion (ADME) in the human body. Therefore, biotransformation plays important roles in the biological activities of orally ingested bioactive compounds. Liver cytochrome P-450 (CYP) isozyme is the key enzyme observed to metabolize PMFs.\(^17\),\(^18\) The \textit{in vivo} animal metabolism study of nobiletin, major PMF isolated from orange peels, reported that 3\(^\prime\)-hydroxynobiletin, 4\(^\prime\)-hydroxynobiletin and 3\(^\prime\), 4\(^\prime\)-dihydroxynobiletin were major metabolites in their tissues and urine after mice or rats were orally administrated nobiletin.\(^19\)–\(^22\) Recently, Zheng
identified the mouse urine metabolites of 5-hydroxynobiletin, as 5, 3’-dihydroxynobiletin, 5, 4’-dihydroxynobiletin and 5, 3’, 4’-trihydroxynobiletin.\textsuperscript{23} Furthermore, stronger bioactivities, such as anti-inflammatory and anti-carcinogenic activity, of metabolites were revealed than their counterparts compounds.\textsuperscript{22,23}

However, most biotransformation studies of dietary flavonoids were focused on the phase I and phase II metabolism in the small intestine and liver. The colonic microbial metabolism of dietary flavonoids is insufficient and less studied. The gut microbes not only contribute to normal digestive function by fermenting unabsorbed nutrients in the upper gastrointestinal tract, but also metabolize xenobiotics, such as dietary flavonoids, drugs, with their respective enzymes. Furthermore, synthesis of B complex vitamins including thiamine, riboflavin, vitamin B\textsubscript{12}, and vitamin K is also correlated with the gut microbiome.\textsuperscript{24} Therefore, the gut microbiome has the ability to act as an organ with more biochemical conversion than, or at least equal to the liver.\textsuperscript{25,26}

The major differences between hepatic and microbial metabolism is that the liver is primarily responsible for metabolism via oxidation and conjugation producing polar high molecular weight metabolites, while the gut microbiome is involved in reduction and deconjugation generating non-polar low molecular weight metabolites. Those microbial metabolites are often better absorbed than the counterpart compounds due to the mechanism of absorption and the large absorptive area available in the gut.\textsuperscript{27} Some microbial metabolites of isoflavones, equol, showed stronger bioactivities than isoflavones. So far, no group has studied and reported the biotransformation of PMFs by the gut microbiome. There is a significant gap in our understanding of the microbial metabolites of PMFs and
how does the biotransformation by the gut microbiome affects biological activities of PMFs.

The biological activities of PMFs isolated from citrus fruits particularly attract the attention, and also their numerous metabolites, characterization of different structure PMFs is important to further applications. Moreover, in the real world, those bioactive components coming from fruits and vegetables are consumed with food. Many potential health benefits and biological fate of flavonoids have been associated with their ability to interact and bind to macronutrients in foods, such as protein, carbohydrates and lipids. Among those interactions, flavonoid–protein interactions are a known phenomenon; those interactions not only play critical roles in food and beverage quality, but also the bioavailability and bioactivity of flavonoids. Particularly, whether the flavonoids–protein interactions increase or decrease or have no effect on bioactivities and bioavailabilities of flavonoids is still debating. The mechanism behind is very complicated, which depends on the different structure of flavonoids and proteins, concentrations, pH, ionic strength and so on. Therefore, molecular characterization of interaction between PMFs and food macronutrients further help us understand how the interactions affect the bioactivities of PMFs

Surface enhanced Raman spectroscopy (SERS) is a novel analytical method that combines Raman spectroscopy and nanotechnology. Raman spectroscopy is a vibrational spectroscopy providing rich characteristic information of molecular structure. It is caused by the interaction between energy change of inelastically scattered incident light and
vibrational mode of chemical bonds within samples. However, only 1 in $10^{6-8}$ of photons undergoes Raman scattering, hence, the Raman scattering signal is relatively weak. Placing samples on or near the noble metal nanostructure, e.g. silver, gold or copper, can enhance the weak Raman scattering more than a million times through the amplification of electromagnetic fields. Raman spectroscopy and IR spectroscopy provide complementary information of chemical structure. Interference of water in Raman spectroscopy is smaller than IR spectroscopy, at the same time, the presence of metallic nanoparticles depress the fluorescence interference, and with its highly sensitive structural detection of trace amounts of analytes, SERS has been rapidly developed as a powerful tool for molecular detection and characterization, with applications in biology, medicine, materials science, biosensor and electrochemistry.\textsuperscript{36–38} SERS has been used to study ligand–protein interactions by dye-labeling methods and label-free methods. The specially designed Raman reporter labeled silver or gold nanoparticle is required for dye-labeling methods, and the detection of Raman reporter signals indicates the interaction between ligand and protein.\textsuperscript{39–41} The label-free methods are based on the intrinsic Raman signals of ligand and protein. The changes in the Raman spectra of ligand protein incubation, either peak shifts or intensity, are a direct indication of ligand-protein interaction. The assignment of Raman peak also provides information of functional group involving in the interaction.

Taken together, we have focused on two significant gaps that currently exist in the study of polymethoxyflavones, 1) the biotransformation of PMFs by the gut microbiome; 2) the interaction of PMFs with the food matrix. Surface enhanced Raman spectroscopy is
introduced to molecularly characterize the PMFs and interactions between PMFs and protein. To achieve those goals, the following specific aims were pursued:

**Aim 1: Investigate the biotransformation of PMFs by the mouse and human gut microbiome.** PMFs are anaerobically fermented by mouse and human gut microbes, and their metabolites will be determined using HPLC. A cell viability assay will be utilized to evaluate the inhibitory effects of microbial metabolites. We anticipate observing PMFs are biotransformed by gut microbiome, probably by deconjugation, and microbial metabolites exhibit stronger bioactivity than parent compounds.

**Aim 2: Characterize monohydroxylated-PMFs by surface enhanced Raman spectroscopy.** The position of the hydroxyl group in PMFs on the interaction with silver (Ag) dendrites (SERS substrate) and its relationship with the SERS enhancement and characterization is elucidated by SERS. HPLC is conducted to evaluate the binding percentage of monohydroxylated-PMFs to Ag dendrites. It is our expectation that SERS will enable us to characterize structure related PMFs, and the different monohydroxylated-PMFs will exhibit different SERS enhancement.

**Aim 3: Characterize the molecular interaction between PMFs and kappa casein by surface enhanced Raman spectroscopy.** The molecular interaction between different structure PMFs and different state of casein (native vs. thermal denatured) are characterized and quantified by SERS and fluorescence.
spectroscopy, respectively. We anticipate that both SERS and fluorescence studies will show consistent results that the interaction between PMFs and casein is influenced by their structure, and also propose a mechanism, such as possible bonding involved in the interaction.
CHAPTER 2
LITERATURE REVIEW

2.1 Polymethoxyflavones

2.1.1 Introduction to polymethoxyflavones

Accumulated epidemiological evidence has consistently indicated that diets abundant in fruits and vegetables may have potential health-promoting effects, including anti-inflammatory, antioxidant, antiallergic, antiestrogenic, antithrombotic, anticarcinogenic activities, cardioprotective and hepatoprotective effects, and this effect has been attributed to bioactive components present in these foods.\(^1,47,48\) Flavonoids are universally found in plants, and are recognized as the pigments responsible for the colors. They are also rich in seeds, citrus fruits, olive oil, tea, red wine, and coffee.

According to the National Agricultural Statistics Service, there has been rapid growth of world citrus production during the last three decades. Production rose from 57.8 million tons in the 1980’s to 115.5 million tons in 2010-2011. China, Brazil and the United States are responsible for three countries produce about half of citrus production in the world. Orange production accounts for 70.7 million tons, then followed by tangerine (25.6 million tons), lemon and limes (12.9 million tons), and grapefruit (6.4 million tons). Citrus fruits are processed, mainly to obtain juice, but also, they are used in the canning industry, and as a source of extract flavonoids and essential oils. During citrus fruits processing, approximately 50% of thick inedible leathery peels is yielded as byproducts.\(^49\) Orange peel has been widely used as a traditional medicine for relieving
stomach upset, skin inflammation, muscle pain, and ringworm infections in some countries. Its major constituents include flavonoids, mainly polymethoxylated flavonoids; terpenoids, such as limonene and linalool; and other volatile oils. Among these, polymethoxyflavones (PMFs) are a unique class of flavonoids, and almost exclusively exist in the citrus genus, particularly in the peels of sweet oranges (C. sinensis) and mandarin oranges (C. reticulata).

2.1.2 Biological activities of polymethoxyflavones

The reason citrus flavonoids, especially polymethoxyflavones (PMFs), have been received particular interest is many of these flavonoids pose a broad spectrum of biological activity, including anti-inflammatory, anti-carcinogenic, anti-atherogenic, antioxidant, and antimicrobial properties. Recent studies have revealed that some hydroxylated PMFs exerted more potent biological activities than those of their methoxylated counterparts, including anti-inflammation and anti-carcinogenesis.

Accumulating findings indicate that PMFs exhibit their anti-inflammatory activity by inhibiting enzymes involved in inflammation. PMFs have a wide range of suppressive activities toward free radical generation in enzymatic and cellular systems, and some of them may be important sources of effective free radical generation suppressors. Nobiletin suppressed the interleukin (IL)-1-induced production of prostaglandin E2 (PGE2) in human synovial cells, and selectively downregulated COX-2 mRNA expression. It also interfered with the lipopolysaccharide-induced production of PGE2 and the gene expression of proinflammatory cytokines including IL-1α, IL-1β, TNF-α.
and IL-6 in mouse J774A.1 macrophages. In addition, nobiletin downregulated the IL-1-induced gene expression and production of proMMP-1/procollagenase-1 and proMMP-3/prostromelysin-1 in human synovial fibroblasts.\textsuperscript{16} Guo also reported that nobiletin showed synergistic anti-inflammatory effects with sulforaphane studied in lipopolysaccharide-stimulated (LPS) RAW 264.7 cells. The combination of nobiletin and sulforaphane produced much stronger inhibitory effects on the production of nitric oxide, and decreased iNOS and COX-2 protein expression levels and induced heme oxygenase-1 (HO-1) protein expression. It also suppressed LPS-induced upregulation of IL-1 mRNA levels and increased HO-1 mRNA levels.\textsuperscript{52}

Numerous \textit{in vitro} and \textit{in vivo} studies have shown protective effects of PMFs against carcinogenesis. Research showed that anti-carcinogenic activities of PMFs included anti-proliferation, induction of cell cycle arrest, induction of cellular apoptosis, inhibited angiogenesis, inhibition of cell mobility, induction of cell differentiation, and inhibition of metastasis cascade. For example, nobiletin and tangeretin induced G1 cell cycle arrest in human breast and colon cancer cells.\textsuperscript{53} In human lung cancer H1299 cells, cell cycle analyses further revealed that hydroxylated PMFs caused significant increase in sub-G0/G1 phase in the H1299 cells, and downregulated iNOS, COX-2, Mcl-1, and K-ras, as well as induced apoptosis evidenced by activation of caspase-3 and cleavage of PARP.\textsuperscript{5} In an animal study, hydroxylated PMFs was administrated to azoxymethane treated mice, and the results showed that hydroxylated PMFs suppressed colonic aberrant crypt foci (ACF) and tumor formation through down-regulation of Wnt/\(\beta\)-catenin and epidermal growth factor receptor (EGFR) signaling pathways.\textsuperscript{12}
Citrus flavonoids have been shown to help reduce cardiovascular disease symptoms as they were capable to influence and reduce hepatic production of cholesterol containing lipoproteins which resulted in the decreased levels of total cholesterol concentration in plasma.\textsuperscript{54} Whitman’s study indicated that nobiletin was able to diminish the circulating concentrations of very low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs) in the blood, and also could directly inhibit macrophage-derived foam-cell formation at the site of the lesion within a vessel wall by modulating macrophage metabolism of the specific class A scavenger receptor (SR-A) ligand, and acetylated LDL.\textsuperscript{55}

Nobiletin, 5- hydroxynobiletin and tangeretin exhibited antimicrobial activities against \textit{Bacillus cereus, Bacillus coagulans, Bacillus subtilis, Staphylococcus aureus, Escherichia coli} and \textit{Pseudomonas aeruginosa}. Moreover, their antimicrobial activities to Gram-positive stains was stronger than Gram-negative.\textsuperscript{7} \textit{In vitro} antibacterial and antifungal activities of several polymethoxychalcones was reported by Rostom, including activity against \textit{S. aureus} and \textit{B. subtilis} as Gram-positive bacteria; \textit{E. coli} and \textit{P. aeruginosa} as Gram-negative bacteria. The antimicrobial activity was correlated to structural variations and modifications of those polymethoxychalcones. One polymethoxychalcone they synthesized was four times superior to ampicillin against \textit{P. aeruginosa}.\textsuperscript{8}
2.1.3 Chemistry, isolation and identification of polymethoxyflavones

Over 4000 structurally unique flavonoids have been identified in plants. Flavonoids are low molecular weight compounds, consisting of phenylbenzo-pyrones (phenylchromones) with an assortment of structures arranged in a C6–C3–C6 configuration. Essentially, the structure consists of two benzene rings, A and B, linked through a heterocyclic pyran or pyrone ring, C, in the middle. The benzene ring A is derived from the acetate/malonate pathway, while ring B is derived from phenylalanine via the shikimate pathway. Flavonoids are usually subdivided into 12 subclasses, e.g. flavonols, flavones, flavanones, flavanols (or catechins), isoflavones, flavanonols, chalcone, and anthocyanidins.

Polymethoxyflavones are a unique class of dietary flavonoids bearing two or more methoxyl groups on their basic heterocyclic C₆–C₃–C₆ skeleton with a carbonyl group at position 4. So far, more than 20 polymethoxylated flavonoids have been isolated and identified from different tissues of citrus plants, including hydroxylated PMFs, PMFs, polymethoxyflavanones, and polymethoxychalcone. (Figure 2.1) The types and contents of PMFs vary among different varieties of citrus species.
Figure 2.1 Hydroxylated PMFs, PMFs, polymethoxyflavanones, and polymethoxychalcones isolated from sweet orange peel.²

Owing to the particularly interesting biological activities of PMFs, separation, identification and characterization of PMFs have been studied. PMFs are mainly isolated and separated from citrus fruit peels,⁵⁸–⁶⁵ oil,⁶⁶–⁶⁹ juice,⁷⁰ leaves,⁶³,⁷¹ and mouse urine after administrated PMFs²⁰,²¹,²³,⁷² by thin-layer chromatography (TLC),⁵⁸ capillary
electrochromatography (CEC), \textsuperscript{66} flash column chromatograph,\textsuperscript{2,62,65,73} supercritical fluid chromatography (SFC),\textsuperscript{21,60,67,72} gas chromatography (GC),\textsuperscript{68} and high pressure liquid chromatograph (HPLC).\textsuperscript{2,20,23,59,61–64,69–71,74–76} HPLC is the most commonly used separation method for PMFs. Some research groups reported the use of reversed phase column for separation.\textsuperscript{2,23,61,63,64–76} The detectors used including photodiode array detectors,\textsuperscript{20,62,64,69} refractive index detectors,\textsuperscript{71} with UV-detectors\textsuperscript{2,21,59,61,63,74,75} being the most commonly used. Recently, an electrochemical detector was also reported to detect 5-hydroxy polymethoxyflavones, and it exhibited over 160 times higher sensitivity in comparison with a previously reported method using UV detection.\textsuperscript{23,76} Mass spectrometry (MS) and nuclear magnetic resonance (NMR) are widely used after separation by chromatography for identification of PMFs.\textsuperscript{2,21,23,61,62,65,68,69,71,73,77}

2.1.4 Metabolism of polymethoxyflavones

The beneficial biological properties of dietary flavonoids are believed to correlate to their absorption, distribution, metabolism and excretion (ADME) in the human body. Even though the ADME of dietary flavonoids is not well understood, considerable information is available regarding the metabolism of dietary flavonoids in animals and to a very limited extent in humans. Generally speaking, the dietary flavonoids may exist in the food as flavonoids glycosides, polymers. Therefore, the first step upon ingestion is release of dietary flavonoids from their food matrix by deglycosylating and cleaving those compounds to aglycones. This step is prerequisite for absorption through the intestinal barrier. Aglycones, monomers to trimers of flavonoids can rapidly be absorbed by enterocytes through passive diffusion. During intestinal absorption and passage in the
liver, dietary flavonoids undergo phase I and phase II metabolism. Phase I metabolism mainly involves oxidation, reduction, and hydroxylation. It introduces reactive and polar group into their substrates, e.g. hydroxylation catalyzed by the cytochrome P-450 (CYP). Phase II metabolism mainly involves sulphation which is catalyzed by sulfotransferases (SULT), glucuronidation which is catalyzed by uridine-5′-diphosphate glucuronosyltransferases (UGT), and methylation which catalyzed by catechol-O-methyl transferase (COMT). Partial aglycones, phase I and phase II metabolites enter into systemic circulation, tissue disposition, and excreted by urine. The dietary flavonoids that are unabsorbed in the small intestine, as well as those compounds absorbed and metabolized in the liver, and excreted with bile enter into the colon. The gut microbiome hydrolyze the unabsorbed glycosides and cleave the conjugates (glucuronides and sulfates) to aglycone, then break larger aglycones to simpler molecules in the colon. The microbial metabolites are either excreted by feces, or re-excreted via bile and can undergo enterohepatic circulation. Kemperman summarized ADME of dietary flavonoids, and illustrated the metabolic fate of dietary flavonoids in the Figure 2.2.
Figure 2.2 Metabolic fate of dietary flavonoids. Partial absorption of flavonoids occurs in the small intestine, with modification by phase I and phase II enzymes, excretion with bile back into the small intestine, deconjugation by microbial enzymes and microbial conversion of the flavonoids in the colon, absorption of microbial metabolites or fecal excretion, deconjugation and excretion via bile or urine.78
Compared to other dietary flavonoids, the metabolism of PMFs is less studied. However, there is evidence that PMFs undergo biotransformation in vivo and produce metabolites with different bioactivities and pharmacological properties. Therefore, the metabolism and identification of metabolites of PMFs are of special interest. Like other dietary flavonoids, the interaction between PMFs and liver cytochrome P-450 (CYP) isozymes has been observed. CYP is the key enzyme system involved in metabolism of PMFs, and is capable of catalyzing hydroxylation and demethylation reactions.

