CHEMOPREVENTIVE EFFECTS OF APIACEOUS VEGETABLES, CRUCIFEROUS VEGETABLES, AND THEIR PHYTOCHEMICALS AGAINST DIETARY CARCINOGEN PHIP IN RAT COLON

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DEDICATION

I dedicate this dissertation to

my father, Young Beom Kim and my mother, Young Sook Lee

for their love and dedication throughout their lives.

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CHAPTER 1. LITERATURE REVIEW

Section 1-1. Diets and Colon Cancer Risk

Colon cancer overview

Colon and rectum cancers are the third most common type of cancer in the world. Globally, it is estimated that around 600,000 deaths occur per year from colorectal cancers. This makes colorectal cancer the fourth cause of cancer death worldwide (1). The incidence and mortality of colon (and rectum) cancer significantly differ by many factors (e.g., sex, race, ethnicity, and degree of industrialization) (1, 2). Of note, the rates of this cancer increase with the degree of industrialization as well as urbanization, leading many researchers to emphasize the importance of diet as a modifying factor in the etiology of colon cancer (3).

Colon cancer is developed through multistage processes over many years (Figure 1-1). The first step of carcinogenesis is the development of neoplastic polyps in colonic mucosa. Adenomatous and hyperplastic polyps are the two most common types (4). The earlier model for colon tumorigenesis is referred to as "adenoma to carcinoma sequence" while the later pathway is known as "serrated adenoma to carcinoma theory" (4, 5). In the adenoma to carcinoma sequence, it was suggested that colon cancer is derived from a result of mutational inactivation of tumor suppressor genes (e.g., *APC*) along with activation of oncogenes (e.g., *p53*) thereby losing the balance between cell proliferation and apoptosis. It is estimated that this sequence is involved in approximately 85% of sporadic colon cancer (4). On the other hand, serrated adenoma which constitutes about 15% of sporadic colon cancer, seems to transform to

colon cancer through a different pathway compared to that of conventional adenoma. It is characterized by *B-Raf* mutation, extensive DNA methylation, and mismatch repair gene malfunction (4, 5). This pathway is also well known for its implication in the development of hereditary nonpolyposis colorectal cancer (2, 6).

As discussed above, carcinogenesis, not limited to colon cancer, is a long and multistage process, meaning that there are a number of chances to modulate this sequence. And, it has been well accepted that diets and nutrient factors could act as either pro- or anti-tumor risk modifiers across colon cancer development. For instance, while pyrolysis products from cooked foods and myoglobin in red meat can act as an initiator (7) and promoter (8) respectively, certain phytochemicals and nutrients may inhibit colon carcinogenesis at different stages (9, 10). According to the latest report published by the World Cancer Research Fund and the American Institute for Cancer Research (WCRF/AICR), there is convincing evidence that certain food items (e.g., milk, garlic, and dietary fiber) are protective against colon cancer whereas red meat, substantial consumption of alcoholic drinks, and factors that lead to greater body fatness and abdominal fatness are causes of this cancer, reinforcing the idea that diet is one of the critical risk modifiers in colon cancer development (2).

Meat consumption and colon cancer

Accumulating epidemiologic evidence indicates that incidence of colorectal cancer is positively related to high consumption of animal fat and meat (11, 12). In Larsson and Wolk's meta-analysis, the relative risk of colorectal cancer was 1.28 (95% confidence

intervals (CI) = 1.15-1.42) for subjects in the highest category of red meat consumption compared to those in the lowest category (13). The association with red meat consumption was stronger for rectal cancer than colon cancer; the relative risk for colon and rectum was 1.21 and 1.56, respectively. In like manner, the relative risk of colorectal cancer was 1.20 (95% CI = 1.11-1.31) for individuals in the highest relative to the lowest category of processed meat consumption. For subsites in the colon, high consumption of processed meat is associated with distal colon cancer risk only (not with proximal colon cancer; (13)). In another meta-analysis, Norat et al. compared relative risk for the highest quartile of red and processed meat intake versus the lowest quartile (14). High intake of red meat was significantly associated with colorectal cancer risk. Average relative risks for the highest quartile of red meat consumption and processed meat were 1.35 and 1.31, respectively. Total meat consumption (including fish and eggs) was significantly associated with colon cancer risk, but not with rectal cancer (14). These results from two separate meta-analyses support the hypothesis that high consumption of red meat and processed meat is positively related with risk of colon cancer and possibly rectal cancer.

One of several mechanistic hypotheses relates to cooking methods because cooking meat at high temperatures is known to produce potent carcinogens such as heterocyclic aromatic amines (HAA) and polycyclic aromatic hydrocarbons (PAH) (15, 16). Butler et al. investigated the correlation between meat intake and colon cancer in a population-based, case-control study (17). It was observed that well-done, very well-done, and pan-fried red meat were positively associated with colon cancer.

A similar association between HAA and colorectal adenoma incidence was observed in other studies as well (18, 19).

However, at the same time, there are also some mixed results with regard to HAA and their colon carcinogenicity. Although experimental studies demonstrated colon carcinogenicity of HAA using different animal models (20), diets containing up to 60% cooked meat (beef, pork, and chicken) did not show any effects on aberrant crypt foci (ACF), putative precancerous lesions in colon (21). Furthermore, recently Ferrucci et al. found the significant positive association between 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and rectal adenoma but no differences were observed between highest and lowest quartile in terms of both distal adenoma and descending/sigmoid colon adenoma (P= 0.89 and 0.98, respectively). Neither 2-amino-3,4,8-dimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) nor 2-amino-3,8-dimethylimidazo[4,5,-f]quinoxaline (MeIQx) were associated with incidence of distal colorectal adenoma (22). In Wu et al.'s study, the highest exposure of MeIQx increased the incidence of colon adenoma compared to the lowest quartile (odds ratio = 1.28; 95% CI = 0.95-1.71), while DiMeIQx and PhIP did not seem to be associated with such risks (23).

These mixed results may be due to several reasons. Of note, dietary intake of HAA and meat consumption was not estimated accurately in many epidemiological studies. HAA level in cooked meats is affected by cooking duration and temperature, cooking methods, as well as types of meats (15). However in some studies, no consideration was given to such factors in regard to HAA formation (24, 25). Further, even if

studies factored in such modifiers, classification of HAA exposure can also be variable; for example, categorization of meat doneness can be somewhat subjective if clear examples and criteria (e.g., photographs) are not provided for study participants (17, 26).

Levels of HAA in foods

HAA are ubiquitous in the environment from tobacco products, various meats, as well as drinks such as wine and beer (27-29). Some HAA (MeIQx, DiMeIQx, and PhIP) were detected in commercial pet foods as well (30). As aforementioned, the amounts of HAA formed in foods are affected by many factors including the type of meat, cooking methods, temperature resulting in a wide range of HAA in various foods (31-33).

Sinha et al. measured five predominant HAA in various pork products, cooked by different cooking methods (e.g., pan-frying, oven-broiling, and microwaving) and doneness levels (i.e., just until done, well-done, or very well-done, (15)). As a result, the types and levels of HAA varied dramatically depending upon types of products, cooking methods, and doneness levels. Specifically, both MeIQx and PhIP levels were much higher in very well-done and well-done bacon than in samples cooked just until done (up to 10-fold and 20-fold, respectively, depending on cooking methods). Of the cooking methods, oven-broiling produced the highest level of PhIP compared with other methods in bacon; however, bacon cooked to a very well-done level in the microwave oven contained non-detectable levels of 2-amino-3-methylimidazo[4,5-

f]quinoline (IQ), MeIQ, and DiMeIQx (15). In general, cooking time and temperature were positively associated with the level of HAA formation in foods (15, 32, 34).

There are two major classes of HAA, the pyrolytic HAA and aminoimidazoarenes (AIA). The pyrolytic HAA are formed during high temperature pyrolysis (higher than 300°C) whereas AIAs are formed in meats that are cooked at lower temperatures (between 100 and 300°C) (35). Non-enzymatic browning reactions are known to be involved in AIA formation and PhIP is one of the most abundant AIA formed in well-done meat (31, 35).

Mechanisms of PhIP formation

As briefly mentioned, PhIP often occurs in the highest concentration among HAA (31, 35, 36). PhIP was firstly isolated and identified in 1984 by the group of Felton et al. from fried ground beef (37). In order to understand the underlying mechanisms of the formation of PhIP, controlled model systems were utilized in multiple studies; these model systems were employed to simplify the food matrix. PhIP was formed in the heated model systems consisting of a mixture of creatinine, phenylalanine, sugars, and aldehydes (i.e., formaldehyde, and acetaldehyde) (38).

Murkovic et al. utilized ^{13}C-labelled phenylalanine as a reaction substance in the model system to elucidate the reaction mechanism. In the system with phenylalanine, creatinine, and diethylene glycol, phenylacetaldehyde (which is formed by pyrolytic degradation from phenylalanine) underwent an aldol addition reaction with creatinine

to form an aldol addition product (39). By the same research group, it was demonstrated that this aldol addition intermediate further loses a molecule of water through the aldol condensation reaction to produce a secondary intermediate compound. In the final reaction, the nitrogen atom of the pyridine moiety of the secondary intermediate product (i.e., aldol condensation product) was incorporated either from creatinine or amino acids thereby forming PhIP (40).

Levels of PhIP exposure in humans

Augustsson et al. estimated the daily intake of PhIP between 0 and 865 ng/day, with a mean value of 72 ng/day (41). This represented 42% of the estimated average of total HAA (170 ng/day). Recently, Puangsombat et al. assessed the content of PhIP in ready-to-eat products (i.e., beef hot dog, deli roast beef, deli ham, rotisserie chicken, and pepperoni). Of the measured HAA, PhIP was one of the most abundant HAA in all products tested and the level of PhIP was up to 13.5 ng/g of rotisserie chicken meat and skin (42). According to this estimation and depending on the serving size, an individual can be exposed to approximately 1.53 mg of PhIP from one serving size (i.e., 1/4 lb) of rotisserie chicken. In another study, Ni et al. also estimated the HAA levels by difference in level of doneness. It was estimated that very well done barbecued chicken contained 304.7 ng of PhIP/g which is 22.5-fold higher than Puangsombat's assessment of rotisserie chicken (31).

PhIP metabolism, mutagenicity, and colon cancer risk

Metabolism of PhIP

Although PhIP can be directly excreted from the body, it is extensively metabolized into a variety of metabolites; in humans, only 0.5-2% of parent compound (unchanged PhIP) is found in urine from 0-24 hours post-meal (43). The excretion times of PhIP vary but it is mostly excreted within the first 24 hours (44).

Once PhIP is ingested, both uptake and efflux back to the intestinal lumen are mediated by transporters, such as ATP-binding cassette (ABC) transporters (45). In the intestinal epithelium, there are multiple transporters (e.g., *P*-glycoprotein, and multidrug resistant-associated protein (MRP 2) found in the apical membrane of the enterocytes; these are responsible for efflux of xenobiotics (including PhIP) to the intestinal lumen (45) thereby preventing the entry of them. In contrast, there are also other ABC transporters (e.g., MRP3), localized in the basolateral membrane, mediating the efflux of compounds into portal blood. Once PhIP is absorbed from the gastrointestinal tract, it is principally metabolized in the liver (46).

PhIP is a procarcinogen, meaning it requires an activation step in order to exert its carcinogenic potency. Cytochrome P450 (CYP) 1A2 is known as the primary enzyme that generates N^2-hydroxy PhIP (47); other CYPs (e.g., CYP1A1, CYP1B1, and CYP3A4) are also able to catalyze this reaction (48, 49). Hydroxylation of PhIP on N^2 position is known as an initial step to exert genotoxicity of PhIP because this PhIP metabolite (i.e., N^2-hydroxy PhIP) is further esterified by *N*-acetyltransferase (NAT) 2 and sulfotransferase (SULT) 1A1 to N^2-acetoxy PhIP and N^2-sulfonyloxy PhIP. These

are known to create DNA adducts via electrophilic nitrenium ion formation by preferentially binding to guanine (50). In contrast, conjugations by phase II enzymes (e.g., uridine diphosphate glucuronosyl transferase (UGT) 1A1 and glutathione *S*-transferase (GST)) can usually lead to production of safe metabolites and efficient excretion of PhIP (20, 51-53).

Species differences in PhIP metabolism were found between rodent models and humans (54, 55). For instance, a larger proportion of *N*-hydroxylated metabolites was found in human urine and plasma when compared to those of rodents. Further, it was seen that humans possess the lower capacity to ring hydroxylate such metabolites (i.e., detoxification processes) relative to rodents, indicating that extrapolating data from one to another should be done carefully.

Mutagenicity of PhIP and colon cancer risk

In the 1970s, Japanese researchers demonstrated that the smoke produced by broiling meat and fish had mutagenic and carcinogenic activities; the hypothesis was developed because cigarette smoke condensate was known to contain many mutagenic compounds (56). The same research group further showed mutagenic activities of extracts from the burned surface of fish and meat toward *Salmonella typhimurium* TA98 after microsomal metabolic activation (57). These pioneering findings have been leading the extensive research on the mutagenicity of HAA (including PhIP), from various food sources. More than 20 HAA have been identified as mutagens so far (58). Although the specific mutagenicity of PhIP is less potent than

other HAA such as IQ and MeIQ, it does possess its own rationale for investigation which is that PhIP is one of the most mass abundant HAA in foods (20, 34, 37).

In vitro studies

It was demonstrated that incubation of PhIP in liver microsomes from 3-methylcholanthrene (known inducer of CYP expression) treated mouse and rabbit resulted in the formation of mutagenic PhIP metabolite (i.e., *N*-hydroxy PhIP (59)). In a subsequent study, the authors incubated DNA with this mutagenic PhIP metabolite alone or with mouse liver cytosol and found no PhIP-DNA adducts. In contrast, addition of SULT substrate (3'-phosphoadenosine 5'-phosphosulfate) or NAT substrate (acetyl coenzyme A) into the same incubation mixture containing *N*-hydroxy PhIP resulted in PhIP-DNA adducts, indicating that sulfation- and acetylation-dependent metabolic pathways are critical in genotoxic actions of PhIP (50).

Thereafter, it has been demonstrated by a number of studies that PhIP has mutagenic and genotoxic activities in various cell lines under different experimental conditions. To be specific, Otsuka et al. reported that PhIP induces structural chromosomal aberrations and sister chromatid exchanges in human cell lines (lymphocytes and diploid fibroblasts) with the presence of rat liver S9 fraction (60). Further, it was found that HAA, including PhIP, are able to cause morphological transformation in C3H/M2 mouse fibroblasts and affect cell viability when incubated with rat liver S9 fraction (61). In the same study, clastogenic, DNA strand breaking, and mutagenic effects of HAA were observed in MCL-5 cells as well. Although PhIP was most

potent in DNA strand-breaking activity, it was not directly related to its mutagenic potencies and clastogenic activity (61). Similarly, Katic et al. evaluated 10 selected environmental xenobiotics, including PhIP, using in vitro micronucleus cytokinesis-block assay; this assay was done in five different laboratories independently. The authors isolated peripheral human lymphocytes and genetic damage events were measured such as chromosome breakage, DNA mis-repair, as well as elimination of amplified DNA. All testing found a statistical significance in chromosome breakage frequency in binucleated cells with PhIP treatment (62).

Regarding colon cancer risk, Herbst et al. utilized immortalized human colon epithelial cells with hSULT1A1 transfection to test if *N*-hydroxy PhIP is involved with malignant transformation. After five treatment cycles of PhIP (one cycle was 0.72 μg *N*-hydroxy PhIP/ml for 24 hours), cells lost cell–cell contact inhibition and started piling up and forming foci in the culture flasks. To find tumorigenicity, PhIP-treated cells were injected into SCID mice. Eight animals out of eight injected with PhIP-treated cells developed solid, undifferentiated tumors at the site of injection within six weeks, indicating high tumorigenic potential of PhIP-treated cells (63). There are also multiple in vitro studies demonstrating the genotoxicity of PhIP in different organs or organ derived cell lines (e.g., liver and mammary gland (64, 65)).

Animal studies

The in vivo carcinogenic potential of PhIP has been demonstrated using various animal models. Specifically, in multiple animal studies, it was demonstrated that PhIP

induced chromosomal aberrations and sister-chromatid exchanges in mice (66), formed PhIP-DNA adducts and ACF in colon mucosa (67), and caused colon and mammary carcinomas (68, 69).

The colon is one of the preferred target organs of PhIP. In F344 male rats, approximately 50% of rats developed colon carcinoma when 400 ppm PhIP was given for 52 weeks (68). The same research team also showed that 13 out of 30 rats developed colon adenocarcinomas if 100 ppm PhIP was provided for 104 weeks, whereas no colon carcinomas were found in the group with 25 ppm PhIP, indicating dose dependent carcinogenicity of PhIP (69).

The mode of action of PhIP in animal models might be due to its genotoxic potency; it forms PhIP-DNA adducts in multiple target tissues such as colon, mammary gland, and prostate (67, 70-72). For example, when C57BL/6 male mice were treated with isotopically labeled PhIP, the detected level of PhIP, likely due to covalent binding to DNA or other macromolecules, was the highest in the intestine followed by stomach, liver, and kidney (73). Interestingly, even though the genotoxicity of PhIP has been assumed to be the major mode of action in vivo, Gooderham et al. found that PhIP possesses estrogenicity which is possibly significant given the implications of estrogens in the etiology of various cancers including colorectal cancer (74) and prostate cancer (75). In a following study, a low dose of PhIP increased the proliferation in the estrogen receptor-negative MCF10A cell line and activated the mitogen-activated protein kinase (MAPK) pathway; such proliferative effects of PhIP were effectively blocked by the known inhibitor of the MAPK pathway (i.e.,

PD98059) demonstrating the mitogenicity of PhIP (76). Thus, future studies are warranted with regard to estrogenicity of PhIP and its potential role as a promoting agent in addition to an initiating agent.

Human studies

A few intervention studies have been done to understand PhIP metabolism and its genotoxic potency in humans. Dingley et al. administrated isotopically labeled PhIP to five male volunteers who were about to undergo surgery to remove colon carcinomas. The level of PhIP given to subjects was approximately equivalent to 175 g of very well-done chicken. At various times up to 24 hours after PhIP administration, blood samples were collected and separated into plasma, red blood cells, and buffy coat. Forty eight to 72 hours after dosing, colon carcinoma tissues were collected from subjects. PhIP formed adducts with albumin and hemoglobin in the peripheral blood within 30 minutes of exposure; but the level of PhIP-albumin adducts was approximately 50-fold higher than that of hemoglobin adducts implying that albumin adducts may provide a more sensitive indication of PhIP exposure (77). Further, the authors found that the level of PhIP-DNA adducts in the normal colon tissue was lower than the tumor tissue (P=0.05). It was speculated that differences between normal colon tissue and colon tumor might be due to 1) the capacity to activate PhIP, 2) ability to detoxify the bioactive PhIP metabolites, and 3) DNA repair systems (77). In a subsequent publication by the same research team, the authors compared the PhIP-DNA adducts in human patients and rats; the mean formation of PhIP-DNA adducts in colon tissues as well as PhIP-albumin adducts were

significantly higher in humans compared to rats after the equivalent level of PhIP exposure, indicating species differences in genotoxic potency and metabolism of PhIP (78).

There are mixed outcomes observed regarding whether genetic polymorphisms significantly affect PhIP metabolism and the formation of PhIP-DNA adducts in colon. Malfatti et al. exposed human volunteers to PhIP prior to colon tumor surgery and found that individuals with the rapid CYP1A2 phenotype had the lowest level of PhIP-DNA adducts in colon (79). These subjects also had the highest levels of N^2-hydroxy PhIP N^2-glucuronide. This metabolite was negatively correlated with the level of PhIP-DNA adducts (79). These findings are somewhat contrary to the observation from preceding studies. For example, Lang et al. reported that rapid CYP1A2 activity and rapid NAT2 activity were positively correlated with incidence of colon cancer among individuals consuming HAA via diet (80). In the study, the interaction between cooking method and phenotypes of CYP1A2 and NAT2 was also significant; the odds ratio of subjects with rapid CYP1A2 activity and rapid NAT2 activity went from 3.1 to 6.5 if the meat cooking preference was changed from rare/medium to well-done (80). In another study, colorectal cancer patients were genotyped for common polymorphisms in the NAT1, NAT2, SULT1A1, GSTM1, and GSTA1 genes; overall, none of the genetic polymorphisms were significantly associated with PhIP-DNA adducts, indicating that polymorphisms of PhIP-metabolizing enzymes had limited effects on the formation of PhIP-DNA adducts (81). These differences between studies might be because of 1) different study designs including PhIP dose (exact dose versus estimation through dietary questionnaire), 2)

environmental factors affecting PhIP metabolism (e.g., diet), 3) missing information about other polymorphisms in key genes of PhIP metabolism (e.g., Lang et al. did not look at glucuronidation activity; (80)), and 4) small sample size.

Genetic alterations by PhIP

A number of studies have described the ability of PhIP to induce selective mutations. For example, using the Chinese hamster cell line which is genetically engineered to express human CYP1A2, PhIP predominantly generates guanine to thymine transversions (82). Furthermore, all mutations induced by PhIP were involved with guanine-cytosine pairs. Similar mutations induced by PhIP were demonstrated in different gene loci using other experimental models (e.g., Muta™ mouse and the *LacZ* transgenic rodent model (83, 84)). Such results are in agreement with the preference of PhIP for binding to guanine (50).

Interestingly, it was shown that five out of eight colon tumors induced by PhIP possessed a mutation in the tumor suppressor gene (*APC*); every mutation involved a deletion of a guanine base at the 5'GGGA3' site thereby resulting in the truncation of the APC protein (85). Mutations in this gene have been observed in familial adenomatous polyposis as well as approximately 80% of sporadic colorectal cancer patients, reinforcing the significance of the *APC* gene in the etiology of colon cancer (86).

Another genetic mutation commonly observed after PhIP exposure is *β-catenin* mutation, the downstream gene of *APC.* Ubagai et al. induced large intestinal tumors with either single treatment of PhIP (400 ppm) followed by long term feeding of high fat diet (108 weeks) or repeated PhIP treatments (400 ppm for three times) with four week intervals followed by continuous feeding of the high fat diet for 42 weeks (87). At the end of the studies, three out of 19 rats and nine out of 20 rats developed large intestinal tumors from each treatment, respectively. In this study, the authors found that five of nine tumors harbored mutations in either *β-catenin* (two out of nine tumors) or *APC* genes (three out of nine tumors) and all tumors showed accumulation of β-catenin protein (87). β-catenin is a structural protein at cell-cell adherent junctions and a transcriptional activator of the Wnt-signal transduction pathway. Under normal circumstances, it is recruited in a large cytoplasmic protein complex which contains APC, axin/axin2, casein kinase I and glycogen synthesis kinase 3-β. However, in response to Wnt signaling, or if *APC* gene is mutated, β-catenin is stabilized, accumulates in the cytoplasm, and enters the nucleus. In colonic adenomas and adenocarcinomas, β-catenin was universally localized to the cytoplasm and/or nucleus (88). Once β-catenin enters the nucleus, it binds with transcription factors (e.g., the lymphoid enhancer factor or T cell factor) and activates target genes such as *c-MYC* which are involved in cell proliferation and cancer development (89). On the other hand, it has been demonstrated that overexpression of mutant proteins of APC (i.e., truncated form) maintains an anti-apoptotic mode of action whereas wild type APC (310 kDa) induces apoptosis (88). Interestingly, common mutations in *K-ras* and *p53* genes, induced by other colon carcinogens (e.g., azoxymethane), were rarely observed in PhIP-induced colon cancer (90, 91).

DNA adducts and PhIP

For genotoxic carcinogens, the formation of DNA adducts is essential, and these carcinogens may not induce various cancers without DNA adduct formation (92). As mentioned, PhIP is known to bind preferentially with one of the nucleosides (i.e., guanine; Figure 1-2); further, there is clear evidence indicating that chemical carcinogen-DNA adducts reflect the exposure to xenobiotics (93, 94). Thus, PhIP-DNA adducts are generally regarded as a useful biomarker of PhIP exposure (49, 50).

However, at the same time, it requires caution to utilize DNA adducts as a biomarker in cancer research. That is, firstly, the exact relationship between DNA adducts (including PhIP-DNA adducts) and carcinogenesis, is not fully understood. Recently, Jonsson et al. investigated whether there are differences in DNA adduct levels in mucosa from patients with colon cancer, polyps, and control subjects; further, the authors also tested if DNA adducts could be a marker for colon cancer development. Although PhIP-DNA adducts were one of the most abundant adducts observed in the mucosa (106 out of 150 tissues analyzed), the authors found no differences in the level of total DNA adducts from colonic tissues among control, polyp, and cancer patients (95). In addition, there was no correlation between DNA adduct levels and smoking and drinking habits, exposure to urban traffic and chemicals, histology, or differentiation grade of tumors as well. In another study, it was also demonstrated that the level of DNA adducts in the colon was not directly correlated with precancerous

lesions (i.e., ACF) in rats (67), however the predictive quality of ACF as a surrogate marker remains controversial (96).

It is also important to note whether carcinogen-DNA adducts were measured in the target tissues or in surrogate tissues (e.g., white blood cells). The primary rationale for the measurement of adducts in surrogate tissues is because they are easily obtained from subjects with less invasive means and thus the response rates are higher at recruitment (97). But sampling from surrogate tissues may not accurately reflect DNA adduct levels in the target tissues. Even though some studies have found strong correlations of DNA adduct levels between surrogate tissues and target tissues (e.g., peripheral blood mononuclear cells versus lung tissues; (98)), others were not able to reproduce such correlations (99).The lack of correlations between biomarkers and tumor incidence might be because carcinogenesis is a long process and affected by a number of factors. For example, each tissue represents different cell turnover kinetics (100) as well as different susceptibility against carcinogens including PhIP (52). In addition, only a subgroup of ACF (i.e., dysplastic ACF) progresses to cancer while the remaining non-dysplastic ACF are not directly related to the formation of tumors (101). Lastly, this biomarker itself might be influenced by other factors. For instance, Godschalk et al. found that body composition affects DNA adduct persistence because lipophilic carcinogens from tobacco products were accumulated in adipose tissues (102).

To summarize, although it has been shown that the formation of carcinogen-DNA adducts is a necessary step to cause cancers in experimental models, alone it seems

not sufficient. Therefore it is difficult to utilize PhIP-DNA adducts as a biomarker for cancer risk. However, the exposure level to carcinogens (e.g., PhIP) and the levels of DNA adducts are proportional (71). Hence, it can be justified that measuring DNA adduct levels would be useful to understand carcinogen metabolism in target tissues.

Measurement of PhIP-DNA adducts

Various methods have been developed and published for the measurement of PhIP-DNA adducts. One of the most commonly utilized methods is a ^{32}P-postlabeling method, coupled with either thin layer chromatography (103) or high performance liquid chromatography (HPLC) (104). In addition, immunohistochemistry (IHC) and different types of mass spectrometry (MS; e.g., gas chromatography (GC)-MS and accelerator MS) have been utilized in multiple studies as well (77, 105, 106). IHC as well as ^{32}P-postlabeling methods possess both strengths and limitations. For instance, in the case of the IHC method, despite the fact that it is able to detect DNA adducts in specific types of cells in the tissues (107), the specificity of the antibody as well as sensitivity are questionable (108). Likewise, the ^{32}P-postlabeling method can detect a level as low as 1 modification in 10^{10} nucleotides with a relatively small amount of DNA (less than 10 μg), but does not show the spectral data to identify the types of adducts (109).

In contrast, liquid chromatography (LC)-electrospray ionization multistage MS provides relatively high sensitivity for the detection of DNA adducts (e.g., adducts per 10^9 nucleosides) with the isotopically labeled internal standard. The addition of the

internal standard into DNA samples allows quantification of adducts in complex biological matrices because it elutes at the identical retention time of the unlabeled PhIP-DNA adduct. Further, the instrument can be interfaced with an online switching system for a DNA enrichment step; this makes pre-purification steps much faster and easier as opposed to the off-line purification processes using solid phase extraction (71, 108).

Collectively, it seems that incidence of colorectal cancer is affected by multiple factors; of note, the high consumption of animal fat and meat was found to be positively associated with its etiology (11, 12). One of the hypotheses regarding this association relates to the presence of potent carcinogens (e.g., HAA). Particularly, PhIP often occurs in the highest concentration among HAA, indicating the significance (31, 35, 36). In fact, PhIP has been shown to elicit genotoxicity (66), mutagenicity (110), and carcinogenicity (68, 69) in many animal studies. PhIP requires an activation step to initiate its carcinogenic potency. Once hydroxylated on the exocyclic amine of PhIP, it can be further esterified by NATs and SULTs to produce reactive metabolites that can bind to DNA and form PhIP-DNA adducts (50); in contrast GSTs and UGTs are known to render them more soluble thereby facilitating excretion (20, 51-53). Given the important role of biotransformation enzymes in the metabolism (not only activation but also detoxification) of this procarcinogen, it is very reasonable to postulate that modulation of these enzymes through diet could be an effective chemoprevention strategy.

Section 1-2. Vegetable Intake and Cancer Prevention

An overview of chemoprevention

As briefly discussed above, carcinogenesis is a long and multistage process which can be divided into three general phases: initiation, promotion, and progression (111). Therefore, this multistage process may provide chances for intervention to modulate each phase of carcinogenesis. Chemopreventive agents refer to any natural or synthetic compounds to inhibit, reverse, delay, and/or prevent stages of carcinogenesis prior to the development of invasive malignancy (112). An ideal chemopreventive agent should 1) have little toxicity, 2) represent high efficacy in multiple sites, 3) be able to easily consume, 4) act via known mechanisms of action, and 5) have low cost (113). In this regard, natural products, especially derived from food stuff (e.g., vegetables) would represent several benefits as chemopreventive agents due to their noticeably low toxicity, lack of side effects along with various biological activities (113). Major mechanisms of fruit, vegetable, and their phytochemical constituents in colon cancer prevention are summarized in Table 1-1.

Cancer prevention through vegetable intake

High intake of fruits and vegetables (and their active compounds) has been hypothesized to be protective against several cancers including colon cancer (114). A number of in vivo feeding studies have shown putative biological mechanisms

regarding the chemoprotective effects of vegetables (115-117), yet epidemiological studies are providing inconsistent results.

According to the first report from WCRF/AICR, 20% or more of all cancers would be prevented from diets containing a substantial amount of vegetables and fruits (118). In particular, it was stated that colon cancer (as well as rectal cancer) is mostly preventable via appropriate diet. However, in the following report (i.e., second report published in 2007) the panel judged that there is limited evidence suggesting that non-starchy vegetables are protective against colorectal cancer (2).

In a recent meta-analysis, Aune et al. investigated 19 prospective studies to find evidence of chemopreventive effects of fruit and vegetable intake; the authors were able to include five large prospective cohort studies in the analysis, published since the second WCRF/AICR report. Interestingly, they found a non-linear inverse association between the intake of fruits and vegetables and colorectal cancer risk; there was the greatest risk reduction of colorectal cancer when increasing fruit and vegetable intake from low levels (119). To be specific, the results indicated that the level of between 100 and 200 g of fruit and vegetable intake per day can decrease risk approximately 10% but no additional benefits were observed with increasing intake of them (119). These results are in agreement with previous findings of Schatzkin et al.; in this randomized trial, diets with large amounts of fruits and vegetables did not influence the risk of recurrent colorectal adenoma when compared to the control group with general dietary guidelines that included approximately 200 to 400 g per day of fruits and vegetables (120). Taken together, it can be carefully concluded that

increasing fruit and vegetable intake may elicit cancer prevention benefit in a population with low base levels of consumption. However, assessing epidemiological evidence for the association between cancer risk and fruit and vegetable intake requires more specifics in terms of vegetable types, responsible active compounds, and underlying mechanisms for their chemopreventive potential given a number of different phytochemicals in vegetables as well as anatomical characteristics of colorectal subsites (121), rather than looking at a simple comparison among populations of high or low fruit and vegetable intake.

Cruciferous vegetables and glucosinolates: evidence for chemoprevention

Cruciferae is a family of vegetables named because of their crucifer shape flowers. These vegetables include cabbage, broccoli, Brussels sprouts, and cauliflower. As like other vegetables, this group of vegetables contains various nutrients and phytochemicals including folate, fiber, carotenoids, and chlorophyll but what makes cruciferous vegetables unique is the sulfur containing compounds thereof, called glucosinolates which are responsible for a pungent and spicy taste (122).

It is generally accepted that the chemopreventive effects of cruciferous vegetables are likely due to glucosinolates. So far, more than 120 different glucosinolates have been identified from various plants and the profiles of these compounds vary depending on cultivars and growing conditions. Depending upon their structures, they can be classified as aliphatic, aromatic, ω-methylthioalkyl, and heterocyclic (e.g., indole) glucosinolates (123, 124). Glucosinolates are accompanied in plants by the enzyme,

myrosinase (β-thioglucosidase), which is normally physically separated from glucosinolate substrates. If the plants are damaged from predators (e.g., chewing) or microbial attack, glucosinolates are released from vacuoles and hydrolyzed by myrosinase to bioactive compounds (e.g., isothiocyanates (ITC), indoles, and nitriles; (124)). Humans also can convert glucosinolates to their bioactive metabolites through the action of the microflora of the gastrointestinal tract (125).

A number of in vitro and animal studies have been reported with regard to the chemopreventive potency of cruciferous vegetables through multiple mechanisms (e.g., induction of detoxification enzymes (126, 127), scavenging free radicals (128), and induction of apoptotic cell death (129, 130)). Particularly, in many animal models of colon cancer, cruciferous vegetables and their active compounds inhibited chemical carcinogenesis. For instance, Kassie et al. demonstrated that supplementation of drinking water with Brussels sprout juices provide protection during both cancer initiation as well as promotion in 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) treated rats (115). In another rat feeding study, garden cress and its constituents (i.e., glucotropaeolin and its breakdown product, benzylisothiocyanate) showed significant chemoprotection against IQ-induced DNA damage in colon (131). Similarly, He et al. showed the chemopreventive property of indole-3-carbinol (I3C), another breakdown product of glucosinolates, against PhIP in rats (132, 133). These results from animal studies are supported by many epidemiological studies and meta-analyses, showing the inverse association between cruciferous vegetable intake and risk of various cancers (134-136).

A recent meta-analysis which covers all prospective and case-control studies published up to April 2012, evaluated the relationship between cruciferous vegetable intake and colorectal cancer (137). Using the representative cruciferous vegetables as key words (e.g., broccoli, cabbage, cauliflower, Brussels sprouts, etc.) the authors were able to include 35 articles for the analysis. It was found that intake of cruciferous vegetables may reduce the risk of colorectal cancer in humans when the highest category was compared to the lowest one. Specifically, high intake of cabbage was associated with reduced colorectal cancer risk. The relative risk of colorectal cancer was 0.76 (95% CI = 0.60-0.97) while the effect of broccoli was of borderline statistical significance (the relative risk was 0.82, 95% CI = 0.65-1.02; P=0.05). Although results from case-control studies showed an inverse association between cruciferous vegetable intake and colorectal cancer risk, the results from prospective studies were not statistically significant (P=0.118). Overall, findings of the meta-analysis support the evidence that high intake of cruciferous vegetables might be inversely associated with colorectal cancer. Importantly, the authors found a statistically significant inverse association between cruciferous vegetable intake and colon cancer, especially distal colon cancer (P=0.017). These results are in agreement with another meta-analysis (119) as well as a large cohort study that showed the stronger inverse association for distal colon tumors (138). For future investigations, it seems that possible factors (e.g., cooking methods; (139)) which may affect exposure level of glucosinolates due to inactivation of myrosinases, should be taken into account for better estimation.

