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Structure - degradation relationships of flavonoids and their correlation to human bioavailability

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NOTE TO USERS

Structure – degradation relationships of flavonoids and their correlation to human bioavailability

by

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ABSTRACT

The chemical structure of flavonoids greatly influences the rate of degradation by human gut microflora and their overall bioavailability. To investigate which soy isoflavone structures were needed for rapid gut microbial degradation, 100 μmol of daidzein, genistein and glycitein were incubated in fecal fermentation mixtures consisting of brain heart infusion media (BHI) and fresh feces *in vitro* from 12 human subjects for 24 hours. Genistein, which possesses a hydroxyl group in the 5 position, degraded most rapidly in all subjects with an average $k = 0.43 \pm 0.44\ h^{-1}$ ($p = 0.018$). Glycitein and daidzein degradation rates were not different from each other with an average $k = 0.30 \pm 0.21\ h^{-1}$ and $k = 0.16 \pm 0.17\ h^{-1}$ ($p = 0.074$). Glycitein, the metabolism of which has been less characterized, was metabolized to dihydroglycitein, dihydro-6,7,4'-trihydroxyflavone, 5'-OMe-*O*-desmethylangolensin, 6-OMe-equol and daidzein.

To further determine the relationship between other flavonoids with hydroxyl groups in the 5 position and gut microbial degradation, 80 μM of 14 flavonoids, flavone, apigenin, chrysin, naringenin, kaempferol, genistein, daidzein, daidzin, puerarin, 7,4'-dihydroxyflavone, 6,4'-dihydroxyflavone, 5,4'-dihydroxyflavone, 5,3'-dihydroxyflavone and 4'-hydroxyflavone, were fermented in fecal fermentation mixtures from 11 human subjects. The degradation rates of 5,7,4'-trihydroxyl-flavonoids, apigenin, genistein, naringenin and kaempferol, were significantly faster with an average $k = 0.38 \pm 0.11\ h^{-1}$, compared to flavonoids with other structural motifs with an average $k = 0.08 \pm 0.02\ h^{-1}$ ($p<0.0001$).

The bioavailability of flavonoids that are rapidly degraded by the gut microflora may be significantly reduced compared to flavonoids that are slowly degraded. The bioavailability of rapidly degraded flavonoids *in vitro*, genistein, naringenin, hesperetin, quercetin, and a

slowly degraded flavonoid, daidzein, was analyzed. Five men and five women were fed soymilk containing 28 mg genistein and 16 mg daidzein, grapefruit juice containing 422 mg of naringenin and 8 mg hesperetin, and sautéed onions containing 115 mg quercetin with a 2 week washout period in between feedings. Rapidly degraded flavonoids from *in vitro* fecal fermentation systems in 33 subjects, included the 5,7,4'-trihydroxyflavonoids, genistein, apigenin, naringenin, kaempferol, quercetin and luteolin, with an average $k = 0.46 \pm 0.10\ h^{-1}$, and the methoxylated flavonoids, glycitein and hesperetin with an average $k = 0.24 \pm 0.21\ h^{-1}$, compared to slowly degraded flavonoids with an average $k = 0.07 \pm 0.02\ h^{-1}$ ($p < 0.0001$). The bioavailability of the rapidly degraded flavonoids, genistein, naringenin, quercetin and hesperetin with an average $k = 5.8 \pm 1.9$ % were significantly lower than daidzein with an average $k = 42.6 \pm 15.9$ % ($p = 0.02$) expressed as the amount of flavonoid excreted in urine as a percentage of ingested dose. Subjects with low genistein degrader phenotypes (average $k = 0.11 \pm 0.07\ h^{-1}$, n=4) based on average linkage cluster analysis, experienced a higher genistein bioavailability (11.5 ± 8.0 %) compared to the lower genistein bioavailability (3.6 ± 1.9 %, $p = 0.007$) experienced by subjects with high genistein degrader phenotypes (average $k = 1.28 \pm 0.45\ h^{-1}$, n=3).

The data from the *in vitro* fecal fermentation studies reveal that the chemical structure of flavonoids greatly influences the gut microbial degradation rate. Flavonoids with 5,7,4'-trihydroxyl-flavonoid structures were rapidly degraded by the human intestinal microflora, which in turn, resulted in lesser apparent absorption and excretion in urine over 24 h. Therefore, the chemical structure of flavonoids is a strong determinant of the human bioavailability of flavonoids.

GENERAL INTRODUCTION

Introduction

Flavonoids are polyphenolic compounds that are widely distributed in plants and in the human food supply. Flavonoids are synthesized in plants from the amino acid phenylalanine, and function as pollinator attractors, plant growth regulators, nitrogen fixating bacteria activators and ultraviolet (UV) filters. Fruits, vegetables and herbs are particularly rich sources of flavonoids where these compounds contribute to their color and flavor. Over 5000 flavonoids have been identified to date, and are divided into subclasses including flavones, isoflavones, flavonols and flavonones (Harborne, 1986).

The estimated daily intake of flavonoids in humans can reach up to 1 g. Epidemiological studies have suggested possible links between diets rich in fruits and vegetables and reduced risk of cancer and cardiovascular disease. The antioxidant and estrogenic activity of flavonoids may be responsible for these positive correlations. However, some flavonoids have been reported to possess some toxic properties such as pro-oxidant activity and adverse flavonoid-drug interactions, which warrant further investigation.

The bioavailability of flavonoids is critical in understanding their biological and health effects in humans. The extent of absorption and gut microbial metabolism may significantly alter the bioavailability and activity of flavonoids at their target site. Therefore, determining how these factors influence human bioavailability is the overall objective of my research.

The two major sites of flavonoid metabolism are the large intestine, where gut microorganisms participate in glycosidase activity and anaerobic reductive reactions, and the liver where phase II conjugation and phase I oxidative reactions occur. The extent to which

gut microorganisms metabolize or degrade the flavonoids determines how much of the parent compound is absorbed and ultimately, the bioavailability of flavonoids. Each flavonoid possesses unique structural features that divide them into their respective subclasses. In addition, each flavonoid in each subclass possesses different substitutions on each ring structure such as hydroxyl, methoxyl or glycosidic groups. One of the objectives of my research was to determine how different chemical structures of flavonoids affect the rate of degradation by the gut microflora of humans.

The bioavailability of flavonoids is a significant factor in the determination of an appropriate dose for a desired health effect. If the chemical structure of flavonoids strongly dictates their rate of degradation by human gut microflora, then the flavonoid chemical structure plays a significant role in their bioavailability. Another objective of my research was, to determine by conducting human feeding studies, how different flavonoid chemical structures affect bioavailability *in vivo*.

Dissertation Organization

This thesis dissertation consists of a literature review and three papers. The first paper is titled 'Metabolism of glycitein (7,4'-dihydroxy-6-methoxyisoflavone) by human gut microflora,' which describes the kinetics of glycitein degradation *in vitro* compared to the other two soy isoflavones, genistein and daidzein, and the glycitein metabolites produced by microbial degradation in humans. This paper will be submitted to the Journal of Agricultural and Food Chemistry. The second paper, titled 'Human gut microbial degradation of flavonoids: structure – function relationships,' examines how different chemical structures of flavonoids affect the rate of degradation *in vitro* by the gut microflora in humans. This paper has been accepted by the Journal of Agricultural and Food Chemistry in March 2005. The third paper is titled 'Gut microbial degradation of flavonoids and their correlation to human bioavailability'. This paper reports how different flavonoid chemical structures affect human absorption and bioavailability *in vivo*. This paper will be submitted to the Journal of Nutrition. A general conclusion follows these three papers.

LITERATURE REVIEW

A. Chemistry and Sources of Flavonoids

Flavonoids are polyphenolic compounds that are derived from benzo-γ-pyrone or chromone. All flavonoids possess a common C-15 structure consisting of 2 aromatic rings, identified as A and B, joined together by a 3 carbon atom chain, that form an oxygenated heterocyclic C ring (Croft, 1998, Figure 1A). Although flavonoids are structurally similar, they can be divided into 6 main groups with shared characteristic structural features such as flavones, flavonols, flavanones, isoflavones, flavanols, anthocyanidins and proanthocyanidins (Figure 1). Each group differs from each other by variations in the heterocyclic C ring. Additionally, flavonoids within each subclass may be substituted on the A and B rings with *O* and *C*-linked sugars, hydroxyl, methyl, methoxyl, acyl, prenyl, sulphate and glucuronide groups (Cook and Samman, 1996).

Flavones are characterized by a 2,3 double bond, a carbonyl group in the 4 position of the C ring and the B ring attached to the 2 position of the C ring (Figure 1B). Flavones are less common than the other flavonoid subgroups in fruits and vegetables. Parsley and celery are the two most commonly consumed sources of flavones, which contain apigenin (5,7,4'-trihydroxyflavone) and luteolin (5,7,3',4'-tetrahydroxyflavone, Table 1). Some cereals contain flavone with C-linked sugars and the skin of citrus fruits contains polymethoxylated flavones (Manthey and Grohman, 2001, Kawaii et al, 2000).

Flavanones are similar to flavones but do not possess the 2,3 double bond in the C ring (Figure 1C). Flavanones are found in tomatoes and in high concentrations in citrus fruits (Kawaii et al, 1999, Table 1). Examples of commonly consumed flavanones are naringenin (5,7,4'-trihydroxyflavanone) found in grapefruit juice and hesperetin (5,7,3'-trihydroxy-4'-

methoxyflavanone) found in orange juice. Flavanones are usually glycosylated at position 7 of the A ring with disaccharides such as rutinose or neohesperidose (Kawaii et al, 1999a, Kawaii et al, 199b). These flavanone *O*-sugar bonds are able to resist hydrolysis during heat processing even at pasteurization temperatures (Gil-Izquierdo et al, 2002). Flavanone concentrations range from 15 – 600 mg/L in orange and grapefruit juice (Tomás-Barberán and Clifford, 2000). However, the pulp of citrus fruits are rich in flavanones, therefore, consuming the whole fruit consisting of the juice and pulp, can result in a flavanone dose of up to 5 times the amount found in the juice alone (Tomás-Barberán and Clifford, 2000).

Flavonols are the most commonly found flavonoids in foods. Flavonols are structurally similar to flavones, except they have an additional hydroxyl group in the 3 position of the C ring (Figure 1D). Examples of common flavonols are quercetin (3,5,7,3',4'-pentahydroxyflavone or 5,7,3',4'-tetrahydroxyflavonol) and kaempferol (3,5,7,4'-tetrahydroxyflavone or 5,7,4'-trihydroxyflavonol, Table 1). Flavonol concentrations in foods range from 15 mg/kg – 1.2 g/kg. The richest sources of flavonols are onions, broccoli, kale, leeks, red wine and tea (Hertog et al, 1992, Hertog et al, 1993a, Hertog et al, 1993b). Flavonols are found in *O*-glycosylated form with glucose or rhamnose (Aherne et al, 2002). Other sugar moieties also include galactose, arabinose, glucuronic acid and xylose.

Isoflavones are similar to flavones except that the B ring is attached to the 3 position of the C ring (Figure 1E). Isoflavones are usually hydroxylated in the 7 and 4' positions. Because of these features, they are structurally similar to estradiol and are able to bind to estrogen receptors (Kuiper et al, 1998). Therefore, isoflavones possess weak hormonal properties and are classified as phytoestrogens or plant estrogens. Soybeans and soy food products are the main sources of isoflavones such as genistein (5,7,4'-trihydroxyisoflavone),

daidzein (7,4'-dihydroxyisoflavone) and glycitein (7,4'-dihydroxy-6-methoxyisflavone, Table 1) but isoflavones can also be found in red and white clover, chickpeas and alfalfa. The isoflavone concentration of soybeans can range from 1 – 3 mg/g (Wang et al, 1994). Isoflavones can be found in both aglucon and β-*O*-glucosylated form. In soy, isoflavones are also found as acetyl and malonyl-glucosides. The malonyl glucosides are very heat sensitive and are quickly hydrolyzed to glucosides during minimal heat processing (Eisen et al, 2003, Xu et al, 2002, Grun et al, 2001). Fermentation results in the hydrolysis of isoflavone glucosides to isoflavone aglucons, which are very resistant to heat (Song et al, 1998). Fermentation is used in the manufacture of soy products such as miso and tempeh.

Flavanols, also referred to as catechins, may exist in monomer form or as polymers, referred to as proanthocyanidins (Scalbert and Williamson, 2000). Flavanols are structurally similar to flavonols except they do not possess the 2,3 double bond or the carbonyl group in the 4 position of the C ring (Figure 1G). Flavanols are not glycosylated in foods, which is in contrast to other flavonoid subgroups. The richest sources of flavanols are green tea, chocolate and apricots (Table 1). Green tea infusions may contain up to 200 mg/cup of flavanols, while flavanol concentrations in apricots may reach up to 250 mg/kg fresh weight (Hara et al, 1995, Lee et al, 1995). Examples of flavanols in fruit are (+)-catechin and (-)-epicatechin. Gallocatechin, (-)-epigallocatechin and (-)-epigallocatechin gallate are found in grapes, leguminous plants and tea (Yilmaz et al, 2004). Proanthocyanidins may be dimers, oligomers or polymers of flavanols which are linked together at the C-4 position of one flavanol to the C-8 position of another flavanol (Figure 1H).

Anthocyanidins are structurally similar to flavanols, however, anthocyanidins possess a positive charge on the oxygen of the C ring (flavylium cation), a double bond between the

charged oxygen and C-2, and between C3-C4 of the C ring (Havsteen, 2002, Figure 1F). Anthocyanidins are water-soluble pigments found in the vacuoles of epidermal tissues of plants and flowers. These compounds are responsible for the coloration of flowers and fruit depending on the pH. Anthocyanidins are found in glycosylated form because they are highly unstable as aglucons. Between pH 3 and 6, anthocyanidins are rapidly hydrated to colorless carbinol pseudobases. Glycosylation at position 3 of the C ring suppresses this hydration so that a higher pH (4 – 5) is needed for hydration. Additional biochemical reactions in the vacuoles help to suppress hydration and keep the flower coloration. Also, formation of complexes between the flavylium cation and other flavonoids called copigmentation, helps to prevent hydration (Havsteen, 2002).

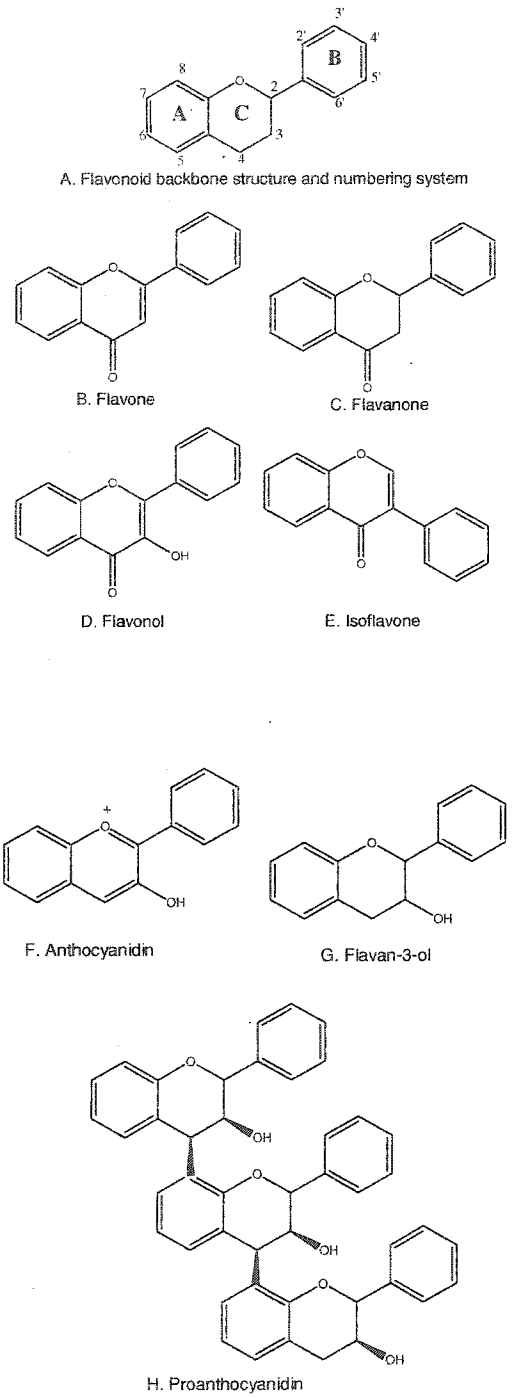


Figure 1. Structures of flavonoid subclasses

Table 1. Sources of Common Flavonoids[a]

Flavonoid Subclass	Common Flavonoids	Major Food Sources
Flavone	apigenin, luteolin	parsley, celery
Flavanone	naringenin, hesperetin	citrus
Flavonol	quercetin, kaempferol	onions, tea, broccoli, apples, red wine
Isoflavone	genistein, daidzein	soybeans
Flavan-3-ol	catechin, epicatechin	apples, tea
Anthocyanidin	cyanidin	cherries, grapes, cocoa powder
Proanthocyanidin	procyanidin C2	red wine, blueberries, strawberries

[a]Information taken from USDA flavonoid database

B. Function of Flavonoids

Flavonoids are a large family of structurally diverse polyphenolic compounds. Many flavonoids are pigments, which contribute to the color of plants (Winkel-Shirley, 2001, Havsteen 2002). Flavonoids are found in all green plant cells, and due to their color, attract pollinators such as birds and insects, which help in seed dispersion (Harborne, 1986).

Flavonoids function as plant growth regulators which are associated with auxin stimulation. Flavonoids prevent the transport of auxins such as indoyl acetic acid (IAA) out of the plant cell. This prevention allows the accumulation of IAA, which leads to gene expression and longitudinal growth of the plant cell (Stenlid, 1976, Jacobs et al, 1988).

Flavonoids play a significant role in the nitrogen metabolism of nitrogen-fixating plants by inducing root nodulation. Exudates containing flavonoids are released from leguminous plants that are in need of nitrogenous substances (ammonia and amino acids) into

the soil (Srivastava et al, 1999). The flavonoids enter bacterial cells, e.g. *Rhizobacterium* strains, which contain nitrogen fixation and nodulation-inducing genes (Perret et al, 1999). The bacteria are taken up into the plant root nodules by chemotaxis where they release enzymes that are vital for nitrogen fixation such as nitrogenase reductase, coenzyme FeMe-co, and nitrogenase (Mortenson et al, 1979).

Convincing evidence has suggested that flavonoids provide protection of arial plant parts against plant damage by ultraviolet (UV) radiation (Murphy, 1997, Ormrod et al, 1995, Reuber et al, 1996, Liu et al, 1995, Gitz et al, 1998). UV-B radiation (280-315 nm) is one of the three bands of UV radiation, which possesses the lowest wavelength and highest energy. Flavonoids are able to act as UV filters and provide UV-B resistance because they absorb in the 280–315 nm region, protecting the photosynthetic tissues from damage. Most of the experimental evidence supporting the role of flavonoids in UV-B protection has been derived from using plant mutants, in which the synthesis of flavonoids are eliminated or greatly reduced. Ormrod et al, 1995 has shown that mutants of *Arabidopsis thaliana*, which lack epidermal flavonoids compared to its wild type counterpart, were very sensitive to UV-B radiation. Reuber et al, 1996 has shown that mutants of *Hordeum vulgare* contain 7% of the flavonoids that are present in the wild type. Photosynthesis was significantly decreased in the mutant plants, where as the wild type plants photosynthesized normally and also increased their flavonoid production. Flavonoid subclasses that are particularly strong UV filters and UV-B protectors are flavone or flavonol glycosides with hydroxycinnamyl acylation linked through sugars. These flavonoid structures absorb most strongly in the 280–320 nm region (Harborne and Williams, 2000).

C. Biosynthesis of Flavonoids

The flavonoid biosynthesis pathway is conserved over a wide variety of plants. Even mosses are able to carry out the initial steps of flavonoid biosynthesis (Stafford, 1991). The diversity of flavonoids that are found in nature however, depends on certain reactions carried out by different plant species. The biosynthetic pathway has almost been completely elucidated with many of the structural and regulatory genes cloned from several plants including *Antirrhinum majus*, *Petunia hybrida* and *Arabidopsus thalia*, *Zea mays* and tobacco (Holton and Cornisch, 1995).

The biosynthesis of flavonoids is illustrated in Figure 2. The first reactions of the flavonoid biosynthesis pathway are called the phenylpropanoid pathway. The phenylpropanoid pathway begins with the amino acid phenylalanine, which is synthesized from the shikimate biosynthesis pathway. Phenylalanine is the primary substrate for the synthesis of flavonoids. Phenylalanine-ammonia lyase (PAL) is the key enzyme of the phenylpropanoid pathway. PAL converts phenylalanine into cinnamic acid and is hypothesized as being the limiting step in this pathway (Creasy et al, 1974). Supporting data by Lister et al, 1996 has shown that the activity of PAL was proportional to the total flavonoid concentration in apples. Cinnamic acid is converted to p-coumaric acid by cinnamic acid 4-hydroxylase then converted to 4-coumaryl-CoA by 4-coumaryl-CoA ligase.

The first committed step in the flavonoid biosynthesis pathway is catalyzed by chalcone synthase (CHS). This enzyme condenses 4-coumaryl-CoA with 3 molecules of malonyl-CoA to form chalcones such as isoliquiritigenin (trihydroxychalcone) and naringenin chalcone (tetrahydroxychalcone) (Tanaka et al, 1998, Holton and Cornisch,1995, Forkmann and Heller, 1999). Studies have shown that suppression or down regulation of the

chalcone synthase genes causes a blockage of the flavonoid pathway. As a result, the flower color is modified or white, as a consequence of the absence of flavonoids (Napoli et al, 1999, Deroles et al, 1998). Chalcone isomerase rapidly transforms the chalcones into the first flavonoids of the pathway, liquiritigenin and naringenin respectively which are both of the flavanone subgroup (Joung et al, 2003, Holton and Cornisch, 1995). In the absence of chalcone isomerase, naringenin chalcone still spontaneously isomerizes into naringenin, but at a slower rate (Holton and Cornisch, 1995).

Several pathways may be taken from the formation of the first flavonones. Firstly, naringenin and liquiritigenin can be converted to genistein and daidzein, respectively, which are of the isoflavone subclass. This reaction is catalyzed by isoflavone synthase which is responsible for the 2-3 migration of the B ring of naringenin and liquiritigenin (Yu et al, 2000). These isoflavones may be further modified by enzymes such as isoflavone *O*-methyltransferase, to form biochanin A and formononetin, and P450 hydroxylase, isoflavone reductase, vestitone reductase and dehydratase, to form other isoflavone compounds. Secondly, naringenin may be converted to another flavanone called eriodictyol by the enzyme flavonoid 3'-hydroxylase. Third, the flavanones may be converted to flavones by flavone synthase, and lastly, both naringenin and eriodictyol can be converted to dihydroflavonols such as dihydrokaempferol, dihydroquercetin and dihydromyricetin by flavanone 3-hydroxylase (Britsch et al, 1993).

Dihydroflavonols can be transformed into flavonols such as quercetin, kaempferol and myricetin by flavonol synthase (Holton et al, 1993, Nielsen et al, 2002, Forkmann and Heller, 1999), or dihydroflavonols may be transformed into flavan-3,4,-diols (leucoanthocyanidins) by dihydroflavonol 4-reductase (Kristiansen and Rohde, 1991).

Flavan-3-ols and anthocyanidins are formed from flavan-3,4,-diols by enzymes including leucoanthocyanidin reductase and leucoanthocyanidin deoxygenase, respectively (Martin et al, 1991, Bradley et al, 1998, Tanaka et al, 1998).

Flavonoids and intermediates in the flavonoid biosynthesis pathway may be modified by glucosylation, acylation, alkylation, methylation or hydroxylation. Some modifications such as sulfation, C-glycosylation and prenylation, are restricted to certain flavonoid subgroups (Heller and Forkman, 1994). Flavonoid methylation occurs with *S*-adenosyl-L-methionine catalyzed by methyl transferases (Wengenmayer et al, 1974). Flavonoid glycosylation is catalyzed by UDPG-flavonoid-glucosyltransferases. Although glycosylation may occur at any hydroxyl group on the flavonoid skeleton, glycosylation occurs usually at position 7 of the A ring, position 3 of the C ring and position 4' of the B ring. Flavonoids with a free hydroxyl group at the 3 position of the C ring are not found in nature because they are unstable under physiological conditions (Forkmann and Heller, 1999). Glycosylation by UDPG-flavonoid-3-O-glucosyltransferase is an especially important step in the biosynthesis of anthocyanidins so that they may be stabilized and accumulate as water soluble pigments in vacuoles of plant cells (Schijlen et al, 2004).

Flavonoid biosynthetic genes are regulated at the transcriptional level and are responsible for the levels of flavonoids in different plant species (Ranish and Hahn, 1996). These regulatory genes have been identified in many plants and are dependent on tissue type and signals such as hormones, microbes and UV radiation (Holton and Cornisch, 1995, Vom Endt et al, 2002).

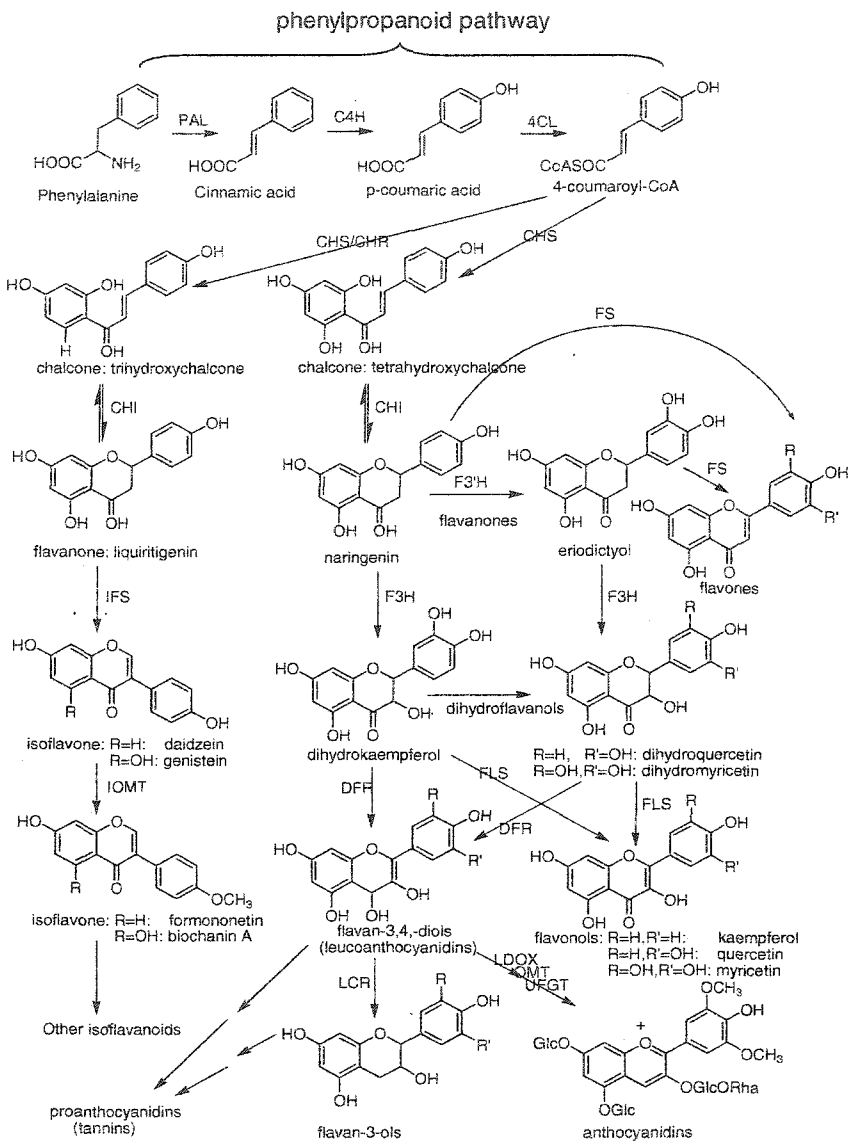


Figure 2: Major pathways of flavonoid biosynthesis in plants. Enzyme names are abbreviated as follows:cinnamate-4-hydroxylase (C4H), chalcone isomerase (CHI), chalcone reductase (CHR), chalcone synthase (CHS), 4-coumaroyl:CoA-ligase (4CL), dihydroflavonol 4-reductase (DFR), flavanone 3-hydroxylase (F3H), flavone synthase (FS), flavonoid 3' hydroxylase (F3'H), isoflavone *O*-methyltransferase (IOMT), isoflavone synthase (IFS), leucoanthocyanidin dioxygenase (LDOX), leucoanthocyanidin reductase (LCR), *O*-methyltransferase (OMT), phenylalanine ammonia-lyase (PAL), UDPG-flavonoid glucosyl transferase (UFGT). Adapted from Winkel-Shirley et al, 2001

D. Dietary Intake

For many years, the data of Kuhnau et al, 1976 has served as a reference for flavonoid dietary intake. They calculated that the flavonoid dietary intake was around 1 g flavonoid glucosides per day, which is equivalent to around 650 mg flavonoid aglucons. They reported that this 1 g/day value consisted of 45% biflavones, 20% catechins, 17% anthocyanidins and 16% flavones, flavanones and flavonols (Kuhnau, 1976). These intake values, however, were based on the analysis of very few foods. Since then, other studies have made more progress in making accurate estimates of the intake levels of various flavonoid subclasses.