In an in vitro experiment, when tangeretin was incubated with Aroclor-induced rat liver microsomes, the major metabolites were identified as 4′-dihydroxynobiletin, 3′, 4′-dihydroxynobiletin and 5, 4′-dihydroxytangeretin. However, in another in vitro metabolism study with human CYP, four of five metabolites were demethylated at position 4′, and the major metabolite was 4′-demethyltangeretin. Interestingly, 5, 6-dihydroxynobiletin was found to be a second abundant metabolite. In the in vivo biotransformation study of tangeretin by repeatedly gavage feeding on rats, 10 metabolites were identified. Among 10 identified metabolites, 7 metabolites were demethylated at position 4′ including the major metabolites, 4′-demethyltangeretin and 3′, 4′-dihydroxynobiletin. Position 6 was found to be the second most frequent site for demethylation. Hence, it can be concluded that position 4′ of tangeretin is the primary site, and position 6 is a second primary site for demethylation by COMT. Also, urine analysis determined that 38% of the tangeretin metabolites were excreted as conjugates.
In vitro nobiletin metabolism using rats, pigs and hamster liver microsomes with 12 recombinant rat CYPs, demonstrated that nobiletin was metabolized to 4′-hydroxynobiletin, 7- hydroxynobiletin, 6- hydroxynobiletin, 3′,4′-di hydroxynobiletin and 6,7-di hydroxynobiletin. CYP2C11, CYP2D1, CYP3A1, CYP3A2 and CYP2C12 are responsible for the demethylation at position 6, 7, 3′ and 4′; while CYP1A1 and CYP1A2 preferentially catalyzed demethylation at position 3′ and 4′. Four years later, the same group reported in vitro metabolism of nobiletin by human liver microsomes and cytochrome P450. Human liver microsomes containing CYP enzymes catalyzed nobiletin to 4′- hydroxynobiletin, 7- hydroxynobiletin, and 6- hydroxynobiletin with a relative ratio of 1:4.1:0.5, respectively. 7- hydroxynobiletin is the major metabolite by human liver microsomes. Human CYP1A1, CYP1A2 and CYP1B1 showed the highest activity for demethylation of nobiletin at the position 4′, whereas human CYP3A4 and CYP3A5 were the key enzymes involved in the demethylation of nobiletin at 7- and 6-positions.

In vitro absorption of nobiletin using Caco-2 cell as model showed that nobiletin preferentially accumulated in a differentiated Caco-2 cell monolayer. In vitro metabolism study of nobiletin by treating nobiletin with rat liver S-9 mixture showed that after 24 hours treatment, only 7.0% of nobiletin was metabolized to metabolites, and 3′-hydroxynobiletin was the major metabolite.

An in vivo animal metabolism study of nobiletin was conducted by Murakami, Yasuda, and Li. Nobiletin was orally administrated to mice or rats, their tissue and urine were collected. Murakami investigated the tissue distribution of nobiletin, and found that nobiletin accumulated in a wide range of organs including the stomach, small
and large intestines, liver, and kidney during a 1-to 4-hour period after a single dose. The observed localization of nobiletin in mucous membranes has been due to its high molecular hydrophobicity. Moreover, 3’- hydroxynobiletin was detected as a major metabolite in serum and urine. Subsequently, both Yasuda and Li reported that 4’-hydroxynobiletin was a major metabolite, while Li also reported 3’-hydroxynobiletin, and 3’, 4’-dihydroxynobiletin were minor metabolite. Recently, Zheng identified the mouse urine metabolites of 5- hydroxynobiletin as 5, 3’-di hydroxynobiletin, 5, 4’-dihydroxynobiletin and 5, 3’, 4’-trihydroxynobiletin. Similarly, 5, 4’-dihydroxynobiletin was a major urinary metabolite. Thus, animal studies have shown the methyl groups on the B ring of nobiletin and 5- hydroxynobiletin, and particularly 4’-methyl group, are easily removed by methyl transferase enzymes in mice or rats. When Li and Zheng treated mice urine samples with β-D-glucuronidase and sulfatase, increased level of those metabolites were suggesting that the free hydroxyl groups after demethylation could be deconjugated by deconjugation enzymes such as glucuronidases or sulfates.
2.2 Gut microbiome

2.2.1 Introduction to the gut microbiome

The gastrointestinal tract is populated with 100 trillion ($10^{14}$) microbes which makes the human gastrointestinal tract one of the most populated microhabitats on earth. It has been generally accepted that it contains 500 to 1,000 species. In terms of genetic diversity, the gut microbiome surpasses the human genome by 100 fold. The gut microbiome composition of each individual is unique and develops under key influences of genotype, physiological status of the host, diet, and environment. The human gastrointestinal tract (GIT) is sterile at birth, and colonization of the GIT of neonates starts shortly after birth. Most of this inoculum is derived largely from the mother’s vaginal and fecal microbiome if delivered by conventional birth or from environment if delivered by caesarean delivery. Interestingly, the composition of the GIT microbiome of a 1 years old child is essentially the same as that of an adult. The GIT includes mouth, pharynx, esophagus, stomach, small intestine and large intestine. The cecum, appendix, colon, rectum and anal canal comprise the large intestine. Moving along these different sections of the GIT, like a pyramid, there is a progressive increase in both numbers and species towards the ileocecal junction to the large intestine. Up to $10^{11} – 10^{12}$ CFU/g is estimated in the large intestine. Figure 2.3 summarized spatial and temporal aspects of GIT microbiome composition.
Figure 2.3 Spatial and temporal aspects of GI tract microbiome composition. **A**: variations in microbial numbers and composition across the length of the gastrointestinal tract. **B**: longitudinal variations in microbial composition in the intestine. **C**: temporal aspects of microbiome establishment and maintenance and factors influencing microbial composition.84

The gut microbiome contribute to normal digestive function, and ferment unabsorbed nutrients, such as carbohydrates and proteins in the upper GIT. Moreover, these microbes and their respective enzymes are able to metabolize xenobiotics, such as dietary flavonoids, drugs, far more extensively than any other part of the body. The gut microbiome also play an important role in synthesis of B complex vitamins including thiamine, riboflavin and vitamin B12, and vitamin K.24 Therefore, the gut microbiome has the ability to act as an organ with more biochemical conversion than, or at least equal to the liver.25,26
Members of the human gut microbiome are key players in maintaining human health and well-being. They are implicated in developmental, immunological, and nutritional host functions and have thus a profound impact on human health far beyond the fermentation of non-digestible food compounds. In turn, distortions in gut microbial community structure and/or functionality are thought to cause several intestinal diseases.  

2.2.2 Dietary flavonoids - gut microbiome interaction

2.2.2.1 Microbial metabolism of dietary flavonoids

The diverse gut microbiome plays a critical role in the metabolism of dietary flavonoids. The overall metabolism of dietary flavonoids is discussed in the section 2.1.4. Most human intervention studies on the ADME of dietary flavonoids focused on the phase I and phase II metabolism in the small intestine and liver. However, the microbial metabolism of dietary flavonoids is insufficient. The major differences between hepatic and microbial metabolism is that the liver is primarily responsible for metabolism via oxidation and conjugation producing polar high molecular weight metabolites, while the gut microbiome is involved in reduction and deconjugation generating non-polar low molecular weight metabolites. Those microbial metabolites are often better absorbed than the parent compounds due to the mechanism of absorption and the large absorptive area available in the colon.  

In general, the gut microbes generate O- and C-deglycosidases and hydrolases to cleave the unabsorbed glycosides and polymers from the small intestine. The release of aglycone and oligomers enhances their absorption. Secondly, microbial enzymes, such as
glucuronidase and sulphatase, deconjugate the phase II metabolites. After deconjugation, there are two possible routes available, one is reuptake of the intact dietary flavonoids through the colonocytes and passage into the bloodstream (as free or conjugated forms) and liver. The other route is breakdown of the dietary flavonoids structure into smaller metabolites. The aglycones may be degraded by gut microbiome to produce simpler phenolic compounds by backbone rupture producing phloroglucinol (derived from A ring), and hydroxylated forms of phenylacetate or phenylpropionate are obtained, derived from the B ring. For example, quercetin gives 2-(3,4-dihydroxyphenyl)acetic acid, 2-(3-hydroxyphenyl)acetic acid, and 3,4-dihydroxybenzoic acid from the B ring, whereas phloroglucinol, 3-(3,4-dihydroxyphenyl)propionic acid, and 3-(3-hydroxyphenyl)propionic acid are produced from the A ring. Naringenin gives 3-(4-hydroxyphenyl)propionic acid and phloroglucinol. Dehydroxylation, demethoxylation and demethylation, and isomerization of both intermediates and end products can occur at various points in the pathway. So far, only a limited number of bacterial species have been identified as being involved in the metabolism of dietary flavonoids. Table 2.1 summarizes an overview of the metabolites produced from known flavonoids by gut microbiome strains.
Table 2.1 The gut microbiome involved in the metabolism of certain flavonoids.

<table>
<thead>
<tr>
<th>flavonoid</th>
<th>metabolites</th>
<th>gastrointestinal microbiota</th>
</tr>
</thead>
<tbody>
<tr>
<td>rutin</td>
<td>3,4-dihydroxybenzyldehydro; 3,4-dihydroxyphenylacetate; phloroglucinol</td>
<td>Butyribrio sp. C3; Eubacterium ramulus</td>
</tr>
<tr>
<td>quercetin-3-</td>
<td>quercetin; glucose; acetate; lactate; formate; ethanol; 3,4-dihydroxyphenylacetae; phloroglucinol; butyrate</td>
<td>Enteroococcus caseilflavus; Eubacterium ramulus</td>
</tr>
<tr>
<td>quercetin</td>
<td>taxifolin; alpinin; 3,4-dihydroxyphenyllpyruvate; 3,4-dihydroxyphenylacetilpyruvate; 3,4-dihydroxyphenylacetate; phloroglucinol; acetate; butyrate</td>
<td>Flavonfractor plantii; Eubacterium ramulus; Eubacterium oxidoreducens; Clostridium spp</td>
</tr>
<tr>
<td>(+)-taxifolin</td>
<td>3,4-dihydroxyphenylacetate; alpha,2',3,4',6'-hexahydroxydihydrochalkone</td>
<td>Flavonfractor plantii; Eubacterium sp. SDG-2</td>
</tr>
<tr>
<td>luteolin-7-</td>
<td>3-(3,4-dihydroxyphenyl)propionate</td>
<td>Eubacterium ramulus</td>
</tr>
<tr>
<td>luteolin</td>
<td>eriodictyol; 3-(3,4-dihydroxyphenyl)propionate; phloroglucinol</td>
<td>Flavonfractor plantii; Eubacterium ramulus</td>
</tr>
<tr>
<td>apigenin</td>
<td>3-(4-hydroxyphenyl)propionate; phloroglucinol</td>
<td>Flavonfractor plantii; Clostridium sp; Clostridium scindens; Eubacterium demolans</td>
</tr>
<tr>
<td>naringenin</td>
<td>3-(4-hydroxyphenyl)propionate; phenylacetate; phloroglucinol</td>
<td>Clostridium sp; Eubacterium ramulus</td>
</tr>
<tr>
<td>kaempferol</td>
<td>4-hydroxyphenylacetate</td>
<td>Flavonfractor plantii; Eubacterium ramulus</td>
</tr>
<tr>
<td>eriodictyol</td>
<td>3-(3,4-dihydroxyphenyl)propionate; 3-(4-hydroxyphenyl)propionate</td>
<td>Flavonfractor plantii; Eubacterium ramulus</td>
</tr>
<tr>
<td>phloretin</td>
<td>3-(4-hydroxyphenyl)propionate; phloroglucinol</td>
<td>Eubacterium limosus</td>
</tr>
<tr>
<td>formononetin</td>
<td>genistein</td>
<td>Eubacterium ramulus</td>
</tr>
<tr>
<td>genistein</td>
<td>6'-hydroxy-O-desmethylangolensin; 2-(4-hydroxyphenyl)propionate; phloroglucinol</td>
<td>Eubacterium ramulus; Clostridium spp. H19136; Egerthella spp. Julong732; Clostridium-like strain TM-40; Slades spp. D2E; Egerthella spp. YY7918; Lactobacillus mucosae EPI2; Enteroococcus faecium EPI1; Bregildae magna EPI3; Vellonella spp. EP</td>
</tr>
<tr>
<td>biochan A</td>
<td>daidzein</td>
<td>Eubacterium limosus</td>
</tr>
<tr>
<td>daidzein</td>
<td>dihydrodaidzein; O-desmethylangolensin; tetrahydrodaidzein; 2,3-dihydroneol; equol</td>
<td>Eubacterium ramulus; Clostridium spp. H19136; Egerthella spp. Julong732; Clostridium-like strain TM-40; Slades spp. D2E; Egerthella spp. YY7918; Lactobacillus mucosae EPI2; Enteroococcus faecium EPI1; Bregildae magna EPI3; Vellonella spp. EP</td>
</tr>
<tr>
<td>glycitein</td>
<td>6,7,4'-trihydroxyisoflavone</td>
<td>Eubacterium limosus</td>
</tr>
<tr>
<td>(+)-catechin</td>
<td>(28)-1-(3',4-dihydroxyphenyl)-3-(2',4',6'-trihydroxyphenyl)propan-2-ol</td>
<td>Egerthella lenta rK3; Flavonfractor plantii aK2; Clostridium coccosides – Eubacterium rectale group; Bifidobacterium spp.; Escherichia coli</td>
</tr>
<tr>
<td>(+)-epicatechin</td>
<td>5-(3',4'-dihydroxyphenyl)-4-valerolactone; 5-phenyl-4-valerolactone; phenylpropiolic acid; 4-hydroxy-5-(3,4-dihydroxyphenyl)valeric acid</td>
<td>Eubacterium limosus</td>
</tr>
<tr>
<td>(-)-catechin</td>
<td>(25)-1-(3',4-dihydroxyphenyl)-3-(2',4',6'-trihydroxyphenyl)propan-2-ol</td>
<td>Egerthella lenta rK3; Flavonfractor plantii aK2; Clostridium coccosides – Eubacterium rectale group</td>
</tr>
<tr>
<td>(-)-epicatechin</td>
<td>(25)-1-(3'-hydroxyphenyl)-3-(2',4',6'-trihydroxyphenyl)propan-2-ol; 5-(3,4-dihydroxyphenyl)-4-valerolactone; 4-phenyl-4-valerolactone; phenylpropiolic acid; 4-hydroxy-5-(3,4-dihydroxyphenyl)valeric acid</td>
<td>Eubacterium limosus</td>
</tr>
</tbody>
</table>

*Note: Flavonfractor plantii is the former Clostridium orbiscindens.*
Microbial metabolism therefore not only is a prerequisite for absorption but also modulates the biological activity of dietary flavonoids, leading to the release of more active metabolite in the body. Monagas summarized the microbial metabolites of flavanols, hydroxyphenyl-γ-valerolactones, and several phenolic acids in terms of their antioxidant, anti-proliferative, and anti-inflammatory, and anti-thrombotic effects, as well as their modulation of the gut microbiome. The 3,4-dihydroxyphenylacetic and 4-hydroxyphenylacetic acid metabolites have been reported to exert higher inhibition of platelet aggregation than their precursors rutin or quercetin. In addition, the hydroxyphenylacetic and phenylpropionic derivatives, produced by microbial degradation of many flavonoids, have structural characteristics similar to apocynin, a relevant inhibitor of NADPH oxidase. This enzyme is associated with the endothelial function, and these metabolites could act as inhibitors of this enzyme.