Metabolism of glucosinolates and hydrolysis products

Compared to other dietary nutrients, there are little data available regarding the metabolism of glucosinolates. Most studies have been done with animal models; human studies are rare and mostly focused on the measurement of urinary metabolites.

Once ingested, glucosinolates are exposed to the acidic condition in the stomach. Depending upon the compounds, the stability is highly varying against acidity. For example, I3C is known to form various condensation products (e.g., a dimer, 3,3'-diindolylmethane) under the acidic condition (140). In humans, intact glucosinolates can also be converted into ITC and other derivatives (e.g., nitriles) in the colon by microflora. It was suggested that this conversion of intact glucosinolates is an important step for the metabolism and bioavailability given the results showing that cumulative excretion of representative urinary metabolites (i.e., dithiocarbamates) was significantly greater (six-fold) after consumption of converted products of glucosinolates (i.e., ITC) compared to that of intact glucosinolate (141).

ITC, either ingested or formed in the lumen of the colon, cross the gastrointestinal epithelium via passive diffusion and rapidly bind to thiols of plasma proteins. This binding is reversible and once these complexes get into cells of tissues, ITC are conjugated with glutathione by GST to form glutathione conjugates. The glutathione conjugates are discharged from cells via multidrug resistant-associated proteins and then cleaved by γ-glutamyltransferase and dipeptidase, consecutively. The resulting conjugates are further conjugated by *N*-acetyltransferases in the liver to form *N*-acetyl derivatives or mercapturic acid derivatives, and excreted into urine (124, 142). These

urinary derivatives (i.e., mercapturic acid derivatives) are major metabolites after dietary exposure of glucosinolates in both rodents (143) and humans (144, 145).

Cruciferous and phase I enzymes: CYP1A regulation

As discussed in the previous chapter, a variety of procarcinogens, including PhIP, require an activation step to elicit their carcinogenic potency. In the case of PhIP, it is primarily, but not exclusively, activated by cytochrome P450 (CYP) 1A2 to produce N^2-hydroxy PhIP (47-49), hence modulation of phase I enzymes such as CYP1A2 through dietary components could possibly be critical in regard to carcinogen metabolism and cancer incidence. Modulation by cruciferous vegetables of phase I enzymes has been investigated, mainly in rodent models.

Vang et al. evaluated the effects on specific CYP activities of various broccoli samples containing different levels of glucosinolates, putative active compounds for modulation of CYP (146). Male Wistar rats were fed one week with one of eight types of broccoli (10%, wt/wt) grown in different conditions and CYP activities from liver, colon, and kidney were analyzed. Overall, one-week dietary intervention of different types of broccoli diets significantly increased both CYP1A1 and CYP1A2 activities in the liver and colon, but not in the kidney. The authors also found significantly enhanced PhIP metabolism in the rats; the N^2-hydroxy PhIP level was increased in all tested groups compared to the control ($P< 0.01$), confirming modulating effects of broccoli on PhIP metabolism (146). Interestingly, different levels of CYP activities were observed depending upon the broccoli samples. These results suggest that

modulation of PhIP metabolism may vary significantly based on different growing conditions, cultivars, and other factors that possibly affect glucosinolate profiles in cruciferous vegetables. Other studies also demonstrated similar effects using different cruciferous vegetables. Scholl et al. fed mice with 20% freeze-dried radishes for two weeks and observed significant increase in hepatic mRNA expression of CYP1A1 and CYP1A2, indicating that these effects occur at the transcription level (147). In another study, rats were supplemented with 2.5, 5, and 20% cooked Brussels sprouts for up to four weeks. The authors reported that the activity of CYP1A1 and expression of CYP1A1 and CYP1A2 were markedly increased in a dose dependent manner (148).

Likewise, comparable effects of cruciferous vegetables were observed in humans. Kall et al. administered 500 g of broccoli for 12 days to 16 healthy subjects and assessed CYP1A2 activity using caffeine metabolites (149). The caffeine metabolic ratio (CMR) was determined in urine six hours after ingestion of 100 mg caffeine and the mean CMR increased by 19% when changing from normal diet to broccoli diet ($P< 0.0005$). Lampe et al. demonstrated that a mixture of cruciferous vegetables (i.e., radish sprouts, cauliflower, cabbage, and broccoli) increases CYP1A2 activity as measured using urinary caffeine metabolites as well ($P< 0.04$, (150)).

As aforementioned, such modulation of phase I enzymes through cruciferous vegetables is likely due to a class of compounds, glucosinolates and their hydrolyzed products. There are a few studies demonstrating the effects of glucosinolates and their breakdown products on CYP1As. For instance, Horn et al. showed the effects of different doses of I3C on hepatic mRNA levels and activities of CYP in Sprague-

Dawley rats (151). The activities of CYP1A1, CYP1B1, and CYP2B1/2 were all increased in a dose dependent manner while mRNA transcripts of CYP1A1 were only significantly up-regulated in the highest concentration (i.e., 250 mg of I3C/kg body wt). On the other hand, recently it was found that intact glucosinolates (glucoraphanin and glucoerucin) increased activities and apoprotein levels of CYP1A1 and CYP1A2 in rat liver slices (152).

The mechanism of induction of CYP1A expression via cruciferous vegetables and their active phytochemicals is largely dependent upon the structures of compounds; for instance, acid condensates of I3C (e.g., 3,3'-diindolylmethane) bind to aryl hydrocarbon receptor (AhR) in the cytosol and lead to translocation of AhR into the nucleus where it binds to aryl hydrocarbon nuclear translocator. This complex interacts with the consensus sequence of dioxin or xenobiotic response elements in the CYP1A gene promoter (153). On the other hand, there is indication that some breakdown products (e.g., phenethyl isothiocyanate, PEITC) of glucosinolates directly inhibit CYP1As (154).

Collectively, it seems reasonable to consider that cruciferous vegetables possess the ability to increase CYP1A expression and such modulation might be due to glucosinolates and their hydrolyzed products. Although this may seem to have negative implications given that CYP1As help activate PhIP, an understanding of the broader effects of the vegetables on biotransformation is necessary to fully appreciate the net potentially beneficial implications.

Cruciferous and phase II enzymes: UGT and SULT regulation

The phase II biotransformation enzymes are known to conjugate heterocyclic aromatic amines (HAA). Specifically, uridine diphosphate glucuronosyl transferase (UGT) 1A1 plays a primary role in conjugating activated PhIP (i.e., N^2-hydroxy PhIP) and rendering HAA safe for excretion (155). The anti-carcinogenic properties of cruciferous vegetables and their active compounds have been attributed to the ability to facilitate detoxification of carcinogens (156). On the other hand, even though sulfotransferases (SULT) have been considered as detoxification enzymes, it was demonstrated that sulfation of PhIP might be another possible activation pathway as it produces the electrophilic metabolite that binds to DNA (50).

Many studies have been done with regard to induction of UGTs by food constituents including cruciferous vegetables. Recently, Robbins et al. investigated induction of phase I and II enzymes and compared these effects between blanched (i.e., inactivation of myrosinase) or non-blanched Brussels sprouts feeding (126). C3H/HeJ mice were exposed to either blanched or unblanched Brussels sprout diets (20% freeze-dried vegetable) for two weeks. The plasma concentrations of sulforaphane and expression levels of UGTs in the lung were higher in the group with unblanched Brussels sprout diets, but not in the liver (126).

Navarro et al. also recently tested if cruciferous vegetable feeding alters UGT1A1 activity in humans, as measured by serum bilirubin concentration (127). Subjects consumed four different controlled diets for two weeks each (basal, single dose

cruciferous, double dose cruciferous, and cruciferous plus apiaceous) with a washout period (three weeks) between diet interventions. The single dose of cruciferous vegetables was 7 g/kg body wt, and the mixed diet included 7 g/kg of cruciferous with 4 g/kg of apiaceous vegetables. There was a significant decrease in serum total bilirubin concentrations in participants, supporting the hypothesis that consumption of cruciferous vegetables increases UGT1A1 activity in humans as well. Additionally, the magnitude of effects from vegetables varied depending upon genotypes of UGTs and glutathione *S*-transferase (GST) (127).

As described earlier, some breakdown products (e.g., I3C) of glucosinolates present in cruciferous vegetables are thought to act as ligands of the AhR thereby influencing gene expression. Thus, not only phase I enzymes but also other genes that are responsive to AhR would be modulated via a similar mode of action. GSTA as well as UGTs are known to be induced via AhR mediated transactivation (157). On the other hand, ITC, another class of compounds derived from glucosinolates, can activate a range of phase II detoxification enzymes (e.g., GSTs) through a different mechanism; ITC (e.g., sulforaphane and PEITC) dissociate the cytoplasmic protein, Keap I, from the transcription factor Nrf2 and allow it to move into the nucleus. Upon translocating into the nucleus, Nrf2 heterodimerizes with members of Maf family of transcription factors in order to activate transcription via antioxidant/electrophile response element (157).

To our knowledge, no studies have been done with regard to the effects of specific cruciferous vegetables (or glucosinolates or ITC) on modulation of SULT1A1.

Apiaceous vegetables and furanocoumarins: evidence for chemoprevention

The apiaceous (formally *umbelliferae*) plant family consists of more than 2,000 species and includes edible items such as celery, dill, parsley, parsnips, and carrots (158). These vegetables are a good source of various bioactive compounds (e.g., polyacetylenes, phthalides, coumarins, alkenylbenzenes, phenolic acids, flavonoids, terpenes, and carotenoids). For instance, polyacetylenes, one of the representative secondary metabolites found in apiaceous vegetables, have been shown to exert multiple bioactivities including antifungal activity, neurotoxicity, allergenicity, anti-inflammatory, as well as anti-cancer activity (159-161). Further, many studies have described that coumarins and their metabolites (e.g., 7-hydroxycomarin) possess anti-cancer activity in vitro (162, 163) and in vivo (164).

Additionally, apiaceous vegetables also contain a class of bioactive compounds called furanocoumarins. Furanocoumarins are stable in normal cooking processes and their levels can be changed depending on storage conditions (165, 166). Derivatives of the linear skeleton (e.g., psoralen) or angular isomer (e.g., angelicin) can be substituted with hydroxyl, methoxy, alkyl, or hydroxymethyl groups. Of the furanocoumarins detected in vegetables, 5-methoxypsoralen (5-MOP) and 8-methoxypsoralen (8-MOP) are generally present in higher amounts than other furanocoumarins (167).

There are several intervention studies as well as epidemiological studies to understand the association between apiaceous vegetables and various cancer risks but results are

somewhat inconsistent and controversial. Franceschi et al. investigated the effects of 26 types or groups of fruit and vegetables on the risk of cancers. Raw carrots showed risk reductions of 20% or more for both colon and rectum cancers (168). In addition, recently, Boggs et al. examined the relation of fruit and vegetable intake to breast cancer risk among more than 50,000 women aged between 21 and 69 years old. Although none of the total fruit, total vegetable, and total fruit and vegetable intakes were significantly associated with overall risk of breast cancer, there was inverse association noted between breast cancer risk and carrot intake ($P = 0.02$; (169)).

However, unexpectedly, in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study, male smokers who received the 20 mg of supplementation of β-carotene per day showed significant increases in lung cancer incidence (16%) and overall mortality (8%) (170). In another prevention trial (i.e., Beta-Carotene and Retinol Efficacy Trial), the effects of a combination of β-carotene (30 mg per day) and vitamin A (25,000 IU per day) on the incidence of cancers, were investigated. There were no statistically significant differences in the risks of other types of cancers but only lung cancer risk was significantly increased in the treatment group compared to the placebo group (28% increase in lung cancer incidence and 17% increase in total mortality; (171)).

Taken together, there are a few studies demonstrating chemopreventive potential of apiaceous vegetables but such effects were not elicited by the supplementation of β-carotene that are often found at the highest level in carrots (172), suggesting that other active compounds present in carrots (and possibly other apiaceous vegetables) might

be responsible for anti-cancer potency via different mechanisms (e.g., furanocoumarins; (173)). In fact, it was demonstrated that some furanocoumarins may be chemopreventive in animal models for lung cancer, mainly due to inhibitory effects on phase I enzymes thereby lowering activation of procarcinogens (174, 175). However, to our knowledge, no study has been done with regard to chemoprotective effects of furanocoumarins in an animal model for colon cancer.

Metabolism of furanocoumarins

A few studies have been done with regard to the metabolism of furanocoumarins; there are several studies regarding either 8-MOP or 5-MOP since these compounds are widely utilized for the treatment of psoriasis (176). Both 8-MOP and 5-MOP are extensively metabolized. Approximately 71% and 26% of administrated 8-MOP metabolites were recovered from urine and feces, respectively, while less than 1% of unchanged compound was found in the urine (177). The metabolism of these compounds is known to be principally mediated by CYP. Due to their ability to induce CYP, the elimination of 8-MOP was non-linear following pretreatment of rats with 8-MOP, which is called '*self induction*' (177, 178). Mays et al. identified 11 urinary metabolites of 8-MOP and it was indicated that 8-MOP is metabolized via *O*-demethylation of the 8-methoxy group, hydroxylation at position 5 to form a furanoderivative of coumaric acid (possibly by CYP), hydrolysis of the lactone ring, and oxidation of the furan ring (177).

In another study, when human subjects were orally administered 5-MOP (50 mg), 62% and 31% of 5-MOP metabolites were recovered from urine and feces, respectively, over five days; unchanged compound (i.e., parent 5-MOP) was very minor and similar to rats while most urinary metabolites were glucuronic acid conjugates (179). Further, there were other sulfated conjugates of psoralens found in urine, implying roles of SULT in the metabolism of furanocoumarins (180).

Apiaceous and phase I enzymes: CYP1A regulation

The biological roles regarding modulating effects of apiaceous vegetables (as well as furanocoumarins) have not been clearly understood. Several in vitro and in vivo studies using different furanocoumarin derivatives have demonstrated that this class of compounds may either induce or inhibit a wide range of CYPs such as CYP1A1 (181, 182), CYP1A2 (181, 182), CYP 2A5 (183), and CYP 6B4 (184).

Peterson et al. investigated the effects of constituents from apiaceous vegetables on human CYP1A2 activity using the 7-methoxyresorufin *O*-demethylation assay (185). In agreement with other studies, psoralen, 5-MOP, and 8-MOP inhibited hCYP1A2 in a dose dependent manner while other apiaceous constituents such as naringenin, caffeic acid, and chlorogenic acid did not inhibit the enzyme. Two-hour incubations of intact yeast cells with psoralen, 5-MOP, and 8-MOP improved cell viability against four-hour treatment with aflatoxin B1, suggesting that CYP1A2 inhibition by furanocoumarins may reduce mutagenicity of other carcinogens which require CYP1A2-mediated activation like aflatoxin B1 (185). Similar modulating effects of

apiaceous vegetables were demonstrated in humans as well. Lampe et al. administered a mixture of apiaceous vegetables (dill weed, celery, parsnips, parsley, and carrot) for six days and enzyme activities were determined using urinary CMR; the apiaceous feeding group showed a decrease in CYP1A2 activity compared to that of the basal group ($P< 0.02$; (150)).

There are several mechanisms that have been proposed regarding the modulation of CYPs by apiaceous vegetables and their active compounds. It has been suggested that furanocoumarins in apiaceous vegetables are mechanism-based inactivators of various CYPs (186). Interestingly, in addition to being potent inhibitors of CYPs, it was also demonstrated that furanocoumarins can act as inducers of CYP gene expression either via the AhR-pathway or an AhR-independent pathway indicating that these furanocoumarins can be considered as both inducers and inhibitors (187).

Apiaceous and phase II enzymes: UGT and SULT regulation

Few studies have been done investigating apiaceous vegetable effects on UGT1A1. As discussed above, Navarro et al. fed the combination of single dose cruciferous and apiaceous vegetables and observed a trend that combining the two vegetables increased UGT1A1 activity compared to the single dose cruciferous feeding (127). Since furanocoumarins have been shown to induce CYPs via the AhR-mediated pathway, we cannot rule out the possibility that UGT1A1 was induced by these compounds as well as other constituents including flavonoids in the vegetables given that UGT1A1 is also partly regulated by the AhR pathway (187, 188).

On the other hand, it was demonstrated that grapefruit juice can inhibit SULT1A1 and SULT1A3 using recombinant *Escherichia coli*. Of the tested furanocoumarins of grapefruit juice, only dihydroxybergamottin exhibited significant inhibition (189).

Intact vegetables versus purified compounds

There are multiple studies demonstrating that vegetables and active compounds may be chemopreventive via more than one mechanism (190-192); as discussed, modulation of biotransformation enzymes, either enhancing detoxification of carcinogens or suppressing activation of procarcinogens, through vegetable-rich diets might be one explanation. However, little investigation has been done to make a direct comparison in terms of chemopreventive effects between fresh vegetable and purified compound intervention. What makes it difficult to compare such biological potency is most likely due to the complexity of phytochemical profiles in intact vegetables. For example, although carrots showed significant anticancer activity in multiple studies (168, 169), the major compound in carrots (i.e., β-carotene) was shown to rather increase lung cancer incidence and mortality in certain populations (170, 171); such inconsistent results can be interpreted that either 1) beneficial effects of the food item, in this case carrots, may not be solely due to β-carotene, tested in the intervention trials, or 2) single compound intake failed to represent the chemopreventive potency of the intact vegetable consumption because of the lack of synergism achieved from various phytochemicals. In fact, the concept of synergy among compounds present in vegetables has become widely accepted as the

mechanism of chemoprevention against multiple cancers (193). Specifically, Wallig et al. examined if two breakdown products of glucosinolates (i.e., I3C and 1-cyano-2-hydroxy-3-butene) would interact synergistically to reduce the number of preneoplastic biomarkers in the liver after carcinogen exposure (aflatoxin B_1). The authors were able to demonstrate that the combination of these two compounds enhanced protection against aflatoxin B_1 in rats (194).

In addition, there are other factors that should be taken into consideration when interpreting results from the intervention of fresh vegetables and purified compounds. Above all, it is important to note that most active compounds found in vegetables are in the form of esters, glycosides, or polymers which cannot be directly absorbed through the small intestine; these compounds need to be hydrolyzed either by intestinal enzymes or colonic microflora to form aglycones. Furthermore, if the microflora is involved, the absorption efficiency is often altered because aglycones are also degraded by them as well. Consequently, the actual levels of phytochemicals, absorbed and reached at the target tissues would be affected by multiple factors. Therefore it is very possible that purified compounds may represent dissimilar bioavailability compared to that of fresh vegetables (195).

To summarize, it is reasonable to believe that both bioactive dietary components and food sources influence phase I and phase II biotransformation of carcinogens thereby possibly impacting cancer risk. However, it is still unclear 1) how active compounds present in vegetables interact with each other in regards to cancer prevention, 2) which intake forms would elicit more pronounced effects on biotransformation

enzymes and favor HAA detoxification, and 3) if modulation of biotransformation enzymes can fully explain the impacts of vegetables on cancer risk; to answer the questions and elucidate unknown underlying mechanisms, it seems to be logical to take a novel approach such as high throughput '*omics*' technologies which allow looking at the complicated interactions of nutrients within complex matrices and the individual genome (196).

Section 1-3. Metabolomics-Based Approaches

Overview

Metabolomics is a rapidly evolving tool across the biological sciences including the nutritional sciences (so called nutritional metabolomics). According to Jones et al., it was defined as '*the use of small molecule chemical profiling to integrate diet and nutrition in complex biosystems*' (196). Changes in metabolites are derived from differences in either food intake or an individual's metabolic condition. In this sense, it is reasonable to consider the metabolome as a surrogate of metabolic status as well as adaptation to changes in environmental exposures, including diet (197).

In general, there are two metabolomic approaches: an untargeted and targeted approach. In untargeted metabolomics studies (also known as unbiased metabolomics), the primary goal is to identify as many metabolites as possible to classify phenotypes based on the pattern of metabolites. On the other hand, in a targeted metabolomics study (known as biased metabolomics), the focus of the study is limited to either specific classes of chemicals or certain metabolites derived from predetermined metabolic pathways (198).

Applications to nutritional sciences

Diet itself is innately complex. Various constituents are from heterogeneous plant- and animal-based foods; furthermore, storage, processing, and preparation steps will

affect such nutrients as well. For example, as mentioned, the levels of phytochemicals (e.g., furanocoumarins) can be dramatically altered depending upon storage conditions (165, 166). In addition, there are numerous studies indicating that genetic variation of individuals significantly influence interactions between nutrients and the genome (199, 200) which provide a rationale for metabolomics approaches because metabolomics-based approaches allow investigations of the complicated interactions within a complex individual with their unique genome as well as specific environmental exposures. In contrast, the conventional framework in nutritional science is often criticized as it takes a too simplified approach (i.e., reductionism based on *Occam's razor*) (201); such approaches in nutritional science, based upon observed associations between nutrient intake and clinical symptoms with supporting mechanistic studies, could be effective but represent limitations in sensitivity to detect early/subclinical nutritional insufficiencies and therapeutic interventions in complex phenotypes (196, 201).

There are a number of nutritional studies that have taken advantage of metabolomics-based approaches to investigate the efficacy of nutrients as well as identify novel biomarkers for clinical outcomes. Recently, Deo et al. utilized an unbiased pathway analysis in order to understand the metabolic profiles in patients with impaired glucose tolerance (202). To be specific, the authors analyzed plasma metabolite profiles from the oral glucose tolerance test and then transformed the metabolite changes into the metabolic network. By doing that, the authors were able to find active modules of metabolites that were naturally grouped by system A and system L amino acid transporters and the osmolyte transporter SLC6A12. The contribution of

such transporter activities in plasma metabolite profiles was normally ignored in traditional analysis of metabolic pathways. Given that these transporters are involved in multiple human diseases, it was expected that this approach may ultimately contribute to improved disease classification and resolution of disease heterogeneity.

In another recent study, Wang-Sattler et al. also took a metabolomics approach to identify novel biomarkers of diabetes (203). In total, 140 metabolites were identified from fasting serum samples and significant metabolic variations in pre-diabetic individuals were noted (e.g., glycosylated hemoglobin levels). In addition, the authors addressed that altered levels of glycine and lysophosphatidylcholine would be predictors for impaired glucose tolerance as well as type 2 diabetes. They concluded that newly proposed biomarkers would aid to prevent type 2 diabetes at an early stage. There are other nutritional intervention studies that utilized metabolomics approaches in order to link food intake with metabolomic outcomes (e.g., tea (204), cocoa (205), and fish oil (206)).

Nonetheless, metabolomics-based approaches also have some limitations as well. Firstly, even though multiple studies indicate some correlations between metabolites, diets, and health outcome, there are a number of signals and metabolites that remain unknown. Further, targeted metabolomics approaches were often criticized as they only represent a subset of overall metabolites. Lastly, metabolomics applications are generally known as not as comprehensive as transcriptomics approaches (197).

Metabolite profiling for metabolomics

As defined, metabolomics is the comprehensive assessment of endogenous metabolites and identifies systematic metabolites from biological samples. However, one of the challenges of metabolomic studies is the innate complexity of the metabolome in biological matrices which include a number of other compounds including lipids, organic acids, as well as amino acids (198).

Recent technological advances in analytical instruments allowed the measurement of metabolites in small volumes of samples with improved limits of detection. Often chemometric statistical tools (e.g., principal component analysis and partial least squares) are also utilized to derive the integrative picture of metabolisms (198, 207). There are various analytical platforms used in metabolomics but clearly no single analytical method is perfect considering the numbers of metabolites and their different chemical properties (e.g., polarity and molecular weight). Therefore, various combinations of multiple analytical instruments have been widely employed for the analysis of metabolites.

Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) is one of the most common spectroscopic analytical techniques and advantageous for metabolomics studies because it is largely automated, reproducible, and not destructive so that samples can be utilized for further analyses. In addition, little sample preparation is required. Perhaps one of the greatest advantages of NMR analysis is that it provides the structural information,

thus unknown metabolites can be identified (198, 207). Lastly, the sensitivity of NMR does not rely on either pK_a or hydrophobicity of metabolites which makes this analysis a great choice for the wide range of organic compounds. However, compared to mass spectrometry (MS) based analyses, it represents relatively low sensitivity in measurement, making NMR inappropriate for the analysis of low-abundance metabolites (208).

There are several example studies that utilized NMR-based approaches (209, 210). For instance, Jung et al. applied ^{1}H-NMR metabolomics approach along with multivariate statistical analysis (e.g., principal components analysis and orthogonal partial least-squares discriminant analysis) to investigate the altered metabolic pattern in plasma and urine from patients with cerebral infarctions (209). The plasma of stroke patients was characterized by the increased excretion of lactate, pyruvate, glycolate, and formate, and the decreased levels of glutamine and methanol. Further, in the urine collected from stroke patients, citrate, hippurate, and glycine were decreased compared to those of normal subjects. It was concluded that such changes in metabolites detected from plasma and urine of patients were associated with anaerobic glycolysis, folic acid deficiency, and hyperhomocysteinemia. In the study, the metabolomics approach utilizing NMR was found to be useful for the early diagnosis of cerebral infarction and development of novel metabolic biomarkers.

Mass spectrometry

Mass spectrometry (MS), often coupled with other analytical techniques (e.g., liquid chromatography (LC)), has gained increasing interest in high throughput metabolomics. Since MS detects chemicals (including metabolites) as ions in the gas phase, it does present practical limitations in terms of quantification, meaning different chemicals possess different ionization conditions in the gas phase. Furthermore, in order to ionize metabolites, energy is required in the instrument; this might result in structural changes of chemical species. Nonetheless, MS is advantageous and widely utilized because of its high sensitivity as well as wide range of covered metabolites (196, 207).

MS is able to analyze biological samples either via direct injection or following chromatographic separation. Both methods have merits and drawbacks. Direct injection into MS provides a fast analysis for a large number of metabolites which make this suitable for metabolic fingerprinting, a global analysis to discriminate between samples from different biological status (e.g., disease versus control). Yet, the direct injection of biological sample may have low ionization efficiency. In addition, a single metabolite can form more than one ionic form depending upon the presence of other chemicals. These limitations can be overcome by coupling the MS with various separation techniques (196, 207). Gas chromatography (GC) and LC are most commonly utilized for the separation of metabolites with different chemical properties. MS coupled with GC is often used for the analysis of hydrophobic metabolites (e.g., fatty acids (211)) as well as organic acids (e.g., lactic acid (212)). Although GC provides efficient and reproducible analysis, additional derivatization steps are required in order to make analytes volatile. In contrast, LC coupled with MS

does not need sample preparation steps (e.g., derivatization) thus it is more suitable for the analysis of labile and non-volatile metabolites in their native forms (207).

Recently, ultra performance liquid chromatography (UPLC) was introduced, employing porous particles with less than 2 μm internal diameter. It results in higher peak capacity, better specificity, and high throughput capability compared to those of conventional HPLC technology. In particular, quadrupole-time-of-flight (QTOF)-MS interfaced with UPLC is known as a powerful tool to analyze and identify metabolites in complex biological matrices at the levels of less than 5 ppm (207). Multiple studies profiled the metabolic fingerprints using UPLC-QTOF-MS to investigate the efficacy of Chinese traditional medicine (213, 214), evaluate toxicity (215), and develop potential biomarkers for diseases (e.g., Alzheimer's disease (216) and various cancers (217, 218)).

Other analytical approaches

As summarized, NMR, GC-MS, and LC-MS are largely utilized for large scale analysis of metabolites, but metabolomics is not limited to these techniques. There are other alternatives or combinations of existing techniques that have been employed to increase metabolite coverage and improve quantification. For instance, the combination of MS (tandem MS) has become an important option for metabolomics; tandem MS is able to dissociate metabolite ions from biological matrices and identify metabolite structures. Further, other types of analytical platforms such as electrochemical detection (as an alternative to MS; (219)), and fourier transform

infrared spectroscopy (as an alternate spectral method; (220)) were exploited in various studies.

Metabolomics to study PhIP metabolism

To our knowledge, two studies applied a metabolomics-based approach regarding PhIP metabolism; both studies were biased metabolomics studies in humanized mice and humans.

First, Chen et al. investigated the metabolism of PhIP in CYP1A2-humanized mice using a metabolomics approach (51); in the study, urinary metabolites were analyzed using UPLC-QTOF-MS and then the urinary metabolome was characterized in a principal components analysis model. The authors found eight novel metabolites out of 17 urinary PhIP metabolites. Further analysis of the multivariate data showed that there are species differences (i.e., catalytic activity of CYP1A2) in PhIP metabolism between humans and mice. Lastly, CYP1A2-null mice were able to activate PhIP (via N^2-hydroxylation of PhIP) indicating potential roles of other CYP subfamilies (e.g., CYP2C). In summary, the combination of high resolution LC-MS technology along with statistical analysis was effective to investigate the metabolism of xenobiotics as well as elucidate novel metabolites (51).

In another study, Gu et al. utilized a triple stage quadrupole MS to profile PhIP metabolites in human urine. To be specific, enriched urine samples from human subjects who consumed cooked beef patties were subjected to LC-MS/MS analysis.

Similar to other previous observations, the authors confirmed the prominent role of CYP1A2 in the metabolism of PhIP in humans (221). Further, less than 1% of unaltered PhIP was detected in urine indicating the extensive metabolism of PhIP. Interestingly, it was noted that CYP1A2 contributes to other HAA metabolism; although it catalyzed the oxidation of the exocyclic amine group (i.e., N^2 position of PhIP), it catalyzed the detoxification of MeIQx via oxidation of the 8-methyl group.

To summarize, as discussed, metabolomics-based approaches provide promising opportunities to discover and validate novel biomarkers as well as to elucidate the metabolic pathways of carcinogens possibly present in foods (e.g., PhIP). Nutritional metabolomics is rapidly evolving as an experimental approach in the nutrition field and known to be effective to understand complex interactions between diet and nutrients, and individuals' genome as it assesses systematic metabolites from the biological system. Thus, there is a need to expand nutritional metabolomics to cover potential direct or surrogate biomarkers of health and disease along with advances in analytical techniques and instruments.

Section 1-4. Summary and Specific Aims

According to the American Cancer Society's most recent report, there were 141,210 new cases of colorectal cancer (101,700 cases of colon cancer and 39,510 cases of rectal cancer) for 2011, making it the third most common cancer diagnosed in both men and women in the United States (222). Multiple lines of evidence demonstrate the involvement of environmental factors, including diet, in the pathogenesis of colorectal cancer. For example, migrant data shows that in populations coming from low-risk to high-risk countries, the cancer mortality (e.g., colorectal cancer) of the migrants eventually reaches that of the adopted country (223).

HAA are commonly found in the environment, principally through the consumption of overcooked meats and fish. Regarding the impact of HAA on health outcomes, several epidemiologic studies showed a positive association between HAA intake and colorectal cancer risk (17, 224). Of the identified HAA, PhIP is one of the most mass abundant HAA in food (20, 34, 37). As discussed, PhIP is metabolized extensively in the body by various biotransformation enzymes including CYPs, NATs, UGTs, GSTs, and SULTs (225), suggesting that modulation of these enzymes would influence HAA metabolism as well as their carcinogenic potential.

Bioactive compounds present in various vegetables may play important roles in the process of carcinogenesis (226). Specifically, cruciferous and apiaceous vegetables modulate biotransformation enzymes that are responsible for metabolizing various carcinogens (e.g., PhIP) (150, 227). Apiaceous vegetables (e.g., celery and parsnips)

and their bioactive compounds inhibit CYP1As (PhIP-activating enzymes (185)) while cruciferous vegetables (e.g., broccoli and cabbage) and their bioactive compounds induce both activating and detoxifying enzymes (CYP1As and UGTs (127, 147)). To our knowledge, it is unknown if the combination of apiaceous and cruciferous vegetables will exert synergistic effects on PhIP metabolism due to their complementary actions on xenobiotic biotransformation enzymes. Furthermore, it is undiscovered if there are any differences between consumption of intact vegetables and consumption of corresponding doses of active compounds in terms of chemopreventive effects against PhIP. Thus, our specific aims in the following rat feeding experiments were:

1. To determine the effects of cruciferous, apiaceous, and the combination of both vegetable types on PhIP metabolism.
   - ✓ Measurement of activities and expression of CYP1A1, CYP1A2, UGT1A1, and SULT1A1; analysis of PhIP urinary metabolites
2. To investigate the effects of cruciferous and apiaceous vegetables, either alone or combined, on colon cancer risk markers.
   - ✓ Measurement of PhIP-DNA adduct levels in colon tissue
3. To compare the effects of the intact vegetable consumption on the above endpoints with the effects of consumption of the respective purified phytochemicals in the vegetables.

Table 1-1. Representative mechanisms of fruit, vegetable, and their phytochemical constituents in colon cancer prevention

Food item (or phytochemical)	**Mechanism of action**	**References**
Black raspberry and strawberry	Induction of apoptosis	(228)
Grape seed	Inhibition of cell proliferation	(229)
Epigallocatechin gallate	Inhibition of cyclooxygenase pathway	(230)
Black raspberry and soybean	Prevention of angiogenesis	(231, 232)
White currant	Modulation of Wnt/β-catenin pathway	(233)
Garden cress	Modulation of carcinogen metabolism	(131)

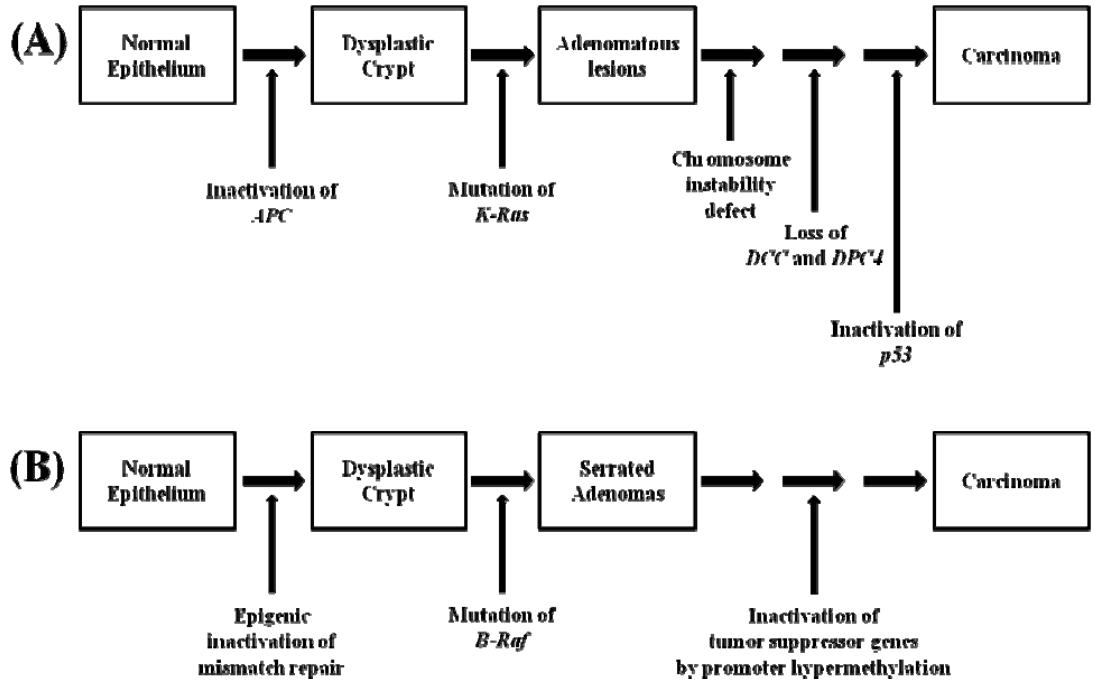


Figure 1-1. Genetic model of colon cancer

The adenoma to carcinoma sequence (A) and serrated adenoma to carcinoma theory (B); Above schemes were adapted and reproduced from the reference (234).