Luteolin and apigenin, found in celery and parsley, respectively, are the main flavones found in the human diet. The intake level of these flavones was determined to be less than 2 mg/day in the U.S, Denmark, Japan, Holland and Finland (Sampson et al, 2002, Arai et al, 2000, Dragsted et al, 1997, Kneht et al, 2002, Hertog et al, 1993). The intake levels of flavonols, such as quercetin, myricetin, kaempferol which have been more extensively studied, were around 15 – 30 mg day in the U.S, Japan, Holland and Denmark (Sampson et al, 2002, Arai et al, 2000, Dragsted et al, 1997, Hertog et al, 1993). The lowest flavone and flavonol intake level was found in Finland of less than 4 mg/day (Kneht et al, 2002). Hertog and colleagues determined that the intake of flavones and flavonols in the Netherlands was around 23 mg/day of which quercetin contributed 16 mg/day (Hertog et al, 1993). The major sources of quercetin in this population were from tea (48%), onions (29%) and apples (7%).

Citrus fruits are the major sources of flavonones (mainly hesperetin and naringenin), however, intake levels were estimated only in Denmark and Finland. Denmark estimated an intake range of 7 – 14 mg/day and Finland estimated an average intake of 20 mg/day

(Dragsted et al, 1997, Kneht et al, 2002). Parts of the U.S. such as Florida and California produce large quantites of citrus so it is surprising that flavanone intake levels were not estimated in the U.S.

The maximum intake of isoflavones from soybeans and soy products in Asian countries can reach around 100 mg/day with an average intake range of 25 – 50 mg/day. In contrast, American and European countries consume less than 1 mg/day because these populations consume substantially lesser amounts of soy compared to Asian countries (Beecher, 2003). For example, in Japan, the average intake of isoflavones is around 47 mg/day compared to the U.S and Holland, with an intake of less than 1 mg/day (Boker et al, 2002, Arai et al, 2000, De Kleijn et al, 2001, Wu et al, 2002). Interestingly, it seems as though cultural habits determine the intake levels of flavonoids especially certain flavonoid subgroups because Asians that have immigrated to the U.S. still consume substantial amounts, around 12 mg isoflavones/day isoflavones (Wu et al, 2002).

If we compare the flavonoid intake levels of the studies described above, with the intake levels of Kuhnau data, it is clear that the Kuhnau estimate was too high. Based on their data, flavonol and flavone intake levels in the U.S. appears to be around 100 mg/day (Kuhnau et al, 1976). However all current data have reported significantly lower values around 23 mg/day (Hertog et al, 1993a).

E. Biological and Health Effects

a. Estrogenic Activity

The estrogenic effects of flavonoids were discovered from cases of infertility in Australian sheep and cattle that grazed on red clover (Schutt, 1976 and Verdeal, 1979). Red clover contains high concentrations (up to 5% dry weight) of the isoflavones, biochanin A

(5,7-dihydroxy-4'-methoxyisoflavone) and formononetin (7-hydroxy-4'-methoxyisoflavone), which are methylated derivatives of genistein and daidzein, respectively (Saloniemi et al, 1995). Sonnenbichler and Pohl in 1980, determined the structure of the complex flavonoid silybin (Figure 3) after an observation that sheep fed fermented clover became sexually aroused. Silybin was described as a flavonolignan and was the first of its kind to be discovered. Structural elucidation revealed that the hydroxyl groups of silybin were positioned similar to that of estradiol and other steroid hormones (Sonnenbichler and Pohl, 1980).

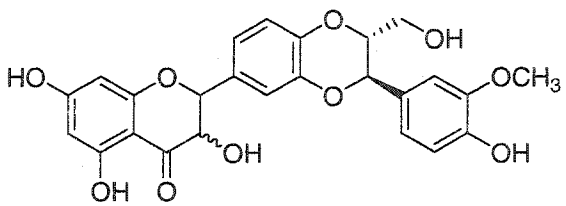
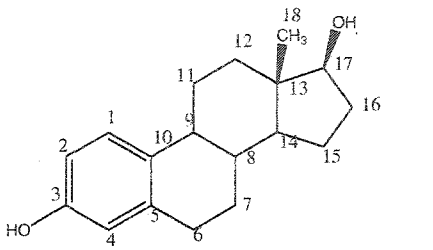
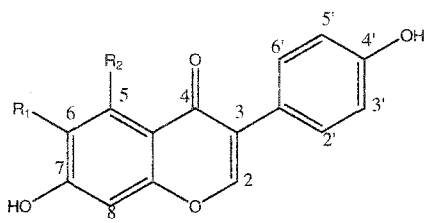


Figure 3. Structure of silybin, a flavonolignan

Additionally, structure similarities with estradiol are observed for genistein and daidzein, which are liberated as a result of demethylation by rumen microbial metabolism (Lundh, 1995). The 7 and 4'-hydroxyls of flavonoids in the isoflavone subclass correspond to the 3 and 17β-hydroxyls of estrogens (Figure 4). Isoflavones have been shown to compete with 17β-estradiol for binding to the estrogen receptor (Fang et al, 2001, Kuiper et al, 1998). Because of this structural similarity, isoflavones have been classified as phytoestrogens.



17-β-estradiol



Soy isoflavones R_1=H, R_2=H: daidzein
R_1=H, R_2=OH: genistein
R_1=OCH_3, R_2=H: glycitein

Figure 4. Structure similarities between 17β -estradiol and isoflavones

Estrogens play a vital role in the growth, differentiation and function of tissues in the reproductive system including the vagina, ovaries, mammary glands, uterus, testes and prostate (Peterson et al, 1998). Estrogens play a role in bone maintainance, the cardiovascular system and the central nervous system (Turner et al, 1994, Farhat et al, 1996 and Iafrati et al, 1997), perhaps because all these tissues possess estrogen receptors which exist in 2 subtypes, ERα and ERβ. These 2 sub-types differ in their C-terminal ligand-binding domain and in the N-terminal transactivation domain (Kuiper et al, 1998). When estrogens bind to the estrogen receptor, the estrogen receptor undergoes a conformational change which activates transcription of target genes (Jensen 1995, Beato et al, 1995). Estrogens, phytoestrogens and

synthetic estrogens may have different relative affinities for ERα and ERβ, but like 17β-estradiol, flavonoids have a preferential affinity for ERβ (Kuiper et al, 1998).

Forty different flavonoids were analyzed for estrogenic activity using a transient transfection assay in HeLa cells and competition binding assay with 17β-estradiol for estrogen receptor expressed in COS-7 cells (Miksicek, 1995). Of the 40 flavonoids, 12 were able to significantly stimulate transcriptional activity, including the isoflavones daidzein, genistein and biochanin A, the flavanones naringenin and 7,4'-dihydroxyflavanone, the flavonol, kaempferol, the flavones apigenin, 6,4'-dihydroxyflavone and 5,4'-dihydroxyflavone and the chalcones phloretin, isoliquiritigenin and 4,4'-dihydroxychalcone. In the competition binding assay, these compounds were able to compete with 17β-estradiol for binding to the estrogen receptor but only in molar concentrations 1000-10,000 fold higher than 17β-estradiol (Miksicek, 1995).

Structure activity relationship (SAR) analysis revealed that the 4'-hydroxyl was extremely important and any deviation from this pattern reduced estrogenic activity as in the lowered activity of 6,3'-dihydroxyflavone compared to 6,4'-dihydroxyflavone. There was some flexibility in the hydroxylation pattern of the A ring but the 7 position was most favorable. Hydroxylation patterns that created catechols were a detriment to estrogenic activity such as 7,8-dihydroxyflavone or 7,8,4'-trihydroxyflavone (Miksicek, 1995). *In vivo*, the estrogenic activity of biochanin A, which is methoxylated in the 4' position, may be explained by the conversion of biochanin A to genistein, but the authors had no explanation, however, for the estrogenic activity of biochanin A *in vitro* (Miksicek, 1995). It has been shown that MCF-7 breast tumor cells can convert biochanin A to genistein (Peterson et al,

1996), therefore HeLa cells, which were used in the Miksicek, 1995 studies, may also be able to make this conversion.

Kuiper et al, 1998, using similar assays as in Miksicek et al, 1995, using human 293 embryonal kidney cells, found that genistein, apigenin, naringenin and kaempferol were stronger competitors with 17β-estradiol for binding to ERβ compared to ERα. They found that genistein activated transcriptional activity at relatively low concentrations (1-10 nM) while the other flavonoids did so at concentrations of around 1000 nM. Genistein and daidzein concentrations in plasma may reach up to 1000 nM after a meal rich in soybeans or soy protein extracts according to Kurzer et al, 1997. These data suggest that these isoflavones may be able to exert estrogenic activity at levels found in the human diet. The ranking of the flavonoids' affinity to ERβ was 17β-estradiol > genistein = coumestrol > daidzein > biochanin A = apigenin = kaempferol = naringenin > phloretin = formononetin = chrysin (Kuiper et al, 1998). From the studies by Miksicek et al, 1995 and Kuiper et al, 1998, it is clear that isoflavones are in general more estrogenic than the other flavonoid structures. However, the latter study did not attempt to determine which structures were important for estrogenic activity (Kuiper et al, 1998).

Fang et al, 2001 suggested 5 main criteria for estrogenic activity, a good H-bond donor that imitates the 3-OH of 17β-estradiol, an H-bond donor that imitates the 17β-OH of 17β-estradiol and the O-O distance between the 3-OH and 17β-OH, a steric hydrophobic center, hydrophobicity and ring structure. This research group studied the affinity of 202 natural and synthetic chemicals, for the androgen receptor. Of all flavonoids studied, the isoflavone genistein was the most estrogenic because its ring structure and hydroxylation pattern met all of the above criteria. The 7,4'-hydroxylation pattern of genistein and most of

the isoflavones, agreed with criteria 1 and 2, which play an important role in binding to the ER receptor. The 7,4'-hydroxylation pattern of the other flavonoid subgroups do not fit these criteria. The 5-OH of genistein forms an intramolecular bond with the 4-carbonyl group, which increases the electron withdrawal capacity of the carbonyl and hydrophobicity. This met criterias 3 and 4. Finally the rigid ring structure of genistein is similar to the ring structure of 17β-estradiol, which is important for fitting into the binding site of the estrogen receptor.

In addition to possessing estrogenic activity, flavonoids have been found to have anti-estrogenic activity. Han et al, 2002 reported that flavonoids possessed anti-estrogenic activity *in vitro*. They observed that genistein, daidzein, luteolin and quercetin at 10 nM suppressed the proliferation of MCF-7 cells caused by industrial chemicals. Genistein caused the greatest suppression (Han et al, 2002). These observations are significant because the flavonoid levels analyzed are physiologically relevant, and have obvious implications for anti-cancer activity. However, they did not analyze the glucuronide forms of these flavonoids, which are the circulating forms in blood of most flavonoids.

Besides binding to the two estrogen receptor subtypes (ERα and ERβ) (Kuiper et al, 1998), flavonoids may compete with endogenous substrates for active sites of estrogen biosynthesizing and metabolizing enzymes, such as aromatase (Jeong et al, 1999, Kellis et al, 1984) and 17β-hydroxysteroid oxidoreductase, type 1 (Mäkelä et al, 1995, Mäkelä et al, 1998). Aromatase is a cytochrome P450 (CYP19) enzyme, which converts C19 androgens such as androstenedione or testosterone, to aromatic C18 estrogenic steroids such as estrone and estradiol, respectively (Dowsett et al, 1993). Estrogenic steroids play an important role in the development of breast cancer cells, therefore the inhibition of aromatase and 17β-

hydroxysteroid oxidoreductase, type 1 lowers 17β-estradiol concentrations in target cells, which may decreases the risk of breast cancer (Mäkelä et al, 1994).

Flavonoids were shown to be potent aromatase and 17β-hydroxysteroid oxidoreductase inhibitors in human placental microsomes by the radioactive measurement of estrone and estradiol produced from radiolabelled (1,2,6,7-^{3}H)-4-androstene-3,17-dione *in vitro*. The IC_{50} values for the flavonoids ranged from 0.2 – 48.0 μM for aromatase and 0.2 – 15.0 μM for 17β-hydroxysteroid oxidoreductase. Structure-activity relationship studies revealed that 7-hydroxyflavone and apigenin were the most effective aromatase and 17*β*-hydroxysteroid dehydrogenase inhibitors, respectively and that a hydroxyl group in position 7 on the A ring of the flavonoid structure was essential for anti-17*β*-hydroxysteroid dehydrogenase activity. For anti-aromatase activity, flavonoids with 7-methoxy or 8-hydroxyl groups on the A ring were most effective (Le Bail et al, 1998). In a similar study, flavonoids such as naringenin, luteolin, chrysin, 7-hydroxyflavone and apigenin were shown to inhibit the formation of radiolabelled 17β-estradiol from radiolabelled androstenedione with IC_{50} values ranging from 0.2 – 0.5 μM in human choriocarcinoma JEG-3 cells and in human embryonic kidney cells HEK 293 transfected with human aromatase gene. However, after oral administration of these flavonoids to immature rats at 50 mg/kg body weight, none of the flavonoids induced uterine growth or reduced estrogen- or androgen-induced uterine growth (Saarinen et al, 2001). These results were interesting because the flavonoids were given at doses that greatly exceed those that are found in the human diet, yet the flavonoids were unable to inhibit aromatase *in vivo*. These results are most likely due to poor bioavailability of these compounds which will be discussed in the 'Metabolism and Bioavailability' section of this dissertation.

The estrogenic activity of isoflavones has been implicated in the prevention and suppression of osteoporosis (Burke, 2000). Osteoporosis is a disease characterized by low bone mass and deterioration of bone tissue (Melton et al, 2004). The mechanism of this effect is thought to be hormonal, by binding to ERβ, which is present in bone (Burke, 2000). Genistein and daidzein dosed orally at 10 μg/g body weight per day suppressed ovarectomized-induced bone loss in adult Wistar rats (Picherit et al, 2000). In a 24 week double blind study with 69 perimenopausal women, a dose of 80.4 mg/d isoflavone aglucons given as isoflavone – rich soy protein isolate, increased bone mineral density (BMD) by 5.6 % and bone mineral count (BMC) by 10.1 % compared to the control group fed whey protein, and the group fed 4.4 mg/d isoflavone aglucons as isoflavone – poor soy protein isolate (Alekel et al, 2000). BMD and BMC decreased by 1.3 % and 1.7 % respectively in the control group (Alekel et al, 2000). The dose of 80.4 mg isoflavone aglucons, which induced the favorable effect in the Alekel et al, 2000 study was an appropriately selected dose, because a diet rich in soy-containing foods, such as 2 or 3 servings of soy foods, can be achieved. More studies are needed in area of suppression of osteoporosis in men and late postmenopausal women.

b. Antioxidant Effects

Several investigators have demonstrated the antioxidant effects of flavonoids in biological systems. It is believed that the antioxidant properties, in conjunction with their weak estrogenic activity of flavonoids, may be responsible for their biological role in decreasing the risk of cardiovascular disease, cancer and chronic inflammation. Many reactive species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are formed from physiological processes in the body, which contribute to aging, mutagenesis,

carcinogenesis, DNA damage, and cardiovascular disease (Croft, 1998). The human body possesses intrinsic antioxidant defense mechanisms that are usually adequate in suppressing the production of free radicals (Halliwell, 1994). It is in situations of oxidative stress, where excess free radicals are formed, and intrinsic antioxidant defense mechanisms are not enough to suppress their formation. For example, the superoxide ion $^{\cdot}O_2^-$ is formed from partial reduction of dioxygen (Havsteen et al, 2002). The hydroperoxide radical $HO_2\cdot$ is formed from protonation of the superoxide anion which leads to the formation of H_2O_2. H_2O_2 may react with nitrogen oxide to form nitrous peroxy acid which is a highly effective oxidant. X- or γ- radiation produces hydroxyl radicals from water which can attack other radicals resulting in nitrous acid, peroxynitrite, superoxide, $NO^{\cdot}$ and H_2O_2 (Haenen et al, 1997). If an excess amount of free radicals are formed, a chain reaction proceeds that leads to the attack of essential unsaturated lipids and the formation of lipid peroxidation products. Nucleic acid bases and other vital cellular compounds may also be damaged. Ultimately, excess production of these highly reactive species result in mutations, disruption of metabolic processes and cancer (Havsteen, 2002).

Flavonoids act as free radical scavengers by breaking the free radical chain reaction. Flavonoids react with free radicals to form a highly stable flavonoid radical (Croft, 1998). This is important because if the flavonoid radical was not stable, it would propagate and attack other molecules causing the formation of more free radicals. Therefore, the formation of flavonoid radicals successfully terminates the chain reaction and prevents injury to cellular components. Other antioxidant defense mechanisms that have been demonstrated for

flavonoids include activating antioxidant enzymes (Havsteen et al, 2002), reducing α-tocopherol radicals (Chen et al, 1990) and inhibiting oxidases (Park et al, 1998).

Flavonoids may also exert antioxidant activity by chelating metal ions. Tissue injury may release copper or iron, which may participate in Fenton-type reactions that produce reactive hydroxyl radicals. Flavonoids react with the metal ions in order to prevent these reactions. This ability of flavonoids to react with metal ions is actually a pro-oxidant effect. Cao et al, 1997 has demonstrated both antioxidant and pro-oxidant activities of flavonoids *in vitro* and found that each activity is highly dependent on the number of hydroxyl substitutions. The prooxidant activity of flavonoids will be discussed further in the flavonoid toxicity section of this review.

Several authors have reported on the flavonoid structural requirements for antioxidant activity (Rice-Evans et al, 1996, Bors et al, 1990, Cao et al, 1997, Van Acker et al, 1996, Chen et al, 1996). As a general conclusion from these studies, structural requirements for antioxidant activity are an ortho-catechol structure on the B ring, a 2,3 double bond together with a 4-carbonyl group and a hydroxyl group in the 3 position. The ortho-catechol structure on the B ring is by far the most important determinant of antioxidant activity especially free radical scavenging (Bors et al, 1990). These hydroxyl groups are important for electron delocalization and donates a hydrogen and an electron to peroxyl peroxynitrite and hydroxyl radicals, which stabilize them and at the same time, form a stable flavonoid radical. For example, luteolin (5,7,3',4'-tetrahydroxyflavone), which possesses a 3',4'-catechol exhibited a higher peroxyl scavenging activity than kaempferol (3,5,7,4'-tetrahydroxyflavone), which possesses only one hydroxyl at the 4' position (Bors et al, 1990).

Flavonoids with a 2,3 double bond and 4 carbonyl group are shown to possess a stronger antioxidant activity in a microsomal system compared to flavonoids without the 2,3 double bond such as flavanones (Ratty et al, 1988). However, the importance of the 2,3 double bond depends on the presence of other structural features. Ratty et al, 1988 has shown that quercetin (3,5,7,3',4'-pentahydroxyflavone), which possess a 2,3 double bond exhibited a stronger peroxyl scavenging activity compared to taxifolin (3,5,7,3',4'-pentahydroxyflavanone), which possesses the same hydroxylation pattern but lacks the 2,3 double bond. However, Rice-Evans et al, 1996, has shown that the radical scavenging ability of apigenin (5,7,4'-trihydroxyflavone), which possesses the 2,3 double bond was not significantly different from naringenin (5,7,4'-trihydroxyflavanone), which lacks this feature. This may be explained by the absence of the catechol structure on the B ring on both apigenin and naringenin, which is an important structural feature for antioxidant activity.

The presence of a 3-hydroxyl on the C ring of flavonoids is important in increasing the stability of flavonoid radicals (Burda et al, 1991). The B ring torsion angle to the rest of the molecule significantly affects the free radical scavenging activity. The catechol hydroxyl groups on the B ring form intramolecular hydrogen bonds with the 3-hydroxyl, which aligns the B ring with A and C rings. Planar molecules allow better conjugation, electron delocalization and overall free radical scavenging ability compared to non-planar molecules. Flavonoids with a 3-hydroxyl group such as flavonols and flavanols are planar, but flavones and flavanones, which do not have this feature are twisted, which diminishes the scavenging activity. Burda et al, 1991 has also demonstrated that methyl or glycosyl substitution in the 3 position diminishes the scavenging activity which is demonstrated by the lowered scavenging

activity of methyl and glycosylated derivatives of quercetin compared to the quercetin aglucon (Burda et al, 1991).

c. Flavonoids and Steroid Hormone Dependent Cancers

Cancer is the second leading cause of death in the U.S. and one in four deaths in the U.S is from cancer (Birt et al, 2004). Evidence for the role of flavonoids in cancer prevention has been demonstrated in many *in vitro* models, animal models and epidemiological studies. Suggested mechanisms of action for cancer prevention include induction of apoptosis by causing apoptotic DNA fragmentation and mitochondrial toxicity, prevention of carcinogen activation by inhibition of drug-metabolizing enzymes, and modulation of gene expression by estrogen receptor binding (Duthie et al, 1999).

Epidemiological Evidence

Epidemiological evidence for the role of flavonoids in cancer prevention is very conflicting and confusing. In the Zutphen Elderly Study, conducted in Zutphen, The Netherlands in 1985, the food consumption of 738 men (without any history of cancer) aged 65 – 84 years was analyzed using a dietary history method (Hertog et al, 1994). The flavonols, quercetin, kaempferol, and myricetin, and the flavones, apigenin and luteolin, were analyzed. Five years of health and mortality data revealed that flavonoid intake was not associated with any cancer type but flavonoid intake from fruits and vegetables was inversely associated with alimentary and respiratory cancer risk (Hertog et al, 1994). These data suggested that other components in fruits and vegetables may be responsible for lowered cancer risk. Additionally, flavonoids may need to be associated with other natural components for a synergistic effect. A Netherlands Cohort Study was conducted in which 120,853 men and women aged 55 – 69 years participated (Goldbohm et al, 1995). After 4

years of follow up, no association was found between flavonol and flavone intake and cancer (Goldbohm et al, 1995). The Netherlands Cohort Study was not complete in that they did not investigate whether other flavonoid subtypes were associated with cancer. In a retrospective cross cultural study (Seven Countries Study) consisting of 12,763 men aged 40 – 59, after 25 years of follow up, no association between flavonoid intake and cancer was found (Hertog et al, 1995). Surprisingly flavonoid intake was positively associated with stomach cancer mortality. In contrast, 2 Finnish studies (the Finnish Mobile Clinic Health Examination Survey and the Alpha-Tocopherol, Beta Carotene Cancer Prevention Study) found inverse associations with lung cancer risk (Hertog et al, 1995). A borderline positive association was also found for colorectal cancer in the latter study (Hertog et al, 1995). Zheng et al., 1999 reported lowered urinary excretion of isoflavones, especially glycitein, in breast cancer patients compared to controls in Shanghai (Zheng et al, 1999). Den Tonkelaar et al., 2001 did not observe this relationship in post-menopausal breast cancer patients (Den Tonkelaar et al, 2001). The inconsistencies in these studies may be a result of other components in fruits, vegetables and soybeans, analysis of only a select number of flavonoids and polyphenols or spontaneous initiation of tumors from environmental factors.

Animal models

Troll et al, (1980) demonstrated that Sprague-Dawley rats fed a raw soybean diet experienced a reduction in mammary tumors caused by X-ray radiation compared to controls fed a casein diet. Although their results could be from other factors in soybeans including trypsin inhibitors, their report started the idea that isoflavones may play a role in this phenomenon (Troll et al, 1980). Since then many studies have shown the protective effects of soy isoflavones in animal carcinogenesis studies (Messina et al, 1994).

The time of flavonoid exposure may be important in cancer prevention (Lamartiniere et al, 1995, Lamartiniere et al, 2002). Lamartiniere et al, 1995 has shown that the number and development of DMBA-induced mammary tumors was reduced on day 50 postpartum in rats, that were injected subcutaneously with 5 mg genistein on days 2, 4 and 6 postpartam. Constantinou et al 1996 reported that the number of *N*-methyl-*N*-nitrosourea-induced mammary tumors in Sprague-Dawley rats was moderately reduced by injections of 0.8 mg genistein daily for 6 months (Constantinou et al, 1996). A 10% fermented soy milk or 0.02% or 0.04% isoflavone mixture was fed to female Sprague-Dawley rats during and after initiation of tumors with 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. Mammary tumor number and size were significantly lower than the control rats (Ohta et al, 2000).

In female CF1 mice treated with azoxymethane, 2 % quercetin or 4 % rutin in the diet significantly reduced tumor incidence and inhibited hyperproliferation of tumor cells (Deschner et al, 1993). Quercetin reduced tumor incidence by 76% and tumor multiplicity by 48% (Deschner et al, 1993). Quercetin inhibited *N*-nitrosodiethylamine-induced lung tumorigenesis during the initiation phase in mice when administered with drinking water at 9 µg/kg (Khanduja et al, 1999). Quercetin and luteolin, fed at 10 g/kg diet, decreased the incidence of fibrosarcomas and tumor size in Swiss albino male mice after injections of 20-methylcholanthrecene (Elangovan et al, 1994).

The citrus flavonoids have been shown to inhibit tumor growth in animal models. Dietary hesperidin, the major flavonone in orange juice, inhibited azomethane-induced colon carcinogenesis during the initiation and progression phases in male F344 rats at an oral dose of 1000 µg/g diet (Tanaka et al, 1997). In addition, concentrated orange juice delayed the development of DMBA-induced mammary cancer in rats (Tanaka et al, 1998). In similar

studies, So et al, 1996 compared the mammary inhibition capacity of concentrated orange juice, concentrated grapefruit juice, naringenin and naringin. Naringin and naringenin were given at a dose that was similar to that provided by the grapefruit juice. The greatest inhibition of cancer was seen with naringin supplemented, and with rats fed concentrated orange juice (So et al, 1996). These results are surprising since grapefruit juice contains significantly higher levels of naringin than orange juice, yet it did not cause the greatest inhibition. Other components in grapefruit juice may inhibit the anti-cancer effects of naringenin. The animal studies analyzing flavanones from orange and grapefruit juice are good studies because these are sources of flavonoids are consumed by humans. Therefore the results from these studies are very relevant to humans. However, the *in vitro* experiments discussed in this section are highly irrelevant to human exposure since they do not take into account the metabolic forms of flavonoids that are present in the blood circulation.