The microbial biotransformation of isoflavones has attracted particular attention for the higher antioxidant activity of the microbial metabolites, equol, than its parent compound, daidzein. Isoflavones, a class of nonsteroidal estrogens that are similar in chemical structure to estrogens, are abundant in soy. Almost all soy isoflavones exist as glucosides, and require the conversion of glucosides into the principal bioactive aglycones (daidzein, genistein, and glycine) by β-glucosidase for absorption. The aglycones are either absorbed intact or further metabolized by gut microbiome. Genistein is metabolized to p-ethylphenol and 4-hydroxyphenyl-2-propionic acid, whereas daidzein is reduced to O-demethylangolensin (O-DMA) and equol. Interestingly, humans produce relatively low levels of equol, in contrast to animal species assayed such as mouse, rat, and monkey.
Furthermore, not all individuals consuming daidzein produce equol and O-DMA. For example, only about 35% of subjects excreted substantial amounts of equol after consuming soy. The inability of some subjects to produce equol is a consequence of the lack of specific microbes of the gut microbiome. In addition to O-DMA and equol, dihydrodaidzein, tetrahydrodaidzein, 3’-hydroxydaidzein, 6-hydroxydaidzein, 8-hydroxydaidzein, 3-(4-hydroxyphenyl)benzopyran-4,7-diol, and 2-dehydro-O-DMA have also been reported to be microbial metabolites of daidzein. Therefore, these isoflavones are transformed by deglucosylation, reduction, C-ring cleavage, and hydroxylation reactions in the gut.

Compared to other subclasses of flavonoids, such as isoflavones and flavonols, the microbial biotransformation of flavones is less studied. The microbial metabolism of homoorientin that is C-glycosylflavone by human fecal suspension was investigated. Homoorientin was catalyzed to 6-C-glucosyleriodictyol. The C-glycosyl bond was then cleaved to produce eriodictyol, which was subsequently converted to 3,4-dihydroxyphenylpropionic acid and phloroglucinol via ring fission. On the other hand, luteolin, the aglycone of homoorientin, was produced in relatively low yields via direct cleavage of the C-glycosyl bond. Although those flavones possessing similar carbon skeletons, different position of ring fission or resistant to ring fission were found. The pig cecum microbiome metabolized hispidulin (5, 7, 4’-trihydroxy-6-methoxyflavone) to scutellarein (5,6,7,4’-tetrahydroxyflavone), and 3-(4-hydroxyphenyl)-propionic acid; apigenin (5, 7, 4’-trihydroxyflavone) to 3-(4-hydroxyphenyl)-propionic acid and 3-phenylpropionic acid, and luteolin (5,7, 3’, 4’-tetrahydroxyflavone) to 3-(3-
hydroxyphenyl)-propionic acid, respectively. Hispidulin was rapidly underwent O-demethylation to scutellarein in 10 hours, and the demethylation occurred at the position 6. Breakdown of the heterocyclic ring of scutellarein, apigenin and luteolin occurred at the position between 4 and 5. Notably, no degradation of chrysin (5, 7-dihydroxyflavone) was observed. Griffiths incubated apigenin, apiin (apigenin 7-apiosylglucoside), chrysin and tectochrysin with microbiome derived from rat intestine, and also found 3-(4-hydroxyphenyl)-propionic acid after apigenin, apiin incubation sample, and no metabolites were found in chrysin and tectochrysin incubation sample. Both Griffins and Labib concluded that 5-, 7- and 4’-hydroxy groups in the molecule are necessary for the microbial breakdown of flavones.

### 2.2.2.2 Modulation of the gut microbiome by dietary flavonoids

Dietary flavonoids are metabolized by gut microbiome, on the other hand, those unabsorbed dietary flavonoids and their microbial metabolites, in addition to their direct beneficial effect on the human tissues, exert significant effects on the gut environment by modulation of the microbiome. However, there are remarkably few studies investigating the effect of polyphenols on the composition and activity of the gut microbial community. There is a significant gap in our understanding of the dietary flavonoids – gut microbiome interactions. Nevertheless, there is evidence from in vitro, animal and human studies that certain doses of selected dietary flavonoids influence gut microbial populations, including inhibition if certain bacterial groups. For example, tea polyphenols, including epicatechin, catechin, 3-O-methylgallic acid, gallic acid, and caffeic acid, which have been identified as responsible for the inhibition
of growth on certain pathogenic bacteria, such as *Clostridium perfringens*, *Clostridium difficile*, and *Bacteroides* spp. growth, whereas commensal anaerobes such as *Clostridium* spp., *Bifidobacterium* spp., and probiotic microbes such as *Lactobacillus* spp. were less severely affected. Similar results were obtained in another *in vitro* study of (-)-epicatechin and (+)-catechin using a batch-culture fermentation system with human feces. (+)-Catechin significantly inhibited growth of *Clostridium histolyticum*, whereas growth of members of the *Clostridium coccoides–Eubacterium rectale* group and *Escherichia coli* was significantly enhanced and growth of *Bifidobacterium* and *Lactobacillus* spp. remained relatively unaffected. Those results indicated that tea polyphenol exerted significant effects on the gut environment by modulation of the gut bacterial population, probably by acting as metabolic prebiotics. The animal study showed that rats treated with red wine polyphenols had significantly lower levels of *Clostridium* spp. and higher levels of *Lactobacillus* spp.. Moreover, a human intervention study with 10 male volunteers who received red wine for 20 days showed consumption of red wine polyphenol for 4 weeks significantly increased the number of *Enterococcus, Prevotella, Bacteroides, Bi-fidobacterium, Bacteroides uniformis, Eggerthella lenta*, and *Blautia coccoides–Eubacterium rectale* groups.

### 2.2.3 Models to study dietary flavonoids - gut microbiome interaction

It is a particularly challenging task to study dietary flavonoids - gut microbiome interactions and their relevance to human health. The high diversity of dietary flavonoids, their bioavailability and the myriad molecular mechanisms by which they may trigger health responses, the large inter-individual variability in both composition and activity of
gut microbiome, all contribute to the complexity. The inaccessibility of the human gut prevents the direct examination of the metabolic and ecological activity of the microbiome, as well as raising ethical issue. Controlling and evaluating all, often interrelated, variables within one experimental setting are not feasible. Consequently, to get the whole picture, a multidisciplinary approach, combining both *in vitro* and *in vivo* animal and human studies, is the key to success.

Currently available *in vitro* gut models range from static batch incubations, semi-continuous culture systems, to multistage continuous culture systems. A more intricate system aiming to study the ecological system present in the GIT was developed, consisting of a five-stage reactor named simulated human gut microbial ecosystem (SHIME).\(^{105}\) A defined bacterial strain, ileostomy fluid or microbes from freshly voided human or animal feces, animal large intestine cultured in a suitable medium to which defined dietary flavonoids was added, and monitoring the disappearance of dietary flavonoids, formation of metabolites or changes in the microbiome.\(^{27}\) Considering the inaccessibility of the human gut does not permit the use of human gut contents for *in vitro* experiments, it is believed that such an environment is more closely simulated with the use of human feces than with the use of gut contents from other animals. Thus, *in vitro* studies carry great value to study a specific class of dietary flavonoids, its metabolism and differentiation with fully controlled variables. The biggest challenge with *in vitro* simulation of the gut environment is the creation of a fermentation system containing microbial numbers and diversity similar to the human gut.
For *in vivo* studies, the use of animal models has some economical and logistical advantages over the use of human models: easier experimental set up and less stringent ethics regulations apply. The simplest approach to elucidate microbial metabolism could be compared bile metabolites with fecal metabolites by collecting animal urine and feces. Another methodology for locating the production of specific metabolites includes the removal of the stomach, small intestines, caecum and colon of rats. Furthermore, inoculation of germ-free rodents with a human fecal microbiome, human-microbiome associated (HMA) animals, effectively mimics human microbial metabolism and thus provides a reliable model to study the human gut microbial ecosystem as well as metabolism. The use of animals has allowed the effects of the animal microbiome and human-type microbiome to be compared. However, there is evidence of variability between animal species and man. This inter-species variability has led scientists to use human volunteers instead of animals to determine dietary flavonoids - gut microbiome interaction in the human intestine. Human *in vivo* studies use volunteers, either free-living or following prescribed diets, and to collect their feces and urine for analysis. Healthy volunteers can be compared with volunteers who have undergone an ileostomy but who are otherwise enabling metabolism in the small intestine to be distinguished from that in the large intestine.
2.3 Surface enhanced Raman spectroscopy

2.3.1 Introduction to Surface enhanced Raman spectroscopy

Raman spectroscopy is a branch of vibrational spectroscopy; both the intensity and frequency of induced molecular vibrations are sensitive to the chemistry and environment around the individual atoms. The Raman scattering was first experimentally observed and reported by Raman and Krishnan in 1928. When monochromatic radiation of frequency \( (v_0) \) is incident on a sample, it generates Rayleigh scattering and Raman scattering. Rayleigh scattering is a elastically scattered radiation that radiation with the same frequency as the incident radiation. While Raman scattering is inelastic scattering, thus, the change in frequencies observed when a photon undergoes Raman scattering is attributed to the excitation (or relaxation) of vibrational modes of a molecule. Since different functional groups have different characteristic vibrational energies, every molecule has a fingerprint Raman spectrum. Raman and infrared (IR) spectroscopy are complementary techniques. Various stretching and bending deformation modes of individual chemical bonds result in the discrete vibrational transitions occurring in the ground electronic state of molecules. In infrared (IR) spectroscopy, the absorption of incident electromagnetic radiation at a particular frequency \( (v_i) \) in the IR region is related to a specific vibrational excitation energy. In contrast, in Raman spectroscopy, the exciting or incident light beam is at a frequency \( (v_0) \) that may correspond to the visible, UV or near-infrared region of the electromagnetic spectrum. The inelastic scattering of the incident radiation, which results in a Raman shift \( (\Delta v = vi) \), is related to the energy of a vibrational transition within a sample molecule. Stokes’ transitions are those in which the molecule is excited by the radiation, whereas anti-Stokes’ transitions are those in
which the molecule is de-excited (Figure 2.4). The intrinsic dipole moment with molecular vibration is changed in order to IR absorption, whereas Raman scattering depends on changes in the polarizability of functional groups as the atoms vibrate. Therefore, polar groups such as C=O, N-H and O-H have strong IR bands, while nonpolar groups such as C=C, C-C and S-S have intense Raman bands. One advantage of Raman spectroscopy is that it produces less water interference. As a consequence, Raman spectroscopy is usually more suitable for the in vivo or in situ study of biological systems, including foods, which are primarily aqueous in nature. However, because Raman scattering is inherently weak, giving signal intensities in the order of 10$^{-9}$ to 10$^{-6}$ of those of Rayleigh scattering, fairly high concentrations of the target analytes are required in samples. Another drawback of Raman spectroscopy is the interference from fluorescence in some samples, which may completely obscure the Raman scattering signals.

Figure 2.4 The relationship between infrared, Rayleigh scattering and Raman scattering.
Because of the inherently small intensity of the Raman signal, the sensitivity limits the applicability of Raman scattering for many years. In 1977, Jeanmaire and Van Duyne demonstrated that the magnitude of the Raman scattering signal can be greatly enhanced when the sample is placed on or near a roughened nanoscale metallic substrate, such as silver, gold, or copper. Therefore, surface enhanced Raman spectroscopy (SERS) is a combination of Raman spectroscopy and nanotechnology. The illustration of SERS enhancement showed in the Figure 2.5.

There are two proposed mechanisms of SERS enhancement, chemical enhancement and electromagnetic enhancement. Chemical enhancement primarily involves charge transfer mechanisms, and a charge-transfer state is created between the metal and molecules. This mechanism is site-specific and analyte-dependent. The molecule must be directly adsorbed to the roughened surface to experience the chemical enhancement. Presently, the experimentally obtained chemical mechanism contributes enhancement factors of only 5 – 10 times. The other mechanism involved in signal enhancement is electromagnetic enhancement. Strong electromagnetic fields are generated when the localized surface plasmon resonance (LSPR) of nanoscale roughness metallic substrate is excited by visible light. When the sample is placed to these intensified electromagnetic

![Diagram of Raman and SERS techniques](Image)
fields, the magnitude of the induced dipole increases, and accordingly, the intensity of the inelastic scattering increases.\textsuperscript{112} The size, shape, aggregation and material of the nanoscale roughness features must be considered in term of electromagnetic enhancement, because the light concentration occurs preferentially in the gaps, crevices, or sharp features of metals. The average for the SERS enhancement is around $10^6$, but the electromagnetic enhancement for SERS is theoretically calculated to reach factors of $10^{10}$ – $10^{11}$.\textsuperscript{113}

\subsection*{2.3.2 SERS substrates}

The noble metal substrate is the key player in the enhanced scattering process. Therefore, the choice and/or fabrication of the noble-metal SERS substrates in most critical aspect of performing a SERS experiment. SERS substrates require chemical and biological compatibility with the samples that have to be measured, chemical and temporal stability, reliability and reproducible, and economical preparation.

Typically, there are 3 SERS substrate categories: (1) Nanoparticles in suspension; (2) Nanoparticles immobilized on solid substrates; (3) Nanostructures fabricated directly on solid substrates.\textsuperscript{114} The commonly used SERS substrates are metal colloids and nanoparticles in diameters between 10 and 200 nm in suspension. The colloids dispersion of metal nanoparticles are prepared by reduction of the respective metal salts with various reduction agents. The fabrication of metal colloids and nanoparticles only require simple chemical laboratory equipment. However, particles in colloidal dispersions often show a size and shape distribution, leading to an undesired broadening of the resonance. Another
drawback for using colloids is their poor reproducibility, and the nanoparticles tend to be randomly aggregated during drying which results in extremely inconstant signals from spot to spot. Immobilizing the nanoparticles on planar platforms is another approach to fabricate a SERS substrate. Modification of the glass surface by derivatized silanes, such as aminopropyltrimethoxysilane (APTMS), could irreversible immobilize gold or silver particles on glass supports.\textsuperscript{115} However, the adhesion of nanoparticles to the solid supports is usually very poor. Electrochemically roughened metal electrodes were the first substrates showing surface-enhanced Raman scattering.\textsuperscript{116} Rough surfaces and island films are prepared by deposition onto inexpensive rough substrates. For example, gold dendrite nanostructures were prepared by using a galvanic exchange reaction achieving distances in the range of the effective plasmon resonance.\textsuperscript{117} Rough surfaces may offer high sensitivity because their larger surface area may provide more target molecules. The SERS substrates could be also prepared by templates to achieve a highly reproducible and large-scale production using photolithography and nanolithography. Different fabrication strategies based on the coating of template structures or, on the other hand, the additional removal of the template. Nanosphere lithography (NSL) is the most prominent example for removal of the template structure for some SERS array fabrication approaches. A solvent containing same size of nanospheres is arranged to dry in a dense monolayer on a substrate serving as a mask for a directed deposition of a metal layer, giving an hexagonal array of triangles.\textsuperscript{118} Photolithography and nanolithography allow the fine control over the size and shape of the nanostructures so that achieve very high reproducibility in the SERS intensities.
Silver dendrites, an easy, convenient, and cost-effective way for SERS analysis was designed by He’s group. Silver dendrites are prepared via a simple replacement reaction: \( \text{Zn} + \text{AgNO}_3 \rightarrow \text{Ag} + \text{ZnNO}_3 \). Basically, a zinc plate is pre-cleaned to remove surface contaminants, then immersed into the AgNO\(_3\) solution. The different morphologies of silver nanostructures are obtained by controlling silver ion concentration and reaction time. Lastly, the formed silver nanostructures are carefully peeled off the zinc plate, rinse several times to remove excessive chemical residues, and stored in the vial containing water. The desired silver dendrites are obtained by immersing zinc plate into 200 mmol/l AgNO\(_3\) solution for 1 minute. Under SEM, the nanostructure is composed of \(~\)50 nm silver nanoparticles that connected with each other to form symmetrical leaves on branches. The length of an individual dendrite varied from 1 to 5 \( \mu \)m. (Figure 2.6) The UV-Vis absorption of silver dendrites exhibits a wider range of wavelengths from 400-800 nm. It shows that the silver dendrites could accommodate a broad selection of laser excitation sources for SERS experiments compared to other different silver nanostructures with optimum absorption around 400-500 nm.
2.3.3 SERS application