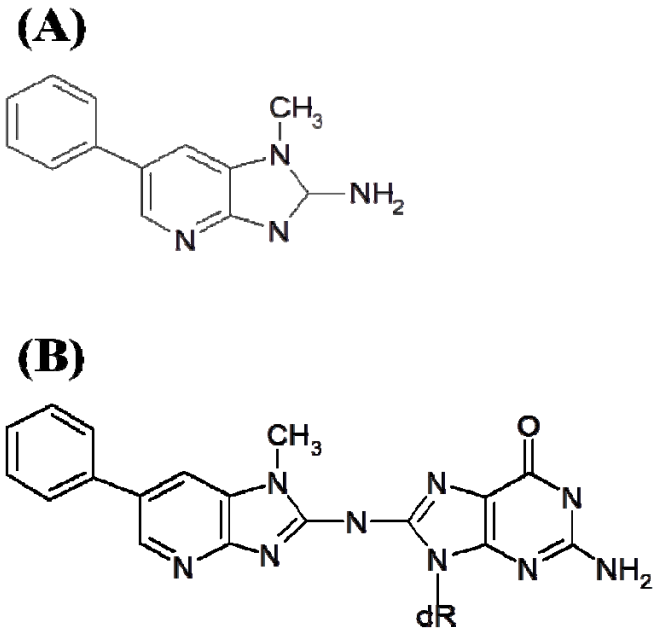


Figure 1-2. The structure of PhIP and PhIP-guanine adduct

PhIP (A) and PhIP-guanine adduct (B)

CHAPTER 2. MANUSCRIPT I: EFFECTS OF CRUCIFEROUS AND APIACEOUS VEGETABLE INTAKE ON PHIP METABOLISM AND GENOTOXICITY IN WISTAR RATS

Introduction

Heterocyclic aromatic amines (HAA) are commonly found in the environment and humans are exposed to them principally through the consumption of overcooked meats and fish (20). Regarding the impact of HAA on health outcomes, a number of epidemiologic studies demonstrated a positive association between HAA intake and colorectal cancer risk (17, 224). Of the identified HAA, PhIP is one of the most mass abundant HAA in food (20, 34). It was demonstrated that PhIP induced chromosomal aberrations and sister-chromatid exchanges (66), formed DNA adducts and aberrant crypt foci in colon mucosa (67), and caused colon and mammary carcinomas (68, 69) in multiple animal studies.

PhIP itself is not genotoxic, but requires an activation step in order to become carcinogenic. Cytochrome P450 (CYP) 1As, and primarily CYP1A2, are known as the primary enzymes that generate N^2-hydroxy PhIP (47). Hydroxylation of the exocyclic amine of PhIP (i.e., N^2 position) is known as an initial step to activate PhIP because it can be further esterified by *N*-acetyltransferase (NAT) and sulfotransferase (SULT) to N^2-acetoxy PhIP and N^2-sulfonyloxy PhIP, respectively; these metabolites create DNA adducts via electrophilic nitrenium ion formation (50). In contrast, conjugation by phase II enzymes (e.g., uridine 5'-diphospho-glucuronosyltransferase (UGT) 1A1 and glutathione *S*-transferase A1-1) can usually lead to production of safe metabolites and efficient excretion of PhIP (20, 52, 53).

Bioactive compounds present in various vegetables are known to play an important role in carcinogenesis (226). One of the proposed mechanisms is via modulation of biotransformation enzymes that are responsible for metabolizing carcinogens (e.g., PhIP; (150, 227)). Specifically, compounds present in apiaceous vegetables (e.g., celery and parsnips) inhibit CYP1As (PhIP-activating enzymes, (185)) while cruciferous vegetables (e.g., broccoli and cabbage) and their active compounds induce both activating and detoxifying enzymes (CYP1As and UGTs, (127, 147)). To our knowledge, it is unknown if the combination of apiaceous and cruciferous vegetables will result in synergistic detoxification of PhIP due to their complementary actions on these biotransformation enzymes. Therefore, in this study, we investigated the effects of cruciferous, apiaceous, and the combination of both vegetable families on PhIP metabolism by analyzing PhIP metabolizing biotransformation enzyme activity and expression, as well as changes in the PhIP urinary metabolite profile. Further, PhIP-DNA adduct levels in the colon were measured to evaluate any protection of the vegetable groups, either alone or combined, against PhIP.

Materials and methods

Chemicals and reagents

Ethoxyresorufin, methoxyresorufin, β-nicotinamide adenine dinucleotide phosphate reduced form tetrasodium salt, resorufin sodium salt, bovine serum albumin, Bradford reagent, 2-naphthol, adenosine 3’-phosphate 5’-phosphosulfate, 4-nitrophenyl sulfate, dimethyl sulfoxide, uridine 5'-diphospho-glucuronic acid, glycerol, DNase I (Type IV,

bovine pancreas), nuclease P1 (*Penicciliumcitrinum*), phosphodiesterase I (*Crotalusadamanteus venom*), and alkaline phosphatase (*Escherichia coli*) were purchased from Sigma-Aldrich (St. Louis, MO). Tris base and ethylenediamine tetraacetic acid disodium salt were from Fisher Scientific (St. Louis, MO). The 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane was purchased from Phoenix Pharmaceutical (Burlingame, CA). All other chemicals were of analytical grade.

Animals

Male Wistar rats, 100-125 g body weight, were obtained from Harlan Laboratories (Indianapolis, IN) and housed in wire-bottom stainless steel cages. Rats were fed the American Institute of Nutrition (AIN)-93G diet, a standard diet, for five days in order to adapt to a powder diet. Animals were allowed free access to water and diets. All experimental procedures were approved by the University of Minnesota Committee on Animal Care.

Experimental design

A total of 52 rats were divided into five groups: negative control, positive control, cruciferous only supplementation (CRU), apiaceous only supplementation (API), and combined cruciferous and apiaceous supplementation (C+A). After six days of feeding, the positive control group and all three vegetable supplemented groups were given an intraperitoneal injection of PhIP (10 mg of PhIP/kg body wt, dissolved in dimethyl sulfoxide). The negative control group received dimethyl sulfoxide only.

Urine was collected from all five groups for 24 hours starting immediately after the injections. On day seven, animals were sacrificed and harvested tissues were stored at -80°C for later analysis.

Study diets

The AIN-93G diet was used for the negative and positive control groups. For the vegetable supplemented diets, vegetables were purchased from a local market. CRU included fresh watercress, broccoli, and cabbage (21%, wt/wt); API consisted of fresh celery and parsnips (21%, wt/wt); C+A included all of the aforementioned vegetables (10.5% cruciferous vegetables and 10.5% apiaceous vegetables, wt/wt). All vegetables used in the study were organically grown. To prepare the diets, vegetables were ground using a food processor (Cuisinart Deluxe, East Windsor, NJ) and added into the powder diet (i.e., AIN-93G). All vegetable-supplemented diets were balanced for macronutrients to minimize any confounders. Detailed diet compositions are summarized in Table 2-1. Once vegetable supplemented diets were made, diets were divided into separate plastic bags for each day of the feeding period and stored at -80°C until use. Each bag was thawed daily and provided to rats. Food intake was determined from spillage and diets remaining in the food cups for 24 hours. Food intake was measured three times.

Analysis of total glucosinolates in cruciferous vegetables

Total glucosinolates were analyzed using a modified method published previously (235). In brief, vegetables (i.e., watercress, broccoli, and cabbage) were frozen in liquid nitrogen, lyophilized, ground, and then treated with 100% hot methanol for 20 minutes at 80°C in a heating block. Water was added to make 80% methanol and extracted for 30 minutes. The samples were centrifuged for 20 minutes at 18,000 × *g*. The remaining pellet was extracted again and pooled with first extracts. An anion exchange column was activated with methanol followed by water, 0.5 M sodium acetate (pH 4.6), and water. Glucosinolates were reconstituted in water and applied to the column. Glucosinolates were eluted with 0.5 M sodium acetate and hydrolyzed with 2 M sodium hydroxide. This alkaline degradation releases 1-thioglucose from glucosinolates which react with ferricyanide. The absorbance was measured at 420 nm at two minutes after addition of the ferricyanide solution; the final values were adjusted for interfering compounds. Sinigrin were used to construct the standard curve.

Analysis of furanocoumarins in apiaceous vegetables

Furanocoumarins were analyzed using a modified method from Ostertag et al. (166). Briefly, celery and parsnips were homogenized in a food processor and extracted with five volumes of 40% diethyl ether in water (v/v). Samples were sonicated for two minutes and centrifuged for 15 minutes at 3,500 × *g*. The extraction and centrifugation processes were repeated four more times and supernatants were pooled. Collected organic phase was evaporated under nitrogen and all sample vials were covered using aluminum foil to avoid light exposure. Extracts were reconstituted in 60% acetonitrile in water (v/v) and applied to C18 columns then to silica columns.

The eluates were dried under nitrogen, re-suspended in 50% acetonitrile in water (v/v), and analyzed using an HPLC equipped with UV diode array detector at 310 nm. Furanocoumarins were separated using a reverse phase C18 column (Agilent Poroshell 120 EC-C18, 150 × 4.6 mm, 2.7 μm). Initial mobile phase composition was 45% of 10 mM phosphoric acid and 55% methanol (v/v). The flow rate was 0.5 mL/min and the isocratic method was used for the separation over 45 minutes. Furanocoumarins were identified using authentic standards, retention time, and UV spectrum. The recovery rate was calculated using one of the furanocoumarins standards (i.e., 8-MOP).

The r-squared values of calibration curves were all greater than 0.999 within the tested range of each furanocoumarin. The recovery rate of internal standard from celery and parsnips was 83.3 ± 6.6% and 87.1 ± 2.7%, respectively. The final quantification of furanocoumarins was adjusted using these recovery rates.

Preparation of hepatic microsomes and cytosol

At the end of the feeding period, rats were fasted for 12 hours prior to sacrifice. Animals were anesthetized with 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane in a glass chamber and liver microsomes isolated as described elsewhere (236). Briefly, livers were perfused with ice-cold 50 mM Tris base-150 mM potassium chloride buffer (pH 7.5) and homogenized in Tris base-potassium chloride buffer containing 1 mM phenylmethylsulfonyl fluoride (final concentration). The tissue homogenates were centrifuged at 10,000 × *g* and supernatants from the first

centrifugation were further centrifuged at 105,000 × *g* for 70 minutes. The lipid layers of the supernatant were aspirated and the supernatants were used for cytosolic assays. The microsomal pellets were re-suspended in 50 mM Tris base-glycerol buffer (pH 7.5) and the microsomal fractions were flushed with nitrogen to prevent oxidation. These cytosolic and microsomal preparations were stored at -80°C until further analysis.

Measurement of protein content

The protein concentration of liver microsome and cytosol preparations were measured by the Bradford method using a protein reagent kit (Sigma-Aldrich, St. Louis, MO; (237)).

Measurement of CYP1A1 and CYP1A2 activity

Microsomal hepatic CYP1A1 and CYP1A2 activity were measured using the 7-ethoxyresorfurin *O*-deethylation and 7-methoxyresorufin *O*-demethylation assays, respectively (238, 239). In brief, stored microsomal fractions were washed by ultracentrifugation at 105,000 × *g* with 100 mM sodium pyrophosphate-10 mM ethylenediamine tetraacetic acid buffer for 70 minutes; pellets were re-suspended in 58 mM Tris base buffer and homogenized in advance of measurement of CYP1A1 and CYP1A2 activity. The enzymatic reaction mixture (1 mL per sample) contained 5μM corresponding substrates, 0.05 M Tris base buffer pH 7.5, 0.025 M magnesium chloride, 500 μM β-nicotinamide adenine dinucleotide phosphate reduced form

tetrasodium salt, and microsomal protein (200 μg/mL, final concentration). The reaction mixture was aliquotted into 96 well microtiter plates (4 wells per sample) and measured using a microplate reader (Biotek Inc., Winooski, VT; excitation: 530/25 nm; emission: 590/35 nm).

Measurement of SULT1A1 activity

Cytosolic SULT1A1 activity was measured using a previously described method (240). The enzymatic reaction mixture (1 mL per sample) contained 50 mM potassium phosphate buffer (pH 6.5), 5 mM magnesium chloride, 20 μM adenosine 3'-phosphate 5'-phosphosulfate, 5mM 4-nitrophenyl sulfate, 0.1 mM naphthol as a substrate, and cytosolic protein (400 μg/mL). The enzymatic mixture was incubated for 15 minutes at 37ºC and the reaction was terminated with 0.25 M Tris base-hydrogen chloride buffer (pH 8.7). *p*-nitrophenol was quantified colorimetrically at 405 nm and calculated using the extinction coefficient (18,200 cm^{-1} M^{-1}) since the concentration of *p*-nitrophenol directly reflects 2-naphthylsulfate, a product of SULT1A1 (Varian Cary 50 UV-Vis spectrophotometer, Agilent Technologies Inc., Palo Alto, CA).

Measurement of UGT1A1 activity

Microsomal UGT1A1 was determined using a slightly modified method (241). Microsomal fractions were washed as described above. The enzyme reaction mixture (200 μL per sample) included 600 μM 4-methylumbelliferone, 5 mM uridine 5'-

diphospho-glucuronic acid, 4 mM magnesium chloride in 100 mM phosphate buffer (pH 7.4) with 50 µg of microsomal protein. The reaction ran for 90 minutes at 37°C and was terminated by addition of 24% perchloric acid. After termination, the mixture was centrifuged at 5,000 × *g* for 10 minutes and the supernatants were analyzed by HPLC (Gilson Inc., Middleton, WI). In order to analyze the final product of reaction (i.e., 4-methylumbelliferone glucuronide), 20 µL of the supernatant fraction was injected to the HPLC system and separation of 4-methylumbelliferone glucuronide was performed on a 5 µm Ultrasphere® ODS C18 column (4.6 × 250 mm; Hichrom, Berkshire, UK). Initial mobile phase composition was 15% acetonitrile in 25 mM phosphate buffer (pH 3.1) and gradually increased up to 50% acetonitrile over 5 minutes with a flow rate of 1.0 mL/min. Column eluant was monitored at 316 nm.

Assessment of protein expression

Protein expression of CYP1A1, CYP1A2, SULT1A1, and UGT1A1 in liver was determined using enzyme-linked immunosorbent assay kits (Uscn Life Science Inc., Houston, TX) according to the manufacturer's instructions. Washed microsomes were prepared to serial dilutions and applied to enzyme-linked immunosorbent assay microtiter plates to measure CYP1A1, CYP1A2, and UGT1A1 expression. Cytosolic fraction was used for measuring SULT1A1 expression. The enzyme-substrate reaction was measured at a wavelength of 450 nm and the protein concentrations were determined using standard curves (Biotek Synergy HT microplate reader, Biotek Inc., Winooski, VT).

LC-ESI-MS/MS analysis of urinary PhIP metabolites

In order to investigate the modulating effects of vegetable feeding on PhIP excretion, the urinary metabolites of PhIP were analyzed using a metabolomics-based approach. Urine samples were centrifuged at 18,000 × *g* for 5 minutes to remove any contaminants. The supernatants were diluted with 50% of acetonitrile in water and centrifuged again at 18,000 × *g* for 12 minutes to precipitate proteins and particles. For LC- tandem mass spectrometry (MS/MS) analysis, a 5 μL aliquot of urine sample was injected into a UPLC quadrupole-time-of-flight (QTOF) system (Waters Corp., Mildford, MA). An Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm; Waters Corp., Mildford, MA) was used to separate urinary PhIP metabolites. The flow rate was 0.5 mL/min with a gradient ranging from water to 95% aqueous acetonitrile containing 0.1% formic acid over 10 minutes. The QTOF system was operated in the positive electrospray ionization (ESI) mode and the capillary voltage and cone voltage were 3.2 kV and 30 V, respectively. Nitrogen was used as both the cone gas (50 L/hr) and the desolvation gas (700 L/hr), and argon was used as the collision gas. Mass chromatograms and mass spectral data were analyzed using MetaboLynx software to identify PhIP metabolites using the mass difference from the PhIP protonated molecular ion ([M + H]+): 225.1140 m/z). The structural identity of metabolites was elucidated based on the fragmentation pattern from MS/MS analysis and confirmed by comparison with reported fragmentation patterns (51) with a collision energy ranging from 15 to 35 eV.

Methyl transferase metabolism of PhIP in human liver S9 fraction

The reaction mixture contained 34 μM of *S*-adenosyl methionine, 1 mM of PhIP and S9 fraction protein (60 μg) in 50 mM Tris base buffer. After 60 minutes of incubation at 37˚C, the reaction was terminated by adding 50% of acetonitrile and then centrifuged at 18,000 × *g* for 12 minutes to remove proteins and particles. Once precipitated, supernatant was analyzed using the QTOF system as described above.

Measurement of DNA adducts

Preparation of colon tissue. After the animals were sacrificed, colon tissue was dissected and flushed with ice-cold phosphate buffered saline (pH 7.4). The tissue was trimmed to remove adipose tissue and opened lengthwise. Harvested colon tissues were frozen in liquid nitrogen and stored at -80°C until analyzed.

Isolation and enzymatic digestion of DNA. DNA was isolated from rat colon tissues using the Qiagen Gentra Puregene DNA Purification Kit with manufacturer's instructions (Qiagen, Hilden, Germany). The concentration of extracted DNA was determined using a UV spectrophotometer (Bio-Rad, Hercules, CA) assuming a concentration of DNA (50 μg/mL) is equal to 1.0 absorbance unit at 260 nm (242). The isotopically labeled internal standard, [^{13}C]-dG-C^{8}-PhIP, was added into DNA at a level of 10 adducts per 10^{7} nucleotides prior to enzymatic digestion. Isolated DNA (50 μg) was treated with DNase I, followed by nuclease P1, and then further digested using alkaline phosphatase and phosphodiesterase, under conditions described elsewhere (243, 244).

Efficacy test of enzymatic digestion of DNA. Hydrolyzed DNA (4 μg) was injected into an HPLC system (Gilson, Middleton, WI) and monitored at 260 nm to validate the efficacy of enzymatic digestion of DNA. A Phenomenex Synergy 4μm Fusion-RP 80Åcolumn (150 × 4.6 mm; Phenomenex Inc., Torrance, CA) was employed for separation with a linear gradient starting at 95% of 20 mM ammonium acetate (pH 4.5) and 5% of acetonitrile increasing up to 15% of acetonitrile over 15 minutes. Each nucleoside standard (50 μg of each nucleosides/mL) as well as all the standards of combined nucleosides (200 μg of combined nucleosides/mL) were prepared and injected into the system. The injection volume was 20 μL and flow rate was 1 mL/min.

Quantification of DNA adducts using online column-switching LC-ESI-MS/MS. The PhIP-DNA adduct levels in colon tissue were determined using the online column-switching LC-MS/MS system as described previously (244). Briefly, the Eksigent nanoLC Ultra system (Dublin, CA) was interfaced with a triple mass spectrometer (Thermo Fisher, San Jose, CA). A Waters Symmetry trap column (180 μm × 20 mm, 5 μm; Mildford, MA) was employed for PhIP-DNA adduct enrichment. The analytical column for separation was a C18 AQ (0.3 × 150 mm, 3 μm) from Michrom Bioresources (Auburn, CA). Digested DNA was injected into the trap column first and washed with 0.2% formic acid in 10% acetonitrile at a flow rate of 8 μL/min for 5 minutes. The trap column was then back-flushed into the analytical column followed by a linear gradient starting at 0.01% formic acid containing 10% acetonitrile and arriving at 0.01% formic acid in 95% acetonitrile at 28 minutes. The flow rate was 8 μL/min. In order to minimize the carry over between runs, the injection needle was

washed with 0.01% formic acid containing 10% acetonitrile in water, 0.01% formic acid containing 25% acetonitrile in water, and 100% dimethyl sulfoxide, consecutively after each analysis. The DNA adduct levels were measured at MS^3 scan stage in the positive ionization mode with an ESI source. The mass spectral parameters were optimized as described elsewhere (245).

Calibration curves and method validation. Calibration curves were constructed with the ratio between unlabeled PhIP-DNA adducts (ranged from 0 to 30 adducts per 10^7 nucleotides) and isotopically labeled internal standard (i.e., [^{13}C]-dG-C^8-PhIP; set at 10 adducts per 10^7 nucleotides). The r-squared value of the calibration curve was higher than 0.995. To validate the method, DNA (50 μg) isolated from the control group without PhIP treatment (i.e., negative control) was spiked with a known amount of labelled internal standard. Then, unlabelled PhIP-DNA adducts were added at levels of 0, 5, 10, and 15 adducts per 10^7 nucleotides into digested DNA. The method validation samples were done in triplicate at each level.

Statistical analysis

All results were expressed as least squares mean ± standard error (SE). The data were analyzed by one-way analysis of variance using SAS (SAS institute, Cary, NC). A *p*-value less than 0.05 was considered statistically significant. The correlation between metabolites and PhIP-DNA adduct was analyzed utilizing linear regression analysis.

Results

Food intakes, tissue weight, and body weight are summarized in Table 2-2. Food intake was determined from the spillage and diets remaining in the food cup for 24 hours; it was measured three times during the one week feeding period. There were differences in food intake for each of the vegetable-supplemented diets (i.e., CRU, API, and C+A diets) when compared to that of the positive control group (Table 2-2).

The total glucosinolate content of the CRU diet was 182.6 mg/kg diet, and thus half that for the C+A diet. The total glucosinolate concentration was highest in watercress followed by cabbage and broccoli (4.58 mg of glucosinolates/g of watercress (fresh wt), 0.46 mg of glucosinolates/g of cabbage (fresh wt), and 0.17 mg of glucosinolates/g of broccoli (fresh wt). The respective concentrations of 5-MOP, 8-MOP, and isopimpinellin were 0.55, 0.51, and 0.69 μg/g in celery (fresh wt), and 11.0, 5.2, and 23.0 μg/g in parsnips (fresh wt). Hence, levels of these furanocoumarins combined in the API and C+A diet were 4.3 mg/kg diet and 2.15 mg/kg diet, respectively.

Initial body weight of animals was between 100-125 g and final body weight was measured right before sacrifice. Liver weight was measured after perfusion with ice-cold Tris base buffer, and colon weight was determined after flushing with ice-cold phosphate buffered saline to remove any contents. Overall, there were no differences in body weight gain or tissue weight amongst all diet groups (Table 2-2).

Effects of vegetables on activity and expression of CYP1A1 and CYP1A2

Compared to the positive control, the CRU diet increased CYP1A1 and CYP1A2 activity (P=0.001 and P<0.0001, respectively, Table 2-3). The C+A diet also increased CYP1A1 and CYP1A2 activity compared to the positive control (P=0.013 and P<0.0001, respectively). The API diet increased CYP1A2 activity (P=0.005), but not CYP1A1 activity. There was no effect on enzyme expression by any vegetable-supplemented diet when compared to the positive control; however, a difference between the negative and positive control in CYP1A1 expression was observed (P=0.0305). When compared to the negative control, CYP1A1 expression was increased by all vegetable diets while CYP1A2 expression was increased by only the API and C+A diets (Table 2-3).

Effects of vegetables on activity and expression of UGT1A1 and SULT1A1

Both the CRU and API diets increased UGT1A1 activity (approximately 20.7% and 27.8%, respectively) while there was no effect observed in the combination group. Further, there was no difference in the expression of UGT1A1 from any vegetable group. For SULT1A1, protein expression was increased 42.8% by the CRU diet but no effect on activity was observed. API and C+A diets had no effect on SULT1A1 activity or expression.

LC-ESI-MS/MS analysis of urinary PhIP metabolites

We identified PhIP (unmetabolized; PhIP (I)) and 16 metabolites in urine samples. Structures of PhIP urinary metabolites and their proposed metabolic pathways are summarized (Fig. 2-1) (51). Metabolites and their retention times are summarized in Table 2-4. Of the 16 metabolites, seven metabolites were glucuronidated (PhIP-N^3-glucuronide (XI), PhIP-N^2-glucuronide (XII), 4'-hydroxy-PhIP-glucuronide (XIII), 5-hydroxy-PhIP-glucuronide (XIV), 4'-hydroxy-PhIP-N^2-glucuronide (XV), N^2-hydroxy-PhIP-N^2-glucuronide (XVI), and N^2-hydroxy-PhIP-N^3-glucuronide (XVII)), three metabolites were sulfated (4'-hydroxy-PhIP-sulfate (VIII), 5-hydroxy-PhIP-sulfate (IX), and 5,4'-dihydroxy-PhIP-sulfate (X)), and three metabolites were methylated (methyl-PhIP (II), N^2-methyl-PhIP (III), and N^2-methyl-4'-hydroxy-PhIP (VII)). Interestingly, metabolite II (methyl-PhIP) did not match any fragmentation pattern from previously reported data. The relative composition of urinary PhIP metabolites was calculated using the percentage of the peak area of each metabolite per total peak area (i.e., PhIP and all identified PhIP metabolites, Fig. 2-2 (A)). The API diet increased two metabolites (VII and XII) and decreased one metabolite (IV) compared to the positive control group. For the CRU diet group, compared to the positive control, two methylated metabolites were lower (II and VII) and six metabolites were higher (X, XII, XIV, XV, XVI, and XVII). Parent compound (I) and five urinary metabolites (III, V, VI, XI, and XIII) did not differ from positive control regardless of which vegetables were supplemented.

Depending upon their metabolic pathways as well as types of biotransformation enzymes involved, urinary PhIP metabolites were categorized into five groups: methylated PhIP metabolites (II, III, and VII), 5-hydroxylated PhIP metabolites (VI,

IX, X, and XIV), sulfated PhIP metabolites (VIII, IX, and X), glucuronidated PhIP metabolites (XI, XII, XIII, XIV, XV, XVI, and XVII), and CYP1A-mediated PhIP metabolites (IV, V, VII, VIII, X, XIII, XV, XVI, and XVII; Fig. 2-2 (B)). Methylated PhIP metabolites overall were increased by the API diet. On the other hand, both the CRU diet and C+A diet decreased methylated PhIP metabolites and increased 5-hydroxylated, sulfated, glucuronidated, and CYP1A-mediated PhIP metabolites. Of note, sulfated PhIP metabolites were highest in C+A group (Fig. 2-2 (B)).

Method validation for PhIP-DNA adducts measurement

The enzymatic efficacy of DNA digestion was assessed using HPLC. Each nucleoside was confirmed with the retention time of reference standards (i.e., deoxynucleosides; Fig. 2-3). Further, the accuracy and precision of the method were tested with the addition of known amount of internal standard into the negative control tissue (Table 2-5). It was unnecessary to estimate the day to day deviation of the instrument in the study as all samples were analyzed in a single sequence. The intra-assay coefficient of variation was 5.5%, 7.3%, and 6.5% for 5, 10, and 15 adducts per 10^7 nucleotides, respectively (Table 2-5).

Measurement of PhIP-DNA adducts in colon tissues

There was a 20% reduction in PhIP-DNA adducts by the API diet compared to the positive control group (P=0.049). The CRU diet trended towards a reduction in PhIP-

DNA adducts (approximately, 17% reduction, P=0.072). No effect was noted by the C+A diet.

We also assessed the correlation between urinary metabolites and PhIP-DNA adducts in the colon tissue. There were common correlations noted, meaning that sulfated PhIP metabolites and CYP1A-mediated PhIP metabolites were generally positively correlated with PhIP-DNA adducts (P=0.0071 and P=0.0326, respectively) while methylated PhIP metabolites were negatively correlated with PhIP-DNA adducts (P=0.005, Table 2-6).

Discussion

In this study, the diet of Wistar rats was supplemented with apiaceous vegetables, cruciferous vegetables, or the combination of them for seven days and an injection of PhIP (10 mg/kg body wt) was administered a day prior to sacrifice. We analyzed activity and expression of CYP1A1, CYP1A2, SULT1A1, and UGT1A1 in the liver. There was no difference between negative and positive controls in activity and expression of measured biotransformation enzymes except for the expression level of CYP1A1; CYP1A1 expression in the positive control was higher than the negative control. In addition, the expression of CYP1A1 and CYP1A2 in the vegetable-supplemented groups was higher when compared to the negative control groups, indicating that the dose of PhIP may have affected the level of protein expression (Table 2-3). HAA, including PhIP, can induce both expression and activity of CYP1A1 and CYP1A2 via the aryl hydrocarbon receptor (AhR)-pathway (246, 247).

However, the dose of PhIP provided to rats did not appear to cause any unintended effects evidenced by no observed influence on general food intake, tissue weight, and body weight in the study.

As expected, cruciferous vegetables increased CYP1A1, CYP1A2, and UGT1A1 activity; however we did not find any difference in protein expression. The CRU diet resulted in approximately 28%, 61%, and 21% increase in activity of CYP1A1, CYP1A2, and UGT1A1, respectively, compared to the positive control. Many studies demonstrated the modulating effects of cruciferous vegetables on both phase I and phase II biotransformation enzymes in humans (127, 149) and animal models (126, 148) with different magnitudes. For example, when Wistar rats were fed with 2.5%, 5%, and 20% of cooked Brussels sprouts for two days, seven days, 14 days, and 28 days, CYP1A1 activity was increased by 20% after two days of exposure; after seven days of exposure of 20% cooked vegetables, Brussels sprouts induced approximately two-fold increase in CYP1A1 activity (148). Although the authors measured CYP1A1 activity using the same animal strain (i.e., Wistar rats) after the same length of exposure with comparable doses (20% versus 21%) used in our study, such a large difference in activity might be due to different vegetables utilized (Brussels sprouts versus broccoli, cabbage, and watercress), diet preparation method (cooking versus fresh), and other experimental conditions that possibly affect experimental results (e.g., age of rats).

Unexpectedly, we did not observe inhibition of CYP1A1 and CYP1A2 activity with the API diet which is inconsistent with previous data regarding the modulating effects

of apiaceous vegetables on CYP (Table 2-3). Peterson et al. demonstrated that constituents (5-methoxypsoralen, 8-methoxypsoralen (8-MOP), and apigenin) in apiaceous vegetables inhibited hCYP1A2 activity in vitro as measured by 7-methoxyresorufin *O*-demethylation assay (185). Similar inhibitory effects on CYP1A2 activity were also observed in humans fed apiaceous vegetables (150). The absence of inhibitory effects of API diet found in our study is likely due to the dose given to rats. In this study, the amount of apiaceous vegetables was 101 g per day based on 2000 kcal intake whereas CYP1A2 activity was inhibited when 265.5 g of apiaceous vegetables (i.e., dill weed, celery, parsley, parsnips, and carrot) were given for six days to humans which is more than twice higher than the dose in the present study (150). In another in vivo study, Wistar rats were exposed to 8-MOP via intraperitoneal injection (25 mg of 8-MOP/kg body wt). Two hours after the injection of 8-MOP, CYP1A1, CYP1A2, and CYP2B1/2 activities were decreased 18%, 17%, and 11%, respectively. However, there are important differences in study design that may account for the inconsistency. Firstly, in our study, we fed rats with fresh vegetables as opposed to the purified compounds. It is well known that the bioavailability of phytochemicals is influenced by the chemical structure (glycosylation, esterification, and polymerization) as well as food matrix of vegetables; native forms of compounds in vegetables need to be hydrolyzed to produce aglycones prior to the absorption through small intestine (195). In addition, the route of exposure also affects the levels of compounds reaching the target tissues; in general, intraperitoneal injection would provide higher systemic availability as it bypasses first pass metabolism (248). Therefore, it is very possible that the actual dose exposed to rats in the study might be lowered than previous studies that

demonstrated the inhibition of CYP1As through either apiaceous vegetables or active compounds thereof.

As mentioned, the API diet induced UGT1A1 activity, which was not expected. Only a few studies have investigated the effects of apiaceous vegetables on UGT1A1. Navarro et al. fed the combination of cruciferous and apiaceous vegetables and observed a trend that the combined vegetable groups increased UGT1A1 activity compared to the cruciferous only feeding, implying that apiaceous vegetables may induce UGT1A1 activity (127). Since furanocoumarins modulate CYPs via the AhR-mediated pathway, we cannot rule out the possibility that UGT1A1 was induced by these compounds and other constituents in the vegetables (including flavonoids) given that UGT1A1 is also partly regulated by the AhR pathway (187, 188).

Interestingly, we found that the overall correlation between enzyme expression and activity was weak. The CRU diet increased SULT1A1expression while no induction in activity was noted. Further, no induction of enzyme expression was found for CYP1A1, CYP1A2, or UGT1A1 in any diet group. This lack of correlation between protein expression and activity has been shown before (152). A possible explanation for discrepancies between enzyme activity and expression might be other mechanisms influenced by the vegetable constituents. There is some indication that CYPs may undergo post-translational modification (249, 250). For instance, van Duursen et al. found that CYPC17 might be modulated via post-transcriptional modification by naturally occurring lignan and structurally related derivatives (250). In the study, the authors treated human adenocorticocarcinoma cell line with lignan (30 μM; final

concentration) for 24 hours and found there is a lack of correlation between expression and activity of this enzyme. The activity of CYP17 was decreased by 37% while mRNA expression was induced by 2.3 fold, indicating that the inhibition was not due to decreased mRNA expression level. It was explained that possibly phosphorylation of CYP17 was affected by the lignan derivative thereby abolishing the effect of induction of mRNA, which was supported by the observation that inhibition of mitogen-activated protein kinase kinase further reduced the enzyme activity. This is in agreement with a previous study showing that phosphorylation is necessary for CYP17 activity (251). A few studies also showed the lack of correlation between protein expression and activity of CYP1As, warranting further investigations regarding the regulatory mechanisms of the enzymes (252).

Metabolomics is a largely evolving tool across the biological sciences including the nutritional science field. Although the original concept of the metabolome includes the complement of metabolites in a cell, it has been expanded to encompass the metabolites modified by diet and other xenobiotics (253). In this study, all urinary PhIP metabolites were analyzed using a metabolomics approach in order to further investigate the effects of cruciferous and apiaceous vegetable feeding on PhIP metabolism. Using QTOF-MS/MS, we identified 16 PhIP metabolites and unmetabolized PhIP in urine samples from Wistar rats. Of the metabolites, seven metabolites were glucuronidated metabolites (XI, XII, XIII, XIV, XV, XVI, and XVII), confirming that UGTs play a crucial role in PhIP metabolism (254). Malfatti et al. demonstrated that the PhIP-DNA adducts in the liver and colon were significantly lower (three-fold and seven-fold, respectively) in β-naphthoflavone-induced (a known

inducer for UGT1A and CYPs) Wistar rats, indicating not only glucuronidation but also hydroxylation of PhIP may facilitate excretion of PhIP and thereby decrease the genotoxic potency (254). As discussed, cruciferous vegetables enhanced CYP1A1, CYP1A2, and UGT1A1 activity. The urinary metabolite profile generally reflected well these changes in enzyme activity, meaning increased glucuronidated (P<0.001) and CYP1A-mediated PhIP metabolites (P<0.001) were observed compared to the positive control (Fig. 2-2 (B)). However, we observed that only a modest reduction of PhIP-DNA adduct formation (approximately 17%, P=0.07) was achieved by the CRU diet in the colon.