In vitro models of carcinogenesis inhibition

More than 30 flavonoids were tested for antiproliferative effects and apoptosis in cell culture models by measurement of the biomarker caspase-3. The cell lines tested included the Caco-2 and HT-29, which are colon cancer cell lines, and MCF-7, which is a breast cancer cell line (Le Bail et al, 1998). All flavonoids except for flavone, myricetin and baicalen, inhibited proliferation in all cell lines without cytotoxicity. The EC_{50} values ranged from 40 – 200 μM, which are extremely high, and not near the maximum plasma flavonoid concentrations found in humans (Le Bail et al, 1998). Quercetin inhibited the normal proliferation of ovarian adenocarcinoma tumor cells, and at a concentration of 10 μM, was able to suppress the proliferation of human ovarian OVCA 433 cells (Scambia et al, 1990). Quercetin reduced cell viability and DNA synthesis in A549 cells in a dose dependent

manner with a dose range of 15 – 58 μM (Nguyen et al, 2004). Genistein, in the range of 15 – 120 μM, inhibited the proliferation and induction of apoptosis in HT29 colon cancer cells (Yu et al, 2004). So et al, 1996 reported that the citrus flavonoids hesperetin and naringenin and baicalein, galangin, genistein and quercetin all inhibited cell proliferation in MDA-MB-435 human breast carcinoma cells with a concentration range of 5.9 – 140 μg/mL (So et al, 1996). Combinations of these flavonoids were more effective at inhibiting cell proliferation and required lower doses to produce the effect compared to individual flavonoids with IC_{50} values of 4.7–9.2 μg/mL. Guthrie and Carroll 1998 reported that naringenin and naringin (glycosylated form of naringenin) were effective in inhibiting the growth of estrogen receptor-positive and estrogen receptor-negative breast cancer cells with IC_{50} values of 1–18 μg/mL. Studies with genistein, quercetin and tangeretin show great promise in their anticancer mechanisms in that they are able to successfully suppress or inhibit growth of malignant cells but have no effects on their untransformed counterparts.

Although many of these studies are conducted at flavonoid concentrations that were not physiologically relevant, some flavonoids, especially genistein, are inhibitory at levels that are comparable to some anticancer drugs such as doxorubicin (Seeram et al, 2003). Another flaw in these studies is that flavonoids are usually glucuronidated in the blood circulation rather than in free aglucon form. All of the above studies have been conducted with flavonoid aglucons and in contrast, *in vivo* cells, either normal or malignant, would never encounter flavonoids in this form. Instead, cell culture studies should be conducted using flavonoid glucuronides because the 2 forms may have different activities (Zhang et al, 1999).

d. Flavonoids and Atherosclerosis

Cardiovascular disease is the number one cause of death in the U.S accounting for more than 40% of deaths. Artherosclerosis, which is the main cause of cardiovascular diseases, refers to the disease process of hardening and thickening of the arteries and mainly affects the coronary arteries that deliver blood to the heart. Atherosclerosis is a disease of inflammation, caused by endothelial dysfunction. Endothelial dysfunction can be caused by low-density lipoprotein (LDL) accumulation in the arteries, which undergo oxidation (Cook and Samman 1996). Oxidized LDL triggers a sequence of events involving the release of cytokines and growth factors, that lead to the accumulation of monocytes and macrophages from the blood to the endothelium (Diaz et al, 1997). Macrophages take up the oxidized LDL resulting in foam cell formation (Henriksen et al, 1981). Foam cell formation results in the development of atherosclerotic lesions and lesion progression (Ross, 1999). Other risk factors for endothelial dysfunction are platelet aggregation and thrombosis, blood pressure and vascular function, which will all be discussed below (Frankel, 1993, Ridker, 1999, Cook and Samman 1996, Birt et al, 2004).

Inhibition of LDL oxidation

It is believed that the antioxidant activity of flavonoids plays a role in inhibiting the development or progression of cardiovascular disease (Kris-Etherton et al, 2002). Mechanisms of inhibition of LDL oxidation are believed to be antioxidant, by reducing the formation of free radicals, inhibiting oxidation of α-tocopherol, and chelating divalent metal ions.

Red wine is a rich source of over 200 phenolic compounds (German and Walzem, 2000). Some of the flavonoids found in red wine are flavonols, monomeric catechins, and

polymeric anthocyanidins. Red wine also contains phenolic acids and resveratrol, which is a stilbene synthesized from phenylalanine, and not classified as a flavonoid. The most abundant flavonoid is catechin, a flavan-3-ol which is present in concentrations up to 300 mg/L, and all of the above named compounds have been shown to possess anti-oxidant properties *in vitro* (Abu-Amsha et al, 1996, DeWhalley et al, 1990, Vinson et al, 1995, Salah et al, 1995).

Red wine was shown to increase the antioxidant capacity of human plasma and inhibit LDL oxidation *in vitro* (Frankel et al, 1993, Duthie et al, 1998). Hayek et al, 1997 has shown that LDL isolated from atherosclerotic apolipoprotein E deficient mice, fed catechin, quercetin (each 50 μg/day) or red wine (0.5 mL/day), were less oxidized than the mice fed the placebo. Fremont et al, 1998 reported that the level of LDL peroxidation products was reduced in rats fed an enriched diet in polyunsaturated fatty acids supplemented with 8 g/kg quercetin and catechin (2:1), and lengthened the lag time of LDL peroxidation in rats fed the same flavonoid supplemented diet but enriched with monounsaturated fatty acids. Textured soybean protein containing 56 mg isoflavones resulted in about 20 % lower concentrations of 8-*epi*-prostaglandin $F_{2\alpha}$ (a biomarker for LDL oxidation) in 19 premenopausal women and 5 men, compared to soybean protein containing only 1.9 mg isoflavones after 17 days (Wiseman et al, 2000).

Structure-activity relationships for inhibition of LDL oxidation activity are not conclusive, but data from De Whalley et al, 1990, suggest that polyhydroxylated flavonoids including quercetin, morin, gossypetin and fisetin, have more inhibition activity compared to a flavonoid that is not hydroxylated, such as flavone (De Whalley et al, 1990).

In vitro models of platelet aggregation

Mechanisms of platelet aggregation inhibitory activity include cyclo-oxygenase and lipoxygenase inhibition, antagonization of thromboxane formation and thromboxane receptor function (Tzeng et al, 1991). Myricetin, fisetin, kaempferol, morin and quercetin inhibited platelet aggregation and ATP release in rabbit platelets induced by arachidonic acid with IC_{50}s ranging from 13 – 300 μM (Tzeng, 1991). Incubation of dilute purple grape juice (7 mL/kg body weight per day for 14 days) with human blood platelets inhibited platelet aggregation, enhanced release of platelet derived nitric oxide and decreased superoxide production (Freedman et al, 2001). *In vitro* platelet aggregation was reduced in platelets from female nonhuman primates fed soy protein isolate with isoflavones compared to animals fed alcohol-washed soy protein isolate, which contained a negligible amount of isoflavones (Williams and Clarkson, 1998).

Blood pressure and vascular function

Systolic and diastolic blood pressure was significantly lower after 24 h in 15 male stroke-prone spontaneously hypertensive rats fed black and green tea, in substitution for water, compared to rats fed tap water. The flavonoids in the 2 teas included 0.5 g/L flavonols and 0.4 g/L catechins in the black tea, and 3.5 g/L catechins, 0.5 g/L flavonols in the green tea (Negishi et al, 2004). Hypertensive patients (n=40) fed 5.5 mL Concord grape juice / kg body wt per day for 8 weeks, experienced significant blood pressure lowering with average systolic and diastolic blood pressure reductions of 7.2 and 6.2 mm Hg respectively compared to patients that received a placebo (Park et al, 2004). In a 15 week randomized, double-blind study, 46 men and 34 post-menopausal women, ages 45 to 75 years, ingested 2 isoflavone

tablets daily, enriched in either biochanin A or formononetin for a total dose of 80 mg/d, randomly crossed over with a placebo in 2 6-week periods. Formononetin enriched isoflavone tablet consumption resulted in a significant reduction of arterial stiffness with improved systemic arterial compliance, reduction in total peripheral resistance and reduction in central pulse wave velocity compared with the placebo. These effects were not observed with the biochanin A enriched isoflavone tablets (Teede et al, 2003). These results may be due to the difference in bioavailability of the demethylation products of biochanin A and formononetin, which are genistein and daidzein respectively. Genistein is much less bioavailable than daidzein therefore enough genistein may not be absorbed to exert a significant biological effect. This phenomenon will be discussed in the metabolism and bioavailability section of this dissertation.

Anti-inflammatory properties

As mentioned before, atherosclerosis is an inflammatory disease and some flavonoids possess anti-inflammatory properties. In particular, intercellular adhesion molecule ICAM-1 is induced during inflammation by cytokines such as IL-1, TNF-α, and IFN-γ. Quercetin inhibited ICAM-1 expression and TNF-α in ECV304 human endothelial cells in a dose-dependent manner (Kobuchi et al, 1999). In a well designed study by Koga et al, 2001, rat plasma metabolites of catechin and quercetin were tested on their effects on modulation of monocyte adhesion to human aortic U937 endothelial cells and on production of reactive oxygen species. Plasma was taken from 7 week old male Wistar rats 1 hr after administration of oral doses of 250 mg/kg body wt. catechin and 120 mg/kg body wt. quercetin. After incubation of the cells with catechin glucuronide and sulfates, cell adhesion to IL-1β-stimulated cells was inhibited. Pre-incubation with intact quercetin did not have this effect.

Opposite results were observed with quercetin, in that quercetin glucuronides, sulfates and methylated derivatives had no effect on cell adhesion, but intact quercetin was able to inhibit cell adhesion (Koga et al, 2001).

Epidemiological Evidence

The concept of the "French Paradox" refers to the association between low cardiovascular disease rates and risk scores for cardiovascular disease in France and Mediterranean countries, which are similar to other populations where the cardiovascular disease incidence was significantly higher. The risk factors accounted for in these analyses were cholesterol, blood pressure, age, sex, smoking and glucose intolerance (Birt et al, 2004). Previous studies have shown that these observations may be due to the increased consumption of red wine in these countries. In fact, studies have shown strong inverse associations between moderate red wine consumption (1 - 3 glasses of wine per day) and coronary heart disease mortality, while mortality from other causes was increased with higher alcohol intake (Klatsky et al 2003, Frankel et al, 1993, Renaud et al, 2004, Renaud et al, 1996 and Mukamal et al, 2005, Gronbaek, 2004). What is important, is that the inverse relationship between moderate alcohol intake and coronary heart disease mortality was stronger for wine, compared to other alcoholic beverages. This suggests that other components of wine besides alcohol are responsible for the observed association. Frankel et al, 1993 suggested that the lowered incidence of coronary heart disease mortality may be from the inhibition of LDL oxidation by flavonoids and other phenolic compounds in the wine.

Tea, chocolate and cocoa are rich in flavonoids, especially flavan-3-ols (Wollgast et al., 2001, Hammerstone et al, 1999, Natsume et al, 2000). Studies have shown inverse

associations between consumption of these foods and incidence of cardiovascular disease (Lagiou et al, 2004, Arts et al, 2001, Geleijnse et al, 2001, Nakachi et al., 2000, and Sasazuki et al., 2000).

The results from the Zutphen Elderly Study mentioned in the above Flavonoids and Cancer section, showed an inverse association between high flavonoid intake (around 30 mg/day) and 50% reduction in coronary heart disease mortality rate compared to people with low flavonoid intake (less than 19 mg/day) (Hertog et al, 1993). Knekt et al, 1996 showed that a high intake of apples and onions (rich sources of quercetin) were associated with significant reductions in coronary mortality in 5,133 men and women in Finland. In Iowa, high flavonoid intake was associated with decreased risk of cardiovascular disease in 34,492 postmenopausal women (Yochum et al, 1999).

F. Metabolism and Bioavailability

Understanding the metabolism and bioavailability of flavonoids is important because the biological activity of flavonoids at any given site of action depends on 3 events: (1) hydrolysis and absorption across the intestinal wall, (2) conjugation/phase II metabolism in the liver and (3) biliary excretion and gut microbial metabolism. The current understanding of flavonoid metabolism is that flavonoid glucosides must be hydrolyzed by microbial and/or mammalian glucosidases before absorption (Griffiths and Barrow, 1972), although recent conflicting reports show the absorption of flavonoid glucosides (Hollman et al, 1995, Talavera et al. 2004, 2003, Boyer et al, 2004), which will be discussed below. A significant amount of flavonoid aglucons (50 – 100%) are absorbed from the stomach and small intestine, and pass to the liver where they are conjugated by phase II metabolism enzymes in the liver hepatocytes (Scalbert et al, 2000). The conjugated flavonoids can be

excreted into the urine or bile (Yasuda et al, 1996). Flavonoid conjugates in the bile can be either reabsorbed by the small intestine after hydrolysis by glucuronidases (enterohepatic circulation), or pass to the large intestine, where the gut microflora can metabolize the flavonoid agucons into smaller phenolic compounds (Turner at al, 2003).

a. Hydrolysis and Absorption

The stomach has been shown to be a probable area of absorption in animal models. Administration of 25 mmol/kg body weight of the isoflavone glucosides, daidzin and genistin, in male Wistar rats, resulted in the appearance of the aglucons, genistein and daidzein in plasma, but not their glucosides 30 min after dosing (Piskula et al, 1999). These results suggest that absorption is possible for isoflavone aglucons but not isoflavone glucosides. These results were confirmed even after absorption was restricted to the stomach, from pyloric ligation (Piskula et al, 1999). However, Talavera et al, 2003 has shown that anthocyanin glucosides were efficiently and rapidly absorbed from the stomach also in male Wistar rats using *in situ* gastric administration. This discrepancy may be because the high gastric pH may be more favorable for anthocyanidins, which are charged molecules, compared to isoflavones and other flavanoids.

Most of the evidence in humans point to the upper small intestine as the site of flavonoid hydrolysis and absorption. Day et al, 1998 reported that quercetin-4'-glucoside, naringenin-7-glucoside, apigenin-7-glucoside, genistein-7-glucoside (genistin) and daidzein-7-glucoside (daidzin) were all hydrolyzed to their respective aglucons when incubated with cell free extracts from human small intestine. However, quercetin-3,4'-diglucoside, quercetin-3-glucoside, quercetin-3-rhamnoglucoside, kaempferol-3-glucoside and naringenin-7-rhamnoglucoside were not hydrolyzed. These results suggest that the human

small intestine shows higher specificity for certain flavanoid glucoside structures. For example, all glucosidic bonds in the 7 or 4' positions were hydrolyzed but not in the 3-position. Flavonoid rhamnoglucosides were not hydrolyzed either. It is possible that the flavonoid glucoside structures that were resistant to hydrolysis may be hydrolyzed by bacterial glucosidase enzymes in the large intestine, and not the small intestine (Crespy et al, 1999).

Murota et al, 2002 used Caco-2 monolayers as a model for the intestinal epithelium to demonstrate the absorption of flavonoid aglucons compared to glucosides. They observed that the isoflavone aglucons genistein and daidzein at 10 μM, were efficiently transported across the Caco-2 cell monolayer compared to their respective glucosides. Less than $1/10^{th}$ of the isoflavone glucosides appeared in basolateral solution. Interestingly, when genistein absorption was compared to the flavones apigenin and luteolin, and the flavonols kaempferol and quercetin, the glucuronidated and sulfated forms of the flavones and flavonols predominated in the basolateral solution compared to the predomination of the genistein aglucon. This suggests that isoflavone aglucons are absorbed more efficiently compared to other aglucons of other flavonoid subgroups and that the intestinal wall is capable of glucuronidation and sulfation. Liu and Hu, 2002 support this observation reporting that genistein and apigenin glucuronides and sulfated were found in a Caco-2 cell model and in a perfused rat intestinal model. Choudhury et al, 1999 reported that after oral dosing of naringenin-7-glucoside in rats, most of the recovered naringenin was that of naringenin glucuronide in urine. After intravenous dosing of naringenin-7-glucoside, the main circulating form was the unchanged glucoside. Supporting data with isolated rat jejunum revealed that naringenin-7-glucoside was not absorbed, but the major metabolite was

naringenin glucuronide. These data suggest that naringenin-7-glucoside is hydrolyzed by glucosidases in the intestinal epithelium before glucuronidation. The absorption of flavonoid glucosides across Caco-2 cell monolayers was reported in Boyer et al, 2004. They showed that quercetin-3-glucoside from onion, apple peel and in pure form were absorbed across the monolayer. However, the peak absorption of quercetin-3-glucoside was only 0.29 nmol when treated with 100 nmol quercetin-3-glucoside, which can be considered minimal or negligible. This supports data by Murota et al, 2002 and Liu and Hu, 2002, who observed only isoflavone glucuronides and sulfates after incubation of flavonoid glucosides with Caco-2 cells. However, Hollman et al, 1995 reported that in healthy ileostomy patients, quercetin glucoside was absorbed more efficiently than quercetin aglucon with an absorption of 52 % for quercetin glucosides from onions and 17 % for rutin. They suggested that the glucose transporter SGLT-1 was responsible for the transport of this glucoside. However, in a more recent study, Walle et al, 2000 in contrast to Hollman et al, 1995 reported that quercetin glucosides such as quercetin-4'-glucoside and quercetin-3,4'-diglucoside from onions were not absorbed in ileostomy patients, and were efficiently hydrolyzed to quercetin before absorption. The Hollman et al, 1995 data may not be reliable because they failed to directly measure quercetin or quercetin glucosides in the plasma, yet they assumed absorption of quercetin glucosides.

Recently, Walle et al, 2005 has shown that quercetin-4-glucoside and genistein-7-glucoside were rapidly hydrolyzed to quercetin and genistein respectively when incubated with human saliva. When flavonoids conjugated with other sugars aside from glucose, such as rutin (quercetin-3-rhamnoglucoside), quercitrin (quercetin-3-rhamnoside) and naringin (naringenin-7-rhamnoglucoside), were incubated with human saliva, hydrolysis did not

occur. These data suggest that human saliva only has the enzymatic capacity to hydrolyze flavonoid glucosides. Also, these data may explain why flavonoids are able to induce apoptosis in oral cancer cells (Hsu et al, 2004). All of the data presented here show the complexity in the understanding of flavonoid absorption. However, some evidence suggests that flavonoid glucosides may be absorbed, but only to an insignificant extent. The majority of the data points to more efficient and rapid absorption of flavonoid aglucons compared to glucosides.

b. Hepatic Metabolism

A large part of the flavonoid metabolism takes place in the liver (Kurzer and Xu, 1997). This is evident by the large amounts of flavonoid conjugates found in the bile and urine (Arts et al, 2004).The liver is responsible for phase I and II metabolism of flavonoids, which aid in their excretion in the urine and bile. This phase of flavonoid metabolism is important because in humans, the conjugated forms of flavonoids are what target sites encounter, not the free aglucon form, unless administered intravenously. Enzymatic activities of the liver include glucuronyl conjugation by UDP-glucuronosyltransferase, sulfate conjugation by phenol sulfotransferase and methyl conjugation by catechol-O-methyltransferase. Whether flavonoids are eliminated in urine or bile is dependent on the nature of their conjugation. For example, glucuronides are preferentially excreted in the bile, while sulfates are preferentially excreted in the urine (Mulder 1992). This is not the only determinant of excretion, however, and other factors such as molecular weight and lipophilicity must be taken into account.

Flavonoids apparently undergo extensive enterohepatic recirculation and excretion, which is common to many drugs (Crespy et al, 1999, Liu and Hu, 2002). The flavonoids are

secreted into the mesenteric vein, metabolized by the liver, partially excreted into the bile (Crespy et al, 2003), and excreted back into the lumen (Liu et al, 2003). Walle et al, 2001 has shown that in healthy volunteers fed 400 mg chrysin (5,7-dihydroxyflavone), most of the dose appeared in feces as free chrysin. In parallel experiments with rats, they reported that high concentrations of chrysin conjugates were in the bile. Walle et al, 2001 assumed that the chrysin appearing in the feces was from enterohepatic recirculation and subsequent microbial deconjugation by glucuronidases or sulfatases in the lumen. This could have been the case, but most of the dose appearing in the feces could have been from poor absorption. In studies with catechin in rats, Donavan et al, 2001 reported that catechin was methylated, sulfated and glucuronidated in the liver and that the circulating forms of catechin were in the conjugated form.

Crespy et al, 2003 observed that flavonoids with catechol groups such as quercetin, luteolin, eriodictyol and catechin were more likely to be methylated by the liver than other flavonoids. Although phase II metabolism is the predominant process in the liver pertaining to flavonoids, phase I oxidative reactions occur. Incubation of daidzein with hepatic microsomes from male Wistar rats resulted in the formation of the oxidative products, 6-hydroxydaidzein, 8-hydroxydaidzein, 5,6-dihydroxydaidzein, 3',6-dihydroxydaidzein, 3'-hydroxydaidzein, 2-hydroxydaidzein, 3',8- dihydroxydaidzein, 6,8-dihydroxydaidzein and 3',5,6- trihydroxydaidzein (Kulling et al, 2000). Incubation of daidzein with human hepatic microsomes only resulted in 6-hydroxydaidzein, 8-hydroxydaidzein, 3',6-dihydroxydaidzein, 3'-hydroxydaidzein and 3',8- dihydroxydaidzein (Kulling et al, 2001). Incubation of genistein with rat and human hepatic microsomes resulted in 6-hydroxygenistein, 8-hydroxygenistein and 3'-hydroxygenistein. Oxidative metabolites of methoxylated

flavonoids such as formononetin, biochanin A and glycitein were analyzed by this group. They reported that formononetin and biochanin A were oxidatively demethylated to daidzein and genistein initially, before the hydroxylation reaction occurred. The main metabolites were 6-hydroxydaidzein, 8-hydroxydaidzein and 3'-hydroxydaidzein for daidzein, and 6-hydroxygenistein, 8-hydroxygenistein and 3'-hydroxygenistein for genistein. 8-Hydroxyglycitein was the main metabolite of glycitein, whereas a small amount of the oxidative demethylated product 6-hydroxydaidzein was detected (Kulling et al, 2000 and Kulling et al, 2001). Glycitein may react differently compared to biochanin A and formononetin because it is methylated in position 6 instead of position 4', as in biochanin A and formononetin. The acidity of the 4'-methoxyl group may be different from the acidity of the methoxyl group in the 5, 6 or 7 position of the flavonoid structure. The products of oxidative metabolism have all been detected in human urine (Heinonen et al, 2003).

c. Gut Microbial Metabolism

Less than 25% of intact flavonoids are excreted in the urine in humans and animal models (Scalbert et al, 2000). This observation indicates that a significant portion of flavonoids are not accounted for. There are 3 possibilities for the flavonoids that are unaccounted for. First, the flavonoids were excreted in the feces, second, the flavonoids were metabolized by the gut microflora or third, some of the flavonoids may have been absorbed into target tissues. While it is possible that target tissues may have taken up some flavonoids, studies suggest that it is unlikely that tissue uptake of flavonoids occur in significant amounts. In rat studies, Coldham and Sauer, 2000 and Chang et al, 2000 reported that the amount of genistein recovered in rat tissues was around 0.5%, which is not a significant amount. Additionally, reports have shown that the amounts of intact flavonoid aglucons in

feces are less than 10% of the ingested dose (Xu et al, 1994, Xu et al, 1995). This suggests that a significant portion of flavonoids are metabolized by the gut microflora in the large intestine. After biliary excretion into the lumen, the flavonoids are deconjugated by bacterial glucuronidases to release the aglucons. The flavonoid aglucons can then be re-absorbed into circulation or be secreted into the large intestine where anaerobic reductive reactions are carried out by the host microfloral population that degrade the flavonoids into smaller phenolic acids. The flavonoid metabolites produced by these reactions are either absorbed and excreted in the urine, or excreted in feces.

The microbial metabolism of some flavonoids has been studied to some extent. Quercetin glucosides such as rutin (quercetin-3-rutinoside) and quercetin-3-glucoside are hydrolyzed to quercetin by bacterial β-glucosidases using various *in vitro* microbial fermentation systems (Rechner et al, 2004, Aura et al, 2002, Winter et al, 1989, Schneider et al, 1999). However, Justesen et al, 2000 did not observe quercetin as a hydrolysis product of rutin in an *in vitro* fecal fermentation system. This may be because they did not sample and analyze the fermentation system between 8 and 24 h after the start of the incubation with rutin. Quercetin may have been formed and rapidly metabolized in this 16 h period (Justesen et al, 2000). The quercetin aglucon is reduced to taxifolin, then further cleaved in the C ring to degradation products such as 3,4-dihydroxyphenylacetic acid and phloroglucinol (Labib et al, 2004, Figure 5). Other quercetin degradation products include 3,4-dihydroxytoluene (Labib et al, 2004), 3-(3-hydroxyphenyl)-propionic acid (Rechner et al, 2004), 3-hydroxyphenylacetic acid (Aura et al, 2002, Justesen et al, 2000) and 3-(3,4-dihydroxyphenyl)-propionic acid (Braune et al, 2001). Most flavonoids are degraded in a similar fashion as quercetin by bacterial C-ring cleavage. The compounds 4-

hydroxyphenylacetic acid, 3-(3,4-dihydroxyphenyl)-propionic acid and 3-(4-hydroxyphenyl)-propionic acid are C-ring cleavage products of kaempferol (Figure 5), luteolin and apigenin (Figure 6) respectively (Winter 1989, Schoefer et al, 2003). The citrus flavonone naringenin was cleaved to 3-(4-hydroxyphenyl)propionic acid and 3-phenylpropionic acid (Labib et al, 2004, Rechner, et al, 2004 and Schoefer, 2003) and hesperetin was initially demethylated to eriodictyol, before C-ring cleavage to 3-(3-hydroxyphenyl)propionic acid (Labib et al, 2004).