SERS clearly presents itself as a highly versatile tool that provides complex chemical fingerprints from a wide range of biological or chemical agents. Comparing with other molecular detection and characterization techniques, the advantages of SERS are: (1) Rich “fingerprint” information based on chemical structure; (2) Highly sensitivity and
selectivity, even a single molecule detection; (3) Rapid detection requiring less than 1 minute for each measurement; (4) The small Raman scattering cross-section of water allow vibrational information to be obtained in their native aqueous environment; (5) The ability to quench fluorescence; (6) The capacity to be used with or without optical labels. Hence, it has been rapidly developed as a powerful tool for molecular detection and characterization, with applications in biology, medicine, materials science, biosensor and electrochemistry.\textsuperscript{36–38} The biomedical application is the most demanding SERS application, since SERS can provide rich biochemical information, such as proteins, peptides, nucleic acids, glucose, lipids and cells, and has been widely accepted as a routine bioanalytical characterization methodology.\textsuperscript{39,120–123} Furthermore, SERS is also used in detection of diseases \textit{in vitro} and \textit{in vivo}, including various cancers, diabetes, Alzheimer’s disease, and Parkinson’s disease.\textsuperscript{124–127}

SERS has been also reported to study ligand–protein interactions by dye-labeling methods and label-free methods. The dye-labeling methods need specially designed Raman reporter labeled silver or gold nanoparticle probes.\textsuperscript{39–41} Dye, such as brilliant blue R-250 (BBR) and brilliant blue G-250 (BBG) was also used as SERS label. The detection of Raman reporter signals indicates the interaction between ligand and protein. The label-free methods are based on the intrinsic signals of ligand and protein. The changes in the Raman spectra of ligand protein incubation, either spectral pattern or intensity, is a direct indication of ligand-protein interaction. (\textbf{Figure 2.7}) It not only provides an insight into the mode of binding of the ligand to the protein, but also the protein conformation change.\textsuperscript{42–46} Jurasenkova studied the interaction between flavonoid luteolin and human
serum albumin (HAS), by comparing the Raman spectra of free HAS and the HAS-luteolin complex, changed in the intensity and the position due to the Trp residue and disulfide bonds were observed.\textsuperscript{43} He reported a different SERS sample preparation method to investigate the interaction between polymethoxyflavones and kappa casein.\textsuperscript{46} The substrate method and solution method were utilized to prepare the SERS samples. Basically, for substrate method, silver (Ag) dendrites (a SERS substrate) were deposited on the glass and air-dried first, then the sample solution was deposited on the dried Ag dendrites and undergo Raman measurement. On the other hand, for the solution method, the Ag dendrites mixed with sample solution for a while, then the mixture of Ag dendrites were washed by distill water for several times to remove non-specific binding, lastly deposited on glass slide and air-dried. The substrate method is a simpler and faster way to collect the SERS ‘fingerprint’ spectrum of sample. While solution method is based on interaction between sample molecules and Ag dendrites. If sample could be bound to Ag dendrites, then the signal of sample is able be enhanced through chemical enhancement, otherwise, sample molecules would be washed away by water, there is little or no SERS signal from sample.
Figure 2.7 Cartoon representation of detection of protein-ligand by SERS. The change in the SERS spectrum is a direct indication of ligand binding to a protein forming a protein-ligand complex.\textsuperscript{44}

Multivariate methods have been applied to SERS to identify the relevant spectral features that distinguish sample classes, such as discrimination between peptide, bacterial and viral species, and certain cancer diagnose. Principal component analysis (PCA) is a common method used for building linear multivariate models of complex data sets using orthogonal basis vectors called principal components (PCs). The first PC describes the maximum amount of variance present in the spectral data set, whereas successive PCs describe features contributing progressively smaller variance. Therefore, PCA identifies
and extracts major trends within a given spectral data set, which variables contribute most to this difference, and whether those variables contribute in the same way (i.e. are correlated) or independently (i.e. uncorrelated) from each other.\textsuperscript{128}
CHAPTER 3

BIOTRANSFORMATION OF POLYMETHOXYFLAVONES BY THE MOUSE AND HUMAN GUT MICROBIOME

3.1 Introduction

Polymethoxyflavones or PMFs are a unique class of citrus flavonoids bearing two or more methoxyl groups on their C₆-C₃-C₆ skeleton with a carbonyl group at the position 4. There is a marked interest in PMFs due to their broad spectrum of biological activities, including anti-inflammatory, anti-carcinogenic, anti-viral, antioxidant, anti-thrombogenic, and anti-atherogenic properties.¹–³,¹¹,¹³ Among different types of PMFs, nobiletin (NBT) is the major component of PMFs isolated from citrus fruits, especially in the peel. Particularly, NBT has been reported as a potential anti-inflammatory agent to inhibit LPS-induced NO generation, phorbolester-induced oxidative stress, skin inflammation and tumor promotion.¹²⁻⁹ It also exhibited synergistic anti-inflammatory effect with sulforaphane in decreased iNOS and COX-2 protein expression levels and induced heme oxygenase-1 (HO-1) protein expression, and suppressed LPS-induced upregulation of IL-1 mRNA levels and synergistically increased HO-1 mRNA levels.⁵² In term of anti-carcinogenic activity, NBT induced G1 cell cycle arrest in human breast and colon cancer cells,⁵³ and protected the development of adenocarcinoma of the prostate in transgenic mice and inhibited the growth of human prostate cancer cells.¹³⁰ Saito et al. reported that NBT had anti-atherogenic effects, such as enhanced both differentiation and lipolysis of adipocyte through activation of signaling cascades mediated by
cAMP/CREB. 5-hydroxynobiletin (5HN) is the most abundant hydroxylated PMFs isolated from citrus peel, and it can be formed via auto hydrolysis of NBT. 5 5HN receives growing interests on its more potent biological activities than NBT. 5,51,132–134 Particularly, 5HN revealed more potent in growth inhibition of lung cancer by causing increase in sub-G0/G1 phase and downregulating iNOS, COX-2, Mcl-1, and K-ras. 5 The colon cancer was also inhibited by 5HN by inducing apoptosis and cell-cycle arrest. 51,134 5HN also exhibited diverse anti-atherogenic bioactivities in inhibiting monocyte-to-macrophage differentiation and foam cell formation. 133 Furthermore, anti-inflammatory study of 5HN demonstrated it reduced the edema formation, cell infiltration, and tissue damage in the inflammation induced by 12-O-tetradecanoylphorbol 13-acetate in mouse ears, along with the acute edema induced by carrageenan, as well as the acute phospholipase A2-induced edema in mouse paws. 132

The biological activities of PMFs have been extensively studied in recent years, but the knowledge of their metabolic profile is less studied and insufficient. Dietary flavonoids undergo biotransformation in vivo and produce metabolites altering their bioactivities and pharmacological properties. 135 Generally speaking, partial absorption of dietary flavonoids occurs in the small intestine, with modification by phase I (oxidation, reduction and hydrolysis) and phase II (glucuronidation, sulphation, and methylation) enzymes, and be excreted with bile back into small intestine or in the urine. Microbial conversion of the dietary flavonoids in the large intestine, absorption of microbial metabolites or fecal excretion. 78 For dietary flavonoids that are not easily absorbed in the upper gastrointestinal tract, and persist to the large intestine, where they are exposed to
the gut microbiome community. The gut microbiome can be acted as an organ with more biochemical conversion than, or at least equal to liver.\textsuperscript{25,26} It has been reported that particular beneficial effects of wine polyphenolics are dependent on biotransformation by gut microbiome to bioactive metabolites.\textsuperscript{136} Soy isoflavones also showed different biological activities between parent compound and its microbial metabolites.\textsuperscript{137} Thus, the gut microbiome may play a crucial role in the potential health benefits of dietary flavonoids.

PMFs have been reported to be hydroxylated and demethylated by cytochrome P450 (CYP) in the liver.\textsuperscript{138} The 3’ and 4’ positions on the ring B of PMFs are the primary sites for biotransformation. The number and position of the hydroxyl and methoxyl groups on the B ring of PMFs have a great influence on the metabolism of PMFs. Several metabolic studies of NBT have identified its metabolites as 3’-hydroxynobiletin, 4’-hydroxynobiletin, and 3’, 4’-dihydroxynobiletin.\textsuperscript{20–22,139} Zheng also reported that major metabolites of 5HN found in mouse urine were 5, 3’-dihydroxynobiletin, 5, 4’-dihydroxynobiletin, and 5, 3’, 4’-trihydroxynobiletin.\textsuperscript{23} However, no group studied and reported the biotransformation of PMFs by gut microbiome. This paper is the first paper to report the biotransformation of PMFs by gut microbiome.

3.2 Materials and Methods

3.2.1 Chemicals

Nobiletin (NBT) was purchased from Quality Phytochemicals LLC (Edison, NJ). Its three major metabolites in the mice have been identified as 3’-hydroxynobiletin (M1),
4’-hydroxynobiletin (M2), and 3’, 4’-dihydroxynobiletin (M3), and synthesized following previous reported method as metabolites standards. 5-hydroxynobiletin (5HN) was obtained directly through the acid hydrolysis of NBT. Three major metabolites of 5HN in the mice have been identified as 5, 3’-dihydroxynobiletin (M4), 5, 4’-dihydroxynobiletin (M5), and 5, 3’, 4’-trihydroxynobiletin (M6), and synthesized by previous reported method as metabolites standard. The purity of these metabolites was >98%, and their chemical structures have been confirmed by MS and NMR. The chemical structure of 8 PMFs are shown in the Figure 3.1

Figure 3.1 Chemical structures of nobiletin (NBT), 3’-hydroxynobiletin (M1), 4’-hydroxynobiletin (M2), 3’, 4’-dihydroxynobiletin (M3), 5-hydroxynobiletin (5HN), 5, 3’-dihydroxynobiletin (M4), 5, 4’-dihydroxynobiletin (M5), and 5, 3’, 4’-trihydroxynobiletin (M6).

Organic solvents and all regents for isolation, chemical synthesis, and HPLC analysis were purchased from Fisher Scientific (Fairlawn, NJ). Ammonium acetate was a product of EMD Chemicals Inc. (Gibbstwon, NJ). The mixture of β-D-glucuronidase and sulfatase was from Sigma-Aldrich Ltd. (Castle Hill, NSW, Australia)
3.2.2 Animal experiments

The protocol for the animal experiment was approved by Institutional Animal Care (permission number, 2011-0066) and Use Committee of the University of Massachusetts Amherst. The CD-1 male mice (6 week of age, weighing 18-20 g) were obtained from Charles River Laboratories (Wilmington, MA). Upon arrival, the animals were housed in a temperature-controlled environment with a 12-h light/12-h dark cycle, receiving a standard diet and water ad libitum. Mice were randomly divided into 3 groups of treatment and control, with 2 mice in each group. After 1 week acclimation, mice were maintained on NBT or 5HN enriched food. NBT or 5HN was mixed with semi-purified AIN93M (with 15% casein protein, Research Diets, Inc., New Brunswick, NJ) diet to formulate 0g/kg and 1g/kg NBT or 5HN enriched diet, which were orally administrated to the control and NBT or 5HN group correspondingly for 5 weeks, respectively. Small intestine (ileum part II) of treatment groups, cecum and colon of control group were removed, and their content were collected immediately after sacrifice. The small intestine content were stored at −80 °C until further analysis. The cecum and colon content were conducted to gut microbiome collection and incubation immediately.

3.2.3 Preparation of anaerobic culture medium

The anaerobic techniques of Hungate (1969) were used, unless specified otherwise. The Revised Darcy Medium (RDM) was prepared as described previously, and it contained 2.0 g/L NaH$_2$PO$_4$, 10.0 g/L K$_2$HPO$_4$, 1.0 g/L (NH$_4$)$_2$SO$_4$, 1.0 g/L L-cysteine hydrochloride monohydrate, 5 g/L Bacto Yeast Extract, and 10 ml/L Bach’s trace element (BTE) solution. Resazurin (1 mg/L) was added as an oxidation/reduction
indicator, and 5 g/L pectin was added to RDM. The pH was adjusted to 7.2, and the final volume was adjusted to 1000 mL with distilled water. The medium were sterilized by autoclaving at 121°C for 20 min.

3.2.4 Mouse and human gut microbes collection and incubation

After sacrificing control group mice, the cecum and colon content were collected and suspended into 10 mL culture medium with 0.4 mL DMSO on the Hungate. Fresh fecal samples were obtained from 3 healthy free-living volunteers with no dietary restrictions (1 female, 2 male; aged 20s - 50s; Caucasian, Asian, and Hispanic; no recent history of gastrointestinal disease). Fresh human feces (0.5 g) of three volunteers were weighed and suspended into 10 mL culture medium with 0.4 mL DMSO on the Hungate. Then the mouse and human gut microbiome suspension were homogenized adequately using a vortex-mixer, and 1 mL of aliquots was stored at -80°C until use.

1 mL aliquots of mouse or human gut microbiome suspension was inoculated into 9 mL culture medium, and anaerobically incubated at 37°C for 24 h.

3.2.5 Exposure of PMFs to the mouse and human gut microbiome

3.2.5.1 Exposure of mouse small intestine content fed with NBT or 5HN to the mouse and human gut microbiome

The small intestine content of 2 mice with NBT treatment were combined together, and were extracted by butanol 3 times. The combined butanol extracts were dried under vacuum, and dissolved in DMSO to achieve final total concentration of NBT with its
metabolites (free form + conjugated form) was 10 mM – 15 mM. The small intestine content stock solution from mouse fed with NBT is named as SI-NBT. Same extraction procedure was applied to the small intestine content stock solution from mouse fed with 5HN, and it was named as SI-5HN. 10 μL SI-NBT or SI-5HN was added to 9.5 mL medium, followed by addition of 0.5 mL of gut microbiome inoculum as treatment group. 10 μL SI-NBT or SI-5HN was added to 10 mL medium without gut microbiome inoculum as positive control group. 10 μL DMSO was added to 9.5 mL medium, followed by addition of 0.5 mL of gut microbiome inoculum without SI-NBT or SI-5HN as negative control group. The mixture was incubated at 37°C for 24 hours in anaerobic chamber containing 80% N₂, 10% CO₂, and 10% H₂. All the groups were prepared in triplicates.

3.2.5.2 Exposure of individual PMFs to human gut microbiome

10 μL 10 mM individual NBT and its synthesized metabolites (M1-M3); individual synthesized 5HN and its metabolites (M4-M6) were added to 9.5 mL medium, followed by addition of 0.5 mL of gut microbiome inoculum as treatment group. 10 μL 10 mM individual NBT and its synthesized metabolites (M1-M3); individual synthesized 5HN and its metabolites (M4-M6) were added to 10 mL medium without gut microbiome inoculum as control group. The mixture was incubated at 37°C for 24 h in anaerobic chamber containing 80% N₂, 10% CO₂, and 10% H₂. All the groups were prepared in triplicates.
3.2.6 Sample preparation for HPLC analysis

3.2.6.1 Mouse small intestine content fed with NBT or 5HN fermentation sample

Two aliquots of fermentation samples (200 μL each) from the control groups or treatment group were added into 200 μL 60% methanol, and centrifuged at 8000 rpm for 5 min. Then methanol in sample supernatant was dried under vacuum. The first aliquot was incubated with 20 μL of mixed β-D-glucuronidase (250 U) and sulfatase (1 U) solution at 37 °C for 45 min, and the second aliquot was incubated with 20 μL of distilled water at 37 °C for 45 min. The samples were then extracted with 400 μL of ethyl acetate for 3 times. The combined ethyl acetate extracts were dried under vacuum, and dissolved in 100 μL of mobile phase A for HPLC analysis.

3.2.6.2 Individual PMFs fermentation sample

200 μL fermentation samples from the control group and treatment group were added into 200 μL 60% methanol, and centrifuged at 8000 rpm for 5 min. Then methanol in sample supernatant was dried under vacuum. The samples were then extracted with 400 μL of ethyl acetate for 3 times. The combined ethyl acetate extracts were dried under vacuum, and dissolved in 100 μL of mobile phase A for HPLC analysis.