Although we did not detect inhibitory effects of apiaceous vegetable feeding on CYP1A1 activity, one of the major metabolites mediated by the enzyme (i.e., IV) was significantly decreased by the API diet (Fig. 2-2(A)). Such inconsistency between enzyme activity and excretion of urinary metabolites could be explained as urine samples were cumulatively collected over 24 hours while enzyme activity measurements only capture the ability to convert the substrate to the final product for 10 minutes in the method. In order to look at the net effects from dietary intervention, it seems more reasonable to address both results, enzyme activity and urinary metabolite profiles.

When looking at sulfated PhIP metabolites, they were highest in the C+A diet group compared to others. This was due to the contribution of metabolite VIII, as the other sulfated PhIP metabolites (i.e., IX and X) were present only in trace amounts. Further, metabolite VIII is derived from metabolite IV which can be subsequently conjugated

by UGTs as well. Given the more pronounced effects of CRU diet on the glucuronidated metabolites (e.g., XIII and XV), higher abundance of sulfated PhIP metabolites, particularly metabolite VIII, in the C+A diet could be due to the availability of substrate, meaning that it was more favorable for metabolite VIII to be glucuronidated in the CRU group compared to that of C+A group. It seems the effect of the CRU diet on the expression of SULT1A1 was minor or negligible.

On the other hand, we identified three methylated PhIP metabolites from rat urine samples. All methylated PhIP metabolites were highest in the API diet group followed by the positive control, CRU, and C+A diet group, respectively. No difference was found between the CRU and C+A diet groups. Recently, Telang et al. observed that oral administration of PEITC in Sprague Dawley rats down-regulated the nicotinamide *N*-methyltransferase (NMT) in the liver (255). Thus, decreased abundance of methylated PhIP metabolites by the CRU diet could possibly be due to down-regulation of NMTs. Another possibility regarding the effects of vegetables is a difference in available substrates for the methylation reactions, meaning, as observed, the phase I (i.e., CYP1A1 and CYP1A2) and phase II (UGT1A1) activity was elevated with CRU diet which could decrease the chance of conjugation by NMT; however, this does not explain the higher methylated PhIP metabolites with API diet. Further studies in regard to modulation of NMTs via apiaceous vegetables and their active compounds are warranted.

Few studies have been published regarding the role of methylation in PhIP metabolism. Methylation is generally considered as a toxification step since both *O*-

and *N*-methylation lose their positive charge from sulfonium ion and make methylated metabolites more lipophilic than the parent compound. Moreover, it masks the functional group of the parent compound that can be conjugated by other phase II enzymes. The exception is that if the pyridine type nitrogen is methylated by NMTs, which form a quaternary ammonium group holding the positive charge on the nitrogen. This makes metabolites markedly more hydrophilic thereby facilitating excretion (256). In our study, two out of three metabolites were methylated on the N^2 position of PhIP thus, possibly methylation of PhIP would not favor excretion. However, we found that methylated PhIP metabolites were negatively correlated with PhIP-DNA adducts (except in rats in the API diet group; (Table 2-6)), implying an alternative role of NMTs in regard to PhIP metabolism. One of the possible explanations is that methylation of PhIP, especially on the exocyclic amine of PhIP, may suppress the activation of this procarcinogen. Taken together, given the abundance of methylated PhIP metabolites, it seems to be one of the major metabolic pathways of PhIP, at least in rats. We also confirmed that human liver S9 fraction is able to conjugate PhIP with a methyl group, implying the possibility of methylation of PhIP in human as well (data not shown). To our knowledge, this is the first study demonstrating that methylated PhIP metabolites are produced in the human liver fraction. Therefore, in addition to the structural analysis of the novel metabolite detected here (i.e., metabolite II), further studies will be needed to address the significance of methylation in HAA metabolism.

We detected 5-hydroxylated PhIP metabolites which are particularly interesting because they are produced via a CYP-independent pathway. Both PhIP-DNA adducts

and metabolite VI are strongly correlated with *N*-hydroxylated PhIP in Wistar rats, indicating that 5-hydroxylated PhIP metabolites may be utilized as a urinary biomarker for PhIP genotoxicity (257, 258). In our study, 5-hydroxylated PhIP metabolites were higher in the CRU diet group and C+A diet group compared to the positive control group (Fig. 2-2 (B)). But we found no significant correlation between these metabolites and PhIP-DNA adducts (Table 2-6). Although it is not clearly understood, Frandsen et al. suggested that 5-hydroxylated PhIP metabolites were derived from the degradation of either PhIP-DNA adducts or PhIP-cellular constituent adducts (257). However, this does not account for the different susceptibility of tissues towards PhIP genotoxicity due to different properties of each tissue (e.g., varying levels of biotransformation enzymes (259)). Therefore it is somewhat unclear if 5-hydroxylated PhIP metabolites are suitable to predict the genotoxicity of PhIP.

As briefly mentioned, once activated, PhIP can bind to the C^8 position of guanine and form PhIP-DNA adducts in target tissues. For genotoxic carcinogens, the formation of DNA adducts is essential, and these carcinogens may not induce cancers without DNA adducts (92). Further, the dose of PhIP and PhIP-DNA adduct levels are proportional (71). Thus, as a surrogate biomarker, we measured PhIP-DNA adducts in colon to find the effects of vegetable supplementations on PhIP metabolism and genotoxicity via the modulation of biotransformation enzymes. However, at the same time, it is important to note that there is no direct correlation between PhIP-DNA adduct levels and tumor incidence (100, 260); this might be because carcinogenesis is a long process and influenced by various factors. For example, each tissue represents different susceptibility against carcinogens including PhIP (52).

We found the lowest level of PhIP-DNA adducts in the API diet group followed by CRU and C+A diet groups, respectively (Fig. 2-4). Of them, only the API diet group showed statistically significant reduction of PhIP-DNA adducts in colon (approximately 20% reduction; P=0.049) while the CRU diet group was of borderline statistical significance (approximately 17% reduction; P=0.072). We did not find a synergistic effect of C+A diet in reducing PhIP-DNA adduct levels. Interestingly, there was no significant correlation noted between urinary metabolites and PhIP-DNA adducts in the API diet group; furthermore, the effect of the API diet on CYP1A2 activity, which is the primary enzyme responsible for the PhIP activation (47), was not significant, indicating that modulation of biotransformation enzymes that are related with PhIP activation as well as detoxification cannot fully explain the results in our study. It is likely that there are different mechanisms (e.g., phase III detoxification) influenced by the apiaceous vegetables. For example, PhIP can be extruded from cells via ATP-binding cassettes (e.g., multidrug resistance-associated protein 2 (MRP 2)) in the colon (261). Interestingly, it was demonstrated that flavonoid compounds present in vegetables can modulate phase III detoxification via more than one mechanism. They can 1) induce protein expression level of MRP 2, 2) inhibit the activity of MRP 2, and 3) act as substrates thereby modulating biliary or renal excretion of MRP 2 substrates (262).

In summary, we hypothesized that the combination of cruciferous and apiaceous vegetable feeding may exert synergistic protection against PhIP genotoxicity in colon via complementary actions of the vegetables on biotransformation enzymes involved

in PhIP metabolism. Our results indicate that the effect of vegetable feeding against PhIP genotoxicity was most pronounced by apiaceous vegetables. Given the lack of effects of API diet on CYP1A activity and N^2-hydroxylated PhIP metabolites (i.e., V, XVI, and XVII), the protection of API diet is not limited to the inhibition of the activation process of the carcinogen, but other additional mechanisms might be involved such as phase III detoxification. Lastly, using a metabolomics approach, we detected a novel methylated PhIP metabolite. It was further confirmed that these methylated PhIP metabolites can be produced by human liver S9 fraction as well. Considering the abundance of methylated PhIP metabolites in rat urine, further investigation is warranted regarding structure elucidation of the novel metabolite and the significance of methylated PhIP metabolites in terms of carcinogenic potency.

Table 2-1. Diet composition

Diet ingredients (g/kg)	AIN-93G (g/kg)	21% Cruciferous (g/kg)[a]	21% Apiaceous (g/kg)[b]	21% Combination (g/kg)[c]
Cornstarch	397.5	391.8 (5.7)[d]	382.3 (15.2)	387.1 (10.4)
Dextrinized cornstarch	132.0	132.0	132.0	132.0
Casein	200.0	195.5 (4.5)	198.0 (2.0)	196.8 (3.2)
Sucrose	100.0	100.0	100.0	100.0
Fiber	50.0	46.1 (3.9)	43.2 (6.8)	44.6 (5.4)
Mineral mix	35.0	35.0	35.0	35.0
Vitamin mix	10.0	10.0	10.0	10.0
L-Cystine	3.0	3.0	3.0	3.0
Choline bitartrate	2.5	2.5	2.5	2.5
Soybean oil[e]	70.0	69.6 (0.4)	69.5 (0.5)	69.5 (0.5)
Apiaceous	Not applicable	Not applicable	210.0	105.0
Cruciferous	Not applicable	210.0	Not applicable	105.0
Total (g)	1000.0	1195.5	1185.5	1190.5

[a]Cabbage, watercress, and broccoli were used for the cruciferous vegetables. [b]Celery and parsnip were used for the apiaceous vegetables. [c]Cabbage, watercress, broccoli, celery, and parsnip were used for the combination vegetables (10.5% wt/wt of cruciferous, 10.5% wt/wt of apiaceous). [d]Values in parentheses represent respective macronutrients from the vegetables. All diets were balanced for macronutrients using USDA National Nutrient Database. [e]The antioxidant, t-Butylhydroquinone (tBHQ; 0.02%) was included in soybean oil.

Table 2-2. Food intake, tissue, and body weight

	Food intake (g)	Weight gain (g)	Tissue weight (g)				
			Liver	Colon	Prostate	Lung	Pancreas
Basal diet (AIN-93G) + No PhIP	19.18±0.58	102.19±2.45	7.11±0.19	0.94±0.05	0.21±0.02	1.13±0.03	0.35±0.03
Basal diet (AIN-93G) + PhIP	19.99±0.58	97.26±2.44	7.18±0.19	1.02±0.05	0.20±0.02	1.18±0.03	0.36±0.03
Cruciferous diet (21%) + PhIP	23.21±0.60**	99.17±2.50	7.30±0.19	0.97±0.05	0.20±0.02	1.17±0.03	0.36±0.03
Apiaceous diet (21%) + PhIP	21.74±0.58*	99.75±2.44	7.14±0.19	1.04±0.05	0.19±0.02	1.14±0.03	0.36±0.03
Combination diet (21%) + PhIP	22.46±0.58*	94.56±2.44	6.94±0.19	1.01±0.05	0.23±0.02	1.08±0.03	0.34±0.03

All results were expressed as least squares means ± SE. Data were analyzed by ANOVA (SAS, Cary, NC). * denotes a statistical difference in food intake compared to positive control group ($P \leq 0.05$). ** denotes a statistical difference in food intake compared to positive control group ($P \leq 0.01$). There were no differences in weight gain, and tissue weight between diet groups and positive control group.

Table 2-3. PhIP metabolizing enzymes activity and expression[a]

	CYP1A1		CYP1A2		UGT1A1		SULT1A1	
	Activity[b]	**Expression[c]**	**Activity**	**Expression**	**Activity[d]**	**Expression**	**Activity[e]**	**Expression**
Basal diet (AIN-93G) + No PhIP	7.43±0.71	0.84±0.08*	5.20±0.58	4.31±0.75	20.21±1.24	1.71±0.23	11.60±0.41	0.73±0.10
Basal diet (AIN-93G) + PhIP	9.36±0.70	1.09±0.08	6.20±0.58	5.88±0.74	16.91±1.23	1.91±0.23	11.26±0.41	0.66±0.09
Cruciferous diet (21%) + PhIP	12.82±0.72**	1.13±0.08	10.00±0.60***	6.09±0.76	20.42±1.26*	1.40±0.23	11.63±0.42	1.04±0.10**
Apiaceous diet (21%) + PhIP	10.56±0.70	1.16±0.08	8.70±0.58**	7.56±0.74	21.57±1.23*	1.88±0.23	10.99±0.41	0.90±0.09
Combination diet (21%) + PhIP	12.15±0.76*	1.25±0.08	10.11±0.62***	7.23±0.74	18.26±1.23	1.80±0.23	11.69±0.45	0.83±0.09

[a]All results were expressed as least squares means ± SE. Data were analyzed by ANOVA (SAS, Cary, NC). * denotes a statistical difference compared to positive control group ($P \leq 0.05$). ** denotes a statistical difference compared to positive control group ($P \leq 0.01$). *** denotes a statistical difference compared to positive control group ($P \leq 0.001$). [b]The unit of CYP1A1 and CYP1A2 activity was pmol

resorufin/min/mg protein. [c]The expression unit of each enzyme was ng of protein/mL. [d]The unit of UGT1A1 activity was nmole 4-methylumbelliferone glucuronide/min/mg protein. [e]The unit of SULT1A1 activity was nmole *p*-nitrophenol/min/mg protein.

Table 2-4. Urinary metabolites of PhIP in Wistar rats measured using the UPLC-QTOF-MS/MS system[a]

Identification	Metabolites	[M+H]+	Retention time (min)	Formula
I	PhIP	225.1162	3.63	C13H12N4
II	Methyl-PhIP	239.1323	3.38	C14H14N4
III	N^2-methyl-PhIP	239.1320	4.19	C14H14N4
IV	4'-hydroxy-PhIP	241.1111	2.52	C13H12N4O
V	N^2-hydroxy-PhIP	241.1105	3.57	C13H12N4O
VI	5-hydroxy-PhIP	241.1107	3.75	C13H12N4O
VII	N^2-methyl-4'-hydroxy-PhIP	255.1266	2.93	C14H14N4O
VIII	4'-hydroxy-PhIP-sulfate	321.0664	2.38	C13H12N4O4S
IX	5-hydroxy-PhIP-sulfate	321.0673	2.68	C13H12N4O4S
X	5,4'-dihydroxy-PhIP-sulfate	337.0618	2.47	C13H12N4O5S
XI	PhIP-N^3-glucuronide	401.1482	2.98	C19H20N4O6
XII	PhIP-N^2-glucuronide	401.1463	3.42	C19H20N4O6
XIII	4'-hydroxy-PhIP-glucuronide	417.1422	1.86	C19H20N4O7
XIV	5-hydroxy-PhIP-glucuronide	417.1416	2.58	C19H20N4O7
XV	4'-hydroxy-PhIP-N^2-glucuronide	417.1431	2.82	C19H20N4O7
XVI	N^2-hydroxy-PhIP-N^2-glucuronide	417.1417	3.31	C19H20N4O7
XVII	N^2-hydroxy-PhIP-N^3-glucuronide	417.1418	4.51	C19H20N4O7

[a]Wistar rats were injected with PhIP (10 mg of PhIP/kg body wt) and urine samples were collected for 24 hours after PhIP treatment and analyzed using the UPLC-MS/MS system.

Table 2-5. Validation of PhIP-DNA adducts measurement using the online column-switching LC-ESI-MS/MS

Adducts spiked into negative control				**Mean**	**SD[a]**	**CV[b] (%)**
5 adducts per 10^7 nucleotides	4.8	5.2	4.6	4.9	0.27	5.5
10 adducts per 10^7 nucleotides	10.0	8.8	10.0	9.7	0.71	7.3
15 adducts per 10^7 nucleotides	14.2	16.1	14.8	15.0	0.98	6.5

The extracted DNA from the negative control (i.e., without PhIP) was spiked with unlabeled PhIP-DNA adducts at levels of 0, 5, 10, and 15 adducts per 10^7 nucleotides. PhIP-DNA adducts were not detected in the level of 0 adduct per 10^7 nucleotides. [a]SD = standard deviation. [b]CV = coefficient of variation

Table 2-6. Correlations between urinary metabolites and PhIP-DNA adducts in colon

Groups	Urinary metabolites	Correlation with PhIP-DNA adducts
All groups were combined	I, IV, VIII, and XV	Positive correlation
	Sulfated PhIP metabolites[a]	
	II and III	Negative correlation
	Methylated PhIP metabolites[c]	
Control group	IV	Positive correlation
	II and III	Negative correlation
Cruciferous group (*P*=0.072)[d]	VIII and IX	Positive correlation
	Sulfated PhIP metabolites	
	II and III	Negative correlation
Apiaceous group (*P*=0.049)	None of the metabolites and grouped metabolites was significantly correlated with PhIP-DNA adducts	
Combination group (*P*=0.52)	VIII, XII, and XIII	Positive correlation
	CYP1A mediated PhIP metabolites	
	Sulfated PhIP metabolites	
	II and III	Negative correlation
	Methylated PhIP metabolites	

[a]Sulfated PhIP metabolites: VIII + IX + X. [b]CYP1A mediated PhIP metabolites: IV + V + VII + VIII + X + XIII + XV + XVI + XVII. [c]Methylated PhIP metabolites: II + III + VII. [d]*P*-value in the parenthesis indicates the difference in PhIP-DNA adducts compared to the positive control group.

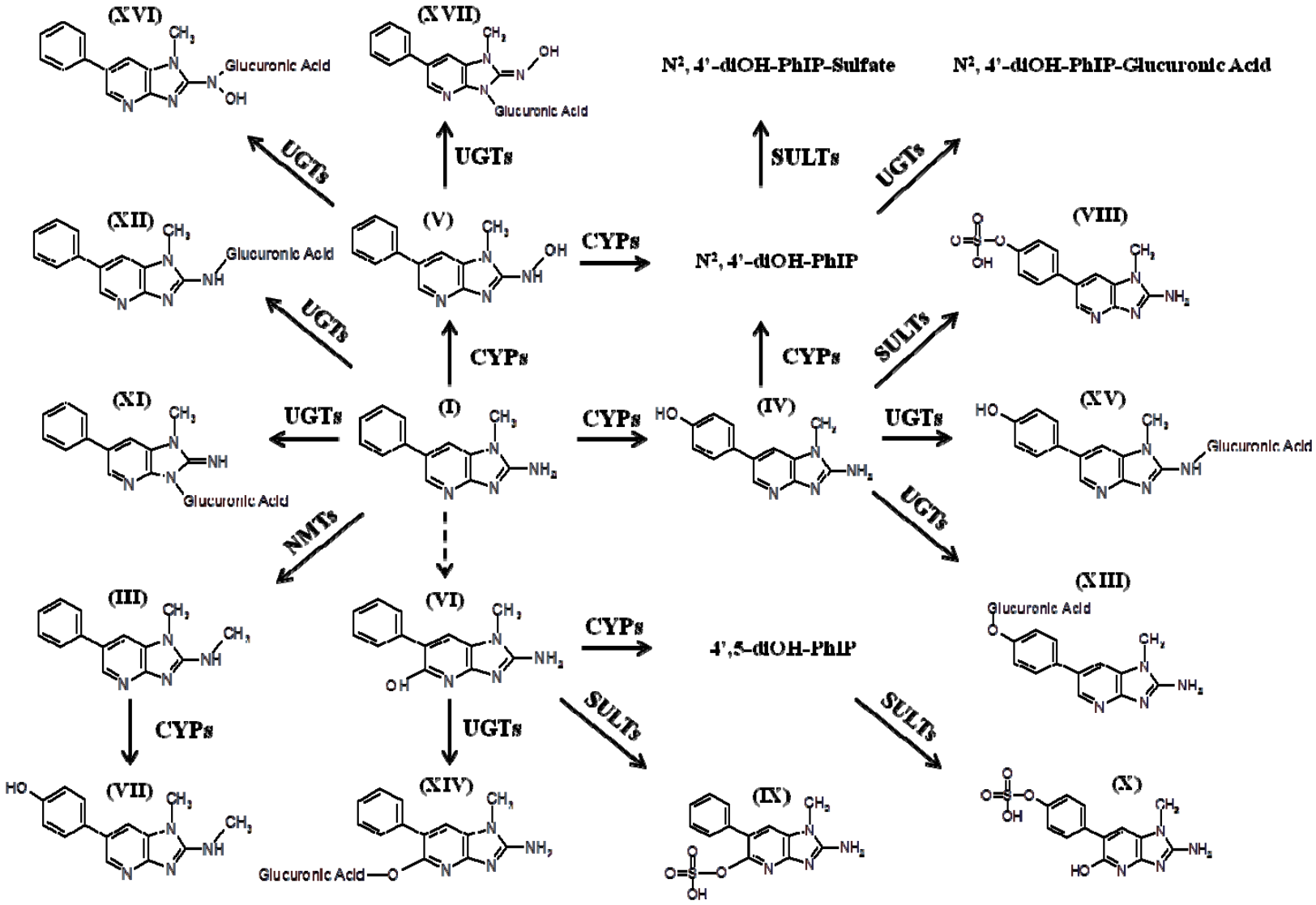
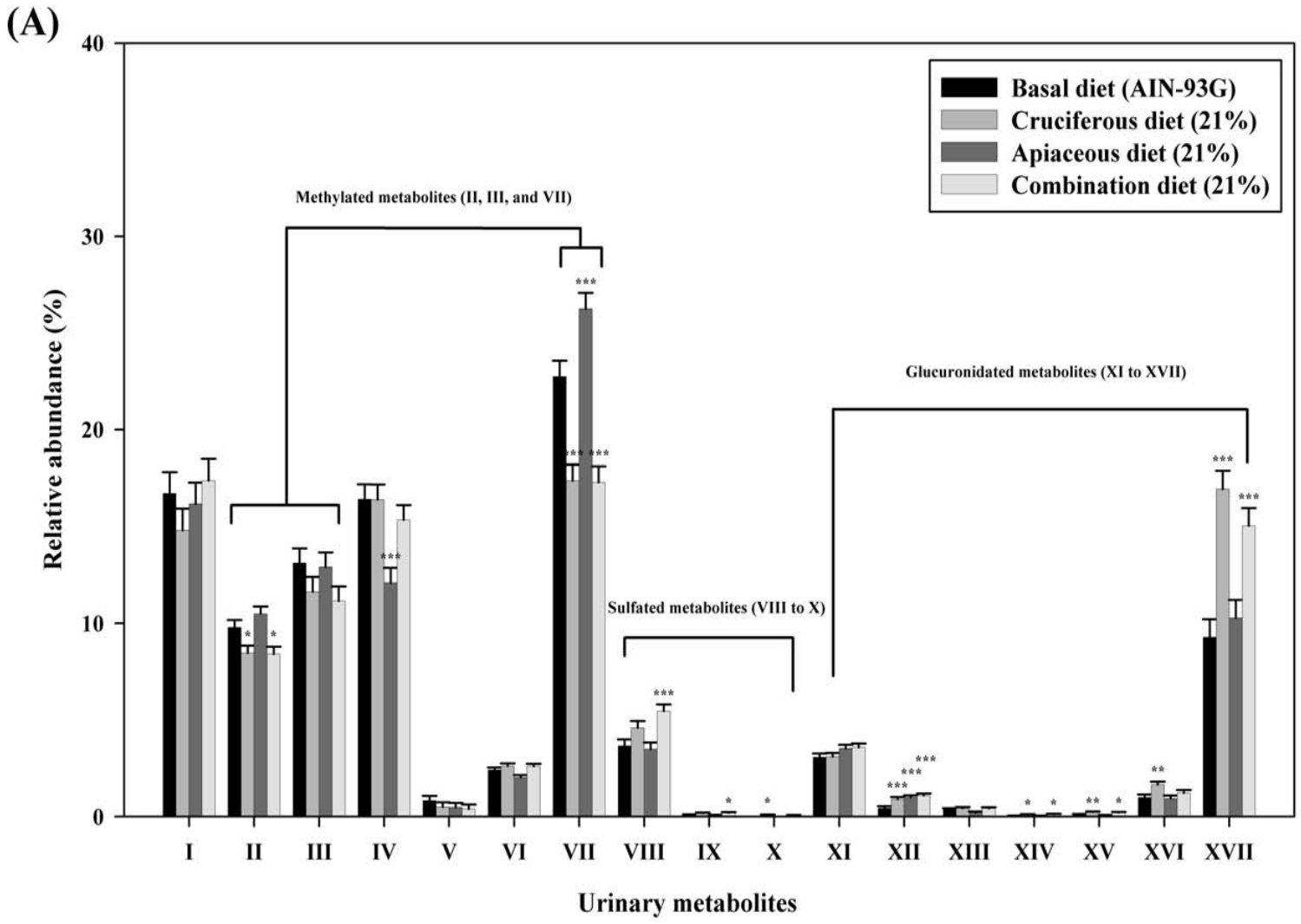

(XVI)
Glucuronic Acid
(XVII)
Glucuronic Acid
N², 4'-diOH-PhIP-Sulfate
N², 4'-diOH-PhIP-Glucuronic Acid
UGTs
UGTs
SULTs
UGTs
(XII)
Glucuronic Acid
(V)
CYPs
N², 4'-diOH-PhIP
(VIII)
UGTs
CYPs
CYPs
SULTs
(XI)
Glucuronic Acid
UGTs
(I)
CYPs
(IV)
UGTs
(XV)
Glucuronic Acid
NMTs
UGTs
(III)
(VI)
CYPs
4',5-diOH-PhIP
(XIII)
Glucuronic Acid
CYPs
UGTs
SULTs
SULTs
(VII)
(XIV)
Glucuronic Acid
(IX)
(X)

Figure 2-1. Structures of urinary PhIP metabolites and metabolic pathways in Wistar rats

For total 17 urinary PhIP metabolites were detected including the parent compound (i.e., PhIP (I)). Solid lines indicate either CYP-dependent oxidation reactions or phase II-dependent conjugation reactions. Dashed line represents CYP-independent oxidation reactions. One of the methylated PhIP metabolite (metabolite II) is not shown above and the structure is to be elucidated.


(A)
Basal diet (AIN-93G)
Cruciferous diet (21%)
Apiaceous diet (21%)
Combination diet (21%)
Methylated metabolites (II, III, and VII)
Sulfated metabolites (VIII to X)
Glucuronidated metabolites (XI to XVII)
Relative abundance (%)
Urinary metabolites
0
10
20
30
40
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII

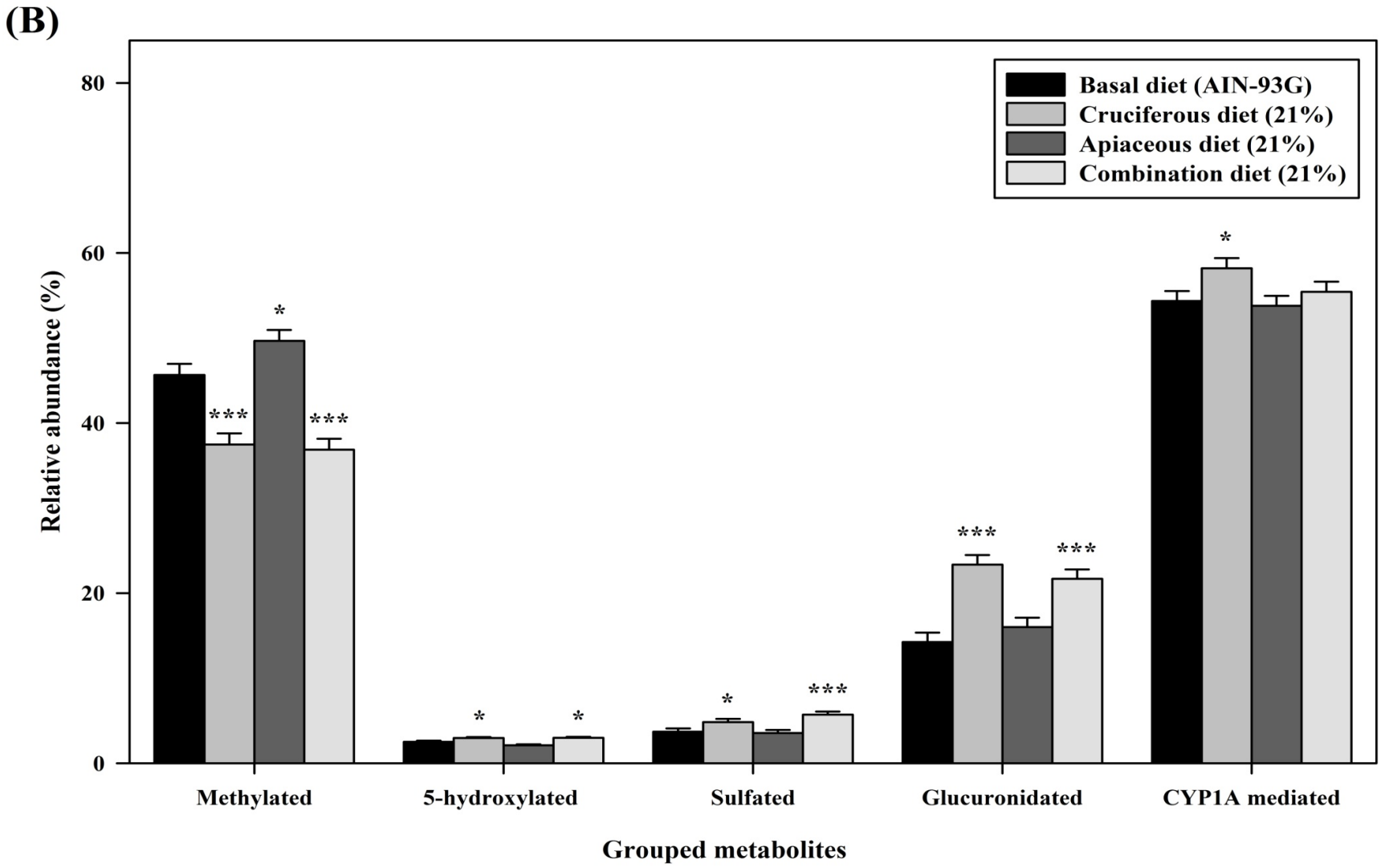

(B)
Basal diet (AIN-93G)
Cruciferous diet (21%)
Apiaceous diet (21%)
Combination diet (21%)
Relative abundance (%)
0
20
40
60
80
***
*
***
*
*
*
***
***
***
*
Methylated
5-hydroxylated
Sulfated
Glucuronidated
CYP1A mediated
Grouped metabolites

Figure 2-2. Urinary metabolites of PhIP in Wistar rats measured using UPLC-QTOF-MS/MS system

Twenty four hours urine samples from each diet group were collected after intraperitoneal injection of PhIP (10 mg of PhIP/kg body wt). The screening and identification of urinary metabolites of PhIP were done by MetaboLynx software. The signal intensity represents relative peak area (area % ± SE) by calculating its percentage in the total peak area of PhIP metabolites profile. Data were analyzed by ANOVA (SAS software package, Cary, NC). *denotes a statistical difference compared to positive control group ($P \leq 0.05$). **denotes a statistical difference compared to positive control group ($P \leq 0.01$). ***denotes a statistical difference compared to positive control group ($P \leq 0.001$).

Each urinary metabolite (A) and grouped urinary metabolites (B) were depicted; methylated PhIP metabolites: II + III + VII; 5-hydroxylated PhIP metabolites: VI + XIV + IX + X; sulfated PhIP metabolites: VIII + IX + X; glucuronidated PhIP metabolites: XI + XII + XIII + XIV + XV + XVI + XVII; CYP1A mediated PhIP metabolites: IV + V + VII + VIII + X + XIII + XV + XVI + XVII.

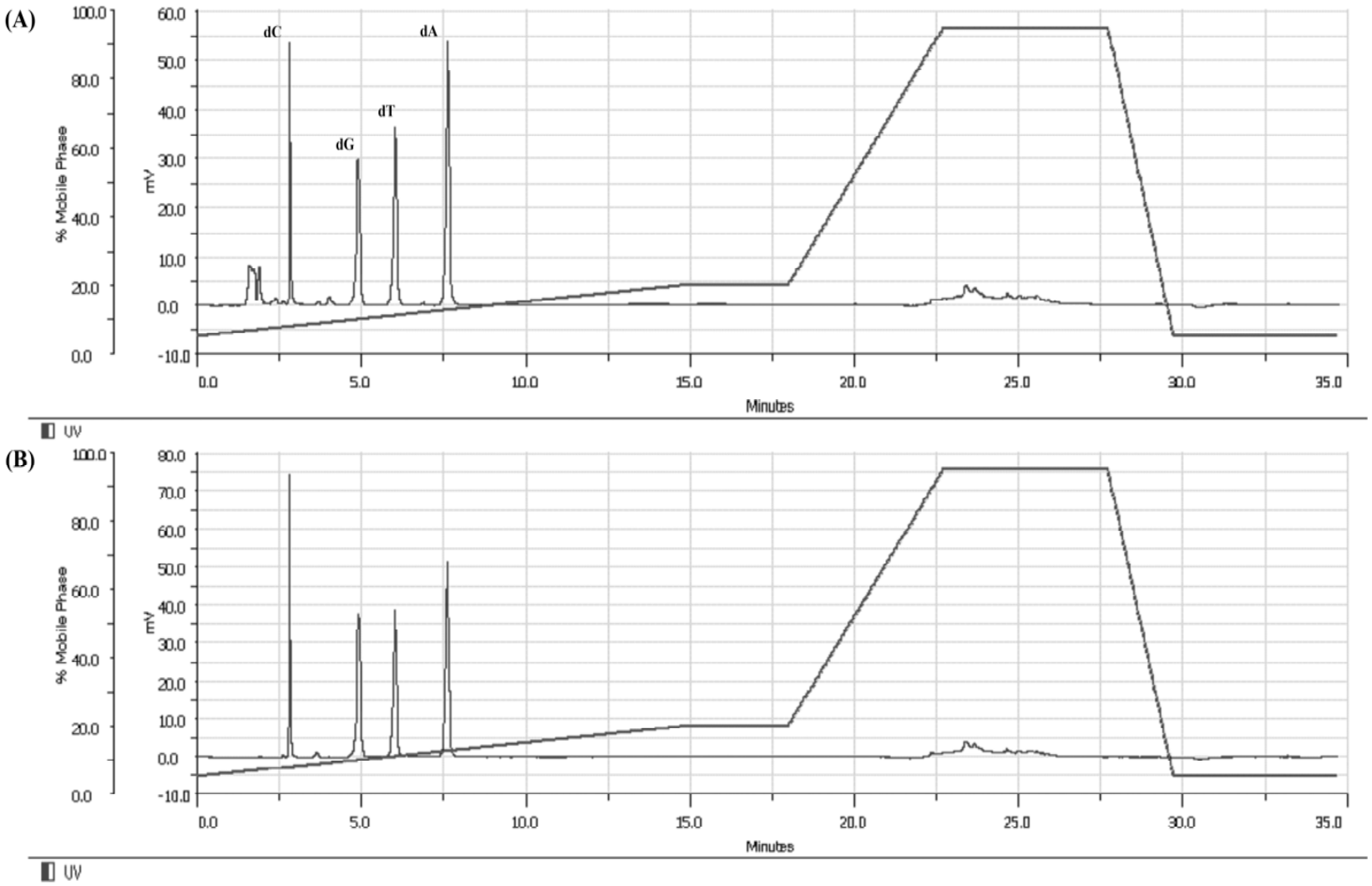

(A)
dC
dG
dT
dA
% Mobile Phase
mV
Minutes
UV
(B)
% Mobile Phase
mV
Minutes
UV

Figure 2-3. Assessment of DNA digestion efficacy using HPLC

The chromatograms of digested colon tissue (4 μg of DNA; (A)) and combined standard nucleosides (1 μg of each; 4 μg of total; (B)). Each nucleoside was injected and their retention time was compared to identify the peaks.