Anaerobic metabolism of the isoflavones genistein and daidzein have been extensively studied and the pathway of degradation is similar to other flavanoid subgroups but with subtle differences. Genistein microbial metabolites have been identified as dihydrogenistein, 6'-hydroxy-*O*-desmethylangolensin, 2-(4-hydroxyphenyl)-propionic acid and phloroglucinol. Genistein is reduced to dihydrogenistein, before partial C-ring cleavage to produce 6'-hydroxy-*O*-desmethylangolensin (6'-OH-ODMA), then fully cleaved to form the products 4-hydroxyphenyl-2-propionic acid and phloroglucinol (Chang et al, 1995, Coldham et al, 1999, Coldham et al, 2002 and Heinonen et al, 1999, Figure 8). All metabolites of genistein have been found to be non-estrogenic compared to genistein (Wiseman, 1999). Daidzein gut microbial metabolism has been shown to result in dihydrodaidzein, *O*-desmethylangolensin (ODMA), equol (7-hydroxyisoflavan) and *cis*-4-hydroxy-equol (Axelson, 1982, Bannwart et al, 1984, Heinonen et al, 1999, Heinonen et al, 2003, Joannou et al, 1995). Daidzein is degraded in the same fashion as genistein but another degradation product is formed from dihydrodaidzein, which is equol (Figure 8). Equol is formed from a decarboxylation reaction from dihydrodaidzein and is more estrogenic than daidzein (Rowland et al, 1999). Only about 30% of humans are able to produce equol from daidzein, which may be because of differences in bacterial populations (Lampe et al, 1998,

Rowland et al, 2000). ODMA has weak estrogenic activity compared to daidzein and equol (Rowland et al, 1999, Joannou et al, 1995). Anaerobic microbial glycitein metabolites have not been well characterized but *in vitro*, glycitein has been shown to be demethylated to 6,7,4'-trihydroxyisoflavone (6-hydroxydaidzein) (Hur et al, 2000, Figure 8). All of the above named isoflavone metabolites have been identified in urine (Axelson, 1982, Bannwart, 1984, Heinonen et al, 1999, Heinonen et al, 2003, Joannou et al, 1995). The gut microflora play an important role in the metabolism of flavonoid compounds and the formation of important metabolites. Antibiotic administration to human volunteers significantly decreased the excretion of bacterial isoflavone metabolites (Kilkkinen et al, 2002). Bowey et al, 2003, reported that germ free administered a soy protein diet did not excrete any bacterial isoflavone metabolites. When the germ free rats were colonized with microflora from human feces, isoflavone metabolites were excreted. Interestingly, when germ free rats were colonized with microflora from equol producers, the rats were able to excrete equol compared to the rats that were colonized with microflora from non-equol producers.

The bacteria that are responsible for flavonoid degradation are still in question. Most *in vitro* fermentation systems used for identification of flavonoid metabolites use fecal slurries that are representative of the many types of bacteria in the gut. Early studies have shown that *Butyrivibrio* spp. from ruminal fluid cleaved the C-ring of the flavonoid glucosides rutin and quercitrin, but not of the aglucon quercetin (Cheng et al, 1969, Krishnamurty et al, 1970, Cheng et al, 1971). In 1985, Morris and colleagues isolated three obligate anaerobic bacteria that were able to hydrolyze flavonoid glucosides to their aglucons: *Bacteroides distasonis*, *Bacteroides uniformes* and *Bacteroides ovatus* (Morris et al, 1985). *Eubacterium ramulus* and *Enterococcus casseliflavus* were identified and isolated

from human feces for hydrolysis activity on genistin, daidzin (Schoefer et al, 2002), rutin, quercetin-3-glucoside, luteolin-7-glucoside and kaempferol-3-sorphoroside-7-glucoside (Schneider et al, 1999, Schneider et al, 2000 and Schneider et al, 2000). C-Ring cleavage activity of flavonoids was identified in *Clostridium orbiscindens* (Schoefer et al, 2003, Hur et al, 2002) and *Eubacterium ramulus* (Schoefer et al, 2002). Recently, Steer et al, 2003 supported the role of *Bacteriodes* spp. and *Clostridium* spp. in flavonoid metabolism. They incubated feces from human volunteers with isoflavones with and without the presence of a prebiotic such as fructooligosacchharide. They hypothesized that the prebiotic would divert bacterial metabolism away from isoflavone metabolism. The addition of the prebiotic caused significant increases in *Bifidobacterium* spp. and *Lactobacillus* spp. and significant reductions in *Bacteroides* spp. and *Clostridium* spp. *Bacteroides*, *Clostridium* and *Eubacterium* spp. are present in high numbers (10^9 – 10^{12} CFU/g) in the colon (Turner et al, 2003) and it is likely, from the above studies, that all of these species play a role in the pathway of gut microbial metabolism of flavonoids. The bacterial species responsible for equol formation has not been identified as of yet.

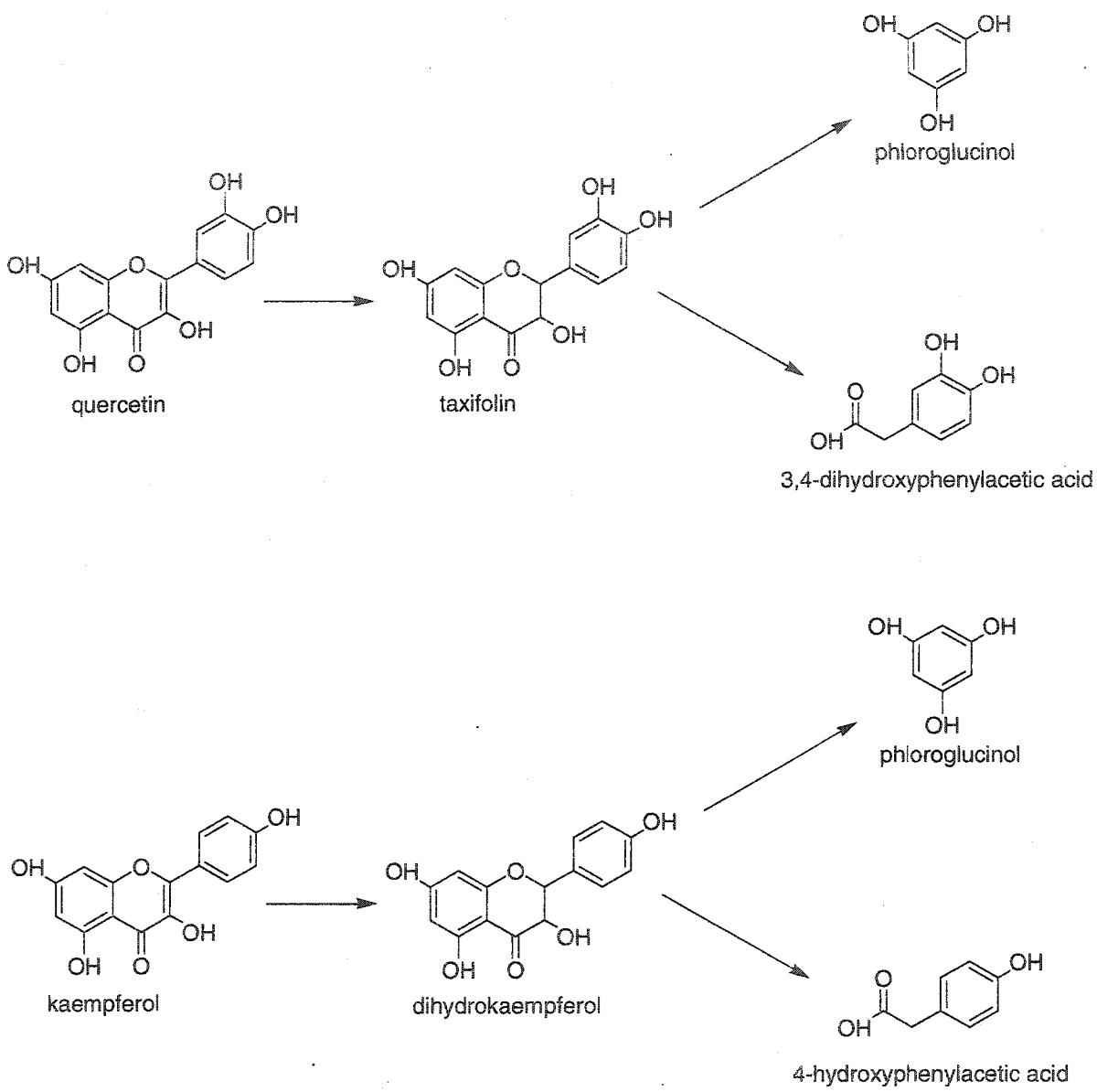


Figure 5. Anaerobic microbial degradation of quercetin and kaempferol (Labib et al, 2004, Schoefer et al, 2003)

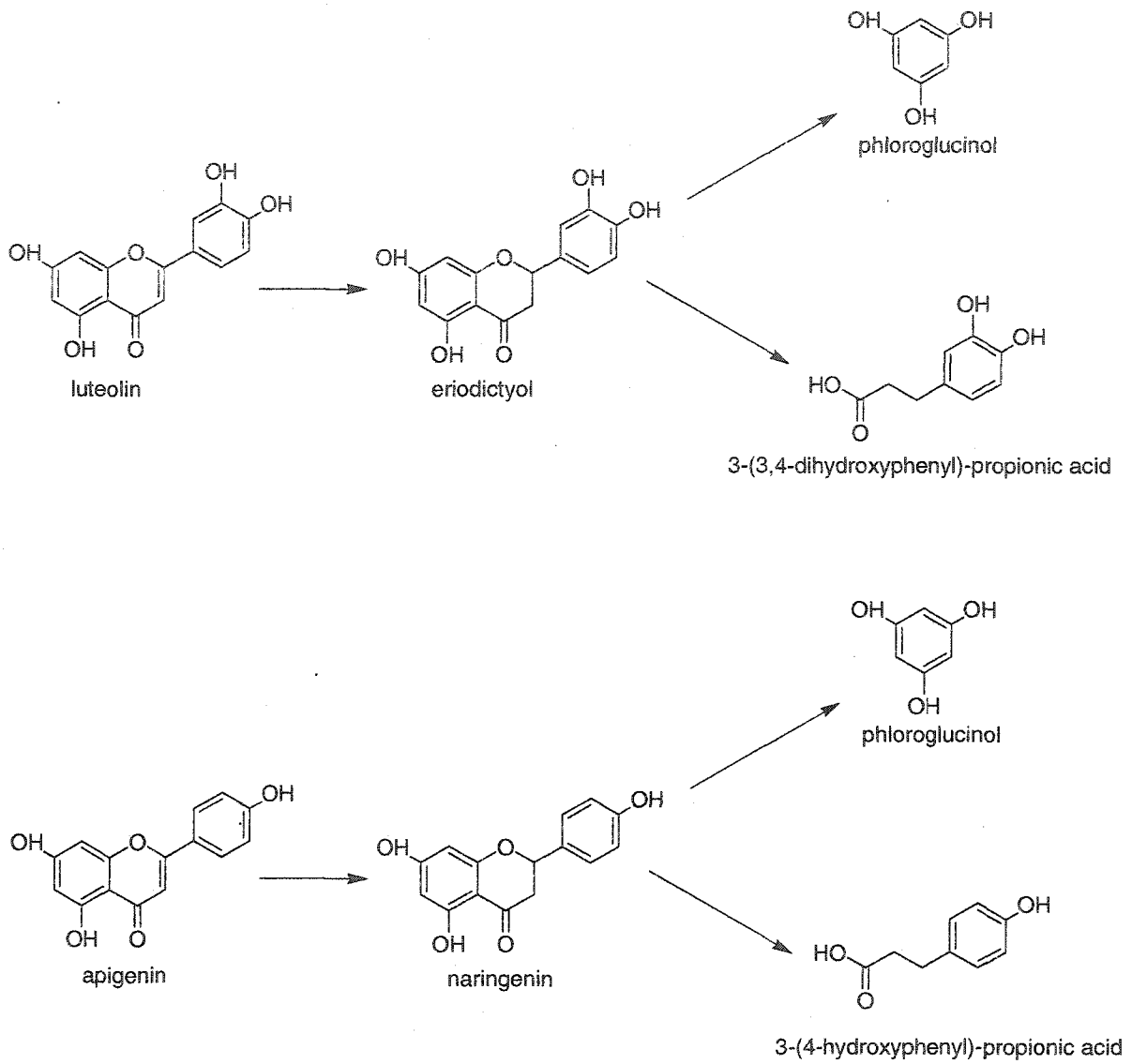


Figure 6. Anaerobic microbial degradation of luteolin and apigenin (Schoefer et al, 2003)

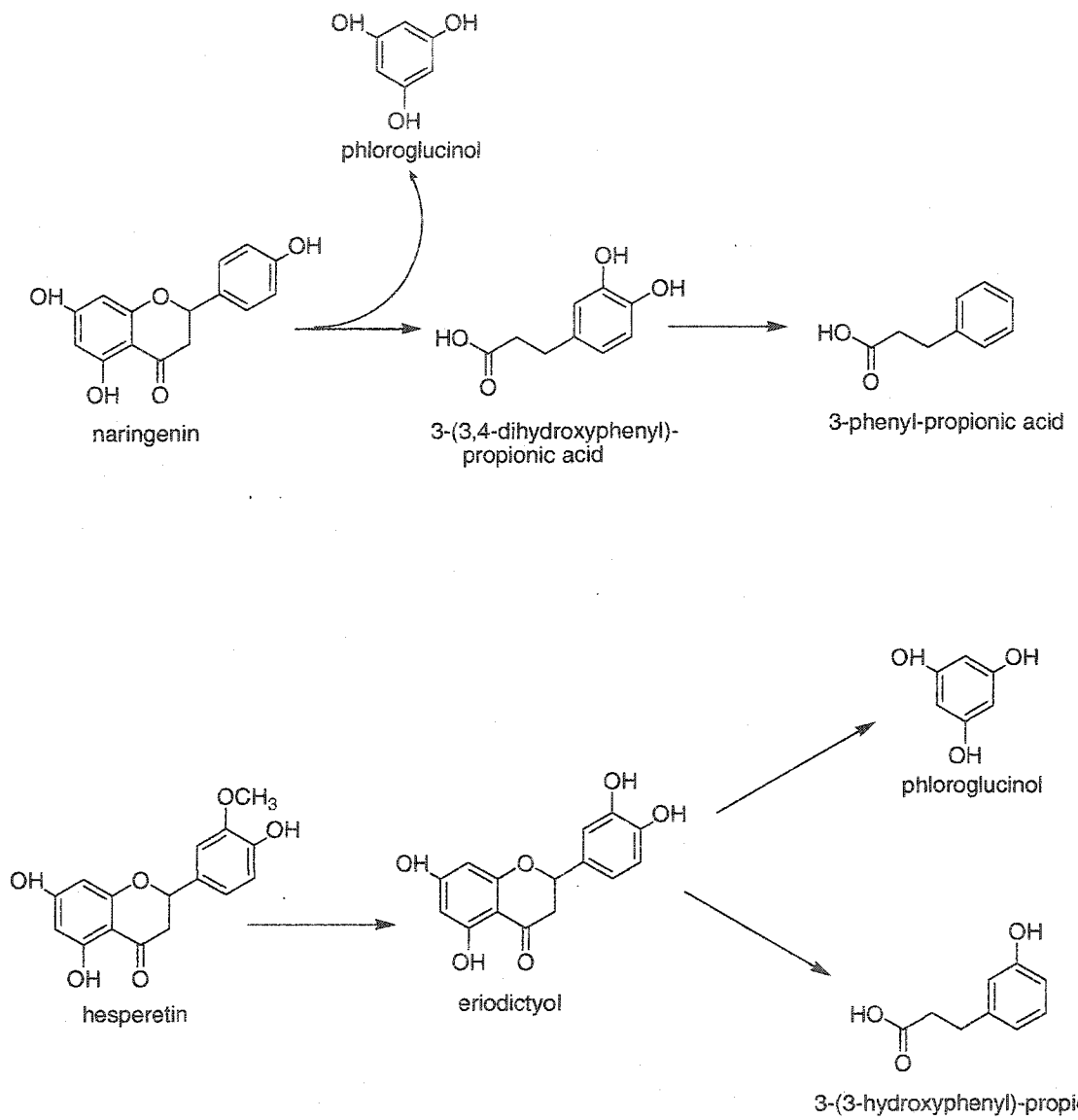
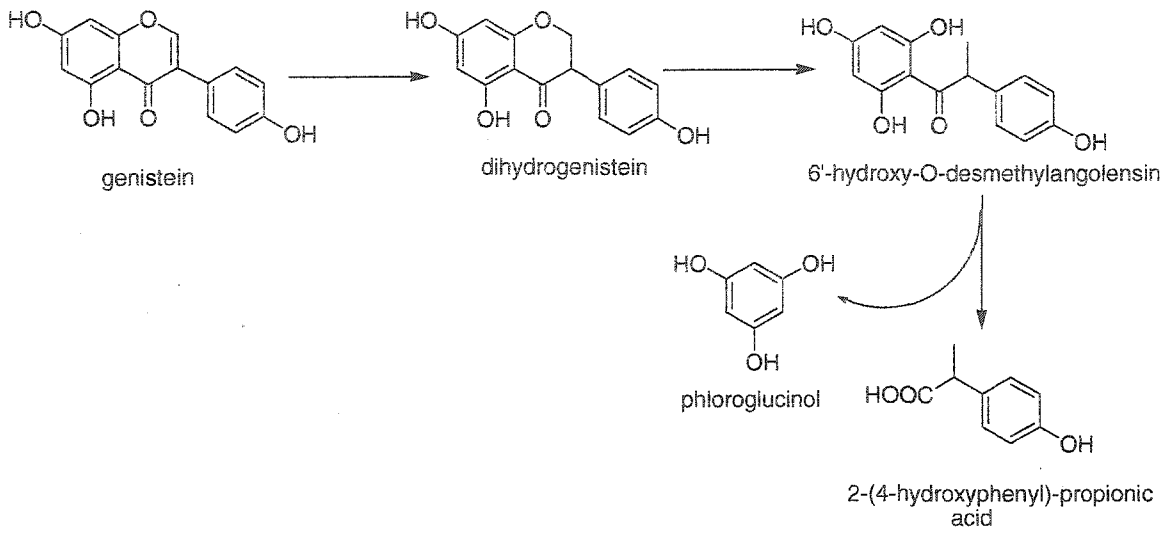


Figure 7. Anaerobic microbial metabolism of naringenin and hesperetin (Labib et al, 2004, Rechner et al, 2004)



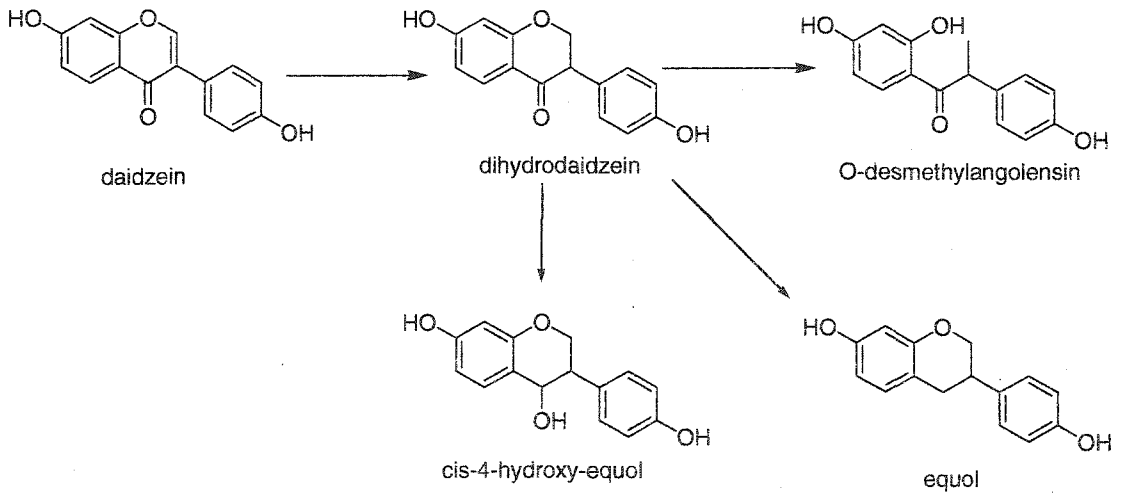


Figure 8. Anaerobic microbial degradation of the soy isoflavones genistein, daidzein and glycitein (Heinonen et al, 1999, Joannou et al, 1995, Hur et al, 2000)

d. Bioavailability

The term bioavailability refers to a proportion of a drug or compound that reaches the systemic circulation unchanged, so that it may exert its pharmacological or biological effect. This term encompasses the absorption, metabolism and excretion of compounds and can be measured in 2 different ways. From a toxicological point of view, bioavailability (F) is measured as the area under the curve (AUC) for a specific test route compared to the AUC for the intravenous route. Therefore, for oral administration of a compound,

$$F = \frac{AUCoral}{AUCiv}$$

An F of 1.0 means that the bioavailability of the compound is 100%.

In contrast, from a nutritional point of view, bioavailability is measured as the amount of the compound excreted as a percentage of ingested dose of the compound. Measurements of AUC using the intravenous route could not be made, therefore, the nutritional definition of bioavailability will be used throughout this disssertation. Therefore,

$$Bioavailability = \frac{mg_{excreted}}{mg_{ingested}} x100$$

The bioavailability of flavonoids varies, and depends on many factors such as chemical structure, lipophilicity, molecular weight, gut microbial metabolism, food matrix and possibly other components of the diet. The bioavailability of flavonols such as quercetin has been extensively studied in humans and animal models. Quercetin is present principally in glucosidic form in foods as quercetin-3-glucoside, quercetin-4'-glucoside and quercetin-3-rutinoside or rutin. Gugler et al, 1975 reported that quercetin was not detected in urine or

plasma after a 6 gram oral dose in 6 volunteers. They recovered 53% of the ingested dose in feces. They concluded that quercetin was not absorbed and was extensively metabolized by gut microorganisms (Gugler et al, 1975). Since then, many quercetin bioavailability studies have been conducted in animal models and human studies.

Hollman et al, 1995 reported that quercetin was absorbed in 9 ileostomy patients after 3 separate feedings, separated by 4 day washout periods, of onions, a rich source of quercetin-4'-glucoside, pure rutin, and pure quercetin aglucon. All quercetin sources provided around 100 mg quercetin aglucon. They reported an average bioavailability of 0.5% (Hollman et al, 1995). Additionally, De Vries et al, 1998 reported that the bioavailability of quercetin from strong black tea and onions was 0.5 and 1.1% respectively after 3 days of feeding in 15 subjects (De Vries et al, 1998). These results suggest that the bioavailability of quercetin is extremely low and could be considered not bioavailable at all.

Since quercetin is found in different glucosidic forms in foods, the bioavailability of the different quercetin glucosides has been compared. Graefe et al, 2001 conducted a 4 way cross over study, in which 12 volunteers ingested 100 mg quercetin-4'-glucoside, 200 mg rutin, onion supplement, whuch was rich in quercetin-4'-glucoside and equivalent to 100 mg querectin aglucon, and buckwheat tea, which was rich in rutin and equivalent to 200 mg quercetin aglucon, in random order. The bioavailability of quercetin from onions was 6.5 %, which was significantly higher than that of quercetin from buckwheat tea 1.0 % (Graefe et al, 2001). This suggests that rutin is less bioavailable than quercetin-4'-glucoside. Additionally, they observed that quercetin was more bioavailable from onions, which was 6.5 %, compared to the pure quercetin-4'-glucoside, which was 4.5 %, that further supports the observations

by Cermak et al, 2003, in that the quercetin form and food matrix affect quercetin bioavailability.

Quercetin-3-glucoside was found to be more than twice as bioavailable when administered with ground beef, compared to a commercial standard diet in pigs (Cermak et al, 2003). These results are likely due to the lipid content of the ground beef. Recently, Lesser et al, 2004 has shown that the lipid content of the diet affected the bioavailability of quercetin in pigs. They fed 30 μmol/kg body weight quercetin, as quercetin aglucon and quercetin-3-glucoside in 3 test meals consisting of 3, 17 and 32 g fat / 100g diet. Similar to previous mentioned studies, quercetin-3-glucoside was more bioavailable than quercetin aglucon in each meal. However, no matter what form quercetin was in, the bioavailability was 50% higher in the 17% fat diet compared to the 3% fat diet. There was no difference between the 17 and 32% fat diets (Lesser et al, 2004). It is possible that flavanoids are more soluble in fat-containing matrices and thus, better absorbed by passive diffusion across the intestinal wall. These results have not been confirmed in humans yet, except for a few studies with isoflavones that have shown that food matrix and background diet did not affect isoflavone bioavailability (Tew et al, 1996, Xu et al, 2000). It would be interesting to determine if the fat content of the human diet play a role in flavonoid bioavailability.

The structure of isoflavones has been suggested to play a role in bioavailability. Zhang et al, 1999 has shown that genistein, with a bioavailability of 29 % was significantly lower than daidzein and glycitein with bioavailabilities of 46 % and 55 % in 14 volunteers fed 4.5 μmol/kg body weight. Data by King et al, 1998 confirmed this observation in rats, in that genistein, with a bioavailability of 12% was lower than daidzein, with a bioavailability of 17% after an oral dose of 74 and 77 μmol/kg body weight. Xu et al, 1995 reported similar

bioavailabilities of 16% and 10% for daidzein and genistein respectively in 7 women given 3 doses of 3, 7 and 10 μmol/kg body weight in soymilk, separated by 2 week washout periods.

Few studies have investigated the bioavailability of the flavanones naringenin and hesperetin. The bioavailability of naringenin was reported to be 30.2 % from grapefruit juice and 1.1% from orange juice in 7 volunteers. Hesperetin bioavailability was 5.3% (Erlund et al, 2001). This obvious difference in naringenin bioavailability between the 2 food matrices may be because of differences in the juice matrix. Manach et al, 2003 reported that the human bioavailability of naringenin and hesperitin in 5 volunteers did not differ after a dose of 444 mg/L hesperetin and 96 mg/L naringenin in orange juice.

More flavonoid bioavailability studies need to be conducted with flavonoid subclasses other than flavonols and isoflavones, which have been extensively studied. Subclasses such as flavanones and catechins are widely and frequently consumed and warrant further studies.

G. Toxicity

Scientific reports of beneficial health effects of flavonoids have led to their increased consumption in health-conscious populations, especially in the form of dietary supplements. Flavonoid dietary supplements usually contain high concentrations of flavonoids and the recommended doses greatly exceed doses that would be obtained from a diet that was rich in fruits and vegetables. The appeal of these dietary supplements is that they cost significantly less than prescribed drugs for certain conditions, they are easily accessed and they are advertised as 'natural' replacements to synthetic drugs for those individuals wary of taking synthetic drugs with side effects. However, manufacturers of these dietary supplements advertise their health effects, which are misleading because most of the flavonoid

formulations have not been studied in human clinical trials. Dietary supplements are regulated by the Federal Drug Administration (FDA) under the Dietary Supplement Health and Education Act (DSHEA) of 1994. This act allows the sale of dietary supplements without the extensive premarket approval process required of new drugs. Therefore, any adverse effects from inappropriate use, side effects or interactions with other drugs are not known.

Pro-oxidant Activity

As mentioned in the antioxidant activity section, reactive oxygen species (ROS) such as peroxyl and hydroxyl radicals, can damage DNA, lipids and other biological molecules. Hodnick et al, 1986, reported that the flavonols quercetin, myricetin and quercetagetin were effective at undergoing autoxidation and causing mitochondrial respiratory bursts resulting in superoxide, hydrogen peroxide and hydroxyl radical formation. Ahmad et al, 1992 supported this finding, demonstrating that flavonols with pyrogallol or catechol rings autoxidized in the presence of transitional metals producing ROS, which were able to accelerate LDL-oxidation. This is important in cardiovascular disease because low levels of copper ions are released during the formation of atherosclerotic lesions or other tissue injury (Smith et al, 1992). Quercetin, naringenin and morin were also able to cause DNA damage through the formation of ROS (Rahman et al, 1989, Sahu and Gray, 1997). Epigallocatechin gallate (EGCG) of the catechin flavonoid subgroup, was shown to cause DNA damage and H_2O_2 formation in the presence of transition metal ions and induced DNA oxidation in HL-60 cells (Furukawa et al, 2003). This pro-oxidant activity was supported *in vivo*, as EGCG enhanced dimethylhydrazine or nitrosamine induced colon carcinogenesis in male F344 rats (Hirose et al, 2001).