3.2.7 HPLC analysis of cultured PMFs

Quantification of biotransformation of PMFs by mouse and human gut microbiome were conducted by an HPLC with an electrochemical detector and UV-vis detector using our previously published method with modification. The CoulArray® HPLC system (Chelmsford, MA) consisted of a binary solvent delivery system (model 584), an
autosampler (model 542), a UV-vis detector (model 526; Waters, Milford, MA) and a CoulArray® Multi-Channel EC detector (model 6210; Waters, Milford, MA). Instrument control and data processing were performed with CoulArray 3.06 software (Chelmsford, MA). Ascentis RP-Amide RP HPLC column (15 cm × 4.6m id, 3 µm; Sigma-Aldrich, MO) was used. Flow rate was set to 1.0 mL/min, and the temperature of auto sampler was set to 4 °C. The injection volume was 30 µL. The UV wavelength of UV-vis detector was set at 330 nm. The electrochemical detector cells were set at the detecting potentials of 100, 200, 300, and 400 mV. The mobile phases consisted of A (75% water, 20% acetonitrile, 5% tetrahydrofuran, and 25 mM ammonium acetate, pH 3.0) and B (50% water, 40% acetonitrile, 10% tetrahydrofuran, and 50 mM ammonium acetate, pH 3.0). The linear solvent gradient consisted of 10% B at 0 min, 100% B at 20 min, and 100% B at 30 min.

Synthesized compounds (M1-M3) were used as standards to identify and quantify the metabolites of NBT in the fermentation samples. Synthesized compounds (M4-M6) were used as standards to identify and quantify the metabolites of 5HN in the fermentation samples.

Standard curves of the standard compounds were constructed by plotting concentrations (x axis, µM) versus peak areas (y axis, µC). Quantification of the metabolite in fermentation sample was performed by comparing their peak areas with the standard stock solutions of serious concentrations. For NBT, M1, M2, M3, linear calibration curves (correlation coefficient, $r^2 > 0.9990$) can be obtained within the range of 0.5 – 10
μM, and their regression equations are as follows: $y = 0.139x + 0.0433$ ($r^2 = 0.9994$), $y = 1.5748x + 0.505$ ($r^2 = 0.9990$), $y = 2.0025x + 0.1996$ ($r^2 = 0.9998$), $y = 2.5992x + 0.3401$ ($r^2 = 0.9998$), respectively. For 5HN, M4, M5, M6, linear calibration curves (correlation coefficient, $r^2 > 0.9990$) can be obtained within the range of 0.5–10 μM, and their regression equations are as follows: $y = 2.0277x + 0.4546$ ($r^2 = 0.9990$), $y = 2.593x + 0.1926$ ($r^2 = 0.9999$), $y = 2.2196x + 0.1181$ ($r^2 = 0.9999$), $y = 2.6472x + 0.1466$ ($r^2 = 0.9999$), respectively.

The HPLC chromatograms of NBT and its metabolites standards, 5HN and its metabolites standards are shown in the Figure 3.2. NBT is detected by UV detector at 330 nm, while 5HN and M1 – M6 are detected by electrochemical detector. The retention time of NBT is 12.3 min, M1 and M2 are detected at potential 400 mV, and the retention time is 10.3 min and 11.0 min, respectively; M3 is detected at potential 200 mV, and the retention time is 9.17 min. 5HN, M4, and M5 are also detected at potential 400 mV, the retention time is 20.9 min, 18.3 min and 19.7 min, respectively; M6 is detected at potential 200 mV, and the retention time is 16.8 min.
Figure 3.2 HPLC chromatograph of nobiletin (NBT), 3’-hydroxylnobiletin (M1), 4’-hydroxylnobiletin (M2), 3’, 4’-dihydroxylnobiletin(M3), 5-hydroxylnobiletin (5HN), 5, 3’-dihydroxylnobiletin (M4), 5, 4’-dihydroxylnobiletin (M5), and 5, 3’, 4’-trihydroxylnobiletin (M6) standards. NBT was showed in dash line, and 5HN, M1-M6 were showed in solid line.

3.2.8 Cell viability assay

The inhibitory effects of NBT/5NN mouse gut microbiome fermentation sample on colon cancer cells were established by our previously published method. Human colon cancer cell line HCT116 (1500 cells/well), HT29 (2000 cells/well) were seeded in 96-well plates. After 24 h, cells were treated with serial concentrations of different fermentation samples in 200 μL of serum complete media. At 48 h, cells were subject to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, media were replaced by 100 μL fresh media containing 0.5 mg/mL of MTT (Sigma-Aldrich, St. Louis, MO). After 1 h of incubation at 37 °C, MTT-containing media were removed and the reduced formazan dye was solubilized by adding 100 μL of DMSO to each well.
After gentle mixing, the absorbance was monitored at 570 nm using a plate reader (Spectramax m2e, Molecular devices, Sunnyvale, CA). Cell viability was expressed as the percentage of absorbance of treated cells relative to that of the control (percentage of control).

3.3 Results and discussions

3.3.1 Biotransformation of SI-NBT/SI-5HN by the mouse gut microbiome

It is very common that flavonoids and their metabolites are in their conjugated forms as glucuronides and/or sulfates after phase II metabolism occurring mainly in the liver. Therefore, we compared the levels of NBT/5HN and its three metabolites in the small intestine content treated with or without a mixture of β-glucuronidase and sulfatase. The treatment of β-glucuronidase and sulfatase converted the glucuronides and/or sulfates of NBT/5HN and its metabolites to their free forms.

The level of NBT and its metabolites change after anaerobic fermentation by mouse gut microbiome were shown in the Figure 3.3. In the negative control group (without adding SI-NBT, but adding mouse gut microbiome), with or without β-glucuronidase and sulfatase treatment, no level of PMFs were detected. Hence, mouse gut microbiome could not produce PMFs by itself. In the positive control group (without adding mouse gut microbiome but adding SI-NBT) in Figure 3.3A, 69.24% of NBT and its 3 metabolites were mainly in their conjugated forms as glucuronides and/or sulfates. Particularly, there was no conjugated form of NBT. The ratio of conjugated form to free form of M1, M2, and M3 was 7.08, 2.34, and 5.74, respectively. In addition, M2 (conjugated form + free
form) was major metabolites, accounting for 75.18% in the total metabolites, then following by M3 (15.52%) and M1 (8.86%), in the small intestine after phase II metabolism. Li and Yasuda also reported that the major NBT metabolite in mouse urine was M2.\textsuperscript{20,21} In the Figure 3.3B, 57.39% of 5HN and its metabolites were presence as conjugates as glucuronides and/or sulfates in small intestine. Unlike NBT, 5HN could form conjugates since it has hydroxyl group at the position 5. The ratio of conjugated form to free form of M4, M5, and M6 was 1.85, 1.26, and 3.72, respectively. M5 (conjugated form + free form) was major metabolites of 5HN in small intestine after phase II metabolism, accounting for 38.40% of total 5HN and its metabolites, then following by M4 (21.39%), and M6 (9.41%). By studying the urine metabolites of 5HN in mice, Zheng found that 5HN could be demethylated at both position 3’ and 4’, and demethylation at the position 4’ was much more preferred.\textsuperscript{23}

However, after anaerobic fermentation by the mouse gut microbiome, no matter NBT or 5HN, the amount of free form of their metabolites dramatically increased, and almost reached to same level of NBT/5HN metabolites (free form + conjugated form) in the control group. When the fermentation groups treated with mixture of β-glucuronidase and sulfatase, no further increased amount of free form of NBT/5HN metabolites were observed (Figure 3.4). Those results demonstrated that the conjugated form of NBT/5HN metabolites in small intestine were fully converted to free form by the mouse gut microbiome. The mouse gut microbiome can largely cleave the glucuronide and sulfate conjugates, deconjugate them to free form. For 2 mice, there were no significant difference between each other in term of total amount of NBT/5HN metabolites, and ratio
of each metabolite. The removal of the polar conjugating groups by the gut microbiome allows NBT/5HN metabolites to be reabsorbed and returned to its site of action. Thus, the gut microbiome may prolong the action of PMFs by allowing their enterohepatic circulation to continue.
Figure 3.3 Metabolic profile of mouse gut microbiome A) small intestine content of mice fed with NBT (SI-NBT); B) small intestine content of mice fed with 5HN (SI-5HN).
Figure 3.4 Metabolic profile of mouse gut microbiome without or with β-D-glucuronidase and sulfatase treatment. A) Small intestine content of mice fed with NBT (SI-NBT); B) Small intestine content of mice fed with 5HN (SI-5HN). Enzyme -: without β-D-glucuronidase and sulfatase treatment; Enzyme +: with β-D-glucuronidase and sulfatase treatment.
3.3.2 Biotransformation of PMFs by the human gut microbiome

3.3.2.1 Biotransformation of SI-NBT, individual NBT and its metabolites (M1-M3) by human gut microbiome

Similarly, there was no PMFs obtained with or without β-glucuronidase and sulfatase treatment in the negative control group without adding SI-NBT and SI-5HN, but adding gut microbiome. The human gut microbiome could not produce PMFs by itself either.

The level of NBT and its metabolites change after anaerobic fermentation by human gut microbiome were also investigated (Figure 3.5). By further treating human gut microbiome fermentation sample with β-D-glucuronidase and sulfatase, no increasing level of free form was observed. (Figure 3.6) First of all, similar to the mouse gut microbiome, the human gut microbiome could deconjugate the glucuronide and sulfate conjugates to free form. Even though the mouse gut microbiome were collected from cecum and colon content directly, while the human gut microbiome were collected from human feces. The composition of gut microbiome of mouse and human is different, and the different species dominate in feces, different parts of cecum and colon. However, in term of deconjugation, no matter mouse or human, microbiome from cecum, colon or feces exhibited same ability.

Secondly, as we discussed in the section 3.3.1, the major metabolite of NBT in small intestine is M2 (75.18%), then following by M3 (15.52%), and M1 (8.86%) had lowest portion. After anaerobic fermented with human subject 1 gut microbiome, there was no significant change of the ratio of every metabolite compared to control. 75.85% of M2,
16.73% of M3, and 6.29% of M1 were observe in human subject 1 fermentation sample, respectively. Interestingly, after anaerobic fermented with human subject 2 and 3 gut microbiome, M3 became major metabolite instead of M2. Particularly, the amount of M1 and M2 decreased largely in the human subject 2 fermentation sample, from 6.29% to 0.54% for M1, and from 75.85% to 2.61% for M2. Whereas M3 increased from 16.73% to 95.83%. The results showed that almost both of M1 and M2 were converted to M3 by human subject 2 gut microbiome. In human subject 3 fermentation sample, M2 dropped to 34.37%, while M3 raised to 57.40%, hence, about half of M2 was converted to M3 by human subject 3 gut microbiome. M1 and M2 both have one hydroxyl group in the different position of B ring, while M3 has two hydroxyl groups in the B ring. Therefore, other than deconjugation, human gut microbiome also caused demethylation at certain positions of flavonoid core structures, such as 3' and 4' positions in the B ring.

Figure 3.5 Metabolic profile of human gut microbiome with small intestine content of mouse fed with NBT.
Figure 3.6 Metabolic profile of human gut microbiome with small intestine content of mouse fed with NBT without or with β-D-glucuronidase and sulfatase treatment. Enzyme -: without β-D-glucuronidase and sulfatase treatment; Enzyme +: with β-D-glucuronidase and sulfatase treatment.

In order to further investigate the demethylation by human gut microbiome, the individual NBT, and its synthesized metabolites M1, M2, M3 (free form) were anaerobic fermented with human gut microbiome (Figure 3.7). When NBT or M3 incubated with human gut microbiome, human subject 1 and human subject 3 fermentation sample showed the similar level of NBT or M3 as control (Figure 3.7A&B). It suggested that both NBT and M3 could not be demethylated by human subject 1 and human subject 3 gut microbiome. On the other hand, human subject 2 gut microbiome revealed different demethylation ability. Level of NBT decreased from 8.26 µM to 2.95 µM, and level of
M3 increased from 0 µM to 1.35 µM. Deduction of M3 from 11.36 µM to 4.01 µM was also observed in the human subject 2 fermentation sample. This results indicated that human subject 2 gut microbiome could demethylate NBT to M3 or other PMFs and small molecules, and M3 to other PMFs or small molecules because the total level of PMFs decreased. Koga et al. reported that NBT could be metabolized to 4′-hydroxynobiletin, 7- hydroxynobiletin, 6- hydroxynobiletin, 3′, 4′-di hydroxynobiletin and 6,7-dihydroxynobiletin by liver microsomes. There was no paper reported directly that human gut microbiome transferred PMFs to small molecules, but there was evidence that flavonoids were transferred by human gut microbiome to small molecules, such as quercetin and rutin to 3,4-dihydroxyphenylacetic acid; luteolin to 3-(3,4-dihydroxyphenyl)propionic acid; and apigenin and naringenin to 3-(4-hydroxyphenyl)propionic acid by cleaving the C ring of flavonoids. When M1 incubated with human gut microbiome, all human subject fermentation samples showed the reduced level of M1, and increased level of M3 compared to control (Figure 3.7C). However, the demethylation degree varied in different human subjects. Human subject 1 only transferred 4.21% M1 to M3, and human subject 3 transferred 85.96% M1 to M3. Particularly, in human subject 2 fermentation sample, there was no M1 but 4.86 µM M3 was detected, which suggested that human subject 2 gut microbiome transferred 100% M1 to M3, and it further transferred M1 to other PMFs, or small molecules. Similar trend of demethylation by human gut microbiome were observed when it incubated with M2 (Figure 3.3D). Human subject 1 only demethylated small portion (3.19%) of M2 to M3. Both human subject 2 and human subject 3 could fully demethylate M2 to M3, and human subject 2 further transferred M3 to other PMFs, or small molecules. When
comparing M1 and M2 metabolic profile by human subject 2 gut microbiome, less M3 was detected in M2 fermentation sample which indicated more M2 was metabolized than M1; comparing M1 and M2 metabolic profile by human subject 3 gut microbiome, M2 was fully metabolized to M3, whereas M2 was partially metabolized to M3 by human subject 3 gut microbiome. Since M1 has one hydroxyl group at the position 3’, whereas M2 has one hydroxyl group at the position 4’, it suggested that compared to 3’ position, 4’ position was much more preferred to be demethylated by human gut microbiome.

Overall, in term of demethylation, human gut microbiome had no effect or very limited effect on NBT and M3. M1 and M2 can be demethylated to M3. The degree of demethylation varied in different human subject gut microbiome that human subject 1 had lowest capacity, while human subject 2 had highest capacity.
Figure 3.7 Metabolic profile of human gut microbiome with A) NBT; B) M3; C) M1; D) M2. Control is PMFs only without human gut microbiome. H1 represents PMFs anaerobically incubated with human subject 1 gut microbiome, H2 represents PMFs anaerobically incubated with human subject 2 gut microbiome, H3 represents PMFs anaerobically incubated with human subject 3 gut microbiome.
3.3.2.2 Biotransformation of SI-5HN, individual 5HN and its metabolites (M4-M6) by human gut microbiome

The metabolic profile of human gut microbiome with small intestine content when mouse fed with 5HN was shown in the Figure 3.8. By further treating human gut microbiome fermentation sample with β-D-glucuronidase and sulfatase, no increasing level of free form was observed. (Figure 3.9) First of all, the glucuronide and sulfate conjugates of 5HN after phase II metabolism could be deconjugated to free form by human gut microbiome. Secondly, as we discussed in the section 3.3.1, the major metabolite of 5HN in small intestine content is M5 (38.4%), then following by M4 (21.39%), and M6 (9.41%) had lowest portion. After anaerobic fermentation with human subject 1 gut microbiome, the ratio of every metabolite (M4-M6) was very similar to control that M5 (39.8%) was major metabolites, then following by M4 (13.7%), and M6 (8.7%). However, human subject 2 and 3 gut microbiome showed different metabolic profile that M6 became major metabolite instead of M5. Particularly, the amount of 5HN, M4 and M5 decreased largely in human subject 2 fermentation sample, from 30.8% to 26.31% for 5HN, from 21.39% to 0% for M4, and 38.4% to 0.85% for M5. While, M6 increased from 9.41% to 72.84%. Additionally, large reduction of total amount of 5HN and its metabolites were observed. The total level of 5HN and its metabolites (conjugates + free) in control was 12.69 µM, while the total level of 5HN and its metabolites in human subject 2 fermentation sample was 6.59 µM. The results suggested that 5HN, M4 and M5 could be converted to M6. 5HN and its metabolites were further metabolized to other PMFs or small molecules by human subject 2 gut microbiome. In human subject 3 fermentation sample, M4 and M5 dropped to 0.05% and 1.09%, while M6 raised to
63.37\%, hence, M4 and M5 were converted to M6 fully by human subject 3 gut microbiome. M4 and M5 both have one hydroxyl group in the different position of B ring, while M6 has two hydroxyl groups in the B ring. Therefore, other than deconjugation, human gut microbiome also caused demethylation at certain positions of flavonoid core structures, such as 3' and 4' positions in the B ring.