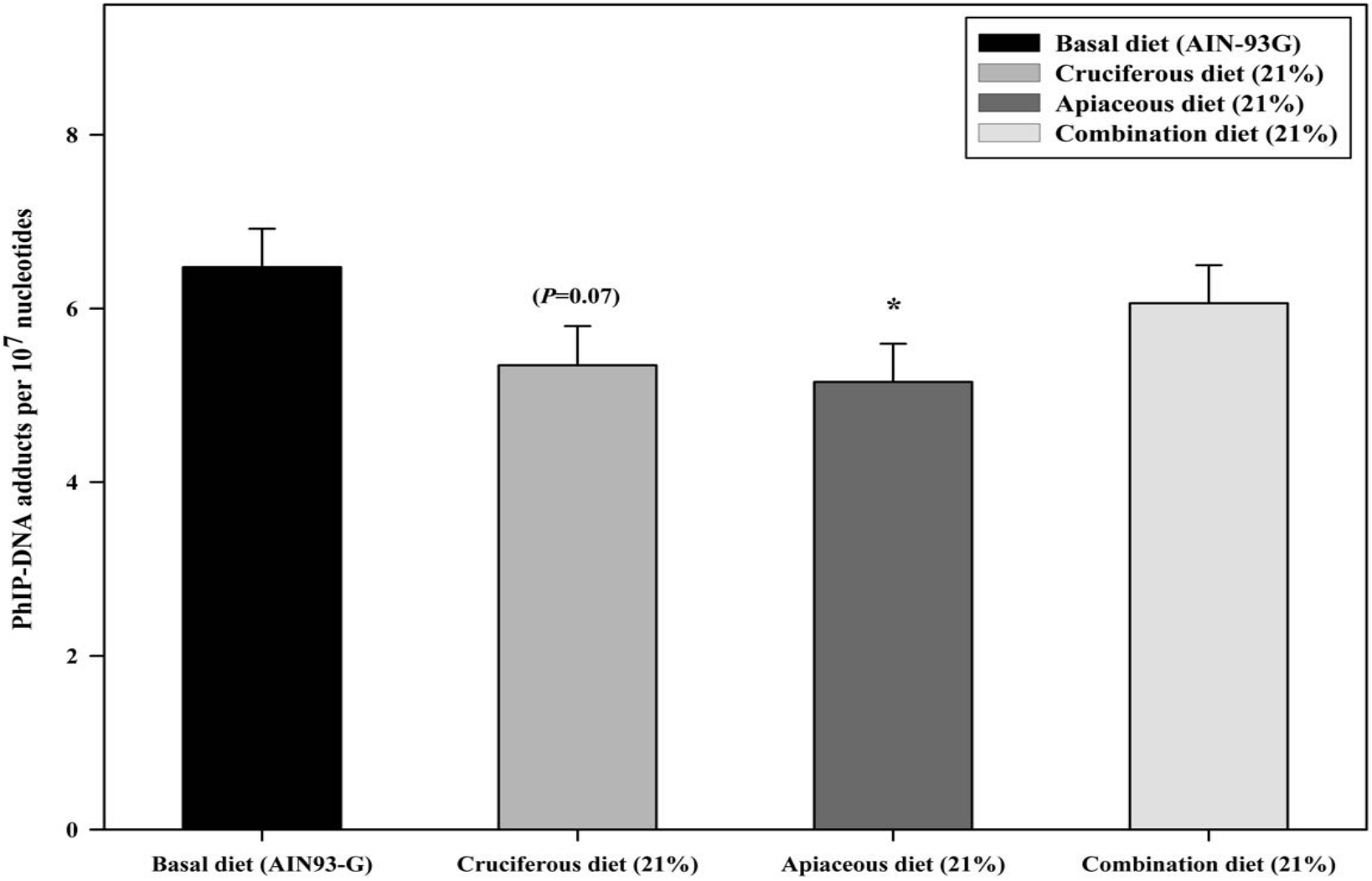

Basal diet (AIN-93G)
Cruciferous diet (21%)
Apiaceous diet (21%)
Combination diet (21%)
PhIP-DNA adducts per 10^7 nucleotides
0
2
4
6
8
(P=0.07)
*
Basal diet (AIN93-G)
Cruciferous diet (21%)
Apiaceous diet (21%)
Combination diet (21%)

Figure 2-4. Measurement of PhIP-DNA adducts in colon tissue

The DNA adducts level of colon tissues was determined using the online column-switching LC-MS/MS system as described in the method. Calibration curve was constructed with the ratio between unlabeled PhIP-DNA adducts, ranged from 0 to 30 adducts per 10^7 nucleotides and isotopically labeled internal standard, [^{13}C]-dG-C^8-PhIP, set at 10 adducts per 10^7 nucleotides. *denotes a statistical difference compared to positive control group ($P \leq 0.05$).

CHAPTER 3. MANUSCRIPT II: EFFECTS OF PEITC, I3C, AND FURANOCOUMARIN INTAKE ON PHIP METABOLISM IN WISTAR RATS

Introduction

Heterocyclic aromatic amines are ubiquitous in the environment from tobacco products, various meats, as well as drinks such as wine and beer (27-29). A number of studies demonstrated a positive association between heterocyclic aromatic amine intake and colorectal cancer risk (17, 263). Among the identified heterocyclic aromatic amines, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is one of the most mass abundant heterocyclic aromatic amines in foods (20, 37). This carcinogen has been shown in animal studies to induce aberrant crypt foci (ACF; (264)) which are precursors of colon cancer, mutations of key genes related to colon tumors (85), and colon cancer (68, 69).

PhIP is a procarcinogen, meaning it needs to be activated to exert carcinogenic potency. To be specific, hydroxylation of PhIP on its exocyclic amine (i.e., N^2 position) by principally cytochrome P450 (CYP) 1A2 is considered as an initial activation step (47). This hydroxylated PhIP metabolite can be further esterified by *N*-acetyltransferase and sulfotransferase (SULT) 1A1 to produce corresponding esters (i.e., N^2-acetoxy PhIP and N^2-sulfonyloxy PhIP). Ultimately, these metabolites can form an electrophilic nitrenium ion on the N^2 position of PhIP and preferentially bind to guanine (50). In contrast, conjugations through phase II enzymes (e.g., uridine 5'-diphospho-glucuronosyltransferase (UGT) 1A1) usually produce non-carcinogenic metabolites thereby enabling more efficient excretion (20, 52, 53).

Increasing evidence indicates that dietary components regulate expression and function of biotransformation enzymes, thus, they can modulate multiple pathological processes including carcinogenesis. Specifically, it has been demonstrated that cruciferous vegetables and their active compounds (e.g., glucosinolates and breakdown products of glucosinolates) induce both activity and mRNA expression of phase I and II enzymes (127, 147, 265) while furanocoumarins in apiaceous vegetables act as mechanistic inhibitors for phase I enzymes (185, 266). In human, if cruciferous and apiaceous vegetables are consumed together, phase I enzyme (CYP1A2) activity was inhibited (267). Hence, it could be postulated that the protective potency of these active compounds would be maximized if they are consumed together as the activation of PhIP would be suppressed while the induction of phase II enzymes would render PhIP metabolites safer and easily excreted.

The objective of the present study was to investigate if the combination of phytochemicals from cruciferous and apiaceous vegetables would elicit synergistic protection against PhIP due to their complementary actions on phase I and phase II biotransformation enzymes. In order to achieve the objective, activity and expression of key biotransformation enzymes (i.e., CYP1As, UGT1A1, and SULT1A1) that are responsible for PhIP metabolism in rats were analyzed. Furthermore, urinary PhIP metabolites were comprehensively analyzed utilizing a metabolomics approach to monitor the modulation of PhIP metabolism through the dietary intervention. Lastly, PhIP-DNA adducts were analyzed as a surrogate biomarker in the target tissue (i.e., colon). The levels of each class of compounds were matched with the dose of vegetables supplemented in the feeding study of our previous study in order to 1)

supplement a dose of phytochemicals that is achievable through habitual diets, and 2) compare more appropriately between intact vegetables and purified compounds in terms of chemopreventive potential as well as effects on PhIP metabolism.

Materials and methods

Chemicals and reagents

Ethoxyresorufin, methoxyresorufin, β-nicotinamide adenine dinucleotide phosphate reduced form tetrasodium salt, resorufin sodium salt, bovine serum albumin, Bradford reagent, 2-naphthol, adenosine 3'-phosphate 5'-phosphosulfate, 4-nitrophenyl sulfate, dimethyl sulfoxide, uridine 5'-diphospho-glucuronic acid, glycerol, DNase I (Type IV, bovine pancreas), nuclease P1 (*Penicillium citrinum*), phosphodiesterase I (*Crotalus adamanteus venom*), alkaline phosphatase (*Escherichia coli*), phenethyl isothiocyanate (PEITC), indole-3-carbinol (I3C), 5-methoxypsoralen (5-MOP), and 8-methoxypsoralen (8-MOP) were obtained from Sigma-Aldrich (St. Louis, MO). Tris base and ethylenediamine tetraacetic acid disodium salt were purchased from Fisher Scientific (St. Louis, MO). The 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane was purchased from Phoenix Pharmaceutical (Burlingame, CA). Isopimpinellin was obtained from Indofine Chemical (Hillsborough, NJ). All other chemicals were of analytical grade.

Animals

Male Wistar rats, 100-125 g body wt, were from Harlan Laboratories, (Indianapolis, IN). Animals were housed in wire-bottom stainless steel cages and adapted to a powder diet (i.e., American Institute of Nutrition (AIN)-93G) for the first five days. Rats had free access to water and were fed ad libitum. All animal experiment procedures were approved by the University of Minnesota Committee on Animal Care.

Study design

Fifty three rats were assigned to five groups: negative control, positive control, PEITC and I3C supplementation (PEITC+I3C), furanocoumarins supplementation (FC), and supplementation with a combination of PEITC, I3C, and furanocoumarins (COMBO). After six days of feeding, the three phytochemical-supplemented groups and the positive control group were given a single injection of PhIP (intraperitoneal injection; 10 mg of PhIP/kg body wt). The negative control was injected with vehicle only (i.e., dimethyl sulfoxide). Urines were collected over 24 hours immediately after the injection. On day seven, rats were killed and tissues were collected. Harvested tissues were frozen in liquid nitrogen and stored at -80°C until analyzed.

Study diets

The AIN-93G diet was used for the negative and positive control groups. The PEITC+I3C group was fed AIN-93G plus 182.6 mg/kg diet each of PEITC and I3C. The FC group was fed AIN-93G plus 1.2, 0.6, and 2.5 mg/kg diet of 5-MOP, 8-MOP,

and isopimpinellin, respectively. AIN-93G was supplemented with 91.3, 91.3, 0.6, 0.3, and 1.25 mg/kg diet of PEITC, I3C, 5-MOP, 8-MOP, and isopimpinellin, respectively, for the COMBO group. Structures of compounds are presented in Fig. 3-1. The concentrations of PEITC, I3C, and furanocoumarins were determined based on the 21% fresh weight of vegetables (wt/wt) that we fed rats in our previous study (Chapter 2); broccoli, watercress, and cabbage (cruciferous vegetables) and celery and parsnips (apiaceous vegetables) were analyzed to measure the levels of total glucosinolates and the individual furanocoumarins, respectively. To prepare the diet, each compound was dissolved in corn oil and then mixed with the powder diet. Detailed diet compositions are summarized in Table 3-1. Once phytochemical-supplemented diets were made, diets were divided into separate plastic bags for each day of the feeding period and stored at -80°C. Each bag was thawed daily and provided to rats. Food intake was determined from spillage and diets remaining in the food cups for 24 hours. Food intake was measured three times.

Hepatic microsome and cytosol preparation

Hepatic microsome and cytosol were prepared as described elsewhere (236). In brief, rats were fasted for 12 hours prior to sacrifice and then anesthetized with 2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane in a glass chamber. Livers were perfused with ice-cold 50 mM Tris base-150 mM potassium chloride buffer (pH 7.5) and tissues were homogenized in Tris base-potassium chloride buffer containing phenylmethylsulfonyl fluoride (1 mM, final concentration) followed by centrifugation at 10,000 × *g*. Supernatants from the centrifugation were further spun at 105,000 × *g*

for 70 minutes. Once the lipid layers were removed, the supernatants were stored for cytosolic assay. The pellets were suspended in 50 mM Tris base-glycerol buffer (pH 7.5) and stored for microsomal assays. Microsomal preparations were flushed with nitrogen to prevent oxidation prior to storage. Both cytosolic and microsomal preparations were stored at -80°C until analyzed.

Measurement of protein content

The protein concentrations of microsome and cytosol samples were determined using the Bradford method (Sigma-Aldrich, St. Louis, MO; (237)).

Measurement of CYP1A1 and CYP1A2 activity

The activity of microsomal CYP1A1 and CYP1A2 was measured using the 7-ethoxyresorfurin *O*-deethylation and 7-methoxyresorufin *O*-demethylation methods as described elsewhere (238, 239). Briefly, microsomal samples were washed at 105,000 × *g* with 100 mM sodium pyrophosphate-10 mM ethylenediamine tetraacetic acid buffer for 70 minutes and then precipitated pellets were suspended in 58 mM Tris base buffer and homogenized prior to measurement of CYP1A1 and CYP1A2 activity. The enzymatic reaction mixture (1 mL per sample) contained 5 μM substrate (for either CYP1A1 or CYP1A2), 0.05 M Tris base buffer pH 7.5, 0.025 M magnesium chloride, 500 μM β-nicotinamide adenine dinucleotide phosphate reduced form tetrasodium salt, and 200 μg of microsomal protein. The reaction mixture was transferred into 96 well microtiter plates (four wells per rat) and measured using a

microplate reader (Biotek Inc., Winooski, VT; excitation: 530/25 nm; emission: 590/35 nm).

Measurement of SULT1A1 activity

Cytosolic hepatic SULT1A1 activity was determined as previously described (240). The enzymatic reaction mixture (1 mL per sample) included 50 mM potassium phosphate buffer (pH 6.5), 5 mM magnesium chloride, 20 µM adenosine 3'-phosphate 5'-phosphosulfate, 5 mM 4-nitrophenyl sulfate, 0.1 mM naphthol, and 400 µg of cytosolic protein. The enzymatic mixture was incubated for 15 minutes at 37ºC and the reaction was terminated with 0.25 M Tris base-hydrogen chloride buffer (pH 8.7). The level of final product (i.e., *p*-nitrophenol) was determined at 405 nm and calculated using the extinction coefficient (18,200 cm^{-1} M^{-1}) as it is proportional to 2-naphthylsulfate, a product of SULT1A1 (Varian Cary 50 UV-Vis spectrophotometer, Agilent Technologies Inc., Palo Alto, CA).

Measurement of UGT1A1 activity

Microsomal UGT1A1 activity was measured using a slightly modified method (241). Microsomal enzymes were prepared as described and then protein contents were determined. The enzymatic reaction mixture (200 µL per sample) contained 600 µM 4-methylumbelliferone, 5 mM uridine 5'-diphospho-glucuronic acid, and 4 mM magnesium chloride in 100 mM phosphate buffer (pH 7.4) with 50 µg of microsomal protein. Once incubated at 37°C for 90 minutes, the reaction was terminated by

adding 24% perchloric acid and the mixture was centrifuged at 5,000 × *g* for 10 minutes. The supernatants were analyzed by HPLC (Gilson Inc., Middleton, WI). Twenty μL of the supernatant fraction was injected to the HPLC system and separation of 4-methylumbelliferone glucuronide (final product) was done on an Ultrasphere® ODS C18 column (5 μm, 4.6 × 250 mm; Hichrom, Berkshire, UK). Initial mobile phase composition was 15% acetonitrile in 25 mM phosphate buffer (pH 3.1) and gradually increased up to 50% acetonitrile over five minutes. The flow rate was 1.0 mL/min and the final product was monitored at 316 nm.

Measurement of protein expression

Protein expression of CYP1A1, CYP1A2, SULT1A1, and UGT1A1 in liver was measured using enzyme-linked immunosorbent assay kits (Uscn Life Science Inc., Houston, TX) according to the manufacturer's instructions. Microsomal enzymes were washed as described above in advance of measurement of protein expression. Washed microsomes were prepared to serial dilutions and applied to enzyme-linked immunosorbent assay microtiter plates. Cytosolic enzyme was used for measuring SULT1A1 expression. The enzyme-substrate reaction was measured at 450 nm and the expression levels of enzymes were calculated using standard curves (Biotek Synergy HT microplate reader, Biotek Inc., Winooski, VT).

UPLC-ESI-MS/MS analysis of urinary PhIP metabolites

Urinary PhIP metabolites were analyzed using a metabolomics-based approach. Collected urine samples were spun at 18,000 × *g* for five minutes to remove contaminants (e.g., feces and hair). The supernatants were diluted with 50% of acetonitrile (1:4) and centrifuged once more at 18,000 × *g* for 12 minutes in order to precipitate proteins and particles.

Five μL of prepared urine sample was injected into a Waters ultra-performance liquid chromatography (UPLC) quadrupole time-of-flight- tandem mass spectrometry (MS/MS) system (Mildford, MA). In order to separate PhIP metabolites, an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm; Waters, Mildford, MA) was employed. The initial mobile phase composition was water containing 0.1% formic acid and it was gradually increased up to 95% aqueous acetonitrile containing 0.1% formic acid over 10 minutes. The flow rate was 0.5 mL/min. The quadrupole time-of-flight-MS/MS system was operated in positive electrospray ionization (ESI) mode. The capillary voltage and cone voltage were 3.2 kV and 30 V, respectively. Nitrogen was used for the cone gas (50 L/hr) and the desolvation gas (700 L/hr), and argon was utilized as the collision gas. The mass chromatograms and spectral data were analyzed using the MetaboLynx software and urinary PhIP metabolites were identified based on the retention time of peaks and the mass difference from the PhIP protonated molecular ion ([M + H]+): 225.1140 m/z). The collision energy for the MS/MS fragmentation ranged from 15 to 35 eV.

Measurement of DNA adducts in colon

Once dissected, colon tissues were flushed with ice-cold phosphate buffered saline (pH 7.4) and then trimmed to remove adipose tissue. Colon tissues were opened lengthwise and frozen in liquid nitrogen prior to storage at -80°C. DNA was isolated from colon tissues using the Qiagen Gentra Puregene DNA Purification Kit with manufacturer's instructions (Qiagen, Hilden, Germany). The concentration and purity of isolated DNA were determined using a spectrophotometer (Bio-Rad Laboratories, Hercules, CA (242)). The isotopically labeled internal standard (i.e., [^{13}C]-dG-*C*8-PhIP) was spiked into DNA at a level of 10 adducts per 10^7 nucleotides. DNA (50 μg) was treated with DNase I, followed by nuclease P1, and then digested using alkaline phosphatase and phosphodiesterase, as described elsewhere (243, 244).

The efficacy of enzymatic digestion was confirmed using HPLC (Gilson, Middleton, WI). A Phenomenex Synergy Fusion-RP 80Å column (4μm, 150 × 4.6 mm; Phenomenex Inc., Torrance, CA) was employed for separation with a linear gradient starting at 95% of 20 mM ammonium acetate (pH 4.5) and 5% of acetonitrile increasing 15% of acetonitrile over 15 minutes. Hydrolyzed DNA (4 μg) and the identical amount of nucleoside standards (50 μg of each nucleoside/mL) were injected and monitored at 260 nm. The injection volume was 20 μL and flow rate was 1 mL/min.

The PhIP-DNA adduct levels in colon tissues were quantified using online column-switching LC-MS/MS (244). The Eksigent nanoLC Ultra system (Eksigent Inc., Dublin, CA) was equipped with a triple mass spectrometer (Thermo Fisher, San Jose, CA). A Waters Symmetry trap column (180 μm × 20 mm, 5 μm; Mildford, MA) and a

C18 AQ column (3 μm, 0.3 × 150 mm; Michrom Bioresources, Auburn, CA) were used for online solid phase enrichment and separation of PhIP-DNA adducts, respectively. Digested DNA was injected into the trap column and washed with 0.2% formic acid in 10% acetonitrile at a flow rate of 8 μL/min for five minutes. The trap column was then back-flushed into the analytical column followed by a linear gradient starting at 0.01% formic acid containing 10% acetonitrile and arriving at 0.01% formic acid in 95% acetonitrile at 28 minute. The flow rate was 8 μL/min. Between analyses, the injection needle was washed with 0.01% formic acid containing 10% acetonitrile in water, 0.01% formic acid containing 25% acetonitrile in water, and 100% dimethyl sulfoxide, consecutively after each run. The PhIP-DNA adducts were measured at MS^3 scan stage in positive ionization mode with an ESI source. The mass spectral parameters were optimized as previously described (245).

Calibration curves were constructed with the ratio between unlabeled PhIP-DNA adducts (0 to 30 adducts per 10^7 nucleotides) and isotopically labeled internal standard (10 adducts per 10^7 nucleotides). To validate the method, DNA, extracted from the negative control group, was spiked with a known amount of labeled internal standard. And then, unlabeled PhIP-DNA adducts were added at levels of 0, 5, 10, and 15 adducts per 10^7 nucleotides. The method validation and calibration curve construction were done in triplicates at each level. The r-squared value of the calibration curve was greater than 0.99 and the coefficient of variation was 5.5%, 7.3%, and 6.5% at the levels of 5, 10 and 15 adducts per 10^7 nucleotides, respectively.

Statistical analysis

All results were expressed as least squares mean ± standard error (SE). The data were analyzed by one-way analysis of variance using SAS (SAS institute, Cary, NC). A *p*-value less than 0.05 was considered statistically significant. The linear regression analysis was used to find the correlation between urinary PhIP metabolites and PhIP-DNA adduct.

Results

Food intake and body weight

Although there was a statistically significant difference in daily food intake between the negative control group and FC group, the actual difference in weight was small (18.80 ± 0.37 g versus 17.74 ± 0.41 g; P=0.04). There was no significant difference in weight gain amongst all groups, indicating that the effect of the difference in food intake is negligible.

Effects of phytochemical supplementation on activity and expression of CYP1A1 and CYP1A2

Compared to the positive control (i.e., AIN-93G + PhIP), the PEITC+I3C group showed increased CYP1A1 and CYP1A2 activity (P<0.0001, Table 3-2). The COMBO group also showed increased CYP1A1 and CYP1A2 activity compared to the positive control (P<0.0001). On the other hand, there was no difference noted in

the FC group for both CYP1A1 and CYP1A2 activity (Table 3-2). We also did not find differences in CYP1A1 and CYP1A2 protein expression for any group.

Effects of phytochemical supplementation on activity and expression of UGT1A1 and SULT1A1

Overall, there were no differences in UGT1A1 activity amongst the intervention groups compared to the positive control. For SULT1A1, PEITC+I3C increased activity (P=0.01) while the effect in the COMBO group was of borderline statistical significance (P=0.07; Table 3-2). There were no differences in UGT1A1 and SULT1A1 protein expression in any group compared to the positive control.

Analysis of urinary PhIP metabolites using UPLC-ESI-MS/MS

The structural identities of metabolites were identified using MS/MS analysis; reported fragmentation patterns of metabolites as well as their retention time were used (51). In total, we found 16 metabolites including unmetabolized PhIP. Structures of PhIP urinary metabolites and their proposed metabolic pathways are summarized (Fig. 3-2) (51). The identification of urinary metabolites is summarized in Table 3-3. In addition to the parent compound, six glucuronidated PhIP metabolites were detected (PhIP-N^3-glucuronide (XI), PhIP-N^2-glucuronide (XII), 4'-OH-PhIP-glucuronide (XIII), 4'-OH-PhIP-N^2-glucuronide (XIV), N^2-OH-PhIP-N^2-glucuronide (XV), and N^2-OH-PhIP-N^3-glucuronide (XVI)), while another six metabolites were either sulfated (4'-OH-PhIP-sulfate (VIII), 5-OH-PhIP-sulfate (IX), and 5,4'-diOH-

PhIP-sulfate (X)) or methylated (methyl-PhIP (II), N^2-methyl-PhIP (III), and N^2-methyl-4'-OH-PhIP (VII)). We also found non conjugated metabolites (4'-OH-PhIP (IV), N^2-OH-PhIP (V), and 5-OH-PhIP (VI)).

The relative abundance of each PhIP metabolite was calculated based upon the ratio of the percentage of each metabolite peak area to the total peak area. In the PEITC+I3Cgroup, all methylated PhIP metabolites (II, III, and VII), and two glucuronidated PhIP metabolites (XI and XII) were lower than the positive control. Metabolites IV, VI, VIII, X, XIII, XIV, XV, and XVI were higher in the PEITC+ I3C group (Fig. 3-3 (A)). Similar to the PEITC+I3C group, the feeding of the combination of phytochemicals decreased all methylated metabolites and one of the glucuronidated PhIP metabolites (i.e., XII) while non-conjugated metabolites (IV and VI) were increased. Notably, the parent compound was significantly increased in the COMBO group compared to positive control ($P<0.001$). In the FC group, none of the metabolites were different compared to the positive control group.

Based on the metabolic pathways of PhIP and biotransformation enzymes responsible for corresponding pathways, urinary metabolites were grouped into five groups: methylated PhIP metabolites (II, III, and VII), 5-hydroxylated PhIP metabolites (VI, IX, and X), sulfated PhIP metabolites (VIII, IX, and X), glucuronidated PhIP metabolites (XI, XII, XIII, XIV, XV, and XVI), and CYP1A-mediated PhIP metabolites (IV,V, VII, VIII, X, XIII, XIV, XV, and XVI; (Fig. 3-3 (B)). In the PEITC+I3C group, 5-hydroxylated, sulfated, glucuronidated, and CYP1A mediated (e.g., CYP1A1 and CYP1A2) metabolites were increased while the methylated PhIP

metabolites were decreased. The COMBO group had increased 5-hydroxylated and sulfated metabolites with decreased methylated metabolites. There was no significant effect noted on any metabolite group by the FC group (Fig. 3-3 (B)).

Measurement of PhIP-DNA adducts in colon tissues

In the PEITC+I3C group, there was a significant reduction in PhIP-DNA adducts in the colon tissues compared to the positive control (approximately 44% reduction; $P<0.0001$). Also, PhIP-DNA adducts were decreased in the COMBO group (approximately 24% reduction; $P=0.006$). There was no effect seen in the FC group (Fig. 3-4).

Discussion

One of the cancer preventive mechanisms of fruit and vegetable intake is to modulate the metabolism of carcinogens via biotransformation enzymes (156). To be specific, it has been demonstrated that cruciferous vegetables and the phytochemicals thereof (e.g., PEITC and I3C) induce expression and activity of phase I and II enzymes (127, 147, 265) whereas furanocoumarins present in apiaceous vegetables inhibit phase I enzymes (185, 266). Therefore, we hypothesized that synergistic effects would be found if these active compounds are provided together because the activation of PhIP will be inhibited while the induction of phase II enzymes may facilitate PhIP excretion.

As expected, we found significant effects of PEITC and I3C feeding on phase I enzyme activity, responsible for PhIP activation. The activity of CYP1A1 was increased by 9.5- and 5.7-fold in PEITC+I3C group and COMBO group, respectively. Similarly, significant effects were found in CYP1A2 activity as well (Table 3-2). In contrast, we were not able to demonstrate the induction of UGT1A1 activity and protein expression in the PEITC+I3C group. Interestingly, SULT1A1 activity was increased by PEITC and I3C feeding; there was a trend for an effect by the combination of phytochemical feeding (P=0.07). Effects of phytochemical supplementation on biotransformation enzymes were mostly in agreement with the results of metabolite profile analysis using the metabolomics-based approach. Specifically, PEITC and I3C feeding increased both phase I and II enzymes (i.e., CYP1A2, CYP1A2, and SULT1A1) and these effects were confirmed in relative abundance of urinary metabolites. Although we didn't see the statistical difference in UGT1A1 activity from glucosinolate hydrolysis product supplementation (i.e., PEITC and I3C), one of the major glucuronidated PhIP metabolites (i.e., XVI) as well as grouped glucuronidated PhIP metabolites were increased (Fig., 3-3 (A) and (B)). This inconsistency between enzyme activity assay and urinary metabolite abundance might be because the urine samples were collected for 24 hours whereas enzyme activity assays only measure activity at the time the tissue was harvested, meaning that effects on biotransformation enzymes would be accumulated in the urine samples. Thus, to understand the net effects from supplementation of phytochemicals on PhIP metabolism, it seems to be more reasonable to address both results, simultaneously.

Unexpectedly, although the PEITC+I3C group had increased CYP1A1, CYP1A2, and SULT1A1 activity, no induction of protein expression was observed. A similar discrepancy was found in the COMBO group as well. This lack of correlation between enzyme expression and activity is not unprecedented. Recently, Szaefer et al. investigated the effect of PEITC and I3C on activity and expression of CYP1A1 and CYP1A2 in Wistar rat liver. This study is somewhat very similar to the present study since both studies utilized the same rat strain (male Wistar rat) with similar age (5 weeks vs. 6 weeks) and provided same test compounds (PEITC and I3C) with comparable duration of exposure (7 days vs. 4, 10, and 30 days) through oral administration. Interestingly, although I3C increased both activity and expression of CYP1A1 and CYP1A2 at all tested time points, PEITC significantly increased CYP1A1 and CYP1A2 activity up to 1.7- and 2.7-fold respectively, without affecting their expression level at any time point (268). Likewise, CYP2B activity was increased by 3-fold after the 30 day treatment of PEITC without changing protein expression level measured by immunoblotting (268).

There are several possible explanations regarding this lack of correlation between enzyme expression and activity. As mentioned, the expression of these enzymes is known to be mainly regulated at the transcriptional level (269). However, more than one study has demonstrated that the expression of these genes might be modulated via other regulatory mechanisms. To be specific, the possibility of post-translational modification of CYPs has been suggested by other studies (249, 250). For instance, it was demonstrated that CYP17 was regulated via post-translational modification by naturally occurring lignan and its derivatives (250). The authors incubated human

adenocorticocarcinoma cell line with lignan (30 μM; final concentration) for 24 hours and found there is a lack of correlation between expression and activity of CYP17. Although activity of the enzyme was reduced by 37%, mRNA expression was increased by 2.3-fold, suggesting that the inhibition was not due to decreased mRNA expression level. It was explained that possibly phosphorylation of CYP17 was affected by the lignan derivative thereby abolishing the effect of induction of mRNA l, which was supported by the observation that inhibition of mitogen-activated protein kinase kinase further reduced the enzyme activity. This is in agreement with a previous study showing that phosphorylation is necessary for CYP17 activity (251). To shed light upon the inconsistency between the expression and activity of enzymes, further investigations, in particular regarding additional regulatory mechanisms of the respective gene expression and possible roles of phytochemicals, would be needed.

As mentioned, hydroxylation of PhIP on N^2 position is known as an initial step to exert genotoxicity of PhIP since it can be further esterified to N^2-acetoxy PhIP and N^2-sulfonyloxy PhIP by NATs and SULTs, respectively. These metabolites are known to create PhIP-DNA adducts via electrophilic nitrenium ion formation (50). This adduct is found to be the major adduct in DNA in in vitro (71), rat (67), mice (71), and human studies (243, 270). Thus, we evaluated PhIP-DNA adducts as a marker of PhIP exposure level in the colon tissues. However, it still requires caution to interpret and utilize as a cancer biomarker because there is no direct correlation between DNA adduct levels and tumor incidence (100, 260). And, it was also demonstrated that the level of DNA adducts in the colon was not directly correlated with ACF formation in rats (67), which are considered to be precursors of colon cancer. The lack of

correlations between surrogate biomarkers and tumor incidence could be because carcinogenesis is a long process and it is affected by a number of factors. For example, each tissue has different cell turnover kinetics (100) as well as different susceptibility against carcinogens (52). However, in this study, we principally hypothesized that activation of procarcinogen and detoxification of genotoxic PhIP would be affected by phytochemical supplementation thereby lowering the level of PhIP that reaches the target tissue (i.e., colon). And it was demonstrated that the level of PhIP exposure and PhIP-DNA adduct levels are proportional (71). Therefore, it can be justified that measuring PhIP-DNA adduct levels in the study would be informative of the net effects on PhIP metabolism from modulation of biotransformation enzymes.

In this study, we found protective effects of PEITC and I3C against PhIP-DNA adduct level in colon tissues, which is in agreement with previous observations (132, 133, 271). When compared to the positive control group, the PEITC+I3C group showed approximately 44% reduction of PhIP-DNA adduct formation while no significant effect was found in the FC group. Although there was also a statistically significant reduction in PhIP-DNA adducts in the COMBO group, given the effects on the metabolite profile as well as the more modest magnitude of reduction in PhIP-DNA adducts, it is likely that the protection of seen by the combination of phytochemicals is due only to the PEITC and I3C supplementation, not because of the synergism we expected. This is further supported by the lack of effect on phase I enzymes (i.e., activity, expression, and the metabolites mediated by CYP1A) in the FC and COMBO groups, indicating that intended suppression of PhIP activation was not achieved by the furanocoumarins.

Despite previous evidence demonstrating inhibition of phase I enzymes by apiaceous vegetables or furanocoumarins, we were not able to find such effects with either furanocoumarin or the combination of phytochemical supplementation. The lack of effects from furanocoumarins might be due to the dose given to rats. Gwang showed the inhibitory effects of 8-MOP, one of the purified compounds we utilized, in rats on CYP1A1, CYP1A2, and CYP2B1/2 activity (182). However, in that study rats were exposed to the compound via intraperitoneal injection as opposed to oral administration as in our study, which may exhibit lower bioavailability than injection. Further, the dose of 8-MOP that Gwang used was 25 mg/kg body wt; given the range of body weight of rats (150-200 g; Sprague-Dawley rat), it is very likely that the level of exposure of 8-MOP was significantly higher than the level provided in our study. In a human crossover study, CYP1A2 activity was significantly inhibited when 265.5 g of apiaceous vegetables per day (including dill weed, celery, parsley, parsnips, and carrot) were consumed over six days (150). In the present study, the levels of furanocoumarins exposure were equivalent to the amount of 101 g apiaceous vegetables (i.e., parsnips and celery) per day based on 2000 kcal intake. Thus, it is possible that the concentration of furanocoumarins given in our study may have been too low to reproduce effects shown in other studies.