Topoisomerase Inhibition

At physiological concentrations (< 1 μM), flavonoids such as genistein, quercetin, myricetin, biochanin A and equol, which is a bacterial metabolite of daidzein, have been shown to be topoisomerase II inhibitors (Austin et al, 1992, Azuma et al, 1995). Topoisomerase II inhibitors cause double strand DNA lesions at topoisomerase-binding sites. Ross, 1998 and Ross et al, 1996 has observed that increasing maternal consumption of topoisomerase inhibitors elevated leukemia risk almost 10-fold in infants. This is important because high flavonoid intake by pregnant women, such as the concentrations found in dietary supplements, may be a significant risk factor for infant leukemia. Microbial and phase II metabolism of flavonoids may play a role in their topoisomerase II inhibitor activity. Equol, which is a microbial metabolite of daidzein, inhibits both topoisomerase I and II activity, while daidzein does not. Metabolites of genistein such as dihydrogenistein, genistein glucuronide, genistein sulfate and 3'-O-methylquercetin have been shown to lose their topoisomerase II inhibitory activity compared to genistein (Chang et al, 1995).

Thyroid Hormone

Endemic goiter in populations where millet is the main dietary staple, has been attributed to the high concentrations of apigenin and luteolin in the millet (Gaitan et al, 1995, Sartelet et al, 1996). The frequent consumption of isoflavones in soymilk in human infants, may explain their high incidence of thyroid disease (Fort et al, 1990). Structure activity studies by Divi et al, 1996 revealed that flavonoids with free resorcinol moieties, including fisetin, kaempferol, naringenin, quercetin and genistein, were the most potent inhibitors of thyroid peroxidase, an enzyme important for thyroid hormone synthesis. Inhibition of thyroid hormone synthesis leads to elevated thyroid stimulating hormone levels, which result in

growth and dysfunction of the thyroid gland. However, in a randomized, double blind, placebo-controlled study, the effect of a daily isoflavone supplement containing 90 mg isoflavone aglucons / day, was compared to a placebo in 38 postmenopausal women ages 64 - 83 years old. Serum thyroid-stimulating hormone, thyroxine, and triiodothyronine levels were measured at baseline and after 90 and 180 days. There were no differences between the isoflavone supplement and placebo groups at each measurement (Bruce et al, 2003). These data show that thyroid hormone levels are not affected by isoflavone supplementation, at least not in postmenopausal adults.

Flavonoid-Drug Interactions

Flavonoids have been shown to inhibit and induce human cytochrome P450 (CYP) enzymes (Tassaneeyakul et al, 1993 and Guengerich et al, 1994). This property is beneficial in chemopreventative cases, because CYPs play a role in activation of carcinogens (Doostdar et al, 2000), but is a toxic property in flavonoid-drug interactions because CYPs are also responsible for the metabolism of therapeutic drugs. However, much of this work has been conducted *in vitro* with flavonoid concentrations ranging from 11 μM – 35 mM, which is not physiologically relevant. Flavonoid-drug interactions may alter the bioavailability/pharmacokinetics of certain drugs resulting in an inhibition of the therapeutic effect, or enhancement, resulting in toxicity depending on the flavonoid structure (Tang et al 2000). Naringenin, the major flavanone in grapefruit juice, inhibits intestinal CYP3A4 within 30 minutes of ingestion, which significantly increased the bioavailability of certain drugs including felodipine, nitrendipine, nisoldipine and verapamil (Fuhr, 1998). Therefore ingestion of grapefruit juice along with certain drugs that must be extensively metabolized should be avoided.

Flavonoids have been reported to overcome multidrug resistance (MDR) to cancer therapy by inhibiting drug efflux transporters such as P-glycoprotein (Kartner et al, 1983). MDR is a phenomenon where cancer cells aquire resistance to anti-cancer agents during cancer therapy, by overexpression of P-glycoprotein and multidrug resistance associated proteins (MRPs), which pump anti-cancer drugs out of cells (Galati et al, 2004). This results in low cellular drug concentrations. Methoxylated flavonoids such as 5,7,3′,4′-tetramethoxyflavone, diosmetin, chrysoeriol, tamarixetin and isorhamnetin were reported to be the best MRP inhibitors *in vitro* with IC_{50} values ranging between 2.7 and 14.3 μM (van Zanden et al, 2005). The inhibition of MRPs may be beneficial when applied to cancer therapy but inhibition of MRPs may possibly disturb the kinetics of other drugs or food constituents that are normally pumped out of cells, resulting in toxicity. This area of flavonoid - drug interactions deserves further study *in vivo*.

Estrogenicity

You et al, 2002 has reported that genistein's estrogenic activity was associated with decreased fertility and increased sexual dysfunction in experimental animals at high doses of 800 ppm (You et al, 2002). Genistein (1 μM) was found to enhance the proliferation of MCF-7 human breast cancer cells *in vitro* (Chen, 2004) and was uterotropic at a high dose of 80 μg/g body weight per day for 91 days in adult Wistar rats (Picherit et al, 2001). Breast tissue proliferation was stimulated in premenopausal women with benign or malignant breast tumors, after short term intake of soybeans (McMichael-Phillips et al, 1998).

Overall, the issue of flavonoid toxicity warrants more attention and research, because of the increasing consumption of flavonoids in dietary supplements in adults and children. However, most of the research published, suggests that flavonoids are non-toxic at doses

consumed in the diet. The interaction that certain flavonoids have with other drugs is a relatively new area in research, and is currently being studied in many research labs.

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METABOLISM OF GLYCITEIN (7,4'-DIHYDROXY-6-METHOXY-ISOFLAVONE) BY HUMAN GUT MICROFLORA

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Abstract

Gut microbial degradation of the soy isoflavone glycitein, 7,4'-dihydroxy-6-methoxyisoflavone, was investigated by incubating glycitein anaerobically, with feces from 12 human subjects. The subjects' ages ranged from 24 to 53 y with a body mass index (BMI) of 20.9 to 25.8 kg/m^2 (mean BMI = 24.0 ± 1.1 kg/m^2) respectively. There were no significant differences between the degradation rates for each subject at glycitein concentrations from 10 – 250 μmol/L (average k = 0.32 ± 0.03 h^{-1}, $p > 0.05$). Glycitein degradation followed an apparent first order rate loss. There were no differences in the degradation rates of glycitein within subjects, when incubated in the presence or absence of daidzein and genistein (average k = 0.30 ± 0.21 h^{-1} vs 0.23 ± 0.19 h^{-1}, $p = 0.72$). Three different groups segregated according to their degradation rates for glycitein described as high (k = 0.67 ± 0.14 h^{-1}), moderate (k = 0.34 ± 0.04 h^{-1}) and low (k = 0.15 ± 0.07 h^{-1}) glycitein degraders ($p < 0.0001$). Four glycitein metabolites characterized by LC-MS (electrospray ionization using positive ionization mode), were dihydroglycitein, dihydro-6,7,4'-trihydroxyisoflavone, 5'-OMe-O-desmethylangolensin. Two subjects produced 6-OMe-equol and 1 subject produced daidzein as an additional metabolite of glycitein.

Introduction

Isoflavones are a subclass of compounds that belong to a much larger group of polyphenolic compounds called flavonoids. Isoflavones mainly occur in plants of the

Leguminosae family (1). However, soybeans and soybean-derived foods are the main sources of the isoflavones genistein (5,7,4'-trihydroxyisoflavone), daidzein (7,4'-dihydroxyisoflavone) and glycitein (7,4'-dihydroxy-6-methoxyisoflavone) where the total isoflavone concentration can reach up to 2 g per kg fresh weight (2). The three soy isoflavones are all hydroxylated in the 7 and 4' positions of the isoflavone skeleton (Figure 1) which makes them structurally similar to estradiol (3). Apparently, because of this similarity, isoflavones possess estrogenic activity, and have been shown to bind to estrogen receptors (4). Isoflavones have been associated with many positive health effects in chronic diseases, such as decreased risk of coronary heart disease and certain types of cancers (5,6), and reduced rates of osteoporosis (7).

Isoflavones are found as both glucosides and aglucons in soy foods (8). Oral ingestion of isoflavone glucosides lead to their hydrolysis in the small intestine by bacterial β-glucosidase activity and β-glucosidases in the cells of the gastrointestinal mucosa (9). The free aglucons resulting from hydrolysis can be absorbed and undergo first pass hepatic metabolism (10) or be metabolized further by the gut microflora. Experiments with germ-free rats showed that isoflavone metabolites were not excreted and antibiotics decreased the production of isoflavone metabolites in humans (11,12). Understanding the metabolism of isoflavones in the gut is important because this process may affect their bioavailability, and thus their absorption and biological activities *in vivo* (13).

The anaerobic bacterial metabolism of genistein and daidzein by gut microflora has been studied to some extent. Genistein metabolites resulting from anaerobic metabolism in the human gut have been identified as dihydrogenistein, 6'-hydroxy-*O*-desmethylangolensin (6'-OH-ODMA), 4-hydroxyphenyl-2-propionic acid, p-ethylphenol and phloroglucinol (14-

17). Anaerobic bacterial metabolism of daidzein has been shown to result in dihydrodaidzein, *O*-desmethylangolensin (ODMA), equol and *cis*-4-hydroxy-equol (18-22). Glycitein comprises less than 10% of the total isoflavone amount in soybeans and soybean foods and is probably the reason why its metabolism has not been well studied. However, glycitein comprises about 50 % of the isoflavone amount in soy germ (23). Glycitein has been shown to be demethoxylated *in vitro* by *Eubacterium limosum* to 6,7,4'-trihydroxyisoflavone (24). Recently, glycitein metabolites such as dihydroglycitein, 5'-O-methyl-*O*-desmethylangolensin (5'-OMe-ODMA) and 6-O-methyl-equol (6-OMe-equol) have been isolated and characterized in human urine (21).

Preliminary studies in our lab have shown that the rate of disappearance of glycitein was significantly slower than that of genistein ($p<0.0001$) in an *in vitro* fecal fermentation system (13) and the human bioavailability of glycitein was significantly higher than genistein *in vivo* (25). The aim of this study was to further characterize glycitein metabolism in humans by investigating the kinetics of the degradation reaction and the metabolites of glycitein using an *in vitro* fecal fermentation system. The variability among human subjects was also investigated.

Materials and Methods

Chemicals

Glycitein was synthesized according to Lang'at-Thoruwa et al, (26). Daidzein and 2,4,4'-trihydroxydeoxybenzoin (THB) were synthesized using the method of Song et al, (27). Genistein was synthesized according to a modification of Chang et al, (28). 6,7,4'-Trihydroxyisoflavone was purchased from Indofine Chemical Co., Inc (Hillsborough, NJ). Dihydrodaidzein, dihydrogenistein, equol and O-desmethylangolensin (ODMA) were

purchased from Plantech U.K. (Reading, England). All other chemicals including HPLC grade acetonitrile, methanol, acetic acid, and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Fairlawn, NJ). All aqueous solutions were prepared using Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water (MQ water).

Subject protocol

Four men and eight women volunteered from Iowa State University and the surrounding Ames area. The subjects were in good health and not taking any medication. The subjects' ages ranged from 24 to 53 y with a body mass index (BMI) of 20.9 to 25.8 kg/m^2 (mean BMI = 24.0 ± 1.1 kg/m^2) respectively. The ethnicities of the subjects included 6 Caucasians, 3 African Americans, 1 Chinese immigrant, 1 Asian-Indian and 1 Latino. Subjects were given written instructions on soy containing foods to avoid for 1 week before providing one fresh fecal sample in sealed sterile containers (Sage Products Inc., Crystal Lake, IL). Approval of the study design was obtained from the Iowa State University Human Subjects Research Committee in 2003.

Isoflavone Fermentation

Brain-heart infusion (BHI) broth media (Difco Laboratories, Detroit, MI) was prepared according to Zheng et al, (25). The media contained a rezasurin color indictator to ensure the fermentation systems were anaerobic. Samples of 1.5 g fresh feces were immediately transferred from their sealed sterile containers into incubation test tubes (Fisher Scientific, Fairlawn, NJ) containing 27 mL BHI media. The incubation tubes were vortexed for 5 s to make a fecal suspension. Daidzein, glycitein and genistein were dissolved in 100% DMSO and added to a fecal suspension for a final concentration of 100 μmol/L. Glycitein dose response was investigated by adding 112 mmol/L glycitein in 100% DMSO to 5 fecal

suspensions for final concentrations of 10, 50, 75, 100 and 250 μmol/L glycitein. An incubation tube consisting of the fecal suspension without isoflavones, served as a negative control. A positive control consisted of BHI media and 250 μmol/L of each isoflavone without the fecal suspension. The incubation tubes were flushed with CO_2, sealed with rubber stoppers, then vortexed for 5 s. One mL of fecal suspension was sampled anaerobically from each tube immediately for time 0 and frozen on dry ice. The tubes were placed in a 37°C incubator. Samples were taken at 3, 6, 9, 12 and 24 h and frozen. All incubations were performed in duplicate.

Isoflavone Extraction

THB was added to the thawed fermentation sample for a final concentration of 0.025 mg/mL to serve as an internal standard. The samples were slowly loaded onto pre-conditioned C_{18} solid phase extraction cartridges (Waters Corporation Milford, MA). The cartridge was washed twice with 2 mL of Milli-Q system water. The isoflavones were eluted with 1 mL 80% methanol, filtered through 0.45 μm filters and analyzed by HPLC.

HPLC analysis

The HPLC system consisted of a Hewlett Packard 1050 Series. Twenty μL of sample was injected onto a reversed-phase, 5 μm, C_{18} AM 303 column (250 x 4.6 mm) (YMC Co. Ltd. Wilmington, NC). The mobile phase consisted of 0.1% glacial acetic acid in water (A) and acetonitrile (B). Solvent B increased from 30 to 50% in 14 min, increased to 100% in 5 min, and recycled back to 30% in 1 min. The flow rate was 1 mL/min. The wavelength used for the detection of isoflavone and metabolite peaks, and for the preparation of isoflavone standard curves was 254 and 280 nm. Chem station^{3D} software (Hewlett Packard Company,

Scientific Instruments Division, Palo Alto, CA) was used to integrate the peak area responses and to evaluate the ultraviolet absorbance spectra.

Mass Spectrometry analysis

Analyses of glycitein metabolites were carried out using a Shimadzu 2010 LC-MS system (Kyoto, Japan) consisting of a Shimadzu 2010 liquid chromatograph with a dual wavelength photodiode array (PDA) detector in series between the chromatograph and a Q-array-Octapole-Quadrupole mass analyzer. Detection of metabolites was performed using electrospray spray ionization (ESI) in the positive ion mode. The mobile phase for sample separation was performed under the same conditions as used for HPLC analysis except the flow rate was 0.2 ml/min. The injection volume was 20 uL. Samples were introduced into the electrospray interface through an untreated fused-silica capillary. A nitrogen gas flow of 4.5 L/min was used as the nebulizing and auxiliary gas for the mass spectrometer. The parameters applied to MS were as follows: block temperature, 200°C; desolvation temperature, 400°C; capilliary voltage, 3.8 kV; cone voltage, 20 V. The mass spectrometer was tuned and calibrated for the range of *m/z* 100-300. Daidzein, glycitein, 6,7,4'-trihydroxyflavone and ODMA standards were dissolved in 100% methanol and analyzed to obtain authentic mass spectra prior to sample analysis.

Data Analysis and Statistics

The rate of disappearance of isoflavones in fecal suspensions was estimated by plotting ln (% remaining isoflavone) versus time. The slope of this line was the apparent first order degradation rate constant. The ratio of peak area of an isoflavone to THB (0.025 μmol/L) versus the isoflavone concentration was used as an internal standard curve to estimate the concentration of isoflavones in the fecal suspensions. Statistical evaluation of

degradation rate differences was performed using the SAS system (version 8.1, SAS Institute., Cary, NC). Differences between the overall and individual degradation rates of isoflavones were estimated using 1-way ANOVA. Isoflavone degradation phenotypes were identified using average linkage cluster analysis (29). The statistical significance of all analyses was set at $\alpha = 0.05$.

Results and Discussion

The glycitein doses of 10 – 250 μmol/L were studied in our fermentation systems because they represent doses of isoflavones that may be seen in the human gut after ingestion of soy-containing foods. There were no significant differences in the degradation rates for each of the glycitein doses in 10 of the 12 subjects with an average $k = 0.32 \pm 0.03\ h^{-1}$, $p > 0.05$ (Figure 2). Two subjects showed significantly higher degradation rates at the 10 μmol/L dose, which can be explained by the low recovery of glycitein at this dose. A plot of the natural log of the remaining percentage of each isoflavone against time resulted in a straight line for all subjects, with coefficients of correlation ranging from 0.75 – 0.99 (data not shown). These findings suggest that isoflavone degradation by gut microflora follow apparent first order kinetics, which supports other investigations reporting isoflavone kinetic data (13, 30).

There were no significant changes in the degradation rate of glycitein when it was degraded in the presence of genistein and daidzein for 10 of the 12 subjects (average $k = 0.30 \pm 0.21\ h^{-1}$ vs $0.23 \pm 0.19\ h^{-1}$, $p = 0.72$, data not shown). These data suggest that the isoflavones may not compete among each other to be degraded by the gut microflora, and different types of bacterial species may degrade each isoflavone. Preliminary studies in our

lab have shown different bacterial profiles of high glycitein degraders compared to other isoflavone degradation phenotypes (Renouf et al, unpublished data).

Cluster analysis was performed on the isoflavone degradation rates to observe any groupings of similar degradation rates. Three significantly different groupings of degradation rates were observed for glycitein. They were called high ($k = 0.67 \pm 0.14\ h^{-1}$), moderate ($k = 0.34 \pm 0.04\ h^{-1}$) and low ($k = 0.15 \pm 0.07\ h^{-1}$) glycitein degraders ($p < 0.0001$, Figure 3). These phenotypes were observed for genistein and daidzein degradation rates (Table 1). These data are supported by Hendrich et al. (31), who reported three isoflavone degradation phenotypes (high, moderate and low) from 9 men and 11 women.

The degradation rate of glycitein with an average $k = 0.30 \pm 0.21\ h^{-1}$ was significantly lower than genistein with an average $k = 0.43 \pm 0.44\ h^{-1}$, $p = 0.018$, but not different from daidzein with an average $k = 0.16 \pm 0.17\ h^{-1}$, $p = 0.074$ (data not shown). We believe that this difference is due to structural differences between the 3 isoflavones. It has been proposed that the hydroxyl group in the 5^{th} position on the A ring of the flavonoid structure is responsible for genistein's rapid degradation by gut microflora (13, 32). We have shown in related studies that flavonoids with hydroxyl groups in the 5, 7 and 4' positions are important for rapid microbial degradation (Simons et al, submitted).

Phenotypic trends were observed in all of the subjects. Subjects 9, 13 and 26 tended to have more rapid or high degradation phenotypes and subjects 5, 8, 17 and 18 tended to have low degradation phenotypes for all three isoflavones (Table 1). We speculate that these differences in degradation phenotypes are because of distinct differences in individual gut bacterial populations. Subjects with low *in vitro* isoflavone degradation phenotypes may experience higher isoflavone bioavailabilty *in vivo*, compared to subjects with high

isoflavone degradation phenotypes. Zheng et al, 2003 has shown that Asian women with low genistein degradation phenotypes experienced greater genistein bioavailability compared to Asian subjects with high genistein degradation phenotypes (33).

We hypothesized that the main glycitein metabolite would be dihydroglycitein based on reports identifying dihydrodaidzein and dihydrogenistein as the main metabolites of daidzein and genistein, respectively (34). Metabolism studies by Hur et al, 2000 reported that glycitein was metabolized to 6,7,4'-trihydroxyisoflavone by *Eubacterium limosum* (24). *Eubacterium limosum* is able to O-demethylate methoxyl derivatives of benzoic acid (35) and is found in the digestive tract (36). From these studies we predicted that 6,7,4'-trihydroxyisoflavone would be one of the metabolites of glycitein.

HPLC chromatograms were analyzed for the formation of new peaks in the fecal fermentation suspensions over the 24 h period. For 10 of the 12 subjects, 2 new peaks (peaks 1 and 4) appeared 6 h after incubation at retention times 14.5 and 19.9 min, respectively (Figure 6A). Two more new peaks (peaks 2 and 3) appeared after 24 h with retention times of 17.5 and 17.7 min, respectively (Figure 6A). Peak 2 was identified in only 2 of the 12 subjects. Two subjects showed no evidence of new chromatographic peaks during the 24 h of fermentation. One subject showed an additional new peak at 15.9 min (peak 5, Figure 6B). All other peaks were not associated with glycitein metabolism and appeared in negative controls.

The UV spectra and retention time of peak 1 was identical to a synthetic reference standard of 6,7,4'-trihydroxyisoflavone (Figure 7.1, Table 2). However, mass spectral analysis using positive electrospray ionization ($M+H^+$) revealed that the molecular weights of

peak 1 and 6,7,4'-trihydroxyisoflavone were 273 and 271 respectively (Table 2). Based on these data, peak 1 was identified as dihydro-6,7,4'-trihydoxyisoflavone.

The UV spectra and retention time of peak 5 and daidzein were identical (Figure 7, Table 2). MS analysis confirmed that this peak was daidzein with a molecular weight ($M+H^+$) of 255 (Table 2).

Peaks 2, 3 and 4 were more hydrophobic than glycitein (Figure 6A-B). The UV spectrums of peak 2, 3 and 4 were similar to the daidzein microbial metabolites, equol, ODMA and dihydrodaidzein respectively (Figure 7). MS analysis for these 3 peaks showed molecular weights of 271, 289 and 287, respectively. We identified peaks 2, 3 and 4 as 6-OMe-equol, 5'-OMe-ODMA and dihydroglycitein, respectively (Table 2) based on the MS and UV data.

Glycitein is similar to daidzein structurally with exception of the methoxyl group at the 6 position. We did expect to have metabolites that were similar to the metabolites of daidzein such as dihydrodaidzein, equol and ODMA (17-19). We did detect dihydroglycitein in the fecal incubation mixture, but the major metabolite to appear in all metabolite-producing subjects was dihydro-6,7,4'-trihydroxyisoflavone at 6 h after incubation. This suggests that the first step in glycitein metabolism is reduction to dihydroglycitein, then O-demethylation to dihydro-6,7,4'-trihydroxyisoflavone. However, we cannot ignore that glycitein may first be O-demethylated to 6,7,4'-trihydroxyisoflavone, then further reduced to dihydro-6,7,4'-trihydroxyisoflavone, although we did not detect 6,7,4'-trihydroxyisoflavone in our incubation mixtures. The latter pathway would be similar to the microbial metabolic pathways of formononetin (7-hydroxy-4-methoxyisoflavone) and biochanin A (5,7-dihydroxy-4-methoxyisoflavone) (24).

Heinonen et al, (21) identified dihydroglycitein in the urine of human subjects fed 3 soy bars, equivalent to a daily intake of 48.4, 40.2 and 4.1 mg of daidzein, genistein and glycitein, respectively, for 2 weeks. These data imply that glycitein was reduced to dihydroglycitein. However, Bowey et al, (12) observed that dihydrogenistein and dihydrodaidzein were produced in the urine of germ-free rats. They concluded that dihydrogenistein, dihydrodaidzein and dihydroglycitein are not products of microbial metabolism. Perhaps, glycitein may be converted to dihydroglycitein by intestinal bacteria and liver enzymes.

The metabolite, 5'-OMe-ODMA, most likely results from C ring cleavage of dihydroglycitein which is consistent with bacterial C-ring cleavage studies of daidzein and genistein (15,16). Heinonen et al (21) also identified 5'-OH-ODMA in addition to 5'-OMe-ODMA in human urine which suggested direct C-ring cleavage of dihydro-6,7,4'-trihydroxyisoflavone or demethylation of 5'-OMe-ODMA. We did not detect 5'-OH-ODMA in our fecal fermentation mixtures during the 24 h incubation period.

Production of daidzein from glycitein would result from direct demethoxylation of glycitein at the 6 position. However, only 1 subject produced daidzein in this study. Additionally, bioavailability studies in our lab have shown that daidzein was produced in 1 out of 10 hamsters that were fed purified glycitein (Lee et al, unpublished data). Setchell et al (37) reported that there was a small rise in the daidzein plasma concentration when glycitin (glycitein-7-*O*-β-D-glucopyranose) was orally given to humans. Taken together, these results suggest that demethoxylation of glycitein may not be a major pathway of metabolism in humans and hamsters.

We observed the *in vitro* degradation of daidzein for each subject and did not observe any equol production. Due to this observation, we speculated that we would not see any equol-like metabolites of glycitein. We assumed that if a subject is not capable of producing equol from daidzein, they were not able to produce an equol-like metabolite from glycitein. However, our *in vitro* fecal fermentation models are poor indicators of equol-producer phenotypes, because equol is only produced in response to chronic soy ingestion over a period of at least 3 days (38, 39). Therefore, we may have had equol-producers among the 12 subjects. We proposed that 6-methoxy-equol and 6-hydroxy-equol would be possible metabolites of glycitein and we were able to identify 6-methoxy-equol in 2 of the subjects.

Based on the metabolites identified in these studies, we have proposed several microbial metabolism pathways for glycitein shown in figure 6. The major pathways of glycitein microbial metabolism are reduction to dihydroglycitein, then demethylation to produce dihydro-6,7,4'-trihydroxyisoflavone, or C ring cleavage of dihydroglycitein to produce 5'-OMe-ODMA. Minor pathways include direct demethoxylation of glycitein to daidzein, and reduction of dihydroglycitein to 5'-OMe-equol. Alternative pathways of glycitein microbial metabolism not observed in these studies are demethylation of glycitein to 6,7,4'-trihydroxyisoflavone, reduction to dihydro-6,7,4'-trihydroxyisoflavone, then either C ring cleavge to 5'-OH-ODMA, or reduction to 6-OH-equol.

It was interesting that we found dihydro-6,7,4'-trihydroxyisoflavone as a microbial metabolite of glycitein because its precursor 6,7,4'-trihydroxyisoflavone has been identified in fermented soybeans and tempe (40). It is believed that the bacteria used in the tempeh fermentation process, *Brevibacterium epidermis* and *Micrococcus luteus*, are responsible for the conversion of glycitein and daidzein to other polyhydroxylated compounds including

6,7,4'-trihydroxyisoflavone (41,42) Kulling et al (43,44) has shown that daidzein was converted to 6,7,4'-trihydroxyisoflavone from oxidative metabolism reactions by rat liver microsomes.

The estrogenic activity of isoflavones may be affected by gut microbial metabolism. For example, equol, a metabolite of daidzein, has been reported to be more estrogenic than daidzein (45). *p*-Ethyl phenol, a metabolite of genistein, has no estrogenic activity even though genistein possesses potent estrogenic activity and numerous other biological effects (46-48). Previous work in our lab has shown that glycitein possessed a lower *in vitro* estrogen receptor-binding affinity compared to genistein, but gave a higher estrogenic response in an *in vivo* mouse uterine enlargement assay (4). This higher estrogenic response may have resulted from the higher bioavailability of glycitein compared to genistein, or the formation of glycitein metabolites in the *in vivo* assays that possess higher estrogenic potencies than the parent compound. 6,7,4'-Trihydroxyisoflavone has been shown to exhibit estrogenic activity (49) but binds the estrogen receptor beta with little or no affinity (49,50). It would be interesting to determine the estrogenic properties of the other glycitein metabolites. Studies in our lab have shown that the gut microflora play a significant role in the bioavailability of isoflavones (13,25,33,51). Plasma daidzein and genistein concentrations were negatively correlated with the daidzein and genistein microbial degradation rate constants, respectively, (33) suggesting that increased isoflavone intestinal microbial degradation, reduced the amount of intact isoflavones appearing in plasma after absorption. Additionally, daidzein and glycitein were more bioavailable than genistein, as reflected in urinary excretion as a percentage of ingested dose, because daidzein and glycitein were degraded at a much slower rate compared to genistein (25).