Figure 3.8 Metabolic profile of human gut microbiome with small intestine content of mouse fed with 5HN.
Figure 3.9 Metabolic profile of human gut microbiome with small intestine content of mouse fed with 5HN without or with β-D-glucuronidase and sulfatase treatment. Enzyme -: without β-D-glucuronidase and sulfatase treatment; Enzyme +: with β-D-glucuronidase and sulfatase treatment.

In order to further investigate the demethylation by human gut microbiome, the free form of 5HN and its metabolites M4, M5, M6 individual were anaerobic fermented by human gut microbiome separately (Figure 3.10). When 5HN incubated with human gut microbiome, no significant change in level of 5HN was observed in the human subject 1 fermentation sample compared to control (Figure 3.10A). While level of 5HN decreased from 10.52 µM to 0.58 µM, and 8.58 µM in the human subject 2 and human subject 3 fermentation sample, respectively. There were deduction of M6 when human gut microbiome incubated with M6, but the degree of deduction were different in those fermentation samples (Figure 3.10B). M6 decreased from 8.19 µM to 3.53 µM, 1.51 µM
and 3.01 µM in the human subject 1, 2, 3 fermentation sample, respectively. Thus, unlike NBT and M3 which had no or limited demethylation by human gut microbiome, 5HN and M6 could be demethylated or metabolized. The chemical structure difference of NBT and 5HN, M3 and M6, is that 5HN and M6 have hydroxyl group at the position 5, while NBT and M3 have methoxyl group at the position 5 (Figure 3.1). The hydroxyl group at the position 5 resulted in 5HN and M6 more active for demethylation and bioconversion by human gut microbiome. When M4 and M5 incubated with human gut microbiome, the demethylated effect on M4 and M5 varied by different human subjects (Figure 3.10C&D). Human subject 1 had no or limited effect on demethylation. The level of M5 slightly decreased from 9 µM to 7.39 µM, and 0.09 µM M6 was detected. In the human subject 2 fermentation sample, both M4 and M5 level dropped dramatically, and increasing level of M6 was detected. At the same time, the total level of PMFs after fermentation was obviously less than control, especially in M5 sample. It suggested that human subject 2 gut microbiome not only demethylated M4 and M5 to M6, but also transferred M1 to other PMFs, or small molecules. Except M4–M6, Zheng also identified new metabolites, 5, 6-dihydroxylnobiletin, 5, 7-dihydroxylnobiletin, 5, 7, 3'-trihydroxylnobiletin, 5, 7, 4'-trihydroxylnobiletin, and 5, 7, 3', 4'-tetrahydroxylnobiletin from mouse urine after mouse administrated. Lack of standards so far, we could not confirm whether the PMFs metabolites by human gut microbiome were same PMFs from urine. Similar trend of demethylation of M4 and M5 were observed when they incubated with human subject 3 gut microbiome. It could partially demethylated M4 and M5 to M6, and further transferred M5 to other PMFs, or small molecules. Additionally, less amount of M5 was observed compared to M4 in fermentation samples, which suggested that
compared to 3’ position, 4’ position was much more preferred to be demethylated by human gut microbiome. In summary, 5HN and M4-M6 can be demethylated and metabolized by human gut microbiome. The effect on demethylation showed high variation among different human gut microbiome.

There were no significant difference between each other for 2 mice, in term of total amount of NBT/5HN metabolites, and ratio of each metabolite (Figure 3.3). On the other hand, inter-individual variations were observed in different human subjects (Figure 3.5, 3.7, 3.8, 3.10). All human gut microbiome could largely cleave conjugates to free form, while the degree of demethylation varied among 3 human subjects as we discussed. 3 human volunteer is Caucasian, Asian, and Hispanic, respectively, 2 male and 1 female, aged from 20s – 50s. It is not surprised because gut microbiome composition varies substantially among individuals, and is influenced by genotype, physiological condition of host, diet, lifestyle and environment. Consequently, each gut microbial community can be expected to display its own characteristic metabolic profile. The bioactivities of PMFs or other dietary flavonoids varies in different human, thus shows different health beneficial effects. Strong inter-individual variability displayed in 10 human subjects in term of specific dietary polyphenol metabolite profile differing in composition and time courses as well as levels of these metabolites was also reported by Gross. Nevertheless, large sample of human volunteers are needed to support the hypothesis at this point.
Figure 3.10 Metabolic profile of human colonic microbiome with A) 5HN; B) M6; C) M4; D) M5. Control is PMFs only without human gut microbiome. H1 represents PMFs anaerobically incubated with human subject 1 gut microbiome, H2 represents PMFs anaerobically incubated with human subject 2 gut microbiome, H3 represents PMFs anaerobically incubated with human subject 3 gut microbiome.
3.3.3 Inhibitory effects on human colon cancer cells

Colon cancer is one of the leading causes of cancer death in the United States, and there is convincing evidence for protective effects of flavonoids from fruit and vegetables against colon cancer. Thus, we studied the effects of gut microbial metabolites of NBT and 5HH on the growth of human colon cancer cells; HCT 116 and HT29 cell lines.

As shown in the Figure 3.11, the isolates from small intestine of mouse fed with NBT after mouse gut microbiome fermentation, demonstrated a dose-dependent inhibition on the growth of 2 human colon cancer cells. First of all, the mouse gut microbiome itself showed no inhibitory effect on 2 colon cancer cells. According to HPLC analysis results and different tolerance of colon cancer cells to PMFs, we determined and prepared the full dose of SI-NBT and mouse gut microbiome treatment as 10 μM and 5 μM total concentration of NBT and its metabolites after SI-NBT fermented with mouse gut microbiome for HCT116 cell and HT29 cell, respectively, then diluted to several lower concentrations. Accordingly, the same concentrated fold used in SI-NBT and mouse gut microbiome treatment was applied in SI-NBT without mouse gut fermentation treatment to achieve full does. In the Figure 3.11A, at full dose of SI-NBT without mouse gut microbiome fermentation, only 13.5% of HCT116 cell was inhibited. On the other hand, 35.7% of HCT cell and 63.5% of HCT116 cell were inhibited by 5 μM (half full dose) and 10 μM (full does) of SI-NBT with mouse gut microbiome fermentation. NBT and its metabolites showed higher inhibitory effects on HT29 cancer cell line compared to HCT 116 cancer cell (Figure 3.11B). When HT29 cell treated with isolates of SI-NBT after mouse gut microbiome fermentation, 5 μM of total concentration of NBT and its
metabolites (full dose) could inhibit 51.9% of HT29 cell growth; while, full dose of isolates of SI-NBT without mouse gut microbiome fermentation only inhibited 21.6% of HT29 cell growth. Overall, SI-NBT after gut microbiome fermentation showed much stronger inhibitory effect on both HCT116 and HT29 colon cancer cell lines than SI-NBT without mouse intestinal microbiome fermentation.

Figure 3.11 Inhibitory effect of SI-NBT with mouse gut microbiome fermentation on the growth of HCT116 (A) and HT29 (B) human colon cancer cells. Data represent mean ± SD (N = 6). All the results described in Section 3.3 showed statistical significance according to Student’s t-test (p < 0.01, n = 6).

Compared to NBT and its metabolites, 5HN and its metabolites exhibited much stronger inhibitory effect on HCT116 and HT29 colon cancer cell (Figure 3.12). Zheng reported that the metabolites of 5HN, M4, M5 and M6 showed stronger inhibitory effects on human colon cancer SW620 and SW480 cells than their parent compound 5HN. Particularly, M4 had IC₅₀ value of 0.18 μM and 0.12 μM after 48 and 72 hours treatment on SW619 cells, and 0.5 μM on SW 480 cells. As shown in the Figure 3.12A, SI-5HN without mouse gut microbiome fermentation only inhibited 17.8% of HCT116 cell at the
full dose. While after mouse gut microbiome fermentation, 5 μM (half full dose) and 10 μM (full dose) of total 5HN, and its metabolites inhibited 45.7% and 76.3% of HCT116 cell growth. Similar to NBT and its metabolites, 5HN and its metabolites showed stronger inhibitory effect on HT29 cell (Figure 3.12B). After mouse gut microbiome fermentation, 1.25 μM of total concentration of 5HN and its metabolites inhibited 47.3% HT29 cell. Once the total concentration increased to 2.5 μM (half full dose), 81% of HT29 cells were inhibited. There was no significant increasing of inhibitory effect at full dose. However, SI-5HN without mouse gut microbiome fermentation only inhibited 20.6% of HT29 cell at the full dose. In sum, SI-5HN showed much stronger inhibitory effect on HCT116 and HT29 cell growth after mouse gut microbiome fermentation.

Mouse gut microbiome mainly cleave the PMFs conjugates to free form. Therefore, the removal of the polar conjugating groups by the gut microbiome not only allows those metabolites to be reabsorbed and returned to its site of action, prolong the action of PMFs by allowing their enterohepatic circulation to continue, but also restore the active compound, and increased the bioactivities of metabolites.
Figure 3.12 Inhibitory effect of SI-5HN with mouse gut microbiome fermentation on the growth of HCT116 (A) and HT29 (B) human colon cancer cells. Data represent mean ± SD (N = 6). All the results described in Section 3.3 showed statistical significance according to Student’s t-test (p < 0.01, n = 6).
CHAPTER 4
SURFACE ENHANCED RAMAN SCATTERING CHARACTERIZATION OF MONOHYDROXYLATED POLYMETHOXYFLAVONES

4.1 Introduction
Polymethoxyflavones (PMFs), a unique class of flavonoids mainly found in citrus fruits, have been reported to have a broad spectrum of beneficial bioactivities including anti-inflammatory, anti-carcinogenic activities and anti-atherosclerosis. More than 20 different PMFs have been isolated and identified from citrus plants. Characterization of different PMFs is critical to further understand and apply these compounds as functional food ingredients in food and beverage products. Many analytical methods have been reported to separate and characterize those PMFs from citrus fruits, e.g. capillary electrochromatography, flashing chromatography, thin-layer chromatography (TLC), supercritical fluid chromatography (SFC), gas chromatography (GC), and high performance liquid chromatography (HPLC). Mass spectrometry (MS) and nuclear magnetic resonance (NMR) were usually conducted after chromatographic system for structural identification and elucidation.

There are growing interests on using an innovative analytical method, surface enhanced Raman spectroscopy (SERS), to characterize PMFs and other flavones. SERS is a novel analytical method that combines Raman spectroscopy and nanotechnology. Raman spectroscopy is a vibrational spectroscopy providing rich characteristic information of molecular structure. It is caused by the interaction between energy change of inelastically
scattered incident light and vibrational mode of chemical bonds within sample. However, only 1 in $10^{6-8}$ of photons undergoes Raman scattering, hence, the Raman scattering signal is relatively weak. By placing sample on or near the noble metal nanostructure, e.g. silver, gold or copper, can enhance the weak Raman scattering more than a million times through the amplification of electromagnetic fields. Due to its highly sensitive structural detection on a trace amount of analytes, SERS has been rapidly developed as a powerful tool for molecular detection and characterization, with applications in materials science, chemistry, engineering, bioscience and medical science.\textsuperscript{36,37} Compared with other analytical methods, the advantages of using SERS for characterizing PMFs are: 1) great capacity to differentiate PMFs with different chemical structures, 2) simple sample preparation, 3) sensitive measurement requiring a trace amount of sample, 4) rapid analysis.

In previous studies, we have explored Raman and SERS for characterizing some PMFs, including nobiletin, 5-hydroxynobiletin and tangeretin, to demonstrate the superior capacity of SERS for characterization and differentiation among different PMFs, in comparison with HPLC.\textsuperscript{151,152} Particularly, we found the number of the hydroxyl groups played an important role in interaction with silver (Ag) dendrites, the SERS substrate used in these studies.\textsuperscript{46,152} However, the molecular behaviors of PMFs interacting with Ag substrate is still poorly understood, which significantly limits the development and applications of SERS for further analyzing the different PMFs. In this study, we aimed to focus on monohydroxylated PMFs to elucidate the role of the hydroxylation position on the interaction between PMFs and Ag, and its relationship with the SERS enhancement.
and characterization. Three monohydroxylated PMFs were studied, namely 3’-hydroxynobiletin (3HN), 4’-hydroxynobiletin (4HN), and 5-hydroxynobiletin (5HN). 3HN and 4HN are the metabolites of nobiletin showed as M1 and M2 in the section 3.2.1. Those monohydroxylated PMFs exhibited much stronger bioactivities than nobiletin. 3HN and 4HN revealed more potent anti-inflammatory activity, and 5HN showed stronger inhibitory effect on different cancer cells. Their enhanced bioactivities are believed to correlate with their unique chemical structures; however, the chemical mechanism remains uncovered. For the first time, this study aims to use SERS to characterize three monohydroxylated PMFs and determine the role of hydroxylation position in the PMF molecules. Understanding the SERS behaviors of these PMFs will further advance the utilization of SERS for the characterization of different flavonoids in future.

4.2 Materials and methods

4.2.1 PMF structures

Three monohydroxylated PMFs, 3’-hydroxynobiletin (3HN), 4’-hydroxynobiletin (4HN), and 5-hydroxynobiletin (5HN) are shown in the Figure 3.1. The major difference is the position of hydroxyl group in the molecule. They were chemically synthesized from nobiletin that was isolated from sweet orange as previously described.
4.2.2 SERS sample preparation and Raman instrumentation

Ag dendrites were synthesized through a simple displacement reaction involving both zinc and silver nitrate in accordance to a previously published method. The PMFs stock solutions were 20 mM in DMSO (Thermo Scientific). 3HN and 4HN were diluted by methanol (Thermo Scientific) to 0.05, 0.1, 0.5, 1, 2, 3 mM, and 5HN was diluted by methanol to 0.005, 0.01, 0.05, 0.1, 0.5, 1 mM due to its poor solubility in methanol. 500 µL serial concentrations of PMFs solutions incubated with 10 µL Ag dendrites for 30 minutes under consistent orbital rotation at room temperature. The Ag mixture was washed by double-distilled water 3 times to remove unspecific binding, and then it was deposited onto a glass slide and air dried for Raman measurement.

The SERS spectra of each sample were collected using a DXR Raman microscope (Thermo Scientific) with a 780 nm excitation laser and a 10× objective confocal microscope (3 µm spot diameter and 5 cm⁻¹ spectral resolution). The Raman measurement was taken with 3 mW of laser power and a 50 µm slit aperture for 2 seconds scanning time. 8 spectra were collected randomly from each sample within 400-2000 cm⁻¹ range using the Thermo Scientific OMNIC software.

TQ analyst software, version 8.0 (Thermo Fisher Scientific), was conducted to analyze the data obtained from the DXR Raman microscope. Second derivative transformation and smoothing (9 nm segment length and 9 nm gap between segments) were achieved to separate overlapping bands and yield spectral noise. Principal component analysis (PCA) was applied to analyze the variance of spectral data, and it provided variance
within a class and between different classes. The PC score revealed the percentage of
data variance. A larger percentage demonstrated more variance within data. In general, if
there is no overlap between two clusters of sample, samples are significantly different.

**4.2.3 HPLC sample preparation and HPLC instrumentation**

HPLC analysis was conducted to directly quantify the concentration difference of PMFs
before and after incubating with Ag dendrites. The same PMFs stock solution was diluted
to 0.005 mM by methanol as the group before incubation. 100 μL PMFs solution (0.005
mM) was incubated with 5 μL Ag dendrites overnight under consistent orbital rotation at
room temperature. After centrifugation, the supernatant of PMFs solution was considered
as the group after incubation. Three replicates of samples were prepared for HPLC
analysis.

The quantitative difference of PMFs before and after incubation with Ag dendrites was
determined by an HPLC with an electrochemical detector using previously published
method described in the section 3.2.7.