In the present study, the levels of each class of compounds were matched with the dose of vegetables in the feeding study of Chapter 2, because 1) it is a realistic dosage to achieve via normal diets (for example, the equivalent amount in human would be approximately 0.42 cup of fresh cruciferous vegetables per day) and 2) initial

comparisons can be made between intact vegetables and purified compounds in terms of chemopreventive potential as well as PhIP metabolism. In Chapter 2, feeding AIN-93G diet supplemented with 21% cruciferous vegetables (wt/wt) increased CYP1A1 and CYP1A2 activity by approximately 35% and 61%, respectively, compared to control whereas in the present study the activity of these same enzymes was induced by 851% and 269%, respectively, by PEITC and I3C supplementation compared to a similar control (Table 3-2). The PEITC and I3C supplementation equaled the amount of total glucosinolates in the cruciferous vegetables fed in our previous study in the Chapter 2. Inconsistencies between intact vegetable feeding and purified compound feeding might be due to the bioavailability of phytochemicals. Most active compounds present in vegetables are in the form of esters, glycosides, or polymers. In order to be absorbed through the small intestine, they need to be hydrolyzed either by intestinal enzymes or colonic microflora to form aglycones (195), suggesting that the actual levels of phytochemicals absorbed and reached in the target tissues would be different. It is likely that purified compounds would exhibit higher bioavailability. On the other hand, in regard to the protective potential against PhIP, apiaceous vegetables significantly reduce PhIP-DNA adducts in colon tissues while no effect was found in the FC group. Furthermore, effects of intact apiaceous vegetable feeding on CYP1A2 were not significantly different compared to the PhIP positive control, implying there are other possible mechanisms regarding the protection than inhibition of PhIP activation via CYP1A2. To note, apiaceous vegetable feeding increased methylated PhIP metabolites while furanocoumarin feeding did not in the present study. Interestingly, methylated PhIP metabolites were negatively correlated with PhIP-DNA adducts in the colon tissues (correlation coefficient=-0.66481; P=0.02). Of the

three methylated PhIP metabolites, two metabolites were methylated on N^2 position of PhIP, indicating physical hindrance of PhIP activation. Thus, it is possible that active compounds in apiaceous vegetables, unlikely furanocoumarins as shown in the present study, are responsible for the induction of methylation of PhIP metabolites on the N^2 position thereby decreasing chances to be activated. Recently, it was found that orally administrated PEITC strongly down-regulated nicotineamide *N*-methyl transferase (NMT) in rats (255), supporting the idea that dietary vegetable intake may modulate NMTs. In fact, cruciferous vegetables, and PEITC and I3C feeding significantly decreased methylated PhIP metabolites in our rat studies. To our best knowledge, no study has investigated the effects of vegetable feeding on NMTs and their implications for PhIP metabolism as well as genotoxicity.

To summarize, we hypothesized that the combination of breakdown products of glucosinolates (PEITC and I3C) from cruciferous vegetables and furanocoumarins from apiaceous vegetables would exhibit synergistic effects against PhIP genotoxicity due to their complementary actions on biotransformation enzymes involved in PhIP metabolism. Although we found protective effects by the combined phytochemical feeding, there was no synergism observed because of the lack of overall effects of furanocoumarins on phase I enzymes (and thus possibly no effect on the suppression of PhIP activation). On the other hand, we also found that previously observed modulation of NMTs by intact apiaceous vegetables was not demonstrated by furanocoumarin supplementation; further investigations are warranted with regard to the compounds present in apiaceous vegetables that are responsible for modulation of methylation and the implications for PhIP metabolism. In addition, it was shown that

the activity of SULT1A1 was increased by PEITC and I3C supplementation. To our knowledge, no studies have been done with regard to the effects of specific cruciferous vegetables (or glucosinolates and their breakdown products) on modulation of SULT1A1. Given the significance of SULT1A1 in drug and xenobiotic metabolism, additional investigations are required.

Table 3-1. Diet composition

Diet Ingredients (g/kg)[a]		AIN-93G (g/kg)	PEITC and I3C (g/kg)[b]	Furanocoumarin (g/kg)[c]	Combination (g/kg)[d]
Cornstarch		397.5	397.5	397.5	397.5
Dextrinized cornstarch		132.0	132.0	132.0	132.0
Casein		200.0	200.0	200.0	200.0
Sucrose		100.0	100.0	100.0	100.0
Fiber		50.0	50.0	50.0	50.0
Mineral mix		35.0	35.0	35.0	35.0
Vitamin mix		10.0	10.0	10.0	10.0
L-Cystine		3.0	3.0	3.0	3.0
Choline bitartrate		2.5	2.5	2.5	2.5
Soybean oil[e]		70.0	70.0	70.0	70.0
PEITC and I3C	**PEITC**	Not applicable	182.6 mg	Not applicable	91.3 mg
	I3C		182.6 mg		91.3 mg
Furanocoumarin	**5-MOP**	Not applicable	Not applicable	1.2 mg	0.6 mg
	8-MOP			0.6 mg	0.3 mg
	Isopimpinellin			2.5 mg	1.2 mg
Total (g)		1000.0	1000.4	1000.0	1000.2

[a]The concentrations of PEITC and I3C, and furanocoumarins were matched with the vegetable contents (21% cruciferous, 21% apiaceous, and 21% combination feeding) used in our previous study (See discussion and Chapter 2). The phytochemicals were dissolved in the vehicle (i.e., corn oil) and mixed into powder diet (AIN-93G). [b]PEITC and I3C were used for the PEITC and I3C supplemented diet. [c]5-MOP, 8-MOP, and isopimpinellin were used for the furanocoumarin supplemented diet. [d]PEITC, I3C, 5-MOP, 8-MOP, and isopimpinellin were used for the combination diet. [e]The antioxidant, t-Butylhydroquinone (tBHQ; 0.02%) was included in soybean oil.

Table 3-2. Activity and expression of PhIP metabolizing enzymes[a]

	CYP1A1		CYP1A2		UGT1A1		SULT1A1	
	Activity[b]	**Expression[c]**	**Activity**	**Expression**	**Activity[d]**	**Expression**	**Activity[e]**	**Expression**
Basal diet (AIN-93G) + No PhIP	6.73±3.09	0.86±0.08	4.53±1.16	6.03±0.54	19.79±0.94*	1.45±0.20	12.10±0.33	0.67±0.09
Basal diet (AIN-93G) + PhIP	6.42±3.31	1.07±0.09	4.49±1.25	6.80±0.58	22.73±1.00	1.47±0.21	11.36±0.35	0.72±0.09
PEITC and I3C diet + PhIP	60.87±3.53***	1.02±0.1	16.62±1.32***	6.00±0.62	21.57±1.07	1.84±0.23	12.68±0.38*	0.91±0.10
Furanocoumarins diet + PhIP	9.66±3.45	1.01±0.09	5.67±1.29	5.73±0.61	20.18±1.05	1.68±0.22	12.12±0.37	0.67±0.10
Combination diet + PhIP	36.23±3.45***	0.91±0.09	12.43±1.29***	7.33±0.61	23.13±1.05	1.49±0.22	12.32±0.37	0.73±0.10

[a]All results were expressed as least squares means ± SE. Data were analyzed by ANOVA (SAS, Cary, NC). * denotes a statistical difference compared to PhIP positive control group ($P \leq 0.05$). *** denotes a statistical difference compared to PhIP positive control group ($P \leq 0.001$). [b]The unit of CYP1A1 and CYP1A2 activity was pmol resorufin/min/mg protein. [c]The expression unit of each enzyme was ng of protein/mL. [d]The unit of UGT1A1 activity was nmole 4-methylumbelliferone glucuronide/min/mg protein. [e]The unit of SULT1A1 activity was nmole *p*-nitrophenol/min/mg protein.

Table 3-3. Identification of urinary metabolites of PhIP in Wistar rats[a]

Identification	Metabolites	[M+H]+	Retention time (min)	Formula
I	PhIP	225.1162	3.63	C13H12N4
II	Methyl-PhIP	239.1323	3.38	C14H14N4
III	N^2-methyl-PhIP	239.1320	4.19	C14H14N4
IV	4'-OH-PhIP	241.1111	2.52	C13H12N4O
V	N^2-OH-PhIP	241.1105	3.57	C13H12N4O
VI	5-OH-PhIP	241.1107	3.75	C13H12N4O
VII	N^2-methyl-4'-OH-PhIP	255.1266	2.93	C14H14N4O
VIII	4'-OH-PhIP-sulfate	321.0664	2.38	C13H12N4O4S
IX	5-OH-PhIP-sulfate	321.0673	2.68	C13H12N4O4S
X	5,4'-diOH-PhIP-sulfate	337.0618	2.47	C13H12N4O5S
XI	PhIP-N^3-glucuronide	401.1482	2.98	C19H20N4O6
XII	PhIP-N^2-glucuronide	401.1463	3.42	C19H20N4O6
XIII	4'-OH-PhIP-glucuronide	417.1422	1.86	C19H20N4O7
XIV	4'-OH-PhIP-N^2-glucuronide	417.1431	2.82	C19H20N4O7
XV	N^2-OH-PhIP-N^2-glucuronide	417.1417	3.31	C19H20N4O7
XVI	N^2-OH-PhIP-N^3-glucuronide	417.1418	4.51	C19H20N4O7

[a]Wistar rats were injected with PhIP (10 mg of PhIP/kg body wt) and urine samples were collected for 24 hours after PhIP treatment (See materials and methods section for details).

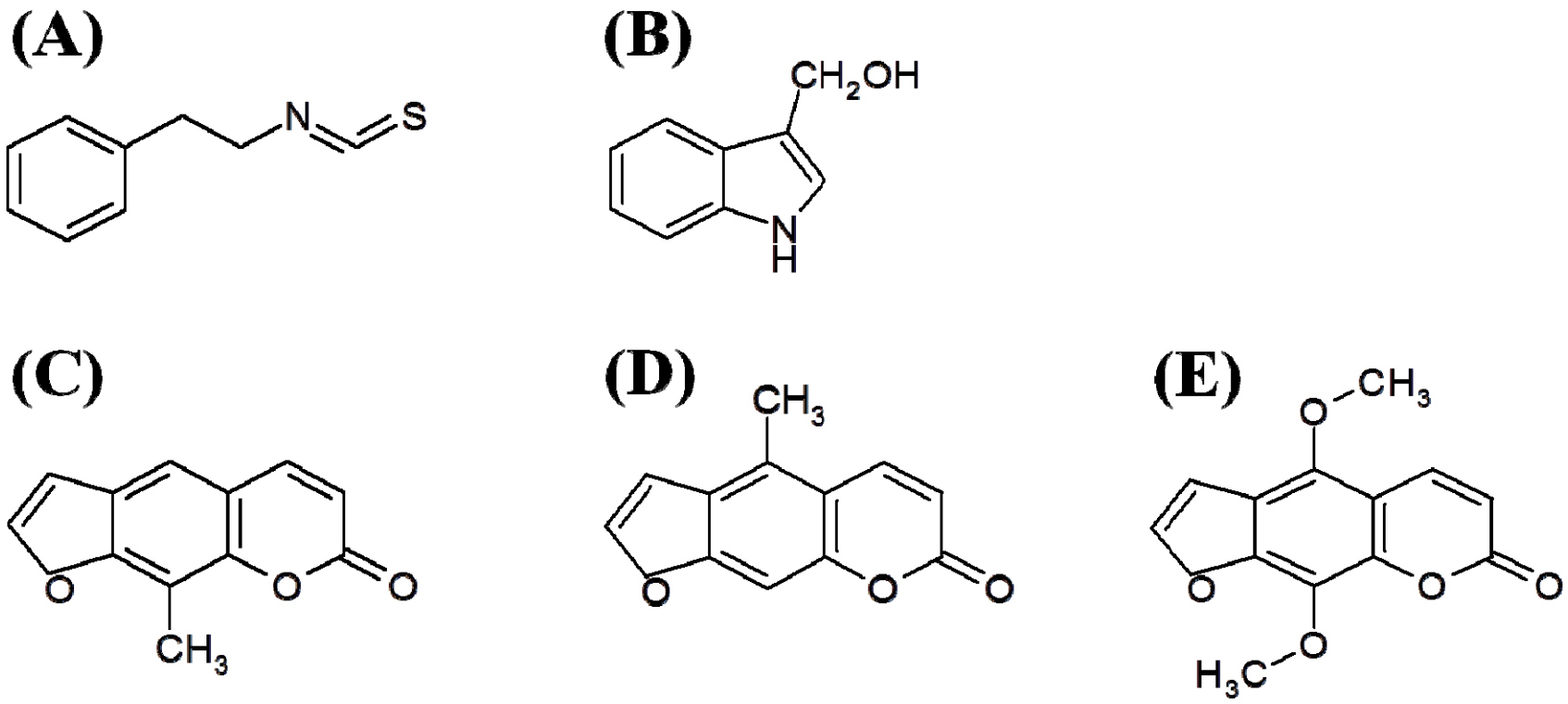


Figure 3-1. Structures of phytochemicals supplemented in diets

PEITC (A), I3C (B), 5-MOP (C), 8-MOP (D), and isopimpinellin (E)

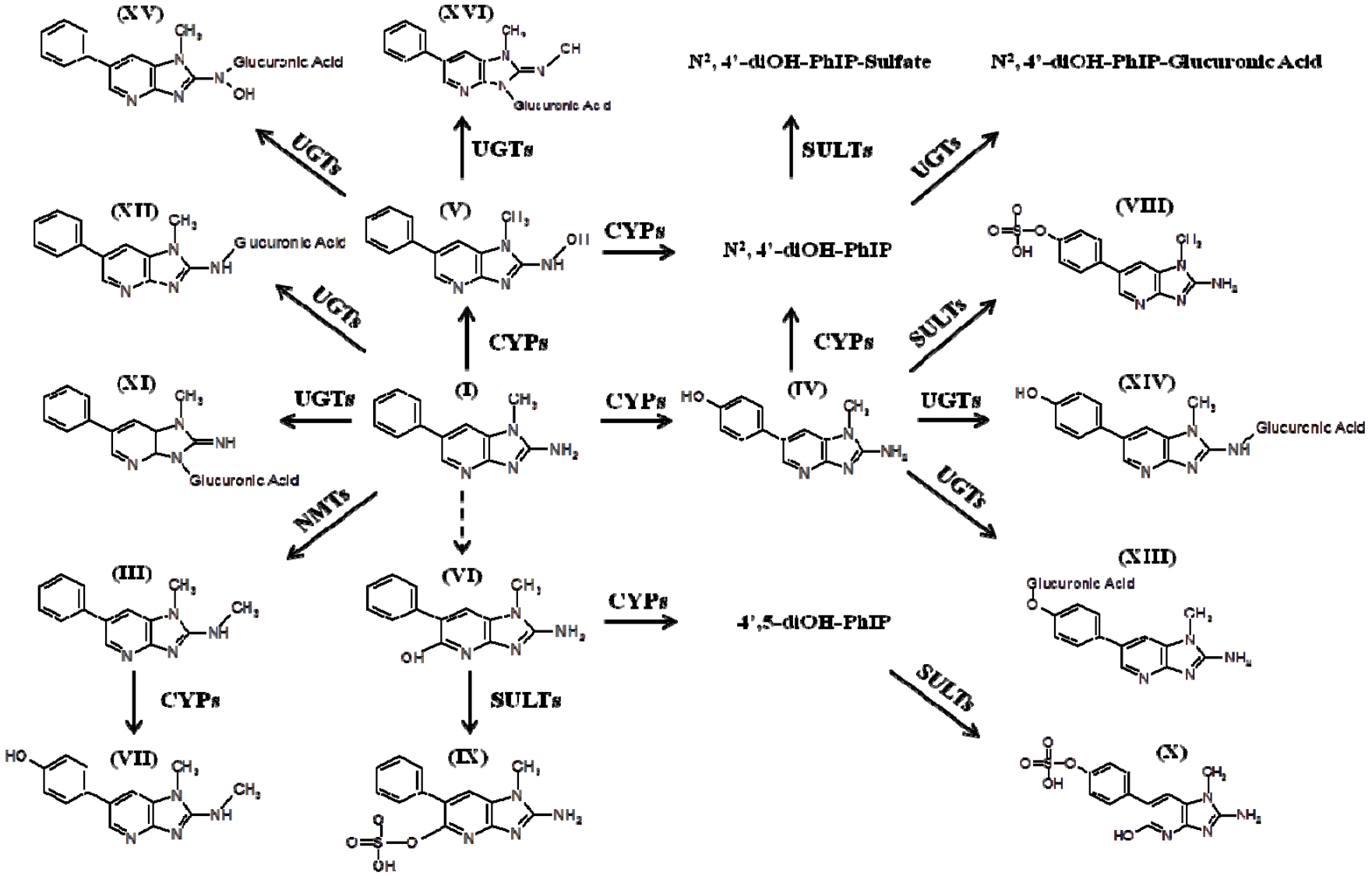
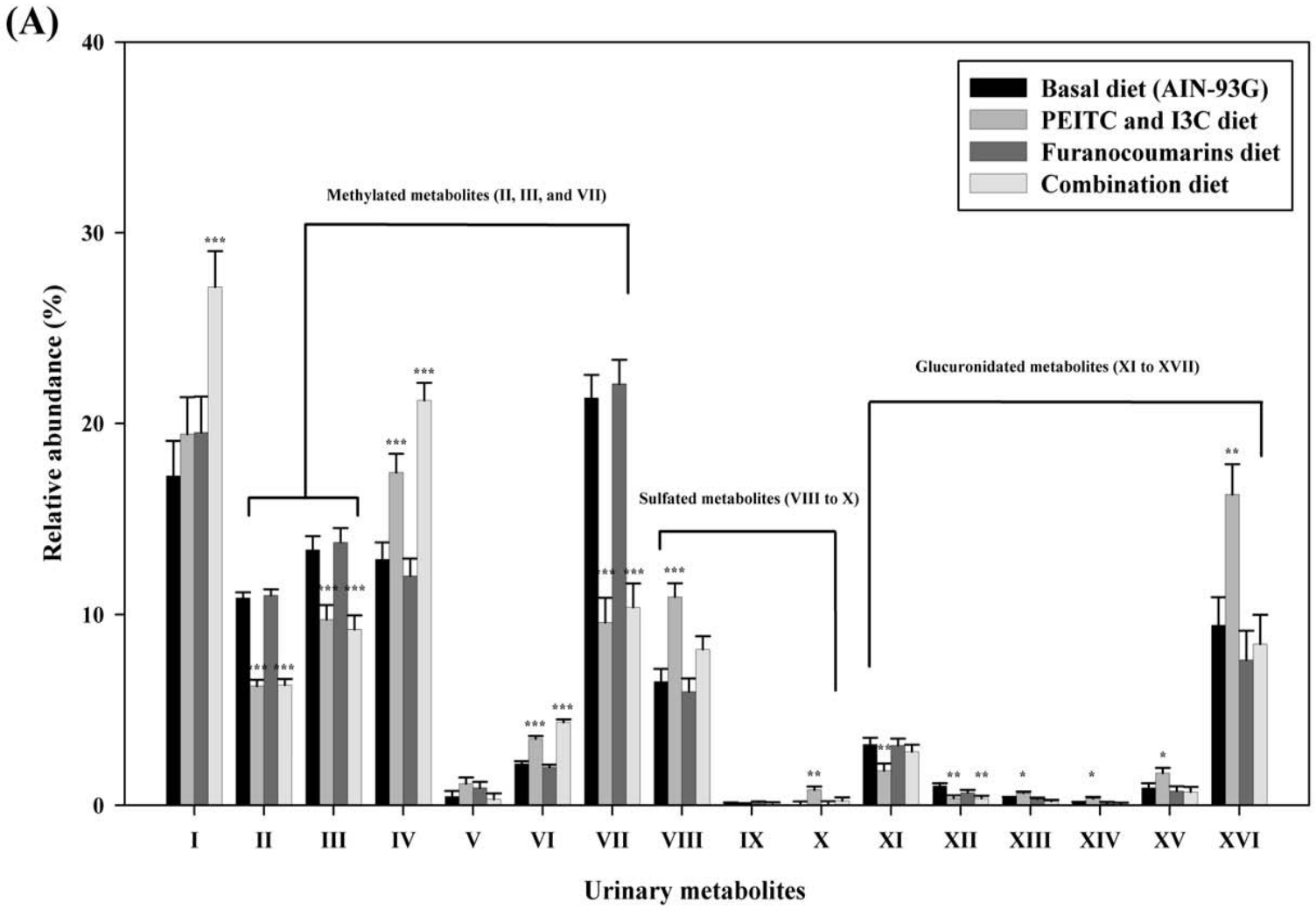

(XV)
Glucuronic Acid
(XVI)
Glucuronic Acid
N², 4'-diOH-PhIP-Sulfate
N², 4'-diOH-PhIP-Glucuronic Acid
UGTs
UGTs
SULTs
UGTs
(XII)
Glucuronic Acid
(V)
CYPs
N², 4'-diOH-PhIP
(VIII)
UGTs
CYPs
CYPs
SULTs
(XI)
Glucuronic Acid
UGTs
(I)
CYPs
(IV)
UGTs
(XIV)
Glucuronic Acid
NMTs
UGTs
(III)
(VI)
CYPs
4',5-diOH-PhIP
(XIII)
Glucuronic Acid
CYPs
SULTs
SULTs
(VII)
(IX)
(X)

Figure 3-2. Structures of urinary PhIP metabolites and metabolic pathways in Wistar rats

For total 16 urinary PhIP metabolites were detected including the parent compound (i.e., PhIP (I)). Solid lines indicate either CYP-dependent oxidation reactions or phase II-dependent conjugation reactions. Dashed line represents CYP-independent oxidation reactions. One of the methylated PhIP metabolite (metabolite II) is not shown above; the structure has yet to be elucidated.


(A)
Basal diet (AIN-93G)
PEITC and I3C diet
Furanocoumarins diet
Combination diet
Methylated metabolites (II, III, and VII)
Sulfated metabolites (VIII to X)
Glucuronidated metabolites (XI to XVII)
Relative abundance (%)
Urinary metabolites
0
10
20
30
40
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI

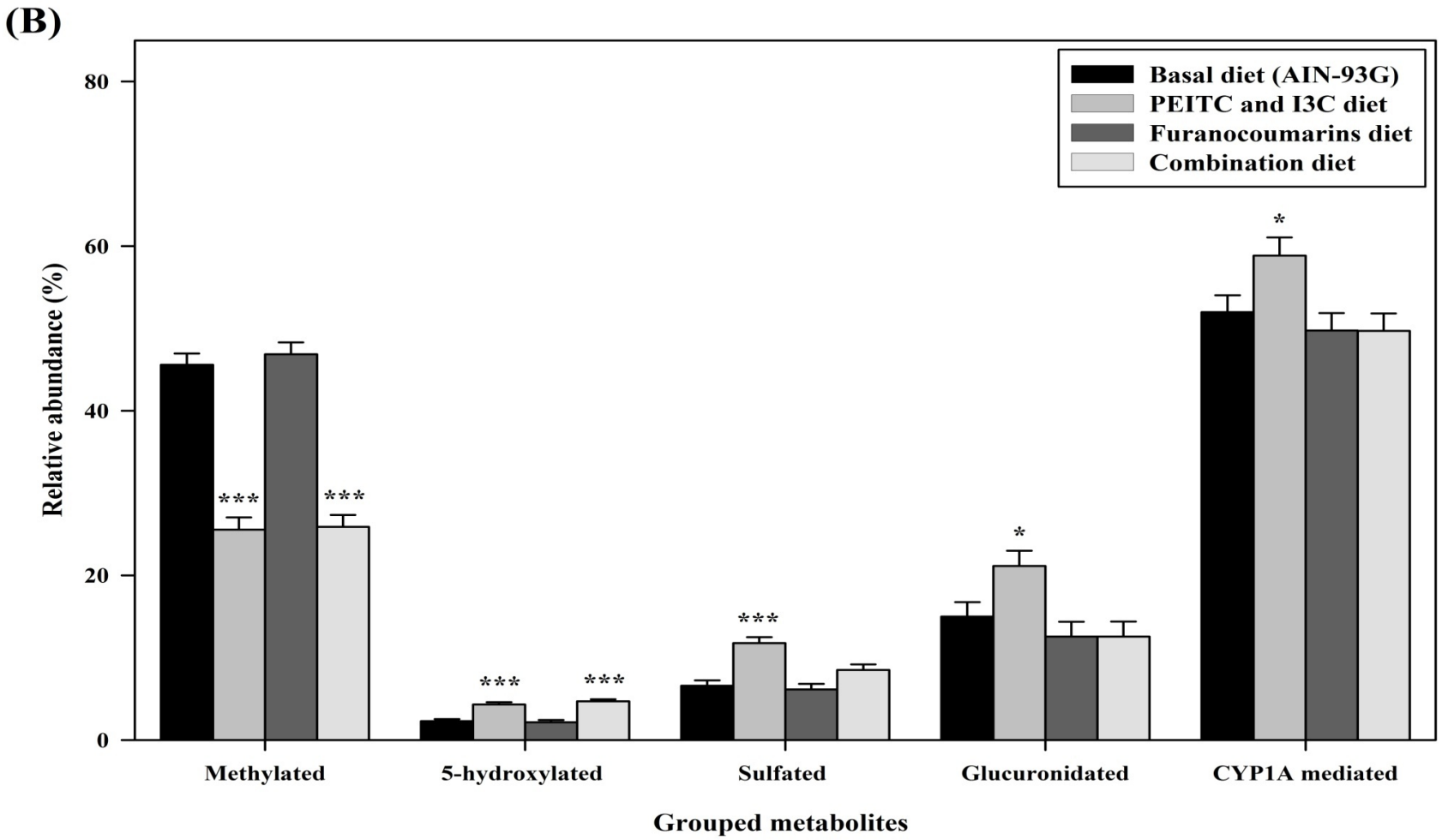

(B)
Basal diet (AIN-93G)
PEITC and I3C diet
Furanocoumarins diet
Combination diet
Relative abundance (%)
0
20
40
60
80
***
***
***
***
***
*
*
Methylated
5-hydroxylated
Sulfated
Glucuronidated
CYP1A mediated
Grouped metabolites

Figure 3-3. Measurement of urinary metabolites of PhIP in Wistar rats using UPLC-quadrupole time-of-flight-MS/MS

Urine samples from each diet group were collected over 24 hours after intraperitoneal injection of PhIP (10 mg of PhIP/kg body wt). The urinary PhIP metabolites were identified using the retention time of peaks and the mass difference from the PhIP protonated molecular ion. The relative abundance of each PhIP metabolites was calculated based upon the ratio between the percentage of each metabolite peak area and the total peak area. Data were analyzed by ANOVA (SAS software package, Cary, NC). *denotes a statistical difference compared to PhIP positive control group ($P \leq 0.05$). **denotes a statistical difference compared to PhIP positive control group ($P \leq 0.01$). ***denotes a statistical difference compared to PhIP positive control group ($P \leq 0.001$).

Each urinary metabolite (A) and grouped urinary metabolites (B) were shown; methylated PhIP metabolites: II + III + VII; 5-hydroxylated PhIP metabolites: VI + IX + X; sulfated PhIP metabolites: VIII + IX + X; glucuronidated PhIP metabolites: XI + XII + XIII + XIV + XV + XVI; CYP1A mediated PhIP metabolites: IV + V + VII + VIII + X + XIII + XIV + XV + XVI.

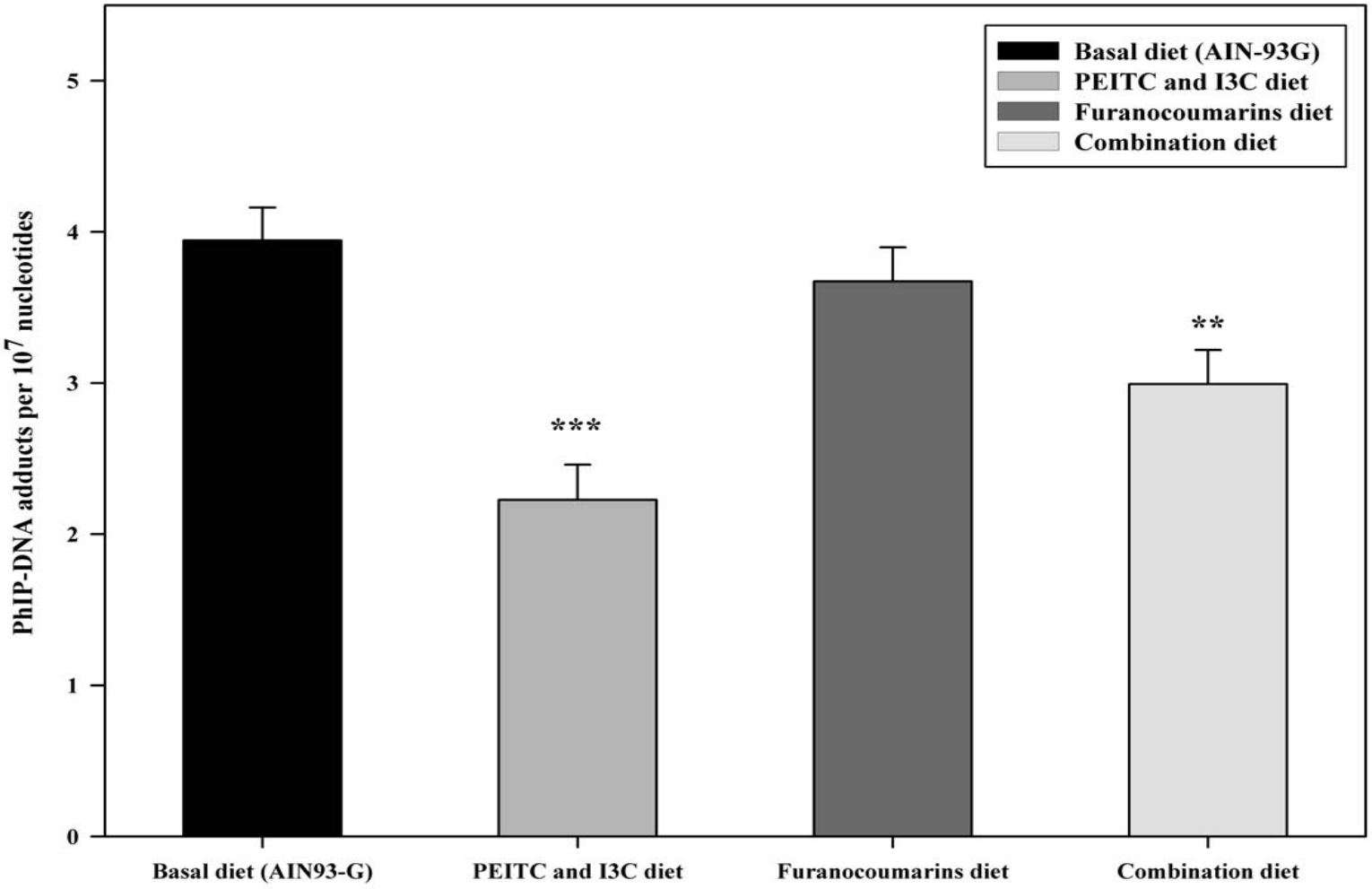

Basal diet (AIN-93G)
PEITC and I3C diet
Furanocoumarins diet
Combination diet
PhIP-DNA adducts per 10^7 nucleotides
0
1
2
3
4
5
***
**
Basal diet (AIN93-G)
PEITC and I3C diet
Furanocoumarins diet
Combination diet

Figure 3-4. Measurement of PhIP-DNA adducts in colon tissue

The DNA adducts level of colon tissues was determined using the online column-switching LC-MS/MS system as described in the method. All results were expressed as least squares means ± SE. Data were analyzed by ANOVA (SAS software package, Cary, NC). **denotes a statistical difference compared to PhIP positive control group ($P \leq 0.01$). ***denotes a statistical difference compared to PhIP positive control group ($P \leq 0.001$).

CHAPTER 4. SUMMARY AND FUTURE DIRECTIONS

Summary and future investigations

Apiaceous vegetables and their furanocoumarins inhibit activating enzymes for the food-born carcinogen PhIP, while cruciferous vegetables and their indole-3-carbinol (I3C) and phenethyl isothiocyanate (PEITC) induce PhIP-activating and -detoxifying enzymes. The experiments described in Chapter 2 and Chapter 3 were conducted to probe if these vegetables and their phytochemicals exert synergistic protection against PhIP genotoxicity owing to their complementary impacts on PhIP metabolism. Further, it was also aimed to make a direct comparison regarding the chemopreventive effects between intact vegetable feeding and purified compound feeding by matching the actual levels of active compounds, exposed to rats in each study.

In the feeding study in Chapter 2, male Wistar rats were fed diets supplemented with cruciferous (21%, wt/wt), apiaceous (21%, wt/wt), and combination of both cruciferous and apiaceous vegetables (10.5% wt/wt of cruciferous and 10.5% wt/wt of apiaceous) for seven days. And it was found that the apiaceous vegetable group had the most protective effect against PhIP genotoxicity (approximately 20% reduction in PhIP-DNA adducts) followed by cruciferous vegetable and combination of vegetable groups indicating that expected synergistic potential through the combination of the vegetables was not shown. However, given the lack of inhibitory effects of apiaceous vegetables on the activity of PhIP-activating enzymes and N^2-hydroxylated PhIP metabolites, the hypothesis on synergism of apiaceous and cruciferous vegetables is not completely rejected. Several experimental approaches can be pursued in future

studies to achieve the suppression of PhIP-activating enzymes and test the hypothesis originally intended: 1) higher dose of apiaceous vegetable supplementation, 2) utilization of specific mechanistic inhibitors of PhIP activating enzymes, and 3) genetically engineered animal strains (e.g., CYP1A1 null mice) (272). However, it should also be considered that such modulation in PhIP metabolism with either high dose of vegetable supplementation or exposure to specific inhibitors is achievable and realistic via habitual diets.

In the experiments described in Chapter 2 and Chapter 3, we utilized the same animal strain (i.e., male Wistar rats) with identical experimental conditions including the level of PhIP exposure in order to compare protective potency between fresh vegetables and purified active compounds present in corresponding vegetable groups; however it was unfeasible to make one-to-one comparison because of statistical difference in PhIP-DNA adduct levels of control groups between studies. Thus, protective potential was estimated by looking at the effects of diet supplementation against the respective PhIP positive control group in each study. Cruciferous vegetable feeding and PEITC+I3C feeding showed approximately 17% (P=0.07) and 44% (P<0.0001) reduction of PhIP-DNA adduct formation, respectively, which is reasonable considering higher bioavailability of purified compounds as opposed to innate forms present in vegetables. However, furanocoumarin supplementation failed to elicit similar protection, demonstrated by apiaceous vegetables in Chapter 2.

On the other hand, using a metabolomics approach, we detected 17 and 16 urinary PhIP metabolites in the vegetable feeding and phytochemical feeding studies,

respectively. Of note, methylation was one of the major metabolic pathways for PhIP based on the relative abundance of the methylated metabolites detected in both studies and one of the methylated PhIP metabolites was not matched with any previously reported reference, suggesting a novel metabolite. As aforementioned in previous chapters, methylation is generally considered as a toxification step since it makes metabolites more lipophilic than the parent compound (256). However, interestingly, it was shown that apiaceous vegetable feeding increased methylated PhIP metabolites and these were negatively correlated with PhIP-DNA adducts in the colon; this association between methylation of PhIP metabolites and PhIP-DNA adducts was not expected but is somewhat biologically plausible. Of three methylated PhIP metabolites, two of them were methylated on the N^2 position of PhIP; it is possible that methylation would influence the substrate-enzyme specificity, thereby providing less chance for PhIP to be activated to carcinogenic metabolites. In contrast, in the feeding study of Chapter 3, with purified compound supplementation, furanocoumarins (i.e., bioactive compounds of apiaceous vegetables) did not change the levels of methylated PhIP metabolites. Considering the abundance of methylated PhIP metabolites, further investigations on *N*-methyl transferases regarding their potential role in PhIP metabolism are warranted.