In this study, we have found that glycitein was degraded according to first order kinetics by human gut microflora, but at a slower rate compared to genistein. We found that there was significant interindividual variation in glycitein degradation but the degradation rates were segregated into 3 significantly different groups. We identified 3 major glycitein metabolites (dihydroglycitein, dihydro-6,7,4'-trihydroxyisoflavone and 5'-OMe-ODMA) in 10 of 12 subjects, 6-OMe-equol in 2 subjects and daidzein in 1 subject. The significance of the glycitein metabolites has yet to be determined.

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Table 1. Cluster Analysis of Subjects' Isoflavone Degradation Rates[a]

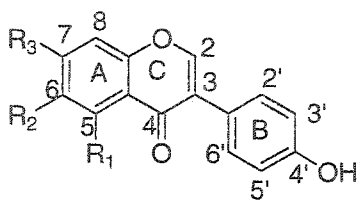
	Degradation rate k (h^{-1})[b]			Subject ID[c]		
Isoflavone	High	Moderate	Low	High	Moderate	Low
glycitein	0.67±0.14	0.34±0.04	0.15±0.07	13,9	26,3,11,2	6,8,18,17,4,5
daidzein	0.34±0.04	0.17±0.04	0.03±0.02	9,26	6,13,2,3,11,4	8,5,18,17
genistein	1.54[d]	0.77±0.01	0.17±0.08	13	26,2,3	8,9,5,17,11,4,18,6

[a]Degradation phenotypes of subjects. Subjects separated into 3 significantly different groups for each isoflavone, named high, moderate and low degraders ($p<0.0001$). [b]Degradation rates expressed as average ± standard deviation of the mean. [c]Whole numbers shown are subjects' identification number and are listed in descending degradation rate order. [d]Standard deviation could not be determined from a measurement of only 1 subject's degradation rate.

Table 2. HPLC and MS characteristics of glycitein metabolites and standards[a]

Standard and Metabolite ID	Retention Time (min)	Molecular Weight $[M+H^+]$[b]	Proposed compound[c]
6,7,4'-trihydroxyflavone	14.5	271	6,7,4'-trihydroxyisoflavone
daidzein	15.9	255	daidzein
1	14.5	273	dihydro-6,7,4'-trihydroxyisoflavone
2	17.5	271	6-OMe-equol
3	17.7	289	5'-OMe-O-desmethylangolensin
4	19.9	287	dihydroglycitein
5	15.9	255	daidzein

[a]HPLC retention time and molecular weight from mass spectrometry (MS) analysis for standards and each metabolite peak. [b]Molecular weight were determined by electrospray spray ionization in the positive ion mode and are represented as M+1. [c]Proposed compounds are based on MS data, HPLC retention time and UV spectral analysis.



Glycitein: R_1=H, R_2=OCH_3, R_3=OH
Daidzein: R_1=H, R_2=H, R_3=OH
Genistein: R_1=OH, R_2=H, R_3=OH

Figure 1. Soy isoflavone structures, substitution patterns and numbering system. A: A ring, B: B ring, C: C ring.

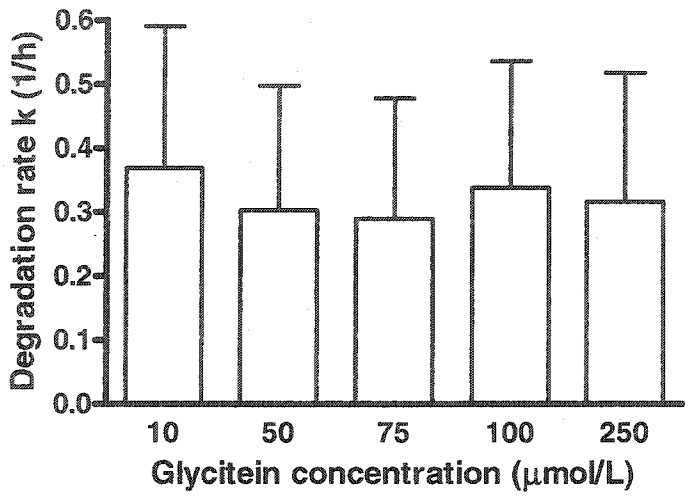


Figure 2. *In vitro* human microbial degradation rates of glycitein at 10, 50, 75, 100 and 250 μmol/L. Error bars represent standard deviation from the mean.

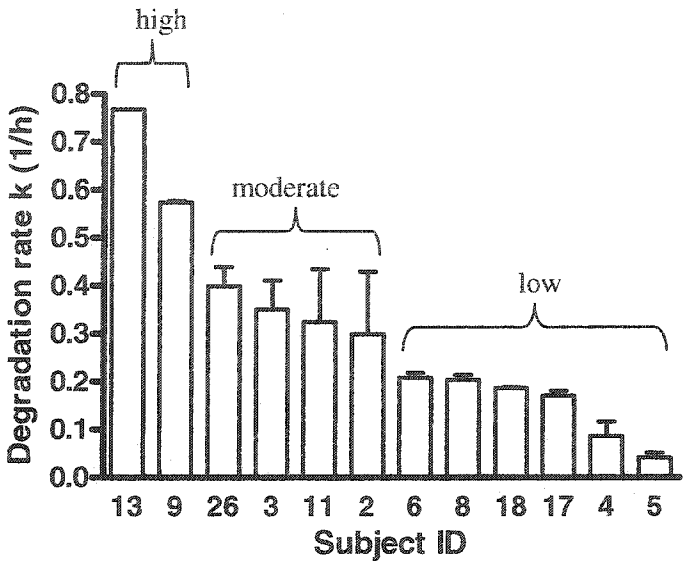


Figure 3. Statistical cluster analysis of subjects' glycitein degradation rates. Subjects segregated into 3 different groups represented as high, moderate and low glycitein degraders ($p < 0.0001$). Error bars represent standard deviation from the mean.

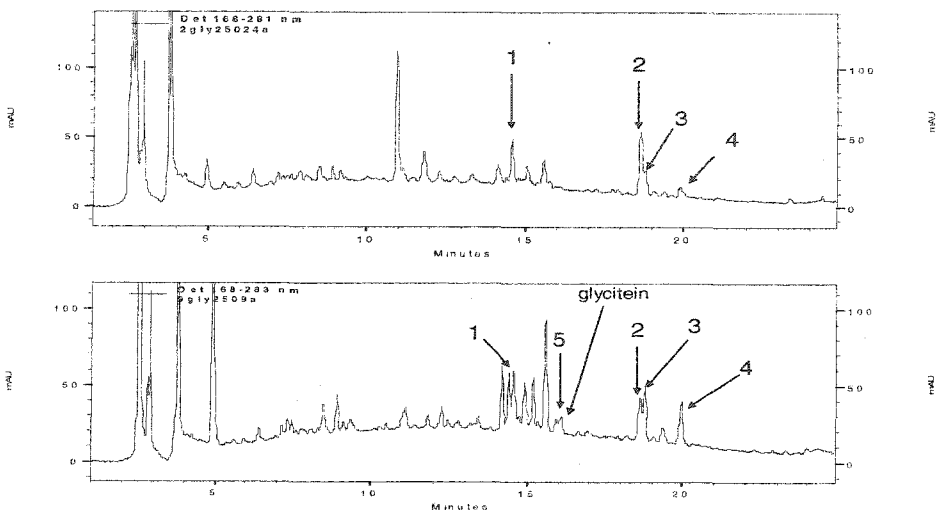


Figure 4. HPLC chromatograms of fecal fermentation extracts after 24 h incubation with 250 μmol glycitein in 2 subjects. Numbers shown are for metabolite peak identification. All other peaks were not associated with glycitein metabolism and appeared in negative controls.

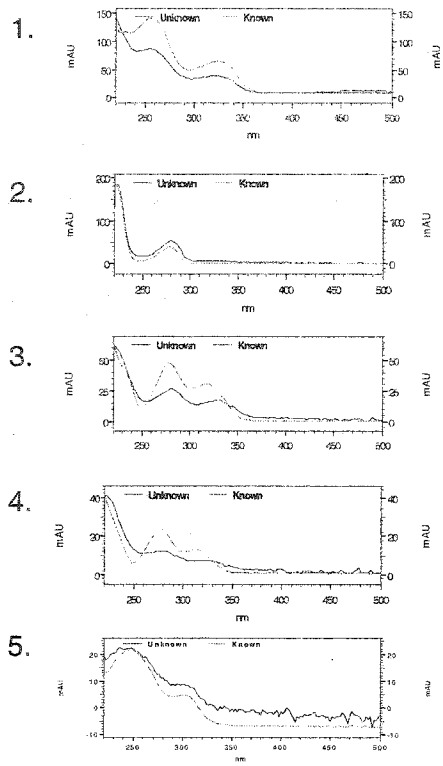


Figure 5. Ultraviolet spectra of metabolite peaks and known standards. Spectra in black are from metabolite peaks and spectra in gray are from standards. Numbers shown are metabolite peak identification numbers. (1) was compared to 6,7,4'-trihydroxyisoflavone, (2) was compared to equol, (3) was compared to ODMA, (4) was compared to dihydrodaidzein and (5) was compared to daidzein.

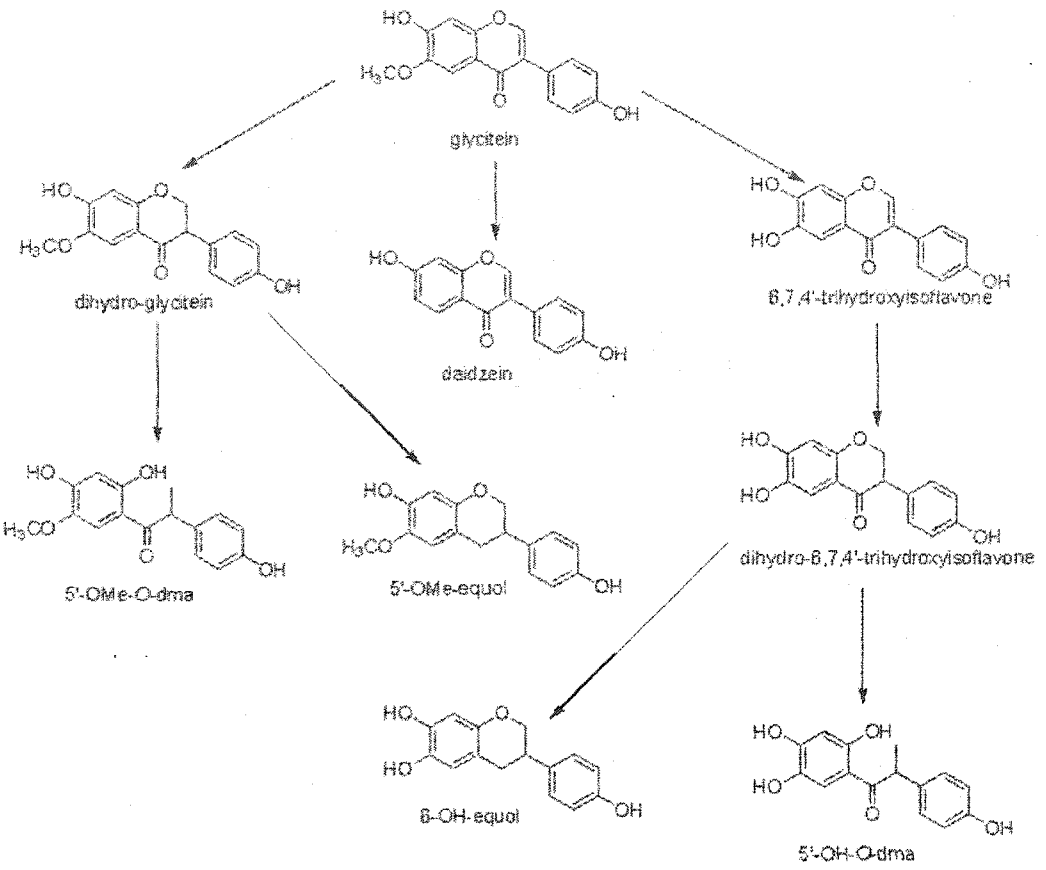


Figure 6. Pathways of anaerobic glycitein metabolism in the human gut.

HUMAN GUT MICROBIAL DEGRADATION OF FLAVONOIDS: STRUCTURE-FUNCTION RELATIONSHIPS

A paper accepted by the Journal of Agricultural and Food Science

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Abstract

The relationship between chemical structure and gut microbial degradation rates of 14 flavonoids, flavone, apigenin, chrysin, naringenin, kaempferol, genistein, daidzein, daidzin, puerarin, 7,4'-dihydroxyflavone, 6,4'-dihydroxyflavone, 5,4'-dihydroxyflavone, 5,3'-dihydroxyflavone and 4'-hydroxyflavone, was investigated by anaerobically fermenting the flavonoids with human gut microflora (n=11 subjects). Degradation rates for the 5,7,4'-trihydroxyl-flavonoids, apigenin, genistein, naringenin and kaempferol, were significantly faster than the other structural motifs. Genistein degraded the fastest of all flavonoids in all subjects. Puerarin was resistant to degradation by the gut microflora. Extensive degradation of flavonoids by gut microflora may result in lower overall bioavailability than those flavonoids that are slowly degraded because rapidly degrading flavonoids are less likely to be absorbed intact.

Introduction

Flavonoids are a large group of polyphenolic compounds that are widely distributed in all plants. Fruits, vegetables and beverages (fruit juices, wine, tea and coffee) are major sources of flavonoids in the human diet and over 4000 of these compounds have been reported to date (1). Adequate intakes of fruits and vegetables are reported to be associated with reduced risks of cardiovascular disease (2) and cancer (3). These observations may be attributed, in part, to the antioxidant effects of flavonoids (4-7).

Flavonoids are diphenylpropanes consisting of 2 phenolic rings, A and B connected by a 3 carbon unit, which along with an oxygen atom, forms the heterocyclic C ring. Flavonoids are systematically classified into subgroups including flavones, isoflavones, flavonols, and flavanones, which are characterized by differences in their C ring structure (8). Differences within these flavonoid subgroups are characterized by substitutions of hydroxyl, methoxyl, methyl and glycosidic groups on the A, B and C rings (Figure 1). Flavonoids are found in foods mainly as *O*-glycosides. Glucose is the most common sugar moiety but other glycosidic units can include galactose, rhamnose, arabinose and xylose. The *O*-β-glucosidic bonds of flavonoids including the isoflavones daidzin (daidzein-7-*O*-β-D-glucopyranoside), genistin and glycitin are hydrolyzed in the gut by microbial and mammalian β-glucosidases to their aglucons, daidzein, genistein, and glycitein, respectively (9-13).

The absorption and metabolism of flavonoid aglucons in humans is not fully understood, but the aglucons may be absorbed, undergo first pass hepatic metabolism (14) and be excreted in the urine or bile (15). Intestinal bacteria can further catabolize the flavonoid aglucons into smaller phenolic compounds that can be reabsorbed by enterohepatic recirculation via the bile duct, or catabolized completely for energy (13, 15-19).

The chemical structure of flavonoids determines the extent and rate of absorption in the gut as reflected in the amounts of flavonoids found intact in the urine and plasma (20-23). Previous studies by Xu et al, (20) have shown that the rate of isoflavone degradation by human gut microflora depends on the structure of the isoflavone. Genistein, which possesses a 5-hydroxyl, was rapidly degraded compared to daidzein which does not have a 5-hydroxyl group. Lin et al, (24) has reported that flavonoids with methoxyl groups, such as diosmetin (5,7,3'-trihydroxy-4'-methoxyflavone), hesperetin (5,7,3'-trihydroxy-4-methoxyflavanone)

and wogonin (5,7-dihydroxy-8-methoxyflavone), were less rapidly degraded compared to flavonoids without methoxyl groups.

Microbial degradation of flavonoids with C-C-linked glucose groups, such as puerarin (daidzein-8-*C*-β-D-glucopyranoside), has not been well studied. Puerarin is structurally similar to daidzin but with the glucose group bound directly to the C-8 position of the A ring. Puerarin is the major isoflavone found in kudzu (*Pueraria lobata*), a plant used in traditional Chinese medicine and as a nutraceutical (25). Puerarin can be found in commercial isoflavone dietary supplements where kudzu was used as the sole source or in combination with soy isoflavones.

The purpose of this study was to determine the structural characteristics of flavonoids that are important for optimal degradation by the human gut microflora. This study compared the relationship of the chemical structure of 14 flavonoids: flavone, apigenin, chrysin, naringenin, kaempferol, genistein, daidzein, daidzin, puerarin, 7,4'-dihydroxyflavone, 6,4'-dihydroxyflavone, 5,4'-dihydroxyflavone, 5,3'-dihydroxyflavone and 4'-hydroxyflavone, with their degradation rate by gut microflora from human subjects.

Materials and Methods

Chemicals

Genistein was synthesized according to modification of Chang et al, (26). Daidzein and 2,4,4'-trihydroxydeoxybenzoin (THB) were synthesized using the method of Song et al, (27). Flavone, apigenin, chrysin, naringenin, kaempferol, puerarin, 7,4'-dihydroxyflavone, 6,4'-dihydroxyflavone, 5,4'-dihydroxyflavone, 5,3'-dihydroxyflavone and 4'-hydroxyflavone were from Indofine Chemical Co., Inc (Hillsborough, NJ). Daidzin was purchased from LC Labs (Woburn, MA). HPLC grade acetonitrile, methanol, acetic acid,

dimethyl sulfoxide (DMSO) and all other chemicals were from Fisher Scientific (Fairlawn, NJ). Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water was used to prepare all solutions.

Subject protocol

Approval of the study design was obtained from the Iowa State University Human Subjects Research Committee in 2003. Three men and eight women volunteered from Iowa State University and the surrounding Ames area. The selection criteria required that the subjects be in good health and not taking any medication. The subjects' ages ranged from 24 to 53 y (mean age = 33.8 ± 3.2 y) with a body mass index (BMI) of 20.9 to 25.8 kg/m^2 (mean BMI = 23.9 ± 0.9 kg/m^2) respectively. The ethnicities of the subjects included 5 Caucasians, 3 African Americans, 1 Chinese immigrant, 1 Asian-Indian and 1 Latino. All subjects provided one fresh fecal sample in sealed sterile containers (Sage Products Inc., Crystal Lake, IL) that was used immediately.

Flavone Fermentation

Brain-heart infusion (BHI) broth media (Difco Laboratories, Detroit, MI) was prepared according to Zheng et al, (21). All flavonoid aglucons were dissolved in 100% DMSO. One and a half grams of freshly voided feces were transferred to incubation test tubes (Fisher Scientific, Fairlawn, NJ) containing 25 mL BHI. The flavonoids flavone, 4'-hydroxyflavone, 5,4'-dihydroxyflavone, 6,4'-dihydroxyflavone, 7,4'-dihydroxyflavone, 5,3'-dihydroxyflavone, chrysin, apigenin, genistein, naringenin, kaempferol and daidzein were added to the incubation test tubes for a final concentration of 78.7 µmol/L. The fermentations were performed in duplicate. The incubation test tubes were flushed with CO_2, sealed with rubber stoppers and autoclave tape, then vortexed for 5 s. One mL was taken

anaerobically from each test tube immediately for time 0 and frozen on dry ice. The tubes were placed in a 37°C incubator. One mL aliquots were sampled from the incubation test tubes at 3, 6, 9, 12 and 24 h and frozen. Negative controls consisted of the fecal suspension without flavonoids. Microbial degradation by the fecal suspension was confirmed by positive controls, which consisted of BHI media and flavonoids without the fecal suspension.

Isoflavone Glucoside Fermentation

Stock solutions of 10 mmol/L puerarin and 5.4 mmol/L daidzin were prepared in 80% methanol. Two g of fresh feces from 2 subjects were transferred to incubation test tubes with 27 ml BHI. Puerarin and daidzin stock solutions were each added to give a final concentration of 4.8 µmol/L. The control incubation contained no isoflavones. Incubations were performed according to the method stated above for flavonoids.

Flavonoid Extraction

THB, as an internal standard, was added at 100 µmol/L to the thawed fermentation sample and slowly loaded onto pre-conditioned C_{18} solid phase extraction cartridges (Waters Corporation Milford, MA). The cartridge was washed twice with 2 mL of Milli-Q system water. The flavonoids were eluted with 1 mL 80% methanol, filtered through 0.45 µm filters and analyzed directly by HPLC.

HPLC analysis

The HPLC system consisted of a Hewlett Packard 1050 Series. Twenty µL of sample was injected onto a reversed-phase, 5 µm, C_{18} AM 303 column (250 x 4.6 mm) (YMC Co. Ltd. Wilmington, NC). The mobile phase consisted of 0.1% glacial acetic acid in water (A) and 0.1% glacial acetic acid in acetonitrile (B). Solvent B increased from 30 to 50% in 8 minutes, increased to 100% in 8 minutes and held for 3 minutes. The gradient was recycled

back to 30% in 1 min for the next run. The flow rate was 1 mL/min. The wavelengths used for the preparation of standard curves, detection and quantification of flavonoid peaks were 254 and 292 nm. The minimum detection limit of all flavonoids ranged from 5 – 7 nM. The gradient elution used to separate puerarin and daidzin was of the method of Song et al (27). Chem station^{3D} software (Hewlett Packard Company, Scientific Instruments Division, Palo Alto, CA) was used to integrate the peak area responses and to evaluate the ultraviolet absorbance spectra.

Data Analysis

The ratio of peak area of a flavonoid to THB (100 μmol/L) versus the flavonoid concentration was used as an internal standard curve to estimate the concentration of flavonoids in the fecal fermentations. The rate of disappearance of flavonoids in fecal fermentation mixtures was estimated by plotting ln (% remaining flavonoid) versus time. The negative slope of this line was the apparent first order degradation rate constant. Statistical evaluation of degradation rate differences was performed using the SAS system (version 8.1, SAS Institute., Cary, NC). Differences between the overall and individual degradation rates of flavonoids were estimated using 1-way ANOVA. Flavonoid degradation phenotypes were identified using cluster analysis (28). The statistical significance of all analyses was set at $\alpha = 0.05$.

Results and Discussion

The structure of four flavonoid subgroups, including the A, B and C rings and the substitution patterns of each flavonoid analyzed are shown in figure 1. Degradation rate differences due to different A ring substitution patterns were investigated by comparing the microbial degradation rates of 4'-hydroxyflavone, 5,4'-dihydroxyflavone, 6,4'-

dihydroxyflavone and 7,4'-dihydroxyflavone. There were no differences when the degradation rates of flavonoids with A ring variations were compared across all subjects with an average $k = 0.060 \pm 0.053\ h^{-1}$ ($p = 0.30$) (Figure 2).

Degradation rate differences due to different hydroxylation patterns on the B ring of flavonoids were analyzed by pairwise comparison of flavone and 4'-hydroxyflavone, 5,3'-dihydroxyflavone and 5,4'-dihydroxyflavone, and chrysin (5,7-dihydroxyflavone) and apigenin (5,7,4'-trihydroxyflavone) degradation rate constants. There were no differences between the degradation rates of flavone and 4'-hydroxyflavone with an average of $k = 0.065 \pm 0.061\ h^{-1}$ ($p = 0.83$), and between 5,4'-dihydroxyflavone and 5,3'-dihydroxyflavone with an average of $k = 0.071 \pm 0.067\ h^{-1}$ ($p = 0.42$). However, the degradation rate of apigenin with an average $k = 0.43 \pm 0.27\ h^{-1}$ was significantly faster than chrysin with an average $k = 0.13 \pm 0.11\ h^{-1}$ ($p = 0.01$) suggesting that the hydroxyl group at the 4' position was important for rapid microbial degradation but only if additional hydroxyl groups were present at the 5, and 7 positions (Figure 2).

The degradation rate differences due to C ring substitution were compared by analyzing the degradation rates of apigenin (5,7,4'-trihydroxyflavone), genistein (5,7,4'-trihydroxyisoflavone), naringenin (5,7,4'-trihydroxyflavanone) and kaempferol (3,5,7,4'-tetrahydroxyflavone). There were no differences between the degradation rates of apigenin, genistein, naringenin and kaempferol with an average $k = 0.38 \pm 0.25\ h^{-1}$ ($p > 0.05$) (Figure 2). These data suggested that the absence of the 2-3 double bond, as found in naringenin, or the addition of a 3-OH group, as in kaempferol, did not affect the rate of microbial degradation.

Apigenin (5,7,4'-trihydroxyflavone) and 7,4'-dihydroxyflavone are the flavone analogues to genistein (5,7,4'-trihydroxyisoflavone) and daidzein (7,4'-dihydroxyisoflavone), respectively. The average degradation rate of genistein was not different from apigenin with an average $k = 0.45 \pm 0.29\ h^{-1}$ ($p = 0.13$) and the average degradation rate of daidzein was not different from 7,4'-dihydroxyflavone with an average $k = 0.076 \pm 0.063\ h^{-1}$ ($p = 0.72$) (Figure 2) suggesting that the attachment of the B ring at the C-3 position for isoflavones, instead of at the C-2 position for flavones, does not affect the rate of bacterial degradation.

Genistein, apigenin, kaempferol and naringenin were the most rapidly degraded flavonoids compared to all other flavonoids examined ($p < 0.0001$) (Figure 2). These flavonoids all have a common structure with hydroxyl groups at the 5, 7 and 4' positions. This observation suggested that these 3 hydroxyls were important for optimal flavonoid degradation. It is evident from the results reported here that any flavonoid missing any one of the 5-, 7-, or 4'- hydroxyls degraded slower than genistein, apigenin, naringenin and kaempferol.

Lin et al, 2003, was the only other investigation of the relationship between chemical structures and microbial degradation of flavonoids (24). Thirteen flavonoids were analyzed including genistein, apigenin, naringenin, kaempferol and daidzein. The other flavonoids included morin (3,5,7,2',4'-pentahydroxyflavone), luteolin (5,7,3',4'-tetrahydroxyflavone), quercetin (3,5,7,3',4'-pentahydroxyflavone), wogonin (5,7-dihydroxy-8-methoxyflavone), baicalein (5,6,7-trihydroxyflavone), hesperetin (5,7,3'-trihydroxy-4-methoxyflavanone), diosmetin (5,7,3'-trihydroxy-4'-methoxyflavone) and neophellamuretin (3,5,7,4'-tetrahydroxy-8-isoprenylflavanone). Fecal incubations from rabbits, rats and 3 humans were

used. Lin et al, 2003 observed that wogonin and diosmetin, which possess methoxyl groups, were the least degraded flavonoids in all 3 species, and concluded that the presence of methoxyl groups on the A or B ring rendered the flavonoid resistant to microbial degradation (24). Our preliminary experiments have shown similar results in which glycitein (7,4'-dihydroxy-6-methoxyisoflavone), which possesses a 6-methoxyl group, was degraded at a slower rate than genistein, $k = 0.30 \pm 0.21\ h^{-1}$ versus $0.43 \pm 0.44\ h^{-1}$ ($p < 0.18$) in fecal fermentations from 12 human subjects. Additional evaluation of the Lin et al data, however, revealed that all of the flavonoids with 5, 7 and 4' hydroxyl groups degraded faster than the flavonoids that were lacking all these hydroxyls in all three species, which is in agreement to our study. The only exception to this generalization was observed with baicalein (5,6,7-trihydroxyflavone), which had no hydroxyl groups at the 4' position. Baicalein was found to be extensively degraded by human fecal microorganisms, but not rabbit or rat fecal microorganisms. Similarly, we found that chrysin (5,7-dihydroxyflavone), which has no 4'-hydroxyl group degraded significantly faster than flavonoids lacking all three 5, 7 and 4' hydroxyl groups (Figure 2). We speculate that flavonoids with 5 and 7 hydroxyls are moderately degraded by human gut microorganisms, but addition of the 4' hydroxyl significantly enhances the microbial degradation rate.