**4.3 Results and discussions**

**4.3.1 Determination of the dose dependent curve of SERS enhancement**

The raw SERS spectra of 3 PMFs were showed in the Figure 4.1. The SERS band
wavenumbers were tabulated in the Table 4.1, along with the assigned brief description
of the major vibrational mode. Even through those 3 PMFs shared very similar
chemical structure, they exhibited their own characteristics bands. Those 3 PMFs spectra
showed similar trend of the region corresponding to Ring A, B, C deformation at 500 -
750 cm\(^{-1}\), and C=O stretch at 1550 – 1650 cm\(^{-1}\). The major differences were in the 1100 – 1500 cm\(^{-1}\) region by appearance of different O–H bend due to the different position of hydroxyl group. According to the Table 4.1, the in-plane vibrational mode were observed in 3 PMFs, especially at the lower wavenumber range, assigned as in-plane ring deformation. According to the surface selection rule,\(^{160}\) when adsorbed perpendicularly, the in-plane bending modes of the molecules are more enhanced than the out of plane bending modes. This indicated that all 3 PMFs were interacted perpendicularly with respect to the Ag surface.
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<td>1613</td>
<td>1618</td>
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<td>C=O stretch; quinoid stretch</td>
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ip: in-plane

Table 4.1. Wavenumbers and assignments of the SERS spectra of 3HN, 4HN and 5HN.
The raw SERS spectra of 4HN in different concentrations were shown in the Figure 4.2. After secondary derivative transformation and normalization based on the NO$_3^-$ peak (1070 cm$^{-1}$) that was generated from Ag fabrication, their spectra were shown in the Figure 4.3. The data preprocessing helped improve the consistency and accuracy for the spectral comparison. It was clearly observed that with the 4HN concentration increased, the overall peak intensity increased rapidly at initial low concentrations (0.1 mM and 0.5 mM). The rate of increase greatly slowed down after 1 mM. Principal component analysis (PCA) of 4HN at different concentrations (Figure 4.4) was used to analyze the variation between different concentrations. The clusters of different concentrations were completely separated up to 1 mM, which demonstrated that the peak intensity of these concentrations below 1 mM were statistically different from each other. Beyond 1 mM,
clusters of different concentrations overlapped, and the variance within cluster became large. It indicated that the difference of peak intensity between those concentrations were not statistically significant. Therefore, based on the SERS spectra and the PCA result, we defined the saturation point of 4HN to be 1 mM. The saturation happened when the Ag surface was covered by the molecules completely, therefore no further increase of peak intensity was observed when the concentration was higher than the saturation point.

Figure 4.2 The raw SERS spectra of 4HN in different concentrations (0.05 – 3 mM).
Figure 4.3 The SERS spectra of 4HN in different concentrations (0.05 – 3 mM) after 2\textsuperscript{nd} derivatives transformation.

Figure 4.4 The PCA of SERS spectra of 4HN in different concentrations (0.05 – 3 mM). PC1 and PC2 revealed 96.26\% of total variance.
Similar method was used to characterize the other two PMFs, namely 3HN and 5HN. The raw SERS spectra and spectra in second derivatives of 3HN and 5HN, and the PCA of 3HN and 5HN were shown in the Figure 4.5 – 4.10. The saturation points of 3HN and 5HN were determined to be 0.5 mM and 0.05 mM, respectively. Figure 4.11 summarized the relationship between PMFs concentrations and relative peak intensity (indicative peak intensity over NO$_3^-$ peak intensity) for the three PMFs. The indicative peak represented ring A, B, C deformation – assigned 3HN, 4HN, 5HN at 579 cm$^{-1}$, 580 cm$^{-1}$, 587 cm$^{-1}$, respectively.$^{153,158}$ Relative peak intensity of 3HN increased smoothly with the 3HN concentration increased and overall peak intensity was much lower than 4HN and 5HN. Relative peak intensity of 4HN and 5HN increased rapidly before saturation, and the increasing rate of 5HN was greater than 4HN. Below the saturation, comparing peak intensity at the same concentration, 5HN had the highest peak intensity, while 3HN had the lowest peak intensity. In addition, 5HN reached saturation peak intensity at relative low concentration (0.05 mM), while 3HN and 4HN reached saturation peak intensity at higher concentrations (0.5 mM and 1 mM, respectively). After becoming saturation, 4HN and 5HN produced similar relative peak intensities that were much higher than that of 3HN. The intensity variance was also larger after the saturation points as demonstrated by the error bars, which was in a good agreement with the PCA results. We didn’t test 5HN for higher concentrations because its solubility is very low and unable to be dissolved at higher concentrations. The concentration and relatively peak intensity at the saturation point of three PMFs were summarized in the Figure 4.12.
Figure 4.5 The raw SERS spectra of 3HN in different concentrations (0.05 – 3 mM).

Figure 4.6 The raw SERS spectra of 5HN in different concentrations (0.005 – 1 mM).
Figure 4.7 The SERS spectra of 3HN in different concentrations (0.05 – 3 mM) after \(2^{\text{nd}}\) derivatives transformation.

Figure 4.8 The SERS spectra of 5HN in different concentrations (0.005 – 1 mM) after \(2^{\text{nd}}\) derivatives transformation.
Figure 4.9 The PCA of 3HN SERS spectra in different concentrations (0.05 – 3 mM). PC1 and PC2 revealed 67.24% of total variance.

Figure 4.10 The PCA of 5HN SERS spectra in different concentrations (0.005 – 1 mM). PC1 and PC2 revealed 96.25% of total variance.
Figure 4.11 The relationship of PMF concentration and relative peak intensity (indicative peak intensity over NO$_3^-$ peak intensity). The indicative peak represented ring A, B, C deformation – assigned 3HN, 4HN, 5HN at 579 cm$^{-1}$, 580 cm$^{-1}$, 587 cm$^{-1}$, respectively.

Figure 4.12 The saturation concentration with its relative peak intensity, and binding percentage of three PMFs.

The characteristic peak of 3 PMFs assigned as – ring A, B, C deformation.
4.3.2 Determination of the binding affinity of three PMFs on Ag by HPLC

In order to characterize the different SERS behaviors of three different PMFs, we determined the binding affinity of PMFs to Ag dendrites before saturation using HPLC. HPLC analysis was conducted to directly compare the PMFs concentration difference in the supernatant before and after incubating with Ag dendrites. By knowing the amount of PMF left in the supernatant, the amount of PMF molecules bound to Ag dendrite could be calculated. The percentage of bound PMFs was obtained from HPLC analysis (Figure 4.12), and the results revealed that 36.13 ± 1.06% of 5HN, 18.40 ± 3.31% of 4HN and 9.66 ± 0.94% of 3HN were bound to Ag dendrites. This result elucidated that higher peak intensity in the SERS spectra might be resulted by higher binding affinity of specific PMFs onto Ag dendrites.

4.3.3 Possible conformations of three PMFs on Ag and their relationships to overall SERS enhancement

Based on our previous studies, the number of hydroxyl group in the structure of PMFs is critical in their binding affinity with Ag dendrites.\textsuperscript{46,152} Although the three PMFs studied here are all monohydroxylated PMFs, their binding behaviors on Ag dendrites are very different, resulting in different SERS enhancement. These results demonstrated that in addition to the total number of hydroxyl groups, the position of the hydroxyl group is also critically important.

Firstly, the overall spatial conformation of PMFs bound on Ag surface is determined by the position of the hydroxyl group. The possible binding site and spatial conformation of
PMFs on Ag dendrites surface was illustrated in the Figure 4.13. All 3 PMFs were attached to the Ag surface perpendicularly as demonstrated by the surface selection rule. 5HN molecule tended to bind onto the Ag surface through the strong chelation of the hydroxyl group in the A ring and the carbonyl group in the C ring, and therefore one single molecule occupied relatively large surface area.\textsuperscript{153,155} Most interestingly, although both 3HN and 4HN had the hydroxyl group in the B ring, they behaved differently when attached on the Ag surface. 4HN has a hydroxyl group at 4’ position, so it tended to bind onto the Ag surface with A and C rings straight up. 3HN has a hydroxyl group at 3’ position, so the A and C rings were more towards to the side. The whole 3HN molecule not only could be turned around by the central axis of C-O bond, but also the subpart of A ring and C ring could be turned round by the C-C bond between the B ring and C ring at certain angle. Once one 3HN molecule bound to Ag surface, the large potential space it occupied might prevent another 3HN binding to nearby binding sites. The uncertainty and flexibility of molecular conformation resulted in less and disorder attachment. The large variance within 3HN sample revealed by PCA (Figure 4.14) also supported the disordered attachment of 3HN on Ag. On the other hand, the 4HN molecule produced smaller space hindrance, even though the C-O bond and the C-C bond linked to the B ring and C ring could be turned around. The 5HN molecule was bound to Ag surface by both A and C rings, only the C-C bond linked to the B and C rings was turnable. The conformation of 4HN and 5HN illustrated those two molecules had more organized and uniform orientation when attached on the Ag surface.
Secondly, our results showed that the position of the hydroxyl group also influenced the binding affinity of the PMFs on Ag. 5HN had the strongest binding affinity because the hydroxyl group which is in the A ring at position 5 could possibly chelate with carbonyl group in the C ring at position 4. 3HN had the lowest binding affinity. It may be due to the highly flexibility of 3HN orientation on the Ag surface and further experiment is needed for supporting this speculation.

Lastly, our results revealed that the different spatial conformations and binding affinity of PMFs bound on Ag surface resulted in observed difference in the saturation point and SERS enhancement. Assuming the area of Ag surface for PMFs binding was the same, and one 5HN molecule occupied larger space, so less 5HN was needed to cover the surface completely compared to 4HN, resulting in a low saturation concentration of 5HN. It is noteworthy that the saturation peak intensity of 5HN was slightly greater than the saturation peak intensity of 4HN in the Figure 4.11. It is potentially due to the fact that the enhancement effect of SERS is distance-dependent, i.e. the maximum SERS enhancing region decreases extremely rapidly when the distance increases. The overall 5HN molecule configuration was closer to the Ag surface than 4HN so that 5HN had higher saturation peak, but further investigation is needed to support this conclusion. Hence, before saturation, due to its stronger binding affinity and larger spatial conformation, 5HN had higher peak intensity than 4HN, and it became saturation at lower concentration. However, when the amount of PMF molecule was more than saturation, all binding sites on Ag surface were occupied by PMFs, both 4HN and 5HN showed similar saturation peak intensities. On the other hand, in the case of 3HN and
4HN, theoretically, they should have similar binding affinity to Ag surface in term of OH-Ag bond in the B ring. However, the spatial conformation of 3HN greatly limited its binding affinity. The potential binding sites were masked not only under large spatial occupation of one 3HN molecule, but also by highly disorder conformational attachment on Ag dendrites surface. Therefore, 3HN had the lowest peak intensity among the three PMFs tested.

Figure 4.13 Illustration of possible binding sites and spatial conformation of PMFs on Ag dendrites surface.
CHAPTER 5
MOLECULAR CHARACTERIZATION OF THE INTERACTIONS BETWEEN
POLYMETHOXYFLANES AND KAPPA CASEIN

5.1 Introduction

Many potential health benefits and biological fate of flavonoids have been associated with their ability to interact and bind non-covalently to macronutrients in foods. Flavonoid–protein interactions are known phenomenon; those interactions not only play critical roles in food and beverage quality, including their taste, texture and other sensorial properties, but also the bioavailability and stability of flavonoids. To probe the interactions between flavonoids and proteins, it is very important to characterize the flavonoids–protein interactions at a molecular level and determine the molecular changes involved in the both molecules.

There are growing interests on using an innovative analytical method, surface enhanced Raman spectroscopy (SERS), to characterize molecular interactions between ligands and proteins. SERS is a combination of Raman spectroscopy and nanotechnology. SERS provides the vibrational fingerprints of biomolecules by placing the analyte molecule onto a nanoscale metallic surface. The abilities to enhance Raman signal and reduce fluorescence background make SERS an emerging method for studying biomolecules. SERS has been used to study ligand–protein interactions by dye-labeling methods and label-free methods. The dye-labeling methods need specially designed Raman reporter labeled silver or gold nanoparticle probes. The detection of
Raman reporter signals indicates the interaction between ligand and protein. The label-free methods are based on the intrinsic signals of ligand and protein. The changes in the Raman spectra of ligand protein incubation, either spectral pattern or intensity, is a direct indication of ligand-protein interaction. It not only provides an insight into the mode of binding of the ligand to the protein, but also the protein conformation change.42–46

Fluorescence quenching is a sensitive, simple, and rapid method to investigate the interaction and binding of flavonoids to proteins.28,162–165 A quencher molecule can decrease the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions between the fluorophore and the quencher molecule. Dynamic or static quenching may be involved in the fluorescence process. Dynamic quenching results from collisional encounters between the fluorophore and quencher, whereas static quenching is due to the nonfluorescent complex of a quencher with a fluorophore. Fluorescence quenching not only reveals the accessibility of fluorophores to quencher, but also the localization of fluorophores in protein and their permeability to quenchers.166

In this study, we aimed to characterize the molecular interactions between polymethoxyflavones (PMFs) and κ-casein using SERS and fluorescence spectroscopy. PMFs, a unique class of flavonoids, are abundant in the citrus peel, and have been received particular interest due to a broad spectrum of beneficial bioactivities including anti-inflammatory, anti-carcinogenic activities and anti-atherosclerosis.3,5,22,50,52,150 Two PMFs molecules, nobiletin (NBT) and 5, 3’, 4’- trihydroxynobiletin (THN) were chosen in this study to investigate the importance of hydroxyl groups in the molecular
interactions with κ-casein. Casein is the major protein found in milk, constituting about 80% of the total protein in milk, and four types of casein (αs1, αs2, β, κ) form casein micelle. K-casein is the outer layer of casein micelle and stabilize the micelle structure. Therefore, κ-casein is considered as the major casein molecules involved in the interactions with flavonoids. Both native and thermal denatured κ-casein were tested to investigate the influence of protein structure and thermal processing on the protein-flavonoid interactions. The results obtained through this study not only can help us to gain the fundamental understanding of the molecular mechanism of protein-flavonoid interactions, but also provide useful information to design a more stable and healthier functional food.

5.2 Materials and methods

5.2.1 Materials

K-casein with purity of 70% was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). The chemical structure of two PMFs, nobiletin (NBT), 5, 3’, 4’-trihydroxynobiletin (THN) are shown in the Figure 3.1. NBT was purchased from Quality Phytochemicals LLC(Edison, NJ), and THN was synthesized from NBT as previously described. Reagents were obtained from Fisher Scientific (Waltham, MA), unless otherwise indicated.

5.2.2 Preparation of stock solutions

The native κ-casein solution (0.1%) was prepared fresh at the day of use. The thermal denatured casein solution was prepared by heating the κ-casein solution in water bath at
95 °C for 30 minutes, then cooling to room temperature. The PMFs stock solutions were 20 mM in DMSO.

5.2.3 Surface enhanced Raman spectroscopy (SERS)

Silver (Ag) dendrites, a SERS substrate, were synthesized through a simple displacement reaction involving both zinc and silver nitrate in accordance to a previously published method.\textsuperscript{119} 250 µL κ-casein solutions were incubated with 5 µL Ag dendrites for 30 minutes under consistent orbital rotation at room temperature. The casein conjugated Ag was washed by double-distilled water 3 times to remove unspecific and oversaturated binding. The PMFs stock solutions were diluted by methanol to 1 mM. 5 µL casein conjugated Ag dendrites were added to 250 µL PMFs solutions, and incubated with for 30 minutes under consistent orbital rotation at room temperature. The mixture was washed by double-distilled water 3 times, and then it was deposited onto a glass slide and air dried for Raman measurement.

The detail of Raman spectroscopy and TQ analyst software were described in the section 4.2.2. The parameters of the Raman measurement was also followed in the section 4.2.2.

5.2.4 Fluorescence spectroscopy

A SpectraMax m2e microplate reader (Molecular Devices, Sunnyvale, CA) was employed to quantify the binding of PMFs with κ-casein. A stock solution of 20 mM PMFs in DMSO was diluted to 0.05-0.5 mM in 50% methanol (v/v). For each data point, 50 µL of the appropriate PMFs solution was added into 450 µL casein solution, to give a
final PMFs concentration in the range 0.005 - 0.050 mM. All measurements were made at room temperature and were conducted in triplicate. Control was casein solutions prepared with 2.5% DMSO in 50% methanol (v/v), without PMFs. The changes in the intrinsic fluorescence of native and thermal denatured κ-casein at varying concentrations of PMFs were measured and fluorescence excitation wavelength was set at 280 nm, and emission spectra were recorded from 300 to 450 nm. Fluorescence quenching was described using the Stern–Volmer equation,\textsuperscript{166}

$$\frac{F_0}{F} = 1 + K_{SV}[Q]$$  \hspace{1cm} [1]

where $F_0$ is the fluorescence intensity of casein control without PMFs, and $F$ is the fluorescence intensity after addition of PMFs, $[Q]$ is the concentration of PMFs, and $K_{SV}$ is the Stern–Volmer quenching constant. Hence, the above equation is applied to determine the $K_{SV}$ using linear regression of a plot of $F_0/F$ against $[Q]$. A linear Stern-Volmer plot generally means that only one mechanism, dynamic or static, when quenching occurs.