In both chapters, enzyme activity results were mostly in agreement with the metabolit e profile analysis while we found overall lack of correlation between enzyme activity and protein expression results. In particular, CYP1A1 and CYP1A2 activity in Chapter 3 were significantly increased in PEITC and I3C feeding (approximately 9.3- and 3.6-fold, respectively) without changes in their protein expression which is

somewhat unexpected. However this lack of correlation between protein expression and enzyme activity was observed in other studies as well. For example, as aforementioned, utilizing a similar study design (e.g., rat strain, supplemented phytochemical components, and duration of exposure), it was found that PEITC supplementation significantly increased both CYP1A1 and CYP1A2 activity while protein expression levels were not changed during 4, 10, and 30 days of feeding periods (268). Such inconsistency was even more pronounced in CYP2B; although 30 days of treatment with PEITC increased enzyme activity approximately 3-fold but did not change the protein expression level, this suggests the possibility that incorporation of PEITC may influence these enzymes via different regulatory mechanisms such as post-translational modification (268). In fact, there is some indication that CYPs may undergo post-translational modification (249, 250). To shed further light on this inconsistency, further studies are warranted, particularly regarding additional regulatory mechanisms of the respective gene expression and possible roles of phytochemicals.

Collectively, we originally hypothesized that chemopreventive effects of vegetables and their active compounds would be achieved via modulation of phase I and phase II enzymes which are responsible for PhIP activation and detoxification. And as a matter of fact, supplementation of cruciferous vegetables and their active compounds resulted in modulation of both phase I and phase II enzymes; these modulations seem to favor PhIP detoxification. Even though apiaceous vegetable supplementation was effective in lowering PhIP-DNA adducts, such protection was not likely owing to furanocoumarins present in them or suppression of PhIP activation, taking into

account the lack of effects of apiaceous vegetables on CYP1A activity and expression. It is not likely that impacts of vegetables are solely limited to their modulation in biotransformation enzymes. For example, there is evidence indicating another possible underlying mechanism of chemoprevention achievable through vegetables against PhIP, modulation of phase III detoxification. In particular, multidrug resistant-associated protein 2 (MRP2) is known to play a crucial role in PhIP metabolism as they extrude parent and PhIP metabolites from the gut mucosa (273, 274). Previously, it was demonstrated that active compounds present in vegetables (e.g., flavonoids) can modulate PhIP metabolism via 1) induction of protein expression level of MRP2, 2) inhibition of the activity of MRP2, and 3) acting as substrates thereby modulating biliary or renal excretion of MRP2 substrates (262). Therefore, investigations in regard to the impact of apiaceous vegetables on phase III detoxification would be meritorious to explain the protection of apiaceous vegetables against PhIP genotoxicity.

There are several limitations of the present experiments to note. Even though utilizing rodent models provides several scientific advantages, caution is required to interpret and extrapolate results. For instance, fresh liver and colon tissues were harvested and analyzed for protein expression, enzyme activities, and marker of genotoxicity (i.e., PhIP-DNA adducts) in the target tissue which are definitely not easy to acquire in human intervention studies. However, there are pronounced interspecies differences in metabolism of HAA, including PhIP. To be specific, it was demonstrated that the pathway of 4'-hydroxylation was shifted to N^2-hydroxylation in genetically engineered mice that express *hCYP1A1* and *hCYP1A2*, suggesting that humans favor

activation of PhIP more than rodents (275). Hence, interspecies differences must be considered when assessing human health risk. In addition, although formation of DNA adducts is an essential step to form various cancers, there are some controversies regarding its validity as a cancer biomarker (95). However, this is not the only case for PhIP-DNA adducts; most biomarkers possess caveats and limitations in regard to their predictive quality (e.g., aberrant crypt foci) (96). And, given the relative weak carcinogenic potency of PhIP, it requires a long-term experimental protocol (e.g., 52 weeks) with a diet containing 400 ppm PhIP for induction of colon cancer. With 100 ppm PhIP in their diet, it takes approximately 2 years to have more than 50% of cancer incidence in the colon which is not feasible for our situation due not only to time constraints but also due to the high cost of this carcinogen (225).

Concluding remarks

Accumulating evidence indicates that incident rates of colon cancer are positively related with high consumption of animal fat and meat (11, 12). And, one of the plausible factors responsible for the association between colon cancer risk and meat consumption might be a class of carcinogens, HAA (18, 19). Of note, PhIP often occurs in the highest concentration among the HAA, emphasizing the significance of PhIP in the etiology of colon cancer (31, 35, 36).

Diet is considered to be a very important risk factor for carcinogenesis. According to the report from the World Cancer Research Fund and the American Institute for Cancer Research, there is an inverse relationship between non-starchy vegetable and

fruit consumption, and colorectal cancer incidence (276). However, the innate complexity of diet makes it difficult to elucidate the underlying mechanisms and exact roles in cancer prevention. The results presented in our feeding studies make additional good examples of these complexities given the discrepant results between intact vegetable feeding and feeding the putative responsible compounds as well as the finding of a novel metabolite. Moreover, knowing that PhIP-induced carcinogenicity is strongly influenced by biotransformation enzymes and the pathways by which the metabolites are processed, the diversity of human genetics (e.g., polymorphisms of biotransformation enzymes) would cause more variations in colon cancer risk and susceptibility to PhIP genotoxicity. In this respect, the present feeding studies may carry an important meaning as they 1) included more than a single food item which is more realistic in daily staples and applicable for the general population, 2) probed potential synergism of two different kinds of vegetables which impact carcinogen metabolism through independent mechanisms, and 3) utilized an 'omics' technique thereby capturing the entire modification of PhIP as well as the complex interactions between dietary active compounds and biotransformation enzymes responsible for carcinogen metabolism.

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APPENDICES

Appendix A: methods and assays

Appendix A-1: method for tissue and microsome preparation

Materials

Need to be prepared a day before

- Homogenizer (large and small probe; make sure those probes are clean)
- Centrifuge and rotors for 20 mL tubes (10,000 × *g*; 6 tubes per each centrifucation)
- Ultracentrifuge + 70Ti rotor (20 mL ultracentrifuge tubes; 8 tubes per each centrifucation)
- Syringes (both 13 ml (with 20 mg of EDTA per each) and 35 mL for blood draw and perfusion respectively)
- 21G needles and butterfly needle (i.e., winged infusion set)
- Glass wool with 13 mL syringes
- Razor blades
- Pipettes (5 mL, 1 mL and 50 μL)
- Kim wipes
- Set aspirator and culture tubes
- Labeled screw cab tubes (6 microsome aliquots, 4 cytosol aliquots and 4 plasma aliquots)
- Labeled plastic bags for tissue storage (i.e., lung, pancreas, prostate, colon, and liver)
- Forms for tissue weight
- Dissection tools

- Centrifuge tube (for plasma prep)
- 15 mL falcon tube with cold phosphate buffered saline (store in the refrigerator; colon prep)
- Positive displacement pipette
- Plastic bags for rat body
- Squeeze bottles
- 10% formalin in PBS (v/v)
- Cassettes for colon tissue fixation
- Absorbent paper

Need to be prepared in the morning

- Glass plate mounted on a ice bucket (for liver mincing)
- Round bottom glass plate with ice-cold saline mounted on an ice bucket (for pancreas process)
- Container with ice to keep the homogenizer probe cold
- Beakers with ice cold water
- Liquid nitrogen with tong

Solutions

- Isolation buffer: 50 mM Tris base/150 mM KCl (pH = 7.5 at 0-4°C)
- Storage buffer: 50 mM Tris base/50% glycerol (pH =7.5 at 0-4°C). Make 100 mM Tris solution and dilute with an equal volume of glycerol. *Adjust the pH the next day in ice water bath and bring to final volume*.
- Phenylmethylsulfonyl fluoride (PMSF) dissolved in ethanol or methanol for a final concentration of 1 mM (wear gloves; Protease inhibitor). *It is kept as a 10 mM stock in ethanol; thus dilute 1:10 before use*.

- Butylated hydroxytoluene (BHT; antioxidant; lipophilic organic compound) for a final concentration of 0.01% (note: *Prepare the 10% BHT; we don't have to dilute this; see the procedure*).
- Wash buffer: 100 mM $Na_4P_2O_7$ (Tetrasodium pyrophosphate; in our lab, Sodium Pyrophosphate; The MW is 446.06.) / 10 mM EDTA (in our lab, Disodium Salt; The MW is 372.24; pH = 7.8 at 0-4°C).
- Phosphate buffered saline
- 10% formalin in PBS
- Normal Saline (0.9% NaCl)

Methods for microsome preparation

NOTE:

✓ For this procedure, it is very important to keep everything cold during all times.

✓ Chill homogenizer probe using a container with ice and keep it cold.

✓ For one ultracentrifuge tube the max is ~10.5g of liver.

1. Anesthetise the animal w/ isoflurane.
2. Cut the animal open, insert the 21G needle into the renal artery, and perfuse the liver with 35 mL of isolation buffer. Liver and kidney will clear if the appropriate vessel has been entered. Cut the blood vessels below heart. This will release the backpressure and further clear the tissues.
3. Remove liver, blot dry with Kim wipes, weigh, and mince with razor blades on a glass plate mounted on a bucket with ice.
4. Rinse the minced liver twice with isolation buffer (use about 1 mL) to further remove residual blood. Use Kim wipes to remove excess buffer.

5. Add 50 µL of PMSF while mincing.
6. Add the minced liver to a centrifuge tube (oakridge tubes) containing isolation buffer (3 g of liver per tube with 15 mL isolation buffer; thus, *1 liver weight : 5 buffer ratio*)
7. Drop the remaining liver into liquid nitrogen for further analysis.
8. Homogenize the minced liver with the pre-cooled probe while keeping the centrifuge tube inside a beaker with ice-cold water. The time for homogenization will depend on how well the liver is minced (10-20 seconds is enough). Use the thicker probe homogenizer.
9. Centrifuge at 10,000 × *g* for 15 minutes at 4°C. (i.e., 8300 rpm)
10. Aspirate off very carefully the lipid layer that forms on the surface of the supernatant.

    Decant supernatant through a syringe (12 mL w/ Luer lock tip) containing glass wool, approximately 1/3 full to remove excess lipids collecting into the ultracentrifuge tubes. Balance the tubes carefully prior to centrifugation by adding isolation buffer.
11. Ultracentrifuge at 100,000 × *g* (i.e., 37,000 rpm) for 70 minutes. Make sure to remove the tubes from the ultracentrifuge as soon as it stops spinning.
12. Aspirate off and discard floating lipid. Keep the supernatant (cytosol), aspirating off the last drops.
13. Add 0.5 mL (for 3 g of liver) or 1.75 mL (for 10.5g of liver) of storage buffer to the tube containing the pellet.
14. Use a cold spatula to remove pellet from the surface of the glycogen pellet, which looks like a yellowish gel. Waiting a few minutes after addition of the

storage buffer might cause the microsomes to slide down the glycogen pellet, making it easier to leave the glycogen pellet behind. Glycogen will be the layer sticking to the tube. Use spatula to remove the reddish layers (where the microsomes are). Try to leave the glycogen in the tube

15. Transfer the microsomes plus the storage buffer to a different tube (culture tubes work fine), and quickly homogenize – small probe (5 seconds), trying to keep the tube in ice-cold water.
16. Add 10% BHT (final concentration should be 0.01%) and homogenize for 5 more seconds. For 1.75 mL you should add 17-18 µL.
17. Transfer the homogenate (about 350 µL) to vials using a positive displacement pipette, flush with nitrogen, and store at –80°C until it is time to run your assays.

Appendix A-2: method for microsomal wash

NOTE

- ✓ Microsomes need to be store with glycerol buffer.
- ✓ This step will wash glycerol, proteases and albumin.

Solution

- Wash buffer: 100 mM $Na_4P_2O_7$ (Tetrasodium pyrophosphate; Sodium Pyrophosphate; The MW is 446.06.)/10 mM EDTA (pH = 7.8 at 0-4 °C).

Methods

1. Chill equipment as necessary: homogenizer, tubes, solutions.
2. Check pH of washing buffer (tetrasodium pyrophosphate/EDTA). It should be around 7.8. (Note: The stored microsome fraction should contain about 40-60 mg protein/mL.)
3. The wash buffer is a chelating buffer, and it removes the soluble proteins by mass action; therefore, the protein concentration of your sample must be less than 2.0 mg/mL of wash buffer.
4. For the first few samples, you should determine the protein concentration so you can figure out the volume of wash buffer to be used.
5. Use a positive displacement pipette to transfer the microsome fraction to an ultracentrifuge tube.
6. Add a small volume of wash buffer to the vials in order to get the microsomes that adhere to the vials.
7. 500 µL of the microsomes in storage buffer: 15 mL of washing buffer
8. Balance the ultracentrifuge tubes by weighing to ± 0.1 g.

9. Ultracentrifuge at 37,000 rpm for 70 minutes.
10. Decant and discard supernatant.
11. Re-suspend washed pellet in 1.0 mL of 50 mM Tris buffer, or whatever buffer is going to be used for your assay.
12. Homogenize gently.
13. Do Bradford and enzyme activity assay.

Appendix A-3: method for protein quantification by Bradford Assay

Methods

1. Gently mix the Bradford reagent in the bottle and bring to room temperature.
2. Prepare a series of protein dilutions
   - 0.5 mg/mL Tris buffer
   - 0.25 mg/mL Tris buffer
   - 0.125 mg/mL Tris buffer
   - 0.0625 mg/mL Tris buffer
   - 0.03125 mg/mL Tris buffer
   - 0.015625 mg/mL Tris buffer
   - 0 mg/mL Tris buffer
3. Prepare microsomes dilutions
   - 1:10 dilution (10 µL of microsome + 90 µL of Tris buffer)
   - 1:20 dilution (40 µL of 1:10 dilution + 40 µL of Tris buffer)
   - 1:40 dilution (30 µL of 1:20 dilution + 30 µL of Tris buffer)
4. Transfer 200 µL of Bradford reagent. No need to place in ice. Cover clear plate.
5. Add 5 µL of BSA STDs and microsome dilutions
6. Wait 10 minutes and read absorbance at 595 nm, Intensity 3, shaking time 10 seconds.

Appendix A-4: method for CYP1A1 and CYP1A2 activity

Methods

1. Supplement (895 μL per sample)

   - 855 μL of Tris buffer (58 mM, pH 7.5 at 37°C; the final concentration will be 0.05 M)

   - 30 μL of $MgCl_2$ (0.833 M stock sol; the final concentration will be 0.025 M)

   - 10 μL of NADPH (the final concentration will be 0.5 mM)

   (Tetrasodium salt form; 50 mM stock sol. (= 4.165 mg/100 μL, or just multiply mg of NADPH × 24.008 μL of Tris buffer)

2. Add Substrate (900 μL per sample)

   (1) For Standard Curve

   - Dilute stock solution of resofurin (100,000 μM (= 100mM) to 1,000 μM)

   - Make serial dilutions from 1,000 μM (i.e., 100 μM, 10 μM, and 1 μM)

   - To dilute resofurin, use DMSO

   895 μL of Supplement + 5 μL of 1,000 μM (final concentration = 5 μM)

   895 μL of Supplement + 5 μL l 100 μM (final concentration = 0.5 μM)

   895 μL of Supplement + 5 μL 10 μM (final concentration = 0.05 μM)

   895 μL of Supplement + 5 μL 5 μM (final concentration = 0.025 μM)

   895 μL of Supplement + 5 μL 1 μM (final concentration = 0.005 μM)

   895 μL of Supplement + 5 μL DMSO

   (2) For microsome samples (1 mM stock sol.; the final concentration will be 5 μM)

   895 μL of Supplement + 5 μL MROD stock sol (for samples; make 35sec)

   895 μL of Supplement + 5 μL MROD stock sol (for control; make one only)

895 μL of Supplement + 5 μL EROD stock sol (for samples; make 35sec)

895 μL of Supplement + 5 μL EROD stock sol (for control; make one only)

3. Make Aliquots (total volume would be 1,000 μL per sample)

(1) For Standard curve

- Add 100 μL of tris buffer

(2) For microsome samples

- Add 100 μL of microsome protein (i.e., 2 mg/mL Tris buffer)

(3) For Control

- Add 100 μL of inactivated microsome protein (i.e., 2 mg/mL Tris buffer; boiled at 100°C)

4. Transfer 200 μL to 96-well plates (4 replicates)

Appendix A-5: method for UGT1A1 activity

Solutions

- Wash buffer: 100 mM tetrasodium pyrophosphate ($Na_4P_2O_7$); 10 mM EDTA-disodium salt; pH: 7.8 at 0 – 4°C
- UGT1A1 Reaction buffer: 100 mM Phosphate buffer; 4 mM $MgCl_2$; pH: 7.4
- 600 µM 4-methylumbelliferone sodium salt (4-MU): 4-MU is delivered in 5 µL of DDH_2O
- 5 mM UDPGA
- 24% perchloric acid
- 25 mM phosphate buffer; pH 3.1 (HPLC mobile phase)
- 4-MU standards (dilute 100 µM STD to 50, 25, 12,5 6.25 and 3.125 µM concentrations)
- 4-MUG standards (dilute this 400 µM STDs to 200, 100, 50, 25, 12,5 6.25 and 3.125 µM concentrations)

Methods

1. Add 50 µg of microsomal protein and bring up to 145 µL with reaction buffer.
2. Add 5 µL of 4-MU
3. Add 50 µL of UDPGA
4. Incubate the tubes for 90 minutes at 37°C
5. Terminate the reaction by adding 10 µL of 24% perchloric acid
6. Centrifuge the tubes at 5000 rpm for 10 minutes to precipitate protein
7. Filter the supernatants through a 0.45 µm syringe tip filter into HPLC vials.
8. Freeze the vials until analysis by HPLC.

Appendix A-6: method for cytosolic SULT1A1 activity

Solutions

- 52.6 mM Potassium phosphate, 5.5 mM Magnesium chloride buffer (pH 6.5)
- 0.1 mM Naphthol (delivered in 100 µL of 50% of EtOH)
- 5 mM ρ-Nitrophenyl sulfate (delivered in 780 µL)
- 20 µM PAPS (delivered in 20 µL)
- Reconstitute the concentration of liver cytosol (4 mg protein/mL)
- 0.25 M Tris-HCl, pH 8.7

Methods

1. Add 0.1 mM Naphthol in 50% EtOH (100 µL per sample) into an EP tube (duplicate).
2. Add 5 mM ρ-Nitrophenyl sulfate (780 µL per sample).
3. Add 20 µM PAPS (20 µL per sample)
4. Add 400 µg of cytosolic protein (100 µL)
5. Vortex and incubate covered for 15 minutes at 37°C
6. Terminate the reaction with 100 µL of 0.25 M Tris-HCl buffer, pH 8.7
7. Read absorbance at 405nm

Appendix A-7: method for in vitro PhIP metabolism in human liver S9 fraction

Solutions

- 20 mM PBS (pH 7.5)
- $MgCl_2$ stock solution
- PhIP stock solution
- β-NADPH
- Microsome stock solution

Methods

1. Wash microsome and reconstitute the protein in PBS
2. Do Bradford and adjust microsome concentration to 5 mg/mL PBS
3. Add 40.7 μL of $MgCl_2$ stock solution per sample
4. Add 44.8 μL of PhIP stock solution per sample
5. Add 20 μL of Microsome stock solution per sample
6. Add 94.5 μL of β-NADPH per sample
7. Incubate the reaction mix in 37°C water bath for 20 minutes
8. Activate Waters Oasis extraction cartridge with 1 mL methanol followed by 1 mL DDW
9. Apply the sample into a column and elute with 1 mL DDW (do not collect this fraction)
10. Elute the column with 1 mL MeOH and collect the fraction
11. Analyze samples using UPLC-TOF-MS

Appendix A-8: method for glucosinolate extraction and analysis

Solutions

- 0.5M Sodium Acetate, pH 4.6
- 0.5N NaCl
- 0.4 M Phosphate Buffer, pH 7
- 2mM Ferricyanide (prepare fresh)
- 1N NaOH (prepare fresh)
- 2N NaOH (prepare fresh)

Vegetable processing

1. Purchase fresh broccoli, cabbage, and watercress from produce department. Check with the produce manager for the freshest batch or when the store receives the vegetables and time your purchase and processing around this.
2. In the lab weigh a fresh sample of each vegetable, taking care to minimally damage. Place in a pilot plant hairnet, secure with a twist-tie and weigh. Flash freeze in liquid nitrogen then transfer the frozen vegetable on dry ice until it goes onto lyophilizer. From experience it was determined that the broccoli and cabbage require about six days to completely dry when using the center drying drum. This was confirmed by weight change over time. Weigh the vegetable when dry.
3. Grind the dried vegetable in a food processor to a powder and transfer to ziplock bags and store at -80°C.
4. Weigh out approximately 200 mg of the ground dried broccoli, or 100 mg watercress, or 200 mg cabbage and place in a 15 mL glass screw-cap tube.

Deactivation of myrosinase and extraction of glucosinolates

1. Pipette 4.5mL cold Methanol into a 15 ml glass screw-cap tube. There should be 1 tube of methanol prepared for each tube containing plant material to extract. Place both sets of tubes in a heating block set at about 65-70°C. Allow time (10min) for the tubes and content to equilibrate with the heating block.
2. Place a flask or capped tube with either ddH_2O or ddH_2O containing 0.5 mg sinigrin/1 mL boiling water to preheat the solutions prior to addition to the extraction tubes
3. Transfer the 4.5 mL of the 100% Methanol solution into the extraction tube by pouring it thru a small glass funnel. Cap the tubes, lightly vortex, and then place it back in the heating block immediately.
4. Reflux the tubes with the methanol added for 20 minutes at 70°C. Do not tighten the caps completely. Pressure will build in the tube and if you open a tightened cap the solution will pop resulting in the loss of sample and methanol.
5. After 20 minutes, carefully open the caps and add either 1 mL of 70°C water or 0.5 mL sinigrin spike using the Microman Positive displacement pipette.
6. Increase the heating block temperature to 80°C and extract for 30 minutes.
7. Remove the tubes from the heating block. Cool the tube.
8. Homogenize the water–vegetable mix at speed 2.5 using the *PRO 200* homogenizer. 30 strokes
9. Transfer the homogenate to a high-speed centrifuge tube.
10. Add 2.5 mL 90% methanol to the extraction tube and rinse the probe with a brief homogenization in this tube.

11. Centrifuge the primary homogenate at 18,000 × *g* for 15 minutes at room temperature. Transfer the supernatant to a 12 mL round bottom Falcon tube.
12. Add the methanol rinse to the tube containing the pellet. Vortex and centrifuge again at 18,000 × *g* for 15 minutes. Add this supernatant to the Falcon tube.
13. Add 2.5 mL 90% methanol to the pellet, vortex, centrifuge and transfer the supernatant.
14. Store the tubes at -20°C until further processing.

Column and sample preparation:

1. Dry the tubes containing the pooled supernatants at 45°C under nitrogen.
2. Prepare the Strong Anion Exchange (SAX) solid phase extraction cartridge (500 mg from Supelco, Cat. #57017) on a vacuum manifold. It is important to never let the columns dry out.
    a. Activate the columns with 3 mL of Methanol
    b. Wash with 3 mL water.
    c. Wash SAX with 2 mL of 0.5M Sodium Acetate pH 4.6
    d. Wash SAX with 2 mL of H_2O
3. Reconstitute the dried extract with 3 mL ddH_2O. Vortex
    a. Place a plug of glass wool into a 3 mL syringe. Then load the supernatant into the syringe and through glass wool onto the SAX column.
    b. Rinse the tube that had contained the extract with 2.5ml of ddH_2O water and load this rinse onto the column.
    c. Place collection tubes in the manifold rack.

d. Elude the glucosinolates with a total volume of 3 mL of 0.5N NaCl. Add the NaCl in 1.5 mL increments and let it move slowly through the column.

Alkaline treatment and reaction with ferricyanide

Alkaline Treatment:

1. Prepare the 1 mM Sinigrin standard in 1N NaOH. This standard will be serially diluted and used for the standard curve in a 12 mL Falcon tube.
2. Label 12 mL Falcon tubes to use for hydrolysis of the extraction. Add 2.0 mL of the eluted glucosinolate with an equal volume of 2 N NaOH to the appropriately labeled tube.
3. Incubate for 30 minutes at room temperature.
4. After about 20-25 minutes, dilute the sinigrin standard preparation as described in the solution preparation portion of the method.
5. After 30 minutes, add 310 µL of concentrated HCl to all tubes, vortex after each addition.
6. Transfer 1.5 mL aliquots of the supernatant to two culture tubes for assaying the total glucosinolate content using the ferricyanide method. Confirm that there are no particulates.

Spectrophotometric Analysis of Glucosinolates using Ferricyanide:

1. Blank the spectrophotometer on water at 420nm.
2. Prepare duplicate tubes with 1.5 mL of either standards or samples.
3. Add the phosphate or ferricyanide solution to the tube containing the sample. Transfer mixture to a glass cuvette, and read absorption at 2 minutes (timed from the start of the addition of the phosphate or ferricyanide)

Appendix A-9: method for furanocoumarin extraction and analysis

Methods

1. Cut at least 4-5 celery stalks in small pieces then homogenize in food processor. Peel parsnip, grate using food processor, and then homogenize parsnip using food processor. Weigh 5 g homogenate directly into 50 mL screw cap glass tubes.
2. Mix 5 g of homogenate with 15 mL water and 10 mL diethyl ether; vortex.
3. Homogenize mixture in Ultra-Turrax homogenizer, at setting 4, completing full up – down motion with tube 10 times.
4. Sonicate samples for 2 minutes.
5. Centrifuge in buckets at ~ 3500 × *g* for 15 minutes (repeat spin if poor separation with parsnip) and collect organic phase in 20 mL screw cap tubes.
6. Repeat extraction/centrifugation four more times, (vortexing for 1 minute; combine organic phases by adding only 10 mL of diethyl ether)
7. Evaporate solvent using N-evap during centrifugation. Use foil under the hood.
8. Resuspend residue in 1.8 mL acetonitrile then, sonicate 3 minutes
9. Add 1.2 mL of water (to make 60% of acetonitrile)
10. Activate reversed-phase (C_{18}) columns with 3 mL methanol followed by 3 mL water (use squirt bottles).
11. Apply the sample into C_{18} column
12. Elute with 3 mL 60% acetonitrile in water, and collect fraction in 15 ml screw top tubes.

13. Wash the tube inside with 3 mL 60% acetonitrile in water and apply this into column as well
14. Remove solvents using N-evap with water bath at 40°C.
15. Reconstitute sample in 1 mL chloroform
16. Activate SiOH column with 3 mL chloroform.
17. Apply the sample into SiOH column.
18. Elute with 3 mL 7.5% ethyl acetate in chloroform, collecting fraction in 15 mL screw cap tubes.
19. Wash the tube inside with 7.5% ethyl acetate in chloroform and apply this into column as well
20. Remove solvent using N-evap.
21. Resuspend in 0.5 mL acetonitrile and sonicate samples for 3 minutes.
22. Add 0.5 mL water (or aqueous mobile phase of your separation; e.g., phosphoric acid); transfer with Pasteur pipet to HPLC vials.
23. Analyze using HPLC; UV diode array detector set at λ = 310 nm; Agilent Poroshell 120 EC-C18 column (150 × 4.6 mm; 2.7 μm); mobile phase: 10 mM phosphoric acid/methanol (45:55, v/v) at 40° C, flow rate 0.5 mL/min. (Isocratic method)

Appendix A-10: method for DNA isolation from animal tissues

Materials

- 1.5 mL microcentrifuge tubes
- Hand homogenizer (chill in liquid nitrogen)
- Water bath (37°C, 55°C and 65°C)
- Ice (in ice buckets)
- Microcentrifuge (24 tubes per run)
- Glass plate with scissors, surgical knife, and forceps

Reagents

- Saline
- Liquid nitrogen
- Isopropanol
- 70% ethanol
- Lysis Solution
- Protein Precipitation Solution
- DNA Rehydration Solution
- Proteinase K
- RNase A

Methods

1. Add 600 µL of Lysis Solution to a 1.5 mL microcentrifuge tube, and chill on ice.
2. Add 30–40 mg of tissue to the chilled Lysis Solution.
3. Incubate the lysate at 65°C for 60 minutes (or more)

4. Add 3 µL of Proteinase K, vortex samples, and incubate at 55°C for 2 hours (or more).
5. Add 3 µL of RNase A, vortex samples, and incubate at 35°C for 25 minutes.
6. Cool down samples on ice for 5 minutes.
7. Add 200 µL of Protein Precipitation Solution and vortex for 20 seconds.
8. Centrifuge for 10 minutes at 18,000 × *g* (the precipitated protein will form a tight white pellet).
9. Carefully transfer the supernatant, containing the DNA to a 1.5 mL microcentrifuge tube
10. Add 600 µL of room temperature isopropanol.
11. Gently mix the solution by inversion until white thread-like strands of DNA form a visible mass.
12. Centrifuge for 1 minute at 18,000 × *g* (DNA will be visible as a small white pellet) and carefully decant supernatant.
13. Add 600 µL of room temperature 70% ethanol and gently invert the tube several times to wash DNA.
14. Centrifuge for 1 minute at 18,000 × *g*.
15. Carefully remove the ethanol using pipette.
16. Air dry the pellet for 10 minutes.
17. Add 100 µL of DNA Rehydration Solution and rehydrate the DNA by incubating at 65°C overnight
18. Check the purity and concentration.

Appendix A-11: method for DNA quantification

Methods

1. Dilute one of your sample (1:50, 2 µL of original solution and add 98 µL of saline).
2. Measure this sample using a spectrophotometer.
3. Dilute this original sample to 100 µg/mL concentration (for total volume 500 µL)
4. Make serial dilutions using the sample from step 3
   (i.e., 100 µg, 50 µg, 25 µg, 12.5 µg, and 6.25 µg/mL, respectively; make sure you will need 200 µL per each concentration for 96 well-plate)
5. Dilute your other samples (1:50, take 5 µL of original sample and add 245 µL of saline).
6. Transfer 200 µL of each sample from step 5 into the 96 well-plate.
7. Measure at 260/280 nm using a microplate reader.

Appendix A-12: method for enzymatic hydrolysis of DNA

Chemicals and Reagents

- 5 mM Tris-HCl/10 mM $MgCl_2$ (pH 7.4)
- DNase I (type IV from bovine pancreas; stored at -20°C)
- Nuclease P1 (from Peniccilium citrinum; stored at 2-8°C)
- Phosphodiesterase I (from Crotalus adamanteus venom; stored at -20°C)
- Alkaline phosphatase (from E. coli; stored at -20°C)

Methods

1. Dissolve 50 µg of DNA in 79 µL of 5 mM Tris-HCl and 10 mM $MgCl_2$.
2. Add 2 µL of internal standard (thus 50 µg of DNA/81 µL concentration).
3. Add 4 µL of DNase I (8 µg (16 U) of DNase I/reaction (50 µg of DNA)).
4. Incubate for 1.5 hours at 37°C.
5. Add 1 µL of nuclease P1 (1 µg (0.2 U) of nuclease P1/reaction (50 µg of DNA)).
6. Incubate for 3 hours at 37°C.
7. Add 10 µL of phosphodiesterase I (0.004 U of phosphodiesterase I/reaction (50 µg of DNA)).
8. Add 4 µL of alkaline phosphatase (0.12 U of alkaline phosphatase/reaction (50 µg of DNA)).
9. Incubate for 18 hours at 37°C.
10. Take 40 µL of mixture (contains 20 µg of DNA) and add 60 µL of 20 mM ammonium acetate.

11. Centrifuge the sample from step #10 for 5 minutes at 18,000 × *g* and transfer 90 µL of supernatant into HPLC vial.
12. Take rest of sample (30 µg of DNA/60 µL) of mixture and add 120 µL of cold EtOH (to precipitate enzymes) and centrifuge for 5 minutes at 18,000 × *g*.
13. Transfer supernatant (150 µL; this fraction contains approximately 25 µg of DNA) and add 5 µL of DMSO.
14. Dry out sample using speedvac (at 20-30 pressure gauge for 2 hours).
15. Add 15 µL of water and sonicate 5 minutes prior to LC/MS analysis.

Appendix A-13: method for assessment of the efficacy of enzymatic DNA digestion

Reagents

- 20 mM ammonium acetate (pH 4.5)

- Acetonitrile

Column

- Phenomenex Synergy 4μ Fusion-RP 80A (150 × 4.6 mm)

Methods for seperation

- Starts at 95% 20 mM ammonium acetate (pH 4.5) and 5% acetonitrile

- Reach 40% acetonitrile over 20 minutes

- Flow rate: 1 mg/min

- Detector wavelength: 260 nm

Sample preparation

- Take 20 μL of DNA hydrolyzed solution and add 60 μL of 20 mM ammonium aceta te (to make 20 μg of DNA/100 μL)

- Inject 20 μL of this mixture (i.e., 4 μg of DNA)

Analytical STD preparation

- Make 50 μg of each nucleotide/mL of ammonium acetate (i.e., aqueous phase of HP LC separation).