Naringenin and kaempferol are structurally similar to apigenin except that naringenin lacks the 2-3 double bond in the C ring and kaempferol has an additional hydroxyl group attached to the C-3 position of the C ring. The lack of significant differences between the average degradation rates of apigenin, naringenin and kaempferol suggests that the 2-3 double bond and a 3-OH group on the flavonoid structure were not necessary for microbial degradation as long as the flavonoid possessed hydroxyl groups at the 5, 7 and 4' positions.

Our results support the work of Lin et al, (24) who reported that these features did not affect the degradation rate.

Puerarin (daidzein-8-C-β-D-glucopyranoside) and daidzin (daidzein-7-*O*-β-D-glucopyranoside) are the two predominant isoflavones found in kudzu root. Daidzin was rapidly hydrolyzed to daidzein with a $k = 1.15 \pm 0.01\ h^{-1}$ and disappeared from the fecal fermentation mixture within 4 h of incubation with fecal microorganisms of 2 subjects. Daidzein was detected at 2 h after incubation with daidzin (Figure 3A). In contrast, puerarin was not hydrolyzed after 48 h with no daidzein being detected in the puerarin incubation (Figure 3B). These results suggest that *C*-glucosides are resistant to β-glucosidase activity compared to *O*-glucosides. It is possible that the position and type of glycosidic linkages of flavonoid glycosides alter their rate of hydrolysis by the gut microflora.

Kim et al, (29) reported that puerarin was converted to daidzein after incubation with fresh feces from a single subject after 48 h, which is in contrast to our observations. No concentration of daidzein formed from puerarin in the Kim et al study was reported, however. Yasuda et al., (30) reported that the urine of rats orally dosed with 100 mg/kg of pure puerarin, contained unchanged puerarin, daidzein and the glucuronide and sulfate conjugates of daidzein and puerarin. The total amount of urine metabolites excreted in 48 h was 3.63% of the puerarin administered, with daidzein comprising less than 0.5%. These data suggest that puerarin might be hydrolyzed to daidzein, but not in significant amounts based on the percentage recovered in urine. Less than 1% of unchanged puerarin was recovered in the urine and bile suggesting that puerarin is minimally absorbed intact in the gut (30, 31). The Yasuda et al, (30) data conflicts other studies reporting that flavonoid glucosides must be hydrolyzed before absorption in the gut. (32-36).

There was considerable variability among the subjects in their degradation rates for each flavonoid ($p<0.0001$). All subjects' degradation rates for each flavonoid were analyzed using cluster analysis. Cluster analysis is a statistical program that is able to group together similar degradation rates. The subjects segregated into 3 different degradation rate groupings for each flavonoid. We described these groups as phenotypic differences in the subjects and called them high, moderate and low flavonoid degraders (Table 1). Most of the subjects remained in their respective flavonoid degradation phenotype groups for all flavonoids examined suggesting that each phenotype may exist as a stable characteristic in these subjects (Table 1). These phenotypes may represent differences in gut microbial populations or enzyme activities. High flavonoid degraders would be more likely to produce flavonoid metabolites as a result of anaerobic metabolism in the gut, and thus absorb less intact flavonoids across the intestinal epithelium. On the other hand, low flavonoid degraders may produce fewer flavonoid metabolites but have greater probability to absorb intact flavonoids. Additionally, low flavonoid degraders may experience greater "in situ" activity from flavonoid aglucons compared to high flavonoid degraders.

We have not determined why the human gut microorganisms prefer to degrade 5,7,4'-trihydroxylflavonoids. Synthesis of flavonoids in plants comes from resorcinol or phloroglucinol synthesis from the acetate pathway. This pathway produces the 5,7 hydroxylation pattern in the A ring. The shikimate pathway produces the B ring and results in 4'-, 3',4'-, and 3',4',5'- hydroxylation patterns (37). Since genistein, apigenin, naringenin and kaempferol are the flavonoid aglucons found predominantly in the food supply, we believe that flavonoids with 5,7,4'-hydroxylation patterns dominate in nature, and the human

gut microflora are more exposed to these dietary compounds and have adapted to metabolize them.

We observed that hydroxyl groups at the 5, 7 and 4' positions of flavonoids are important structural characteristics for optimal flavonoid degradation by human gut microflora. The flavonoid degradation rate ranking is: genistein = apigenin = kaempferol = naringenin > chrysin > daidzein = 5,3'-dihydroxyflavone = 5,4'-dihydroxyflavone = 6,4'-dihydroxyflavone = 7,4'-dihydroxyflavone = 4'-hydroxyflavone = flavone. These observations have great implications for evaluating the potential bioavailability of flavonoids. A prediction of the rate of flavonoid degradation by human gut microorganisms can be made by evaluating the structure of a flavonoid. Genistein, apigenin, naringenin and kaempferol were degraded more rapidly than the other flavonoids without hydroxyl groups at the 5, 7 and 4' positions. Therefore, genistein, apigenin, naringenin and kaempferol may not be as bioavailable in the colon compared to more slowly degraded flavonoids, because they have less time to be absorbed before they are degraded by the gut microflora. However, the degradation products of genistein, apigenin, naringenin and kaempferol may potentially be bioactive metabolites of interest. The other slowly degraded flavonoids examined may be more bioavailable because the gut microflora degraded them at a slower rate, which gives these flavonoids a greater opportunity to be absorbed.

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Table 1. Cluster Analysis of Subjects' Degradation Rates and Segregation into Degradation Phenotypes Groupings[a]

	Degradation rate k (1/h)[b]			Subject ID[c]		
Flavonoid	High	Moderate	Low	High	Moderate	Low
flavone	0.18±0.02	0.06±0.02	0.02±0.01	13,26	5,8,9,18	2,3,4,6,17
4'-hydroxyflavone	0.16±0.03	0.06±0.02	0.02±0.01	9,13,26	5,18	2,3,4,6,8,17
5,3'-dihydroxyflavone	0.16±0.03	0.06±0.02	0.01±0.00	13,17,26	4,5,8,9,18	2,3,6
5,4'-dihydroxyflavone	0.21±0.04	0.10±0.03	0.02±0.02	17,26	5,13	2,3,4,6,8,9,18
6,4'-dihydroxyflavone	0.11±0.02	0.04±0.01	0.02±0.00	13,17,26	2,5 18	3,4,6,8,9
7,4'-dihydroxyflavone	0.18±0.05	0.08±0.03	0.02±0.01	17,26	4,13	2,3,5,6,8,9,18
chrysin	0.28±0.06	0.15±0.03	0.03±0.02	9,13,26	4,5,8	2,3,6,17,18
daidzein	0.17±0.02	0.12±0.01	0.04±0.01	9,13,26	2,4,17	3,5,6,8,18
genistein	1.54±0.00	0.77±0.00	0.18±0.08	13	2,3,26	4,5,6,8,9,17,18
apigenin	0.77±0.00	0.53±0.03	0.17±0.07	9,13,26	4,5,8	2,3,6,17,18
naringenin	0.77±0.00	0.19±0.01	0.08±0.04	13,26	4,5,6,8,9,17,18	2,3
kaempferol	0.70±0.10	0.47±0.07	0.15±0.07	13,26	2,8,9	3,4,5,6,17,18

[a]Degradation phenotypes of subjects. Subjects separated into 3 significantly different groups for each flavonoid, named high, moderate and low degraders ($p<0.0001$). [b]Degradation rates expressed as average ± standard error of the mean. [c]Whole numbers shown are subjects' identification number.

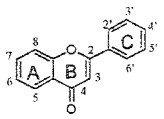
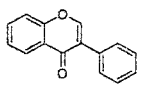
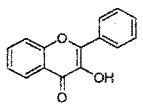
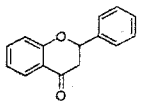
Structure	Flavonoid Subgroup	Flavonoid	R_2	R_3	R_5	R_6	R_7	R_8	$R_{3'}$	$R_{4'}$
	Flavone	Flavone		H	H	H	H	H	H	H
		4'-hydroxyflavone		H	H	H	H	H	H	OH
		5,4'-dihydroxyflavone		H	OH	H	H	H	H	OH
		6,4'-dihydroxyflavone		H	H	OH	H	H	H	OH
		7,4'-dihydroxyflavone		H	H	H	OH	H	H	OH
		5,3'-dihydroxyflavone		H	OH	H	H	H	OH	H
		Apigenin		H	OH	H	OH	H	H	OH
		Chrysin		H	OH	H	OH	H	H	H
	Isoflavone	Daidzein	H		H	H	OH	H	H	OH
		Daidzin	H		H	H	Ogl	H	H	OH
		Puerarin	H		H	H	OH	Cgl	H	OH
		Genistein	H		OH	H	OH	H	H	OH
	Flavonol	Kaempferol		OH	OH	H	OH	H	H	OH
	Flavanone	Naringenin		H	OH	H	OH	H	H	OH

Figure 1. Flavonoid subgroup structures and substitution patterns. Ogl: 7-*O*-β-D-glucopyranose, Cgl: 8-*C*-β-D-glucopyranose. A: A ring, B: B ring, C: C ring

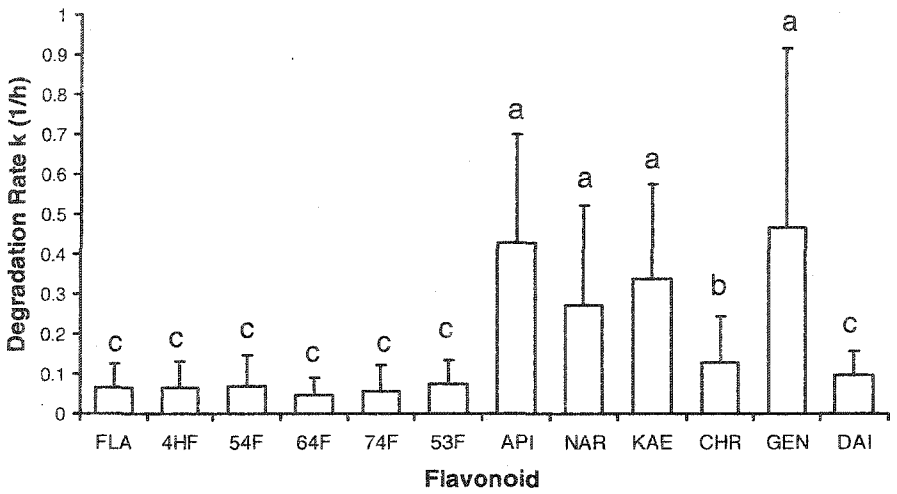


Figure 2. *In vitro* human microbial degradation rates of flavonoids. FLA: flavone, 4HF: 4'-hydroxyflavone, 54F: 5,4'-dihydroxyflavone, 64F: 6,4'-dihydroxyflavone, 74F: 7,4'-dihydroxyflavone, GEN: genistein, API: apigenin, NAR: naringenin, KAE: kaempferol, 53F: 5,3'-dihydroxyflavone, CHR: chrysin, DAI: daidzein. Bars with different letters are significantly different ($p<0.05$, $n = 11$). Error bars represent mean square error.

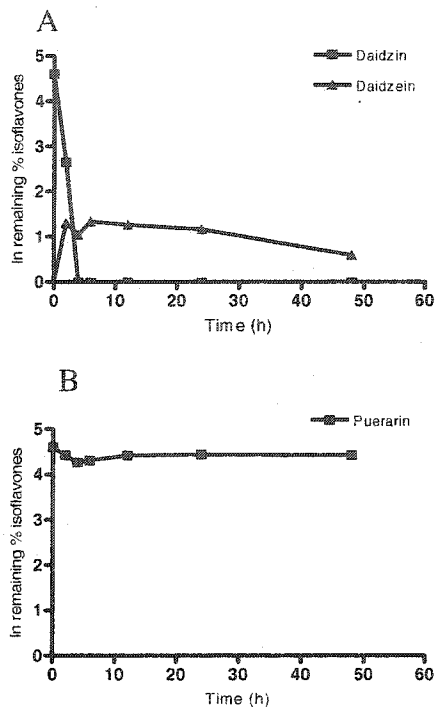


Figure 3. Human microbial degradation of isoflavone glucosides versus time in 2 subjects. A: Disappearance of daidzin and appearance of daidzein. B: Stability of puerarin over time. Mean square error at each time point was < 0.07.

BIOAVAILABILITY OF FLAVONOIDS AND THEIR CORRELATION WITH HUMAN GUT MICROBIAL DEGRADATION

A paper to be submitted to the Journal of Nutrition

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Abstract

The relationship between flavonoid structure, gut microbial degradation *in vitro* and human bioavailability *in vivo* was determined. Gut microbial fermentations with fresh feces from 33 healthy subjects (20 male, 13 female) were conducted with 15 flavonoids, genistein, apigenin, naringenin, kaempferol, luteolin, quercetin, myricetin, hesperetin, chrysin, flavone, daidzein, glycitein, 5,4'-dihydroxyflavone, 6,4'-dihydroxyflavone, 7,4'-dihydroxyflavone, 5,3'-dihydroxyflavone, over a 24 h period. Flavonoids with 5, 7 and 4' – hydroxyl groups genistein, apigenin, naringenin, luteolin, kaempferol and quercetin, rapidly disappeared from the fermentation mixtures compared to the other flavonoids ($k = 0.46 \pm 0.10\ h^{-1}$ vs. $0.07 \pm 0.02\ h^{-1}$, $p < 0.0001$). The methoxylated flavonoids hesperetin and glycitein were rapidly demethylated and disappeared from the fermentation mixtures with an average $k = 0.24 \pm 0.21\ h^{-1}$. Human bioavailability of flavonoids was investigated in 10 volunteers (5 men and 5 women) in 3 feedings separated by a one week washout period. Subjects ingested 28 mg (104 μmol) genistein and 16 mg (62 μmol) daidzein from soymilk, 422 mg (1549 μmol) naringenin and 8 mg (26 μmol) hesperetin from grapefruit juice and 115 mg (381 μmol) quercetin from sautéed onions. Blood and urine samples were collected over a 24 h period and all feces were collected until the excretion of a carmine red dye, which was ingested along with the test meal. Peak plasma concentrations of flavonoids analyzed over the 24 hour

period ranged from 0.01 – 1 μM. The bioavailability, calculated as urinary excretion as the percentage of ingested dose, was significantly lower for the rapidly degraded flavonoids, naringenin (3.2 ± 1.7 %), genistein (7.2 ± 4.6 %), hesperetin (7.3 ± 3.2 %) and quercetin (5.6 ± 3.7 %) compared to a slowly degraded flavonoid, such as daidzein (43.4 ± 15.5 %, p = 0.02). These data reveal that the chemical structure of flavonoids affect their gut microbial degradation rate and thus their overall bioavailability.

Introduction

Flavonoids are polyphenolic compounds that are widely distributed in foods of plant origin (1). Dietary intake levels in humans range from a few hundred mg to 1 g (2). Flavonoids in fruits and vegetables have been suggested to be responsible for lowering the risk of steroid dependent cancers (3) and heart disease (4) in populations consuming high amounts of flavonoids. Over 5000 flavonoid compounds have been identified to date and are divided into subclasses, which differ structurally in their heterocyclic C ring (5). These flavonoid subclasses include flavones, flavanones, flavonols and isoflavones. Commonly consumed flavonoids in these flavonoid subclasses are illustrated in figure 1. Substitution patterns on the A and B rings with hydroxyl, methyl, methoxyl, O and C-sugars, acyl, prenyl, sulphate and glucuronide groups provide additional structural variation in each flavonoid subclass (5).

Flavonoid aglucons have been shown to be absorbed across the intestinal wall and conjugated in the liver with phase II enzymes such as UDP-glucuronosyltransferase, sulfotransferase and catechol-O-methyltransferase (6). The flavonoids may be excreted in the urine or bile. Colonic bacteria hydrolyze the flavonoid conjugates after biliary excretion, which results in reabsorption of the flavonoid aglucons and enterohepatic recirculation (6,7).

The colonic bacteria are also able to further degrade the aglucons into smaller phenolic compounds (8).

Determination of the metabolism and bioavailability of flavonoids is crucial in the assessment of flavonoid health effects. Additionally, investigation of which flavonoids are best absorbed intact, is important. The microbial population of the human gut plays an important role in the metabolism and bioavailability of isoflavones (9) and our lab has extensively investigated this phenomenon. We have developed *in vitro* human fecal fermentation systems that allow us to simulate the gut environment to determine the microbial degradation rate (9), identify microbial isoflavone metabolites and predict human bioavailability (9-12). Zheng et al (10,11) has shown that slow *in vitro* daidzein and genistein microbial degradation rates corresponded to greater daidzein and genistein bioavailability in human subjects, measured by the average amount of isoflavones recovered in urine as a percentage of ingested dose. Xu et al, (9) has shown that the rate of isoflavone microbial degradation *in vitro*, depended on the chemical structure of isoflavones. Genistein, which possesses a hydroxyl group, was degraded significantly faster than daidzein, which does not possess this structural feature (9). This suggests that isoflavone chemical structure may be a determinant of human bioavailability.

Recently we have shown that the chemical structure and substitution pattern of other flavonoids, flavones, flavanones and flavonols, influenced their degradation rate (Simons et al, in press). In the present study, we investigate how the chemical structure of flavonoids determine their overall bioavailability *in vivo*.

Materials and Methods

Subjects

Thirty three subjects (20 male and 13 female) were recruited from Iowa State University for the *in vitro* flavonoid degradation study. The subjects' ages ranged from 18 to 37 y (mean age = 25.6 ± 4.4 y) with a body mass index (BMI) of 18.1 to 46.1 kg/m² (mean BMI = 23.7 ± 4.9 kg/m²) respectively. The ethnicities of the subjects included 15 Caucasians, 7 Asian-Indians, 7 Chinese, 3 African Americans and 1 African.

Ten (5 male and 5 female) of the 33 subjects were selected from Iowa State University to participate in the human bioavailability studies based on a moderate daidzein degradation phenotype. Characteristics of these subjects were age of 18 – 30 y (mean age = 25.0 ± 4.0) and BMI of 18.1 – 29.1 kg/m² (mean BMI = 22.6 ± 3.3). The ethnicities included 3 Caucasian, 3 Chinese, 2 Asian-Indians and 2 African Americans (Table 1). All subjects were healthy and not taking any medication. Approval of the study design was obtained from the Iowa State University Human Subjects Research Committee in 2004. The subjects followed an isoflavone-, flavonol- and flavonone-free diet for 1 week before their respective feedings. All subjects were given oral and written instructions on foods and beverages not to consume during each washout period.

Reagents and Chemicals

Apigenin, naringenin, kaempferol, luteolin, quercetin, flavone, chrysin, 7,4'-dihydroxyflavone, 6,4'-dihydroxyflavone, 5,4'-dihydroxyflavone, 5,3'-dihydroxyflavone were from Indofine Chemical Co., Inc (Hillsborough, NJ). Daidzein and 2,4,4'-trihydroxydeoxybenzoin (THB) were synthesized using the method of Song et al, (12). Genistein was synthesized according to modification of Chang et al (13). HPLC grade acetonitrile, methanol, acetic acid, dimethyl sulfoxide (DMSO) and all other chemicals were

from Fisher Scientific (Fairlawn, NJ). Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water was used to prepare all solutions.

Flavonoid Degradation Study Design

A stock solution of flavonoids was prepared by dissolving the flavonoids together in 100% DMSO. Brain-heart infusion (BHI) broth media (Difco Laboratories, Detroit, MI) was prepared according to Zheng et al, (14). One and a half grams of freshly voided feces and 25 mL BHI were transferred to incubation test tubes (Fisher Scientific, Fairlawn, NJ). The final concentration of all flavonoids was 100 μmol/L. The fermentations were performed in duplicate. The incubation test tubes were saturated with CO_2, sealed with rubber stoppers and autoclave tape, then vortexed for 5 s. One mL was taken anaerobically from each test tube immediately for time 0 and frozen on dry ice. The tubes were placed in a 37°C incubator. One mL aliquots were sampled from the incubation test tubes at 3, 6, 9, 12 and 24 h and frozen. Negative controls consisted of the fecal suspension without flavonoids. Microbial degradation by the fecal suspension was confirmed by positive controls, which consisted of BHI media and flavonoids without the fecal suspension.

Flavonoid Bioavailability Study Design

The genistein, naringenin and quercetin bioavailability studies were performed on three separate days with a 2 week washout period in between each feeding. Genistein, naringenin and quercetin were fed in the form of soymilk, grapefruit juice and sautéed red onions, respectively, purchased from local grocery stores. The ingested amount of soymilk was 2 cups which provided 28 mg genistein, 16 mg daidzein and 3 mg glycitein. The ingested amount of grapefruit juice was 2 cups, which provided 422 mg naringenin and 8 mg hesperetin. One hundred and eighty five grams of sautéed onions were fed in the form of a 3

egg onion omelete, which provided 115 mg quercetin. The subjects consumed each flavonoid source along with a capsule containing 170 mg of non-toxic carmine red dye within 10 minutes for breakfast on the morning of the study. Other breakfast items were provided that did not contain the flavonoid of interest such as toast, bagels, cereal, milk, eggs and cheese. Baseline, urine, blood and fecal samples were collected before each flavonoid feeding. All urine was collected for 24 h into plastic containers in 3 fractions, 0 – 6, 6 – 12 and 12 – 24 h. Blood samples (10 mL) were collected into vacuum tubes containing EDTA at 2, 4, 6, 12 and 24 h after consumption of the test meal. Blood samples were collected 1, 3, 5, 12 and 24 h after the quercetin feeding. All feces were collected until the carmine red dye was excreted. Urine and fecal samples were frozen at -20°C until analysis. Blood samples were centrifuged at 3000 g for 25 min at 4°C within 1 h of collection. The plasma was separated and stored at -80°C until analysis. The subjects remained at the study site until the collection of the 6 hr blood sample, after which they were allowed to leave and come back for collection of the 12 and 24 h blood samples.

Analytical Methods

Flavonoid analysis in foods

Isoflavones in soymilk were extracted according to the method of Murphy et al (15). One gram of freeze dried and ground grapefruit juice was extracted in 12 mL of 1M HCl in a 125 mL screw top Erlenmeyer flask for 2 h at 98°C with stirring. The solution was cooled, and 18 mL acetonitrile added to the solution and stirred for 5 minutes. One mL of this solution was filtered with a 0.45 μm PTFE filter (Alltech Associates, Deerfield, IL) into an HPLC vial. Red onions were chopped and slightly sautéed in vegetable oil, before being

cooked in a 3-egg omelete. Portions of the omelete were freeze dried and extracted according to the method described above for grapefruit juice.

Urine analysis

Urine samples were pooled in 0-6, 6-12 and 12-24 h increments for each subject. Five mL samples were incubated with 5 mL 0.2M sodium acetate buffer at pH = 5.5 and 50 μL β-glucuronidase/sulfatase (H_2 type) for 18 h in a 37°C shaking water bath. Ten mL of 10 mM sodium phosphate buffer at pH = 7.0, was added to the incubation solution and mixed well. The solution was applied to a 20 mL Extralut QE column (EM Sciences) and the column was washed twice with 18 mL ethyl acetate. The ethyl acetate collections were collected, pooled and dried on a rotary evaporator at 25°C. The residue was dissolved in 9.8 mL 20% ethanol in water and 200 uL 1N HCl. Five mL of this mixture was loaded onto a preactivated SepPak C_{18} cartridge and washed twice with 2 mL MQ water. The flavonoids were eluted with 2 mL 80% methanol in water and filtered through a 0.45 μm PTFE filter into an HPLC vial.

Plasma analysis

One mL of plasma was incubated with 1 mL 0.2 M sodium acetate at pH 5.5 and 50 μL β-glucuronidase/sulfatase H2 type for 20 h at 37°C on a shaking water bath. One mL of a 10 mM sodium phosphate buffer at pH 7.0 was added to the incubation solution and mixed. The solution was added to a 5 mL Extralut SE column and the flavonoids eluted twice with ethyl acetate twice into a 20 mL test tube. The solvent was evaporated under N_2, the dried residue dissolved in 200 μL 80% methanol then filtered with a 0.45 μm PTFE filter into an HPLC vial.

HPLC analysis

A Beckman-Coulter HPLC system was used for HPLC analysis consisting of a System Gold 126 solvent module, System Gold 508 autosampler and a System Gold 168 photo-diode array detector. Forty µL of sample was injected onto a reversed-phase, 5 µm, C_{18} AM 303 column (250 x 4.6 mm) (YMC Co. Ltd. Wilmington, NC). The mobile phase consisted of 0.1% glacial acetic acid in water (A) and 0.1% glacial acetic acid in acetonitrile (B). Solvent B increased from 30 to 50% in 10 minutes, increased to 100% in 7 minutes and held for 1 minute. The gradient was recycled back to 30% in 2 min for the next run. The flow rate was 1 mL/min. The wavelengths used for the preparation of standard curves, detection and quantification of flavonoid peaks were 254 nm for isoflavones, flavonols and flavones, and 292 nm for flavanones. Integration of peak area responses and evaluation of ultraviolet spectra was carried out using 32 Karat™ software (Beckman Coulter Inc., Fullerton, CA).

Data Analysis

The ratio of peak area of a flavonoid to THB (0.1 µmol/L) versus the flavonoid concentration was used as an internal standard curve to estimate the concentration of flavonoids in the *in vitro* fecal fermentations. The rate of disappearance of flavonoids in fecal fermentation mixtures was estimated by plotting ln (% remaining flavonoid) versus time. The negative slope of this line was the apparent first order degradation rate constant. Flavonoid degradation phenotypes were identified using cluster analysis (16).

Gut transit time (GTT) was determined by the time it took for the red dye to appear in the feces after ingestion of the carmine red dye capsules. GTT estimates were averaged over the 3 flavonoid feedings. The amount of flavonoids in urine and plasma, were calculated directly from external standard curves constructed in these matrices, extracted in the same

manner as the experimental samples. The flavonoid standard curves were obtained by plotting flavonoid peak area versus flavonoid concentration. Recovery ranged from 60 – 90 % for flavonoids in plasma and urine. Pharmacokinetic parameters were determined using a one compartment linear model. Bioavailability was calculated as average total urinary flavonoid excretion in 24 h, as a percentage of ingested dose. $AUC_{0\rightarrow 24h}$ was calculated using the linear trapezoidal rule (17). The peak plasma concentration (C_{max}) and the time to reach it (T_{max}) were taken directly from the data. The elimination half-life ($T_{1/2}$) was calculated from the equation $T_{1/2} = \ln 2/k$.

Statistical evaluation of all experimental results was performed using the SAS system (version 8.1, SAS Institute., Cary, NC). Differences in flavonoid bioavailability, normalized AUCs, overall and individual degradation rates of flavonoids and daidzein degradation phenotypes were estimated using 1-way ANOVA using general linear models. All analyses were performed in duplicate and all data are reported as mean ± SDM. The statistical significance of all analyses was set at $\alpha = 0.05$.