When the relationship between $F_0/F$ and the concentration of quencher is no longer linear, but exhibits an upward curvature, concave toward the y axis at high $[Q]$, it is described with a modified form of the Stern–Volmer equation,\textsuperscript{168}

$$\frac{F_0}{F} = e^{(K_{app}[Q])}$$  \hspace{1cm} [2]

where $K_{app}$ is the apparent static quenching constant, and it suggests that the fluorophores and quenchers do not actually form a ground-state complex. Instead, the quencher is adjacent to the fluorophore at the moment of excitation, and these closely spaced
fluorophore-quencher pairs are immediately quenched, thus appears to be dark complexes.

5.3 Results and discussion

5.3.1 Surface enhanced Raman scatting characterization

The raw Raman spectra of native and thermal denatured κ-casein were showed in the Figure 5.1. The 2nd derivative Raman spectra of native and thermal denatured κ-casein were showed in the Figure 5.2. The band at 1662 and 1689 cm\(^{-1}\) in the native casein spectrum shifted to 1665 and 1676 cm\(^{-1}\) in the thermal denatured casein spectrum, and those bands were related to amide I region. The band at 1249 cm\(^{-1}\) and 1275 cm\(^{-1}\) in the native casein spectrum shifted to 1242 cm\(^{-1}\) and 1270 cm\(^{-1}\), which corresponded to amide III bond.\(^{108,169}\) Those band shifts in amide I and III indicate the secondary structure change of casein after thermal treatment. Increased band intensity of band 642 cm\(^{-1}\), 824 cm\(^{-1}\), 1169 cm\(^{-1}\), 1203 cm\(^{-1}\), and 1599 cm\(^{-1}\) which assigned as tyrosine (Tyr) were observed in the thermal denatured casein spectrum.\(^{169}\) K-casein contains 9 Tyr residues, 7 of Tyr residues are located between position 35 to 68 which represents an exceptionally hydrophobic area.\(^{170}\) The higher band intensity of Tyr residues might be caused by more hydrophobic area exposed in the thermal denatured casein than the native casein. Therefore, the thermal treatment resulted in conformational change of casein secondary and tertiary structure. Additionally, phenylalanine band (1002 cm\(^{-1}\)) was not sensitive to conformational changes of protein, so it was used for normalization of the Raman spectra of protein for direct comparison.\(^{108}\)
Figure 5.1 Raw Raman spectra of native casein and thermal denatured casein.

Figure 5.2 Raman spectra of native casein and thermal denatured casein after 2\textsuperscript{nd} derivatives transformation with peak assignment.
Different states of casein exhibited different binding affinity to the same PMFs. Raw Raman spectra of casein, casein incubated with NBT, and NBT were shown in the Figure 5.3. Figure 5.4 presented the $2^{nd}$ derivative Raman spectra of casein, casein incubated with NBT, and NBT. In the Raman spectrum of the native casein incubated with NBT, clear phenylalanine band at 1002 cm$^{-1}$ from casein was shown, but no or very little signature band from NBT was observed. Previous study in our group also confirmed it by principal component analysis, and pointed out that there was no or very weak binding between NBT and native casein.$^{46}$ Interestingly, after incubating the thermal denatured casein with NBT, the Raman spectrum exhibited increased NBT bands intensity (578 cm$^{-1}$, 1598 cm$^{-1}$, 1626 cm$^{-1}$). The signature band 578 cm$^{-1}$ comes from A, B, C ring deformation, band 1598 cm$^{-1}$ and band 1626 cm$^{-1}$ are related to B ring quinoid stretch and C=O stretch, respectively.$^{153,157,158}$ The higher band intensity resulted from stronger binding affinity of two molecules. Hence, the binding affinity of NBT to thermal denatured casein was much stronger than native casein.

On the other hand, THN behaved an opposite manner when it interacted with casein. Raw Raman spectra of casein, casein incubated with THN, and THN were shown in the Figure 5.5. Figure 5.6 presented the $2^{nd}$ derivative Raman spectra of casein, casein incubated with THN, and THN. No matter which states of casein, the casein – THN spectra contained both signature bands of casein (1002 cm$^{-1}$) and THN (582 cm$^{-1}$, 1571 cm$^{-1}$) suggesting THN could interact with native casein and thermal denatured casein. The band 582 cm$^{-1}$ and band 1571 cm$^{-1}$ are assigned as A, B, C ring deformation, and 5 OH bend and C=O stretch, respectively.$^{153,158}$ Interestingly, the band intensity of THN
was much higher in the native casein – THN spectrum compared to thermal denatured casein – THN spectrum. Therefore, the binding affinity of THN to thermal denatured casein was lower compared to native casein.

Moreover, Raman band shifts were further observed when THN interacted with casein (Figure 5.7). The band at 1324 cm\(^{-1}\) is assigned as 5, 3’ OH bend, the band at 1571 cm\(^{-1}\) is assigned as 5 OH bend and C=O stretch, and the band at 1620 cm\(^{-1}\) is assigned as 5, 3’ OH bend in the THN spectrum were shifted to 1337 cm\(^{-1}\), 1566 cm\(^{-1}\), and 1612 cm\(^{-1}\) in the casein – THN spectra, respectively.\(^{153,157,158}\) The phenolic hydroxyl group is an excellent hydrogen bond donor and forms strong hydrogen bonds with the amide carbonyl of the peptide backbone or alkaline protein groups.\(^{171}\) Therefore, the hydrogen bond was formed between native casein and THN, which has three hydroxyl groups. The decreased band intensity of those bands in thermal denatured casein-THN spectrum means lower hydrogen bonding between the thermal denatured casein and THN.

Different PMFs also exhibited different binding affinity to the same state of casein. In the Figure 5.4, the ratio of fingerprint band intensity of NBT assigned as A, B, C ring deformation (578 cm\(^{-1}\)) over casein phenylalanine (1002 cm\(^{-1}\)) band intensity was lower than 1. While, the ratio of fingerprint band intensity of THN assigned as A, B, C ring deformation (582 cm\(^{-1}\)) over casein phenylalanine (1002 cm\(^{-1}\)) band intensity was greatly higher than 1 when the THN concentration was 0.1 mM (Figure 5.6). When same amount of PMFs were added to same amount of casein, THN showed higher band intensity indicating that more THN were bound to casein. Hence, THN revealed stronger
binding affinity to casein than NBT. The different structure of flavonoids largely affect their binding affinity with protein. Particularly, methoxylation of flavonoids decreased the binding affinity, while hydroxylation on the A and B ring of flavones increased the interaction. THN has one hydroxyl group at the position 5 on the A ring and two hydroxyl groups at the position 3’ and 4’ on the B ring significantly enhances its binding to casein.

Figure 5.3 Raw Raman spectra of casein, native casein incubated with NBT, thermal denatured casein incubated with NBT, and NBT. Compared to NBT spectrum, other 3 spectra enlarged 4 times.
Figure 5.4 Raman spectra of casein, native casein incubated with NBT, thermal denatured casein incubated with NBT, and NBT after 2nd derivatives transformation. Compared to NBT spectrum, other 3 spectra enlarged 6 times.
Figure 5.5 Raw Raman spectra of casein, native casein incubated with THN, thermal denatured casein incubated with THN, and THN. Compared to THN spectrum, casein spectra enlarged 10 times, native casein-THN spectrum and thermal denatured casein-THN spectrum enlarged 3 times.
Figure 5.6 Raman spectra of casein, native casein incubated with THN, thermal denatured casein incubated with THN, and THN after 2nd derivatives transformation. Compared to THN spectrum, other 3 spectra enlarged 2 times.

Figure 5.7 Raman band shifts of native and thermal denatured casein interacted with THN after 2nd derivatives transformation.
5.3.2 Fluorescence quenching spectroscopy

Figure 5.8 showed the typical fluorescence emission spectra obtained for native casein with the addition of NBT and THN, at 280 nm excitation. The casein exhibited a strong fluorescence emission with a peak at 335 nm. The fluorescence intensity of casein emission peaks inversely decreased with the increasing concentration of NBT and THN. The decline in the fluorescence intensity was caused by quenching, but there was no significant emission wavelength shifts with the addition of NBT and THN. The same trend was observed by NBT and THN with thermal denatured casein shown in the Figure 5.9. Since κ-casein contains one Trp residue at position 76, no significant emission wavelength shifts suggested that there was no change in the immediate environment of the tryptophan residues, other than the fact that the PMFs were situated at close proximity to the tryptophan residue for the quenching to occur. It could be further interpreted that the molecular conformation of casein was not significantly affected by PMF interactions.

The Stern-Volmer plots of $F_0/F$ versus concentration of PMFs did not exhibit good linear relationship, but an upward curvature, concave towards the y-axis. (Figure 5.10.) It was indicative of both dynamic and static quenching involving in the interaction. In this case, the modified Stern-Volmer equation described by Eq. [2] was applied. The concentration dependence of the fluorescence intensity of casein was analyzed using modified Stern-Volmer plot of $\ln(F_0/F)$ as a function of concentration of PMFs shown in the Figure 5.11. The Stern-Volmer constant can be described by an apparent static quenching constant ($K_{app}$), with a nonspecific binding, as both dynamic and static quenching occur. The particular quenching behavior further confirmed that PMFs and casein did not actually
form a ground-state complex, but PMFs was adjacent to the casein at the moment of excitation. PMFs were situated at close proximity to casein but did not enter into the casein molecule.

Table 5.1 summarized the calculated $K_{app}$ for PMFs and $\kappa$-casein. The apparent quenching constants were statistically different between NBT and THN to different states of casein. The NBT showed higher quenching constant to the thermal denatured casein than the native casein. However, the quenching constant of THN to the thermal denatured casein was lower than it to the native casein. In addition, for native casein, the quenching constant of THN was higher than NBT. While, for thermal denatured casein, it did not show significant difference between NBT and THN.
Figure 5.8 Emission spectra of 0.05% native casein at $\lambda_{ex} = 280$ nm showing the quenching effect of increasing concentrations of (A) NBT (0, 5, 10, 15, 20, 25, 30, 40, 50 $\mu$M) and (B) THN (0, 5, 10, 15, 20, 25, 30, 40, 50 $\mu$M).
Figure 5.9 Emission spectra of 0.05% thermal denatured casein at $\lambda_{ex} = 280$ nm showing the quenching effect of increasing concentrations of (A) NBT (0, 5, 10, 15, 20, 25, 30, 40, 50 µM) and (B) THN (0, 5, 10, 15, 20, 25, 30, 40, 50 µM).
Figure 5.10 Stern–Volmer plot. (plot of $F_0/F$ against the corresponding concentrations of (A) NBT for native casein and thermal denatured casein; (B) THN for native casein and thermal denatured casein.)
Figure 5.11 Modified Stern–Volmer plot (plot of ln (F₀/F) against the corresponding concentrations of (A) NBT for native casein (y=0.0246x, R²=0.994) and thermal denatured casein (y=0.0272x, R²=0.9994); (B) THN for native casein (y= 0.0378x, R²=0.991) and thermal denatured casein (y=0.029x, R²=0.991)).
Table 5.1 Modified Stern–Volmer quenching constants, $K_{app}$ (μM$^{-1}$) for the interaction of PMFs with native and thermal denatured casein. Results are the average of three independent experiments. Within a column, values with different superscript same letters are significantly different ($p < 0.05$). Within a row, values with different superscript capital letters are significantly different ($p < 0.05$).

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<th>THN</th>
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<td>Native casein</td>
<td>0.0246±0.0007 $^a^A$</td>
<td>0.0378±0.0016 $^a^B$</td>
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<tr>
<td>Thermal denatured casein</td>
<td>0.0272±0.0012 $^b^A$</td>
<td>0.0290±0.0010 $^b^A$</td>
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**5.3.3 Proposed mechanism of PMFs-casein interactions**

The mechanism of flavonoids-protein interaction has been reported that complex formation results from hydrogen bonding, hydrophobic interaction and ionic interaction for non-covalent bonding.$^{28,165,171–174}$ Hydrogen bonding involves H-acceptor sites of the proteins, such as peptide backbone or alkaline protein groups and the hydroxyl groups of the polyphenols. Hydrophobic interactions results from aromatics rings of polyphenols and hydrophobic sites of proteins, such as pyrrolidine rings of prolyl residues. Ionic bonds occurs between positively charged groups of proteins, such as ε-amino groups of lysine, and negatively charged hydroxyl groups of polyphenols. Casein has negative charge in the neutral pH, while PMFs have net charge in the neutral pH. Therefore, the ionic bond could be ruled out in this specific interaction.

Both SERS study and fluorescence study have consistent results that the binding affinity of NBT to the native casein was lower than it to the thermal denatured casein, while the binding affinity of THN to the native casein was higher than it to the thermal denatured casein.
casein. NBT with no hydroxyl group interacted with casein mainly through hydrophobic interaction. After casein was thermal denatured, it exposed more hydrophobic area as shown in the Raman spectra, therefore, the increased binding affinity was due to the increased hydrophobic interaction. THN with three hydroxyl group interacted with casein through hydrophobic interaction and hydrogen bonding, and hydrogen bonding was the driving force. Increasing temperature can destroy the hydrogen binding site and decrease hydrogen bonding.\textsuperscript{175} Therefore, the binding affinity of THN to the thermal denatured casein decreased. Sastry and Prigent also reported that 5-\textit{O}-caffeoylquinic acid bound less to the polyphenol-free 11S protein of sunflower seed and bovine serum albumin with increasing temperature, suggesting hydrogen bonding dominated the interaction\textsuperscript{176,177} Additionally, THN has a stronger binding affinity than NBT to casein, both the native and the thermal denatured states. This also demonstrated the increased binding affinity of THN compared to NBT with casein was due to the hydrogen bonding.
Polymethoxyflavones (PMFs) have received a considerable interest due to their remarkable bioactivities. In my dissertation, the current two significant gaps in our understanding of the bioactivity of PMFs were investigated. A study of the biotransformation of polymethoxyflavones (PMFs) by the mouse and human microbiome showed that glucuronide and/or sulfate conjugates of NBT, 5HN and their metabolites were largely deconjugated by both mouse and human gut microbiome. Deconjugation of these conjugates enabled reuptake of free active PMFs by enterohepatic circulation. The human gut microbiome also catalyzed the demethylation at certain positions of flavonoid core structures, e.g. 3' and 4' positions in the B ring, and position 4’ was more preferred than position 3’. Inter-individual variations were observed in different human subjects in terms of demethylation. Moreover, our results using cell culture models demonstrated that isolates generated after gut microbiome fermentation showed much stronger anti-carcinogenic effects than isolates without gut microbiome fermentation. Thus, the gut microbiome is a key player in biotransformation of PMFs. We provided the first report of the biotransformation of PMFs by the mouse and human gut microbiome with its potential for enhancing health benefits of PMFs. In the second part of the dissertation, we applied a novel analytical method to characterize monohydroxylated PMFs and their SERS behavior. According to SERS spectra, PCA and HPLC analysis, the position of hydroxylation is a crucial factor for molecular interaction between PMFs and Ag dendrites, the spatial conformation of PMFs on binding sites, the binding affinity, and the
saturation concentration. 5HN exhibited the highest binding affinity and the lowest saturation concentration mainly because of the stronger interaction to Ag through chelation. 3HN produced the lowest binding affinity, primarily due to a highly disorder of molecular conformation. This study, for the first time, reported that the position of hydroxylation in monohydroxylated PMFs was crucial for their interaction with Ag dendrites and advanced the fundamental understanding of the relationship between molecular structure and the interaction with Ag substrate. We further characterized the molecular interaction between PMFs and kappa casein. Both SERS and fluorescence spectroscopic studies revealed the structure of PMFs (i.e. with or without hydroxyl groups) and casein state (i.e. native or thermal denatured) affect their binding affinity. This study demonstrates the significance of flavonoids structure and protein states on the flavonoid-protein interactions. It also shows the feasibility of SERS as a powerful tool to characterize the molecular flavonoid-protein interactions. Overall, this dissertation provides important leads for further investigation on the bioactivities of PMFs.
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