Results

In vitro flavonoid degradation

Degradation rate differences of flavonoids were determined for 16 flavonoids, genistein, apigenin, naringenin, kaempferol, luteolin, quercetin, myricetin, hesperetin, chrysin, flavone, daidzein, glycitein, 5,4'-dihydroxyflavone, 6,4'-dihydroxyflavone, 7,4'-dihydroxyflavone, 5,3'-dihydroxyflavone. Flavonoids with 5, 7 and 4'- hydroxyl groups, genistein, naringenin, apigenin, kaempferol, quercetin and luteolin, rapidly disappeared from the fermentation mixtures, with an average $k = 0.46 \pm 0.10\ h^{-1}$ ($p<0.0001$) (figure 2). However, myricetin (3,5,7,3',4',5'-hexahydroxyflavone), which possesses hydroxyl groups

in the 5, 7 and 4'- position, did not rapidly disappear from the fermentation mixtures with an average k = 0.04 ± 0.03 h^{-1}. The methoxylated flavonoids, hesperetin and glycitein, rapidly disappeared with an average k = 0.24 ± 0.21 h^{-1}. All other flavonoids that were lacking any of the 5, 7 and 4'- hydroxyl groups were slowly degraded with an average k = 0.07 ± 0.02 h^{-1} (figure 2).

Isoflavone bioavailability

Each subject ingested 2 cups of soymilk, which contained 103.6 μmol genistein and 61.8 μmol daidzein (Table 2). There was great interindividual variation for each pharmacokinetic parameter measured (area under curve (AUC), C_{max}, T_{max}, $T_{1/2}$, amount excreted). Plasma concentrations peaked about 5 h after soymilk dosing for both genistein and daidzein with half lives of 2.1 and 1.1 h respectively. Peak concentrations were 0.7 μmol/L for genistein and 1.0 μmol/L for daidzein. Average bioavailability of genistein and dadizein, defined as amount of flavonoid excreted in urine as a percentage of ingested dose, was 7.2 and 42.6 % respectively (Table 2).

In vitro degradation rates of the 10 subjects clustered into 3 significantly different groups ($p<0.0001$), called high genistein degraders (average k = 1.28 ± 0.45 h^{-1}, n=3), moderate genistein degraders (average k = 0.35 ± 0.01 h^{-1}, n=3) and low genistein degraders (k = 0.11 ± 0.07 h^{-1}, n=4, figure 3A). Urinary genistein excretion in low genistein degraders was 11.5 ± 8.0 % and was significantly higher than urinary genistein excretion in moderate (4.9 ± 2.7 %, $p = 0.027$) and high genistein degraders (3.6 ± 1.9 %, $p = 0.007$, figure 3B). There was no difference between AUC values for high, moderate and low genistein degraders ($p > 0.05$, data not shown).

Flavanone bioavailability

Nine subjects ingested 2 cups of grapefruit juice, containing 1549.9 μmol naringenin and 25.5 μmol hesperetin (table 2). One female subject dropped out of the study. Naringenin and hesperetin plasma concentrations peaked at 5.1 and 12 h, respectively, with peak plasma concentrations of 0.3 and 0.05 μmol/L, respectively. Half lives were 5.7 h for naringenin and 3.6 h for hesperetin. The average bioavailability of naringenin was 3.2 %. The average bioavailability of hesperetin across the nine subjects was 4.1 %, but hesperetin was not recovered in the plasma or urine of the 4 females ingesting the grapefruit juice. Therefore, the bioavailability of hesperetin in males was 7.3 % (table 2).

In vitro naringenin degradation rates clustered into 3 significant groups which we called high (average $k = 0.63 \pm 0.20\ h^{-1}$, n=2), moderate (average $k = 0.20 \pm 0.01\ h^{-1}$, n=3) and low naringenin (average $k = 0.05 \pm 0.03\ h^{-1}$, n=4) degraders and were similar to the phenotypic groups for genistein (data not shown). There was no difference between urinary naringenin excretion or AUC values in these 3 phenotypic groups ($p > 0.05$).

Flavonol Bioavailability

Each subject ingested a 3 egg omelete with 185 g sautéed red onions, containing 380.5 μmol quercetin (table 2). Plasma quercetin concentrations peaked at 1.5 h with a half life of around 9 h. Average peak plasma concentrations was 0.8 μmol/L with an AUC value of 6.1 μmol*h/L. Bioavailablity of quercetin was 5.6 % (table 2).

Overall flavonoid-bioavailability comparison

When all flavonoids were compared on a per μmol basis, AUC values were not significantly different for genistein and daidzein ($p = 0.49$, figure 4A). The average AUC for genistein and daidzein was significantly higher than naringenin, quercetin and hesperetin ($p = 0.001$). Naringenin had the smallest AUC value, which was significantly lower than all of the

other flavonoids tested ($p<0.0001$, figure 4A). Overall bioavailability was not significantly different between quercetin, naringenin, hesperetin and genistein (5.8 ± 1.9 %, $p > 0.05$). The bioavailability of daidzein was significantly higher than the other flavonoids ($p < 0.0001$).

Discussion

Influence of flavonoid chemical structure on human gut microbial degradation *in vitro* was illustrated in figure 2. The flavonoids that were most rapidly degraded in the fecal fermentation systems from 33 subjects included the isoflavone, genistein, the flavones apigenin and luteolin, the flavonols quercetin and kaempferol and the flavanone naringenin. All of these flavonoids possessed hydroxyl groups on the 5, 7 and 4' positions of the flavonoid backbone structure. These results support previous data in our lab which have shown that 5,7,4'-trihydroxylflavonoids were most rapidly degraded by the fecal microflora from 11 human subjects compared to flavonoids without these structural features (Simons et al, submitted). However, myricetin, which also possesses hydroxyl groups in the 5, 7 and 4' positions was not degraded rapidly. A possible reason for this observation is because the rapidly degraded 5,7,4'-trihydroxylflavonoids in this study possess between 3 and 5 hydroxyl groups and are similar in their hydrophobicity, when analyzed by reversed phase HPLC (data not shown). Myricetin possesses a 6 hydroxyl group on the flavonoid backbone structure and was much more hydrophilic compared to the other 5,7,4'-trihydroxylflavonoids. The 6 hydroxyl groups on the myricetin structure may hinder the bacterial enzymatic reaction that is necessary to degrade these flavonoid compounds. It is possible that the more hydroxyl groups a flavonoid has, the more likely it is resist degradation by the human gut microflora.

Other flavonoids that were rapidly degraded were the methoxylated isoflavone and flavanone, glycitein and hesperetin, respectively. These flavonoids do not possess a 5,7,4'-

trihydroxylflavonoid structure and do not support our previous findings in that only 5,7,4'-trihydroxylflavonoids were rapidly degraded. An explanation for this is that these methoxylated flavonoids are rapidly demethylated *in vitro* before further microbial degradation and our calculated degradation rates are based on this demethylation reaction, instead of degradation of the demethylation product. We have tested this hypothesis *in vitro* and have shown that hesperetin was rapidly demethylated to eriodictyol, which possesses a 5,7,4'-trihydroxylflavonoid structure. Eriodictyol was then rapidly degraded, which supports our findings that 5,7,4'-trihydroxylflavonoids are rapidly degraded (data not shown). Supporting data has shown that hesperetin was demethylated in an *in vitro* pig caecum model to eriodictyol, then further degraded to 3-(3-hydroxyphenyl)-propionic acid and phloroglucinol (18).

Hesperetin was degraded significantly faster than glycitein, which may be as a result of a more rapid demethylation reaction from the 4' position in hesperetin compared to the 6 position in glycitein. Hur et al, 2000 has reported that the methoxylated isoflavones, formononetin, biochanin A and glycitein were demethylated regardless of the position of the methoxyl group but they did not report the actual demethylation rates. However, they reported that the demethylation rate of bioachanin A was faster than that of formononetin and speculated that the hydroxyl group in the 5 position of biochanin A was responsible for this faster reaction (19). Since hesperetin possesses a 5-hydroxyl group and glycitein does not, the explanation in Hur et al, 2000 may be valid.

We observed a significant amount of inderindividual variation in the degradation rates for all of the flavonoids (Figure 2). For all of the flavonoids, except hesperetin and quercetin, cluster analysis revealed 3 significant flavonoid phenotype groupings ($p < 0.0001$),

which we named high, moderate and low flavonoid degraders. Although the interindividual variation in the quercetin and hesperetin degradation rates was high, there were no clear significant phenotype groupings after cluster analysis (data not shown). Low flavonoid degraders may experience higher bioavailability of flavonoids compared to high flavonoid degraders because the flavonoids have more time to be absorbed from the small intestine before they are degraded in low flavonoid degraders. Zheng et al, 2004 has shown that women with a low daidzein degradation phenotype experienced a greater 24 h urinary daidzein excretion compared to women with high daidzein degradation phenotypes (11). Additionally, since 5,7,4'-trihydroxyflavonoids are rapidly degraded *in vitro*, we hypothesized that that these compounds are not very bioavailable in humans because these flavonoids have less time to be absorbed before they are degraded by the intestinal bacteria. On the other hand, flavonoids lacking any one of the 5, 7 and 4' hydroxyl groups would be more bioavailable because they are degraded at a slower rate and have more time to be absorbed compared to 5,7,4'-trihydroxyflavonoids. Zhang et al, 1999 has shown that subjects with moderate isoflavone degradation phenotypes experienced a significantly lower bioavailbility of genistein, which is a 5,7,4'-trihydroxyl-isoflavonoid, compared to daidzein (14). To test these hypotheses, we conducted 3 human bioavailability studies to compare the bioavailability of the rapidly *in vitro* degraded flavonoids, genistein, naringenin, quercetin and hesperetin, to a less rapidly *in vitro* degraded flavonoid daidzein in humans.

In an attempt to minimize interindividual variation, 5 men and 5 women were chosen from the initial 33 subjects that participated in the *in vitro* fecal fermentation degradation study based on a moderate daidzein degradation phenotype. The average 24 h urinary excretion, and thus, overall bioavailability of daidzein was significantly higher than genistein

with average bioavailabilities of 43 and 7 %, respectively, ($p < 0.0001$) even though the ingested dose of genistein was almost twice as high as daidzein (104 vs. 62 μmol respectively). These data are in agreement with Xu et al 1994 and King et al, 1998, who fed similar doses of genistein and daidzein in soy foods to human subjects (20, 21). Their doses of around 97 μmol daidzein and 71 μmol genistein resulted in an average daidzein bioavailability of 19.8 %, which was significantly higher than the average genistein bioavailability of 5.3 % (20). The average daidzein bioavailabilty of 43 % reported in our data is significantly higher than the Xu et al, 1994 data, but this could be due to differences in the isoflavone degradation phenotypes of the subjects, which may affect the overall isoflavone bioavailability. Peak plasma concentrations were similar in our study and the Xu et al, 1994 study in that they did not exceed 1 μmol/L (20). The AUC of daidzein appeared to be greater than that of genistein when normalized on a per umol basis, but there was no significant difference in the AUC values of genistein and daidzein because of the large variation at each time point. The higher bioavailability of daidzein compared to genistein was expected because daidzein was not a 5,7,4'-trihydroxyisoflavone, and was not degraded rapidly *in vitro*.

The 10 subjects that ingested the soymilk were divided into 3 genistein degradation phenotypes based on their *in vitro* genistein degradation rates. Low genistein degraders exhibited a higher genistein bioavailability compared to high genistein degraders (figure 3). This supports our hypothesis that low flavonoid degraders exhibit a higher flavonoid bioavailability than high flavonoid degraders, at least in genistein. Zheng et al, 2003 has reported that Asian women with low genistein degrader phenotypes experienced a higher genistein bioavailability compared to Asian women with high genistein degrader phenotypes

(10). This phenomenon was not observed for daidzein in the current study because all subjects were moderate daidzein degraders.

Naringenin bioavailability of around 3 %, and ranged from 1 – 7 %, which was surprisingly low, and significantly lower than hesperetin, with an average bioavailability of 7 % with a range of 4 – 10 %, given the high dose of 1550 μmol naringenin ingested in the grapefruit juice. The low naringenin bioavailability is in agreement with data from Ishii et al, 2000 and Ameer et al, 1996, who reported a naringenin bioavailability of 4 - 5 % when a single oral dose of 1837 μmol pure naringin was administered to human subjects (22, 23). Average naringenin bioavailability from grapefruit juice was determined to be 7 %, with an oral dose of 1194 μmol in 4 subjects (24). A wide range of 5 – 57 % naringenin bioavailability was reported in 6 subjects ingesting 26 μmol naringin per kg body weight (25). The range of naringenin reported in our studies was much lower than this and may suggest that our attempts at minimizing inderindividual variation in our subjects was successful, by selecting subjects based on *in vitro* moderate daidzein degradation phenotypes.

Most of the available research on hesperetin bioavailability has previously been determined from ingestion of orange juice, because hesperetin is the major flavanone in orange juice, whereas naringenin predominates in grapefruit juice. Studies have shown that hesperetin bioavailability ranged from 3 – 6 % (23 - 25), which is in agreement with our calculated average hesperetin bioavailability of 7 %, with a range of 4 – 10 %.

We observed significant sex differences in hesperetin bioavailability in that hesperetin was not recovered in the urine or plasma of females. The hesperetin bioavailability reported was thus based on bioavailability in the 5 males that ingested the grapefruit juice. Perhaps all of the hesperetin was excreted unchanged in the feces and is being investigated.

The pharmacokinetics of quercetin was slightly different from the other flavonoids, in that the average time to get to peak plasma concentrations was 1.5 h, and was more rapid than the other flavonoids with T_{max} values of 4 – 5 h. The average elimination half life was 9 hours, and ranged from 1 – 20 h compared to the half lives of the other flavonoids, which ranged from 1 – 6 h. Average quercetin bioavailability was determined to be about 6 % with a range from 1 – 10 %. These data support data from Graefe et al, 2001, who reported average quercetin bioavailability of 6% with an average T_{max} of 0.68 h, and average half life of 10.9 h after feeding a dose of 331 μmol quercetin in onions to 12 human subjects (26). This dose is comparable to the dose of 381 μmol quercetin that we fed in onions. Aziz et al, 1998 has reported a lower average quercetin bioavailability of 1 % after a dose of 300 g of lightly fried yellow onions in 5 subjects compared to our average bioavailability of 6 % (27). The reason for the difference in these bioavailability values is not clear, but may be due to the food matrix. In our study, we fed red onions in the form of an omelette, while the Aziz et al, 1998 study fed lightly fried onions as is (27). It is possible that unknown factors within the omelette facilitated the absorption of quercetin which resulted in a higher urinary recovery and bioavailability.

Overall, when all of the flavonoids were compared on a per umol basis, the AUC values of genistein and daidzein were significantly higher than quercetin, naringenin and hesperetin (figure 4A). Based on appearance of figure 4A, we expected daidzein to be significantly higher than genistein but the standard deviation was high at each time point, which prevented this statistical significance. It was evident from figure 4A that the overall AUC was higher for isoflavones, than for the other flavonoids. Overall bioavailability was not different for genistein, naringenin, quercetin and hesperetin, but they were significantly

lower than the bioavailability of daidzein (figure 4B). From this graph, it is evident that we have supported our hypothesis, in that rapid microbial degradion of flavonoids *in vitro*, such as those with a 5,7,4'-trihydroxylflavonoid structure (genistein, naringenin and quercetin), and hesperetin, which was rapidly demethylated into a 5,7,4'-trihydroxylflavonoid, are less bioavailable than flavonoids that are more slowly degraded *in vitro*, such as daidzein, which does not possess a 5,7,4'-trihydroxylflavonoid structure.

These results give insight into why flavonoids with minor differences in their chemical structure, may have such vast differences in certain biological effects such as estrogenicity and antioxidant activity. Because different flavonoid chemical structures alter their intestinal microbial degradation and thus overall human bioavailability, the biological activity of flavonoids may be altered depending on how much of the intact flavonoid gets absorbed across the intestinal wall to exert its biological effect. More data needs to be done in this are to investigate the relationship of flavonoid chemical structure to human bioavailability in other flavonoid subgroups.

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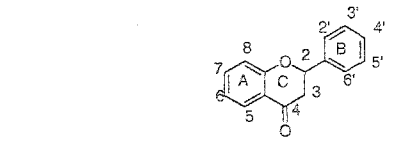
Table 1. Characteristics of subjects in the *in vivo* flavonoid bioavailability study

Subject ID	Sex	BMI (kg/m^2)	Age (y)	GTT (h)	degradation rate k (h^{-1})	Ethnicity
114	F	23.2	22	39.83±9.3	0.054	Caucasian
118	F	18.1	24	53.22	0.120	Asian
119	F	22.7	30	67.48±19.8	0.047	Asian
125	F	29.1	24	50.20	0.037	Af-American
127	F	22.5	26	104.25	0.046	Af-American
206	M	26.3	24	38.67±6.1	0.017	Caucasian
212	M	20.7	18	23.25±15.9	0.036	As-Indian
217	M	20.7	30	23.47±11.4	0.064	Asian
224	M	23.7	25	75.20	0.030	Caucasian
229	M	19.0	27	65.02±9.2	0.074	As-Indian
Male		22.1±2.9	25±4	45.1±23.9	0.044	
Female		23.1±3.9	25±3	63.0±25.1	0.061	
Overall		22.6±3.3	25±4	54.1±25.0	0.053±0.029	

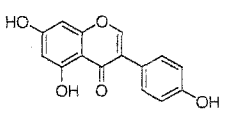
Table 2. Pharmacokinetic parameters of the soy isoflavones genistein and daidzein

Isoflavone	Ingested Dose	AUC	Cmax			Amount excreted	Bioavailability
	μmol	*μmol·h/L*	*μmol/L*	T_{max} *h*	$T_{1/2}$ *h*	*μmol*	%
Genistein	103.6	9.2±4.8	0.7±0.3	4.8±3.0	2.1±1.6	7.9±5.3	7.2±4.8
Daidzein	61.8	12.8±3.7	1.0±0.4	5.3±0.9	1.1±0.8	26.8±10.1	42.6±16.0
Naringenin	1549.5	3.1±1.7	0.3±0.2	5.1±1.6	5.7±5.9	50.0±27.7	3.2±1.7
Hesperetin	25.5	0.7±0.9	0.05±0.09	12.0±5.5	3.6±4.9	0.9±0.4	7.3±3.2[a]
Quercetin	380.5	6.1±5.9	0.8±0.6	1.5±1.3	9.1±8.9	21.1±4.4	5.6±3.7

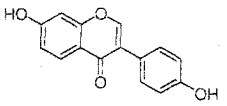
[a]bioavailability in males only



Flavonoid structure and numbering system



Genistein



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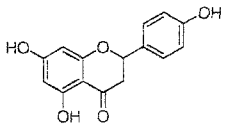

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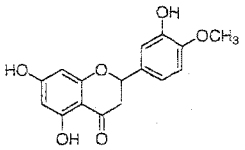
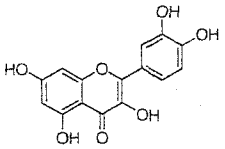


Hesperetin



Quercetin

Figure 1. Structure of flavonoids analyzed

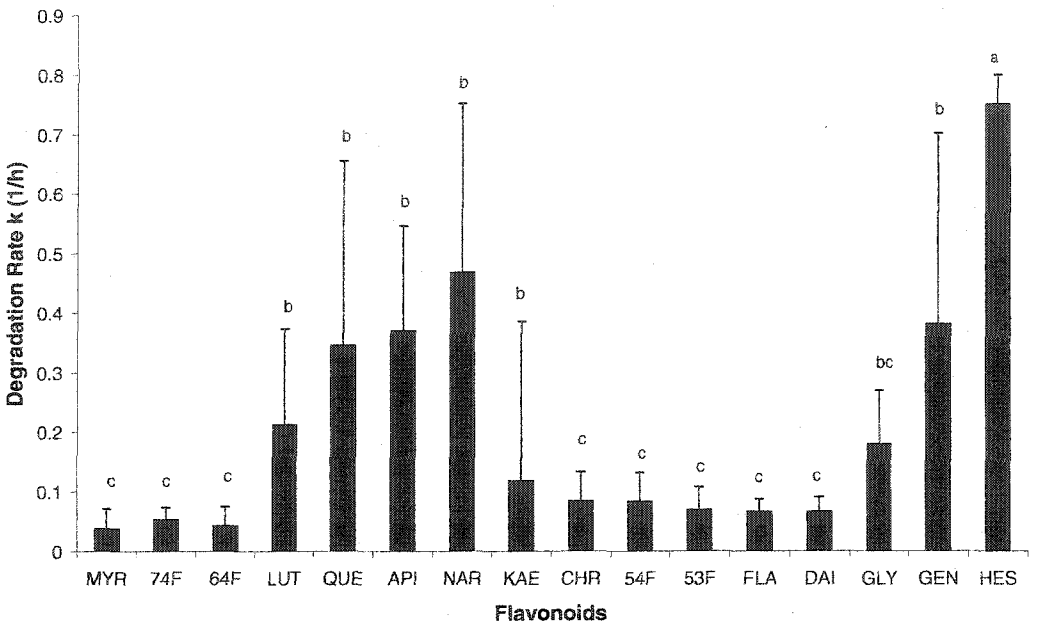


Figure 2. Average *in vitro* degradation rates for flavonoids. MYR: myricetin, 74F: 7,4'-dihydroxyflavone, 64F: 6,4'-dihydroxyflavone, LUT: luteolin, QUE: quercetin, API: apigenin, NAR: naringenin, KAE: kaempferol, CHR: chrysin, 54F: 5,4'-dihydroxyflavone, 53F: 5,3'-dihydroxyflavone, FLA: flavone, DAI: daidzein, GLY: glycitein, GEN: genistein, HES: hesperetin. Error bars represent standard deviation of the mean. Bars with different letters are significantly different.

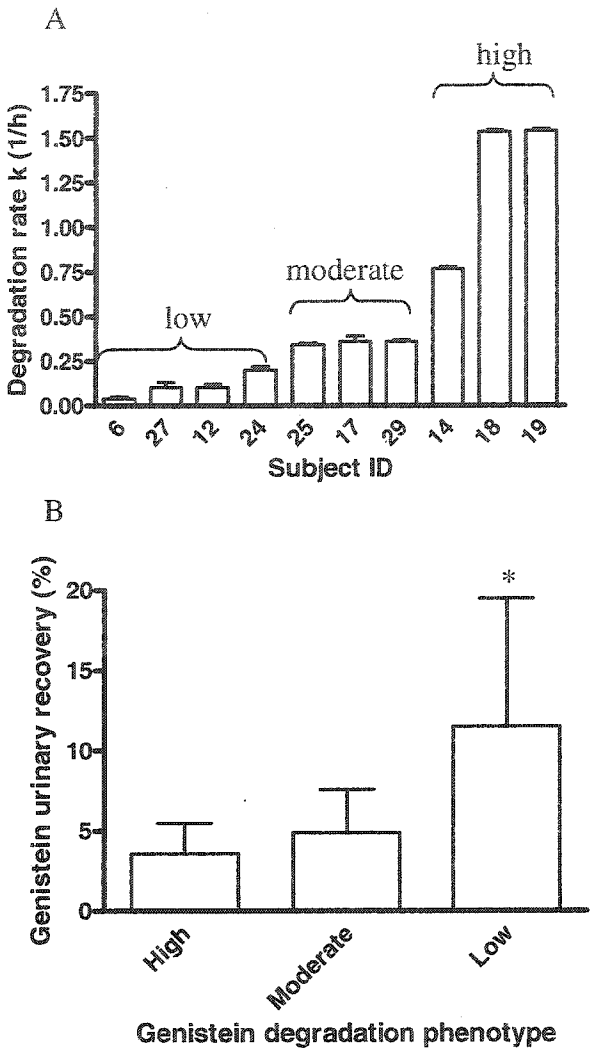


Figure 3. Genistein degradation phenotypes and correlation to urinary exretion. A: Cluster analysis of genistein degradation rates. B: Amount of genistein excreted in urine after 24 h in subjects with high, moderate and low genistein degrader phenotypes

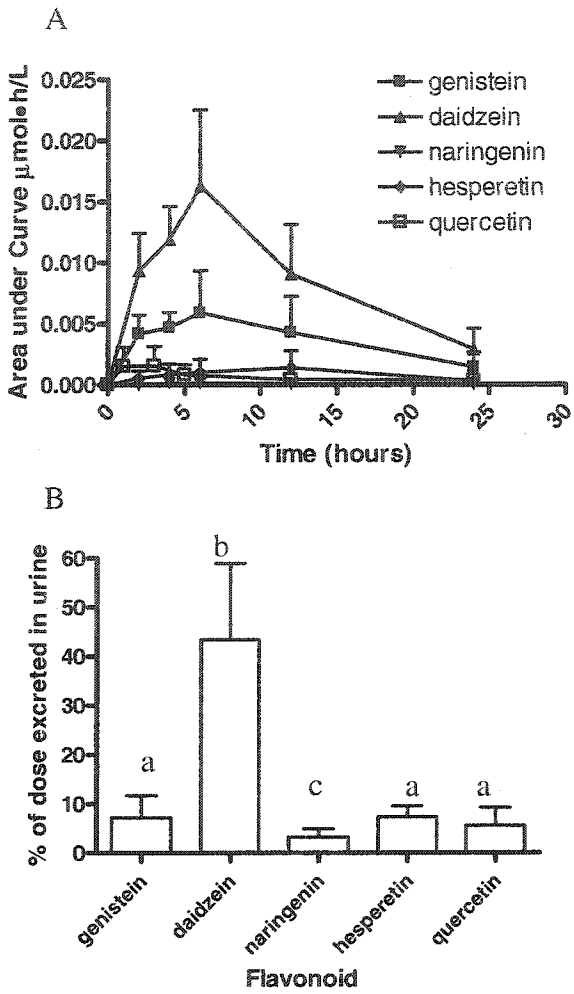


Figure 4. A: Overall AUC for flavonoids compared on a per umol basis. B: Bioavailability of flavonoids expressed as the amount of flavonoid excreted in urine after 24 h as a percentage of ingested dose.

GENERAL CONCLUSIONS

I have hypothesized that the chemical structure of flavonoids influences their gut microbial degradation, and is therefore a strong determinant of bioavailability. The first study, 'Metabolism of glycitein by human gut microflora', suggested that the chemical structure of soy isoflavones did influence the rate of degradation, with genistein being the most rapidly degraded isoflavone. Since isoflavones are only one subclass of flavonoids, the second paper, 'Human gut microbial degradation of flavonoids: structure-function relationships', examines the degradation of a wide variety of flavonoid subclasses including flavones, flavonols, flavanones and isoflavones. This paper supported the first paper, in that the chemical structure of flavonoids, not only isoflavones, influence gut microbial degradation. The flavonoids that tended to be the most rapidly degraded possessed hydroxyl groups at the 5, 7 and 4' positions. Why this is the case, is not clear but it may have to do with the similar hydrophobicities of these flavonoids with these structural features.

Flavonoids that are extensively metabolized in the human gut have reduced bioavailabilities compared to flavonoids that are not extensively metabolized because of limited absorption of the intact flavonoid. This holds true for other pharmacological drugs. This was observed in the human bioavailability studies conducted in the 3rd paper. Here we see that the overall hypothesis was upheld, in that the flavonoid chemical structure did determine human bioavailability. Additionally, we were able to conclude that the *in vitro* fecal fermentation systems in my studies are excellent models for simulating human intestinal microbial metabolism and a good predictor of bioavailability.

Further research should be conducted to determine why 5,7,4'-trihydroxylflavonoids were preferentially degraded over other flavonoids. First, this would require knowledge of

the specific bacteria in the gut that are responsible for degrading these compounds. With this knowledge, many more detailed studies may be conducted, such as identifying and characterizing the enzymes responsible for flavonoid degradation. Maybe there are certain active sites that preferentially bind to 5,7,4'-trihydroxyflavonoids. I also think that *in vitro* fermentation and bioavailability studies should be conducted with bioactive polyphenols and compounds in other herbs and dietary supplements that have not been well studied in human clinical trials and approved for use by the FDA.