- Make 200 μg of all nucleotides/mL

- Inject 20 μL of STDs

Appendix A-14: method for urine preparation for UPLC QTOF analysis

Methods

1. Centrifuge the urine sample at 14,000 rpm (i.e., 18,000 × *g*) for 5 minutes to remove contaminants (e.g., diet, feces, and etc.) and take the supernatant
2. Dilute 50 µL of this supernatant with 200 µL of 50% ACN
3. Spin the sample at 14,000 rpm (i.e., 18,000 × *g*) for 12 minutes to remove protein and particles
4. Transfer supernatant to HPLC vials

Appendix B: data set and SAS codes

```
data HFHL_PhIP_Study (label='all data')
veg_only (label='vegetable diets only')
pure_cmps (label='purified compounds only')
Store1 (label='PCA output - Exp 1')
store2 (label ='Factor output - Exp 1')
store3 (label='PCA output - Exp 2')
store4 (label ='Factor output - Exp 2')
control1 (label= 'cotrol grp exp1')
control2 (label='control grp exp2')
cruciferousdiet (label='Cruciferous diet in experiment 1')
apiaceousdiet (label='Apiaceous diet in experiment 1')
Vegetables_combination (label='Vegetables combination diet in
experiment 1')
ITCdiet (label = 'PEITC and I3C diet in experiment 2')
FCdiet (label = 'Furanocoumarins diet in experiment 2')
Phytochem_combination (label = ' Phytochem combination diet in
experiment 2')
Exp1 (label='Experiment 1 data')
Exp2 (label='Experiment 2 data')
;

Title2 'HFHL PhIP Stdy by Kim (vegetable diet vs pure compound
diet)';
Input Animal      Diet Block  Experiment  Weight_gain Food_intake
      Colon       Pancreas    Prostate    Liver Lung  CYP1A1
      CYP1A2      Plate_number
UGT1A1      SULT1A1     CYP1A1_E    CYP1A2_E    UGT1A1_E    SULT1A1_E
      I     II    III   IV    V     VI    VII   VIII  IX X  XI    XII
      XIII  XIV   XV    XVI   XVII Glucuronidated_p Sulfated_p
Methylated_p Five_hydroxylated_p
N2_hydroxylated_p I_p   II_p III_p  IV_p  V_p   VI_p  VII_p VIII_p
      IX_p  X_p   XI_p  XII_p XIII_p      XIV_p XV_p  XVI_p XVII_p
DNA_adduct DNA_adduct_P
;

Label
Animal= 'Animal number'
Diet= 'Diet type'
Block= 'Block animal was sacrificed'
Experiment= '1. vegetable diet feeding; 2. pure compound diet
feeding'
Weight_gain= 'final weight - initial weight'
Food_intake= 'Food intake per 24 hr'
Colon= 'Colon weight (g)'
Pancreas= 'Pancreas weight (g)'
Prostate= 'Prostate weight (g)'
Liver= 'Liver weight (g)'
Lung= 'Lung weight (g)'
CYP1A1= 'CYP1A1 EROD - pmol_resorfurin/min_mg_protein'
CYP1A2= 'CYP1A2 MROD - pmol_resorfurin/min_mg_protein'
Plate_number= 'Plate number of kinetic assay'
UGT1A1= 'Enzyme UGT1A1 assay'
SULT1A1= 'Cytosolic SULT1A1 Activity (nmol/min/ug prot)'
CYP1A1_E= 'CYP1A1 ELISA'
```

```
CYP1A2_E= 'CYP1A2 ELISA'
UGT1A1_E= 'UGT1A1 ELISA'
SULT1A1_E= 'SULT1A1 ELISA'
I= 'PhIP'
II= 'methylated PhIP - has not idenfied yet'
III= 'N2-methyl-PhIP'
IV= '4 prime-OH-PhIP'
V= 'N2-OH-PhIP'
VI= '5-OH-PhIP'
VII= 'N2-methyl-4 prime-OH-PhIP'
VIII= '4 prime-OH-PhIP sulfate'
IX= '5-OH-PhIP sulfate'
X= '5, 4 prime-DiOH-PhIP sulfate'
XI= 'PhIP-N3-glucuronide'
XII= 'PhIP-N2-glucuronide'
XIII= '4 prime-OH-PhIP glucuronide'
XIV= '5-OH-PhIP glucuronide'
XV= '4 prime-OH-PhIP-N2-glucuronide'
XVI= 'N2-OH-PhIP-N2-glucuronide'
XVII= 'N2-OH-PhIP-N3-glucuronide'
DNA_adduct= 'DNA adduct analysis'
DNA_adduct_P= 'DNA adduct analysis proportion to basal'
Glucuronidated_p = 'All glucuronidated metabolites proportion to
basal'
Sulfated_p = 'All sulfated metabolite sproportion to basal'
Methylated_p = 'All methylated metabolites proportion to basal'
Five_hydroxylated_p = 'All 5-OH metabolites proportion to basal'
N2_hydroxylated_p = 'All N2_OH metabolites proportion to basal'
I_p = 'I proportion to basal'
II_p = 'II proportion to basal'
III_p = 'III proportion to basal'
IV_p = 'IV proportion to basal'
V_p = 'V proportion to basal'
VI_p = 'VI proportion to basal'
VII_p = 'VII proportion to basal'
VIII_p = 'VIII proportion to basal'
IX_p = 'IX proportion to basal'
X_p = 'X proportion to basal'
XI_p = 'XI proportion to basal'
XII_p = 'XII proportion to basal'
XIII_p = 'XIII proportion to basal'
XIV_p = 'XIV proportion to basal'
XV_p = 'XV proportion to basal'
XVI_p = 'XVI proportion to basal'
XVII_p = 'XVII proportion to basal'
;

DNA_adduct_t=sqrt(DNA_adduct);
CYP1A1_metabolites_P = IV_P + VIII_P + XV_P + XIII_P + X_P;
CYP1A2_metabolites_P = V_P + XVI_P + XVII_P;
CYP1A1_metabolites = IV + VII + VIII + X + XV + XIII;
CYP1A2_metabolites = V + XVI + XVII;
CYP1A_metabolites = IV + V + VII + VIII + X + XIII + XV + XVI + XVII;
Glucuronidated = XVI + XVII + XI + XII + XIV + XV + XIII;
Sulfated = IX + X + VIII;
Methylated = II + III + VII;
Five_hydroxylated = VI + XIV + IX + X;
N2_hydroxylated = V + XVI + XVII;
```

```
if experiment = 1 and diet = 2 then basalPhIP = 1;
if experiment = 2 and diet = 2 then basalPhIP = 2;

if experiment = 1 and diet = 1 then basalnoPhIP = 1;
if experiment = 2 and diet = 1 then basalnoPhIP = 2;

output HFHL_PhIP_Study;

if experiment = 1 then output Exp1;
if experiment = 2 then output Exp2;
if diet=2 and experiment =1 then output control1;
if diet=2 and experiment =2 then output control2;
if diet=3 then output cruciferousdiet;
if diet=4 then output apiaceousdiet;
if diet=5 then output Vegetables_combination;
if diet=6 then output ITCdiet;
if diet=7 then output FCdiet;
if diet=8 then output Phytochem_combination;

datalines;

1     2     1     1     83.90 20.80 0.82  0.34  0.11  6.55  0.95
      10.55 6.48  1.00  13.7323216  11.63 1.63  2.84  2.23  0.72
      27.72 8.43  9.36  18.15 0.91  3.32  15.73 7.02  0.00  0.00
      3.64  1.00  0.21  0.00  0.00  0.77  3.74  0.69  0.89  0.79
      0.59  0.81  1.58  0.89  0.76  1.11  1.18  1.37  0.71  1.66
      0.00  1.00  1.09  1.93  0.56  0.00  0.00  0.87  0.39  7.06
      0.95
2     2     1     1     85.10 19.40 0.99  0.34  0.22  7.50  1.23
      6.66  5.56  1.00  18.28637531 10.88 1.16  3.64  1.80  0.76
      14.18 9.09  11.06 15.01 2.74  2.31  22.70 4.25  0.20  0.00
      4.20  1.13  0.28  0.00  0.22  1.40  11.23 1.29  1.39  0.96
      1.03  2.10  0.81  0.96  0.90  0.91  3.54  0.95  1.03  1.00
      2.18  1.00  1.26  2.18  0.75  0.00  2.09  1.57  1.19  8.46
      1.14
3     2     1     1     90.20 20.70 1.14  0.36  0.15  7.09  1.28
      6.57  6.65  1.00  19.84298795 10.39 1.32  5.96  2.89  1.13
      16.52 7.91  8.63  23.95 1.02  2.62  19.19 3.79  0.16  0.00
      2.90  1.03  0.38  0.14  0.29  1.15  10.31 1.95  1.21  0.80
      2.12  1.23  0.94  0.84  0.70  1.46  1.32  1.08  0.87  0.90
      1.74  1.00  0.87  1.99  1.02  4.67  2.75  1.29  1.09  11.11
      1.50
4     2     1     1     102.70      24.25 1.06  0.54  0.31  7.36
      1.38  7.91  6.97  1.00  20.41603208 7.91  0.91  5.60  3.31
      0.63  21.90 8.27  7.65  18.27 0.00  2.12  23.89 4.77  0.00
      0.00  3.72  0.00  0.00  0.00  0.00  0.54  8.87  0.38  0.71
      0.86  0.47  0.51  1.25  0.88  0.62  1.11  0.00  0.87  1.08
      1.13  0.00  1.00  1.12  0.00  0.00  0.00  0.00  0.61  0.94
      10.26 1.39
5     3     1     1     86.20 24.10 1.00  0.59  0.24  6.37  1.14
      11.34 12.67 1.00  18.01404274 10.98 1.18  5.96  2.40  0.93
      19.49 7.10  9.09  15.15 0.00  2.55  17.40 7.94  0.40  0.32
      3.27  0.89  0.39  0.00  0.34  0.91  14.75 1.36  2.18  0.76
      1.43  0.86  1.11  0.75  0.74  0.92  0.00  1.05  0.79  1.88
      4.36  0.32  0.98  1.72  1.05  0.00  3.22  1.02  1.56  8.75
      1.18
6     3     1     1     99.90 25.10 0.87  0.39  0.20  6.77  1.25
      14.22 10.56 1.00  29.73717412 10.83 1.72  8.31  1.59  0.93
      15.82 6.35  6.66  16.76 0.00  2.48  12.72 6.84  0.33  0.16
```

```
      3.74  1.11  0.50  0.24  0.43  2.18  23.68 3.09  1.79  0.60
      3.19  1.65  0.90  0.67  0.54  1.02  0.00  1.02  0.58  1.62
      3.59  0.16  1.12  2.14  1.34  8.00  4.08  2.45  2.50  7.94
      1.07
7     4     1     1     92.60 23.25 1.05  0.35  0.21  6.44  1.23
      8.08  8.26  1.00  30.57892557 6.85  0.93  7.24  1.55  0.79
      17.95 10.44 9.60  15.28 0.34  2.55  25.61 3.41  0.00  0.00
      3.54  1.07  0.20  0.00  0.00  1.12  8.88  0.84  0.27  1.02
      0.26  0.88  1.02  1.11  0.78  0.93  0.44  1.05  1.16  0.81
      0.00  0.00  1.06  2.06  0.54  0.00  0.00  1.26  0.94  7.58
      1.02
8     5     1     1     83.80 23.20 0.97  0.56  0.23  6.74  1.09
      10.84 8.65  1.00  20.95646556 10.55 1.70  4.76  4.52  0.99
      16.61 6.87  6.92  15.13 0.00  2.37  17.36 9.56  0.35  0.18
      3.98  1.35  0.60  0.27  0.49  0.64  17.32 3.09  2.08  0.69
      3.49  0.85  0.95  0.73  0.56  0.92  0.00  0.98  0.79  2.26
      3.81  0.18  1.20  2.61  1.61  9.00  4.65  0.72  1.83  8.99
      1.21
9     1     2     1     92.50 21.00 1.02  0.46  0.23  7.13  1.21
      14.60 7.07  2.00  25.06246331 12.30 1.02  5.02  2.16  0.82  .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
10    1     2     1     78.90 18.25 1.07  0.49  0.19  6.02  0.99
      9.38  8.88  2.00  24.60134288 12.31 1.00  7.07  2.84  0.79  .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
11    1     2     1     91.50 19.05 0.94  0.35  0.20  6.65  1.18
      7.88  6.37  2.00  21.34384669 11.96 0.84  3.91  2.65  0.35  .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
12    1     2     1     86.70 19.95 0.99  0.31  0.21  7.12  1.06
      6.86  5.06  2.00  22.80551532 9.08  1.00  3.33  3.01  0.03  .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
13    3     2     1     76.80 19.95 0.94  0.27  0.18  5.76  0.99
      17.19 16.77 2.00  17.13533962 10.70 0.91  7.51  1.67  0.88
      18.86 5.93  8.73  19.80 0.37  2.15  13.80 5.39  0.21  0.00
      3.03  1.12  0.56  0.21  0.33  1.65  17.88 2.63  1.19  0.66
      2.54  1.41  1.07  0.63  0.71  1.21  0.48  0.89  0.62  1.27
      2.29  0.00  0.91  2.16  1.50  7.00  3.13  1.85  1.89  5.21
      0.70
14    3     2     1     99.80 26.85 0.92  0.28  0.16  7.47  1.11
      11.57 15.08 2.00  16.62474981 11.11 1.75  8.76  2.11  1.01
      12.92 8.24  11.60 17.67 0.59  2.93  14.86 5.09  0.34  0.33
      2.61  0.78  0.43  0.25  0.42  2.14  18.80 2.88  1.74  0.83
      3.39  1.72  0.74  0.87  0.95  1.08  0.76  1.21  0.67  1.20
      3.70  0.33  0.78  1.51  1.15  8.33  3.98  2.40  1.98  4.72
      0.64
15    4     2     1     92.60 21.90 1.01  0.30  0.14  6.77  1.22
      8.18  9.13  2.00  25.24394104 11.26 1.56  8.13  1.83  0.62
      14.19 10.16 9.45  12.58 1.31  2.40  27.73 4.03  0.15  0.00
```

```
      3.55  0.97  0.07  0.01  0.21  1.18  12.02 1.15  0.86  1.03
      0.74  1.43  0.81  1.08  0.77  0.77  1.69  0.99  1.25  0.95
      1.63  0.00  1.07  1.87  0.19  0.33  1.99  1.33  1.27  4.23
      0.57
16    5     2     1     75.20 25.85 0.89  0.27  0.22  5.68  1.00
      14.75 13.31 2.00  13.90565743 11.87 1.11  12.36 1.26  0.81
      14.64 8.23  13.27 15.81 0.13  2.69  16.53 5.87  0.23  0.00
      2.35  0.83  0.31  0.16  0.30  2.23  16.42 2.22  1.30  0.90
      2.24  1.47  0.83  0.87  1.08  0.96  0.17  1.11  0.75  1.39
      2.50  0.00  0.71  1.60  0.83  5.33  2.84  2.51  1.73  4.18
      0.56
17    1     3     1     121.20      20.65 1.09  0.34  0.26  7.05
      1.13  8.12  3.15  3.00  11.38907186 11.77 0.84  2.58  1.53
      1.85  .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .
18    1     3     1     98.00 19.05 0.99  0.34  0.23  6.98  1.13
      6.54  4.45  3.00  14.05985302 11.20 1.13  10.00 2.12  1.18  .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
19    2     3     1     110.30      20.65 1.10  0.37  0.27  7.17
      1.01  12.27 4.61  3.00  14.71334846 11.76 1.11  10.93 2.90
      0.44  15.62 9.29  11.92 13.89 0.12  1.81  27.06 3.91  0.26
      0.00  3.29  1.06  0.50  0.12  0.22  0.74  10.18 1.77  1.59
      1.06  2.14  0.69  0.89  0.99  0.97  0.85  0.16  0.75  1.22
      0.92  2.83  1.00  0.99  2.05  1.34  4.00  2.09  0.83  1.07
      7.61  1.03
20    2     3     1     96.30 22.35 1.05  0.29  0.17  6.18  1.08
      11.02 4.96  3.00  10.31559623 12.27 1.56  13.02 2.02  1.08
      14.51 7.94  12.67 17.58 2.52  2.22  22.51 4.25  0.23  0.00
      2.83  0.75  0.26  0.00  0.22  1.15  10.35 1.07  1.50  0.96
      1.11  1.88  0.83  0.84  1.03  1.07  3.26  0.92  1.02  1.00
      2.50  1.00  0.85  1.45  0.70  0.00  2.09  1.29  1.09  9.97
      1.35
21    3     3     1     94.70 23.25 1.00  0.32  0.21  7.12  1.17
      14.14 8.40  3.00  15.70114864 11.49 1.29  11.24 2.20  1.06
      15.09 8.16  10.28 18.69 1.11  3.52  17.95 7.19  0.26  0.00
      2.84  0.94  0.25  0.00  0.35  1.01  12.35 1.30  1.51  0.84
      1.07  1.29  0.86  0.87  0.84  1.14  1.43  1.45  0.81  1.70
      2.83  0.00  0.85  1.81  0.67  0.00  3.32  1.13  1.30  6.64
      0.90
22    3     3     1     116.90      25.30 0.93  0.30  0.25  9.02
      1.19  10.12 5.65  3.00  12.41499346 10.35 1.70  4.62  1.31
      1.11  11.06 9.82  15.24 15.59 0.31  2.17  20.96 4.53  0.18
      0.00  2.95  0.84  0.36  0.15  0.29  1.24  14.31 2.02  1.01
      1.08  1.96  1.10  0.63  1.04  1.24  0.95  0.40  0.90  0.95
      1.07  1.96  0.00  0.89  1.62  0.97  5.00  2.75  1.39  1.51
      4.80  0.65
23    4     3     1     101.30      21.40 0.87  0.32  0.20  7.16
      1.09  9.99  6.04  3.00  22.24948234 12.57 1.22  8.22  2.30
      1.22  16.53 12.52 13.56 9.66  0.00  2.01  25.63 5.08  0.00
      0.00  4.28  1.26  0.26  0.00  0.00  0.00  9.21  0.77  0.40
      1.20  0.21  0.32  0.94  1.33  1.11  0.59  0.00  0.83  1.16
      1.20  0.00  0.00  1.29  2.43  0.70  0.00  0.00  0.00  0.97
      4.94  0.67
24    5     3     1     103.40      24.55 1.06  0.37  0.31  7.18
```

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      1.07  13.56 10.44 3.00  18.3898655  11.32 1.52  10.00 1.13
      1.44  19.22 6.98  10.18 16.16 0.04  2.04  17.20 7.28  0.31
      0.00  3.39  1.04  0.43  0.12  0.29  0.99  14.33 1.94  1.70
      0.78  2.05  0.89  1.09  0.74  0.83  0.98  0.05  0.84  0.78
      1.72  3.38  0.00  1.02  2.01  1.15  4.00  2.75  1.11  1.51
      7.30  0.99
25    1     4     1     107.10      21.80 1.08  0.43  0.26  7.06
      1.40  7.81  4.07  4.00  18.87360893 10.71 0.73  7.16  2.49
      0.76  .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .
26    4     4     1     112.40      22.40 1.00  0.43  0.21  7.30
      1.29  9.66  5.48  4.00  12.44771346 12.53 1.29  6.67  1.75
      0.79  11.23 12.11 10.91 12.55 0.00  1.89  29.01 2.74  0.00
      0.00  4.43  1.24  0.23  0.00  0.00  1.13  12.53 0.99  0.22
      1.16  0.19  0.86  0.64  1.28  0.89  0.76  0.00  0.78  1.31
      0.65  0.00  0.00  1.33  2.39  0.62  0.00  0.00  1.27  1.32
      3.83  0.52
27    4     4     1     123.90      20.05 1.17  0.46  0.20  9.16
      1.29  8.37  5.92  4.00  17.14747293 12.19 0.93  7.07  1.93
      0.99  19.67 10.69 10.56 9.77  1.41  1.93  31.54 2.11  0.00
      0.00  3.04  1.07  0.00  0.00  0.00  0.78  7.42  0.66  0.17
      1.14  0.20  1.16  1.12  1.13  0.86  0.60  1.82  0.80  1.43
      0.50  0.00  0.00  0.91  2.06  0.00  0.00  0.00  0.88  0.78
      6.85  0.93
28    4     4     1     120.00      20.00 1.07  0.35  0.30  7.80
      1.09  10.82 6.48  4.00  18.05825484 11.10 1.36  8.04  1.76
      0.95  13.72 9.95  15.27 12.69 0.59  1.93  27.35 2.95  0.14
      0.00  2.29  0.69  0.26  0.08  0.00  0.99  11.10 1.10  0.74
      1.18  1.25  1.02  0.78  1.06  1.25  0.77  0.76  0.80  1.24
      0.70  1.52  0.00  0.69  1.33  0.70  2.67  0.00  1.11  1.17
      4.94  0.67
29    5     4     1     107.90      23.55 1.15  0.43  0.20  7.86
      1.11  11.84 6.72  4.00  14.29804896 12.48 1.34  5.82  0.94
      0.75  18.31 9.50  18.00 13.75 0.67  2.73  16.92 3.33  0.19
      0.00  3.00  0.65  0.18  0.00  0.00  1.24  11.54 0.75  0.95
      1.08  0.80  1.16  1.04  1.01  1.47  0.84  0.87  1.13  0.77
      0.79  2.07  0.00  0.90  1.25  0.48  0.00  0.00  1.39  1.22
      2.89  0.39
30    5     4     1     111.20      20.90 0.98  0.41  0.25  7.76
      1.34  12.04 6.63  4.00  13.14025652 11.67 0.75  4.58  1.35
      0.94  13.87 10.98 12.28 16.36 0.15  2.35  20.03 3.70  0.15
      0.00  3.44  0.97  0.42  0.17  0.17  1.02  13.95 1.99  0.84
      1.02  2.07  0.94  0.79  1.17  1.00  1.00  0.19  0.97  0.91
      0.87  1.63  0.00  1.03  1.87  1.13  5.67  1.61  1.15  1.47
      5.59  0.76
31    5     4     1     117.80      21.70 1.08  0.30  0.24  7.89
      1.09  11.08 6.61  4.00  14.53915934 8.48  0.98  6.18  1.68
      1.03  13.43 10.29 12.01 17.02 0.54  3.05  19.55 4.33  0.00
      0.00  3.12  1.44  0.65  0.00  0.00  1.23  13.33 1.18  0.34
      0.99  0.31  1.16  0.76  1.09  0.98  1.04  0.70  1.26  0.88
      1.02  0.00  0.00  0.94  2.78  1.74  0.00  0.00  1.38  1.41
      5.56  0.75
32    1     5     1     90.10 18.15 0.96  0.29  0.16  6.75  1.08
      8.11  5.45  4.00  15.53454967 11.39 1.00  3.91  0.66  0.12  .
      .     .     .     .     .     .     .     .     .     .     .
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      .     .     .     .     .     .     .
33    1     5     1     103.80      19.50 1.09  0.31  0.21  7.70
      1.29  6.03  4.01  5.00  18.19247844 10.82 1.07  1.29  1.56
      1.05  .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .
34    2     5     1     86.40 19.35 0.94  0.25  0.14  7.20  1.14
      11.58 6.97  5.00  17.61130071 8.62  1.11  4.22  1.77  0.86
      15.22 10.18 11.89 16.41 1.20  2.39  22.71 4.17  0.16  0.00
      2.75  0.73  0.28  0.07  0.21  0.95  10.68 1.36  1.24  1.03
      1.52  1.25  0.87  1.08  0.97  1.00  1.55  0.99  1.03  0.98
      1.74  1.00  0.83  1.41  0.75  2.33  1.99  1.07  1.13  5.73
      0.77
35    2     5     1     77.80 17.45 0.95  0.34  0.21  6.33  0.88
      9.55  6.22  5.00  16.76645752 10.08 1.50  4.84  1.46  0.60
      15.23 9.99  14.26 15.18 0.00  2.82  19.17 4.24  0.00  0.00
      3.27  0.00  1.21  0.00  0.00  1.09  13.55 0.98  0.67  1.03
      0.54  0.88  0.87  1.06  1.16  0.92  0.00  1.16  0.87  1.00
      0.00  1.00  0.98  0.00  3.25  0.00  0.00  1.22  1.43  5.13
      0.69
36    3     5     1     85.10 24.50 0.98  0.28  0.17  6.73  1.15
      18.38 8.03  5.00  23.29977329 12.42 0.93  2.40  1.62  1.00
      11.39 9.89  12.87 18.16 0.71  2.68  19.73 4.48  0.00  0.00
      3.36  1.18  0.40  0.00  0.22  1.51  13.41 1.37  0.35  1.00
      0.28  1.34  0.65  1.05  1.05  1.11  0.92  1.11  0.89  1.06
      0.00  0.00  1.01  2.28  1.07  0.00  2.09  1.70  1.42  3.37
      0.45
37    3     5     1     89.60 21.15 0.99  0.36  0.15  7.20  1.22
      13.15 9.76  5.00  20.61237862 10.45 1.09  6.31  1.19  1.13
      17.71 8.51  12.32 12.53 0.00  3.05  15.40 3.38  0.00  0.00
      3.87  0.94  0.62  0.00  0.00  1.79  19.88 1.25  0.27  0.87
      0.31  1.37  1.01  0.90  1.01  0.76  0.00  1.26  0.70  0.80
      0.00  0.00  1.16  1.81  1.66  0.00  0.00  2.01  2.10  5.47
      0.74
38    4     5     1     87.00 20.75 1.13  0.29  0.14  6.72  1.09
      14.98 9.08  5.00  19.83116282 7.62  1.50  7.16  2.66  0.92
      14.21 10.54 15.99 11.55 0.00  1.99  24.97 3.25  0.00  0.00
      3.80  1.19  0.30  0.00  0.00  1.29  10.89 0.98  0.26  1.18
      0.21  0.87  0.81  1.12  1.30  0.70  0.00  0.82  1.13  0.77
      0.00  0.00  1.14  2.30  0.80  0.00  0.00  1.45  1.15  5.42
      0.73
39    5     5     1     77.70 23.85 1.05  0.20  0.21  6.19  1.06
      11.99 11.50 5.00  17.82440987 12.81 1.29  7.02  3.54  0.66
      23.49 8.42  8.03  17.98 0.00  3.35  16.45 3.82  0.00  0.00
      5.62  1.34  0.54  0.00  0.00  0.00  10.97 0.98  0.30  0.76
      0.35  0.39  1.34  0.89  0.66  1.10  0.00  1.38  0.74  0.90
      0.00  0.00  1.69  2.59  1.45  0.00  0.00  0.00  1.16  6.28
      0.85
40    1     6     1     95.90 18.35 1.06  0.03  0.17  6.85  1.06
      8.52  10.56 5.00  24.68423311 13.31 0.70  8.76  0.33  0.41  .
      .     .     .     .     .     .     .     .     .     .     .
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      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
41    1     6     1     100.30      18.05 0.09  0.31  0.13  6.70
      1.04  6.36  4.35  6.00  22.51421979 13.04 0.75  1.69  0.49
      0.70  .     .     .     .     .     .     .     .     .     .
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      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .
42    2     6     1     91.70 17.65 0.96  0.50  0.30  7.00  1.12
      9.61  6.65  6.00  19.9983649  12.30 1.04  2.93  1.34  0.45
      20.07 10.71 15.44 12.42 0.00  1.67  24.31 3.55  0.00  0.00
      3.23  0.00  0.21  0.00  0.00  1.03  7.34  0.50  0.61  1.17
      0.42  0.64  1.14  1.14  1.26  0.76  0.00  0.69  1.10  0.84
      0.00  1.00  0.97  0.00  0.56  0.00  0.00  1.16  0.77  6.72
      0.91
43    2     6     1     99.20 20.05 1.01  0.34  0.20  7.10  1.02
      6.65  5.61  6.00  19.97859624 12.43 0.75  3.07  1.51  0.67
      15.64 11.50 16.57 15.21 0.00  2.76  19.00 3.66  0.00  0.00
      4.02  0.00  0.45  0.00  0.00  0.00  11.18 0.51  0.62  1.14
      0.53  0.39  0.89  1.22  1.35  0.93  0.00  1.14  0.86  0.86
      0.00  1.00  1.21  0.00  1.21  0.00  0.00  0.00  1.18  4.85
      0.66
44    3     6     1     86.60 23.15 1.02  0.39  0.24  6.79  1.12
      10.93 8.76  6.00  25.4883268 13.28 0.91  3.78  0.76  1.22
      14.45 8.64  12.08 15.53 0.46  2.51  16.94 3.07  0.06  0.00
      2.93  0.76  0.51  0.14  0.00  1.86  20.07 1.80  0.46  0.89
      1.59  1.60  0.82  0.92  0.99  0.95  0.59  1.04  0.77  0.72
      0.65  0.00  0.88  1.47  1.37  4.67  0.00  2.09  2.12  4.69
      0.63
45    3     6     1     94.10 23.00 0.98  0.38  0.16  7.24  1.03
      10.70 8.40  6.00  30.95127498 12.71 0.84  5.29  0.81  1.53
      13.69 8.30  11.83 13.51 1.24  2.25  16.34 3.17  0.14  0.00
      3.90  1.22  0.35  0.14  0.18  2.36  21.39 2.25  0.76  0.86
      1.78  2.17  0.78  0.88  0.97  0.82  1.60  0.93  0.74  0.75
      1.52  0.00  1.17  2.35  0.94  4.67  1.71  2.65  2.26  6.15
      0.83
46    4     6     1     95.70 24.65 1.15  0.50  0.21  7.44  1.07
      13.40 12.88 6.00  25.96102066 12.75 0.86  9.02  1.64  0.78
      14.23 9.42  18.42 10.20 0.38  1.75  25.84 2.60  0.00  0.00
      2.90  1.03  0.15  0.05  0.00  1.22  11.81 1.08  0.20  1.22
      0.60  1.04  0.81  1.00  1.50  0.62  0.49  0.72  1.17  0.61
      0.00  0.00  0.87  1.99  0.40  1.67  0.00  1.37  1.25  4.90
      0.66
47    5     6     1     88.20 21.00 1.17  0.32  0.16  6.98  1.09  .
      .     6.00  20.86204989 13.05 1.41  7.07  0.60  1.04  18.38
      7.12  9.87  9.94  1.10  2.42  15.39 5.51  0.35  0.27  3.75
      1.06  0.35  0.20  0.25  2.18  21.85 2.56  1.79  0.75  2.94
      2.06  1.05  0.76  0.81  0.61  1.42  1.00  0.70  1.30  3.81
      0.27  1.13  2.05  0.94  6.67  2.37  2.45  2.31  6.02  0.81
48    2     7     1     111.40      20.40 1.13  0.35  0.12  7.95
      1.34  7.49  7.42  6.00  26.20358584 12.40 0.70  5.20  1.23
      0.64  16.54 10.36 15.38 14.45 0.00  2.63  26.83 2.97  0.00
      0.00  2.75  0.00  0.32  0.00  0.00  0.97  6.80  0.50  0.57
      1.19  0.52  0.60  0.94  1.10  1.25  0.88  0.00  1.08  1.21
      0.70  0.00  1.00  0.83  0.00  0.86  0.00  0.00  1.09  0.72
      4.50  0.61
49    4     7     1     104.90      20.50 1.02  0.33  0.17  7.15
      1.23  8.71  9.42  7.00  18.6172415  12.79 0.66  5.33  0.96
      0.87  17.69 11.41 13.97 14.92 0.00  1.53  25.69 2.56  0.14
      0.00  3.25  0.00  0.31  0.00  0.00  0.59  7.93  0.47  0.71
      1.17  0.54  0.50  1.01  1.21  1.14  0.91  0.00  0.63  1.16
      0.60  1.52  0.00  0.98  0.00  0.83  0.00  0.00  0.66  0.84
      2.42  0.33
50    4     7     1     114.60      20.20 1.10  0.31  0.19  7.57
      1.12  11.12 9.48  7.00  18.31735023 11.19 0.70  6.71  1.56
```

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      0.93  19.56 10.68 15.36 12.12 0.94  2.11  25.29 2.30  0.00
      0.00  3.04  1.00  0.17  0.00  0.00  0.97  6.48  0.72  0.18
      1.18  0.22  1.00  1.11  1.13  1.25  0.74  1.22  0.87  1.14
      0.54  0.00  0.00  0.91  1.93  0.46  0.00  0.00  1.09  0.68
      3.64  0.49
51    5     7     1     116.80      21.05 1.00  0.31  0.22  7.67
      1.10  10.27 10.44 7.00  23.65973145 12.92 0.84  5.56  0.91
      0.37  18.89 7.46  11.21 18.83 0.46  2.44  16.52 3.90  0.12
      0.00  3.37  1.04  0.45  0.10  0.23  1.11  13.88 1.78  0.74
      0.82  1.41  1.10  1.08  0.79  0.91  1.15  0.59  1.01  0.75
      0.92  1.31  0.00  1.01  2.01  1.21  3.33  2.18  1.25  1.46
      7.61  1.03
52    5     7     1     111.10      16.65 0.92  0.33  0.36  7.55
      1.16  11.22 11.13 7.00  17.76665445 12.69 0.98  6.98  1.15
      0.20  14.14 11.27 13.76 12.84 1.04  2.54  23.16 3.47  0.14
      0.00  2.71  0.75  0.25  0.06  0.12  1.49  12.27 1.29  0.78
      1.12  1.14  1.44  0.81  1.20  1.12  0.78  1.34  1.05  1.05
      0.82  1.52  0.00  0.81  1.45  0.67  2.00  1.14  1.67  1.29
      3.38  0.46
53    1     8     2     95.90 18.65 1.00  0.38  0.22  7.52  1.05
      4.93  3.24  1.00  25.55829698 9.18  0.34  3.02  0.78  0.25  .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
54    1     8     2     78.70 16.00 0.93  0.27  0.19  6.82  1.06
      8.14  4.31  1.00  23.90471727 12.32 1.07  4.84  1.03  0.47  .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
55    2     8     2     85.30 15.55 0.99  0.32  0.20  7.12  1.16
      6.85  4.01  1.00  26.63873791 10.53 0.70  6.13  0.83  0.29
      19.73 8.91  10.72 15.72 0.36  2.39  15.71 6.64  0.20  0.00
      3.52  1.25  0.88  0.00  0.29  1.00  12.67 1.71  0.95  0.80
      0.99  1.28  1.06  0.83  0.80  1.21  1.05  1.09  0.75  1.01
      1.85  0.00  1.04  1.27  2.62  1.00  3.26  1.34  1.46  5.74
      1.43
56    2     8     2     90.50 18.50 1.03  0.25  0.19  6.62  1.22
      7.22  3.21  1.00  20.43312442 13.05 1.22  7.51  0.43  0.49
      18.80 8.67  9.49  18.50 0.48  2.78  14.84 9.58  0.23  0.09
      3.21  1.05  0.37  0.00  0.27  0.99  10.65 1.39  4.86  0.74
      3.85  1.32  1.01  0.81  0.71  1.42  1.40  1.27  0.71  1.45
      2.13  11.00 0.94  1.07  1.10  1.00  3.03  1.33  1.22  5.21
      1.30
57    6     8     2     91.10 17.45 0.96  0.22  0.18  8.12  1.17
      17.00 6.02  1.00  27.1032187  12.94 0.70  6.31  1.80  0.98
      28.11 4.51  8.87  15.23 0.00  3.63  6.40  13.72 0.00  0.54
      3.50  0.00  0.60  0.00  0.00  0.00  14.89 0.65  22.69 0.46
      16.92 0.57  1.51  0.42  0.66  1.17  0.00  1.66  0.31  2.08
      0.00  66.00 1.03  0.00  1.79  0.00  0.00  0.00  1.71  2.96
      0.74
58    6     8     2     94.50 19.30 0.97  0.35  0.15  7.92  1.06
      53.10 17.59 7.00  22.85534092 13.45 0.86  4.98  1.99  1.03
      20.10 5.67  7.34  20.40 2.14  4.05  11.11 11.30 0.00  1.15
      0.00  0.00  0.54  0.00  0.00  2.29  13.91 0.90  47.42 0.54
      35.60 3.64  1.08  0.53  0.55  1.57  6.26  1.85  0.53  1.71
      0.00  140.56       0.00  0.00  1.61  0.00  0.00  3.07  1.60
      1.56  0.39
```

```
59    7     8     2     86.60 15.25 0.91  0.28  0.17  6.33  1.04
      8.68  4.21  1.00  27.22666431 12.85 1.00  7.96  1.52  0.90
      30.41 9.28  11.92 11.07 0.00  2.20  12.87 10.27 0.21  0.11
      4.45  1.27  1.02  0.00  0.23  0.00  4.68  1.25  5.65  0.79
      4.10  0.18  1.64  0.86  0.89  0.85  0.00  1.01  0.62  1.56
      1.94  13.44 1.31  1.29  3.04  0.00  2.58  0.00  0.54  5.00
      1.25
60    8     8     2     80.60 17.85 1.07  0.25  0.19  6.69  1.00
      49.22 5.86  1.00  26.62944966 10.20 0.91  6.13  1.18  0.60
      32.00 6.73  8.57  17.55 0.00  5.04  11.20 11.81 0.00  0.50
      3.27  0.00  0.00  0.00  0.00  0.00  3.32  0.19  20.97 0.60
      15.85 0.13  1.72  0.63  0.64  1.35  0.00  2.31  0.54  1.79
      0.00  61.11 0.96  0.00  0.00  0.00  0.00  0.00  0.38  4.38
      1.09
61    1     9     2     90.70 18.30 0.86  0.34  0.15  6.93  0.99
      8.60  4.04  2.00  20.90643119 13.21 0.84  8.58  1.13  0.55  .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
62    1     9     2     95.20 16.90 0.99  0.35  0.16  7.70  1.07
      8.53  2.92  2.00  21.00985842 10.51 0.52  6.67  1.81  1.15  .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
63    2     9     2     96.10 18.75 1.09  0.24  0.18  8.20  1.19
      6.82  3.45  2.00  27.21612492 11.32 0.68  6.36  1.58  0.95
      18.96 9.89  13.62 11.80 0.33  2.33  19.89 7.89  0.21  0.00
      2.66  0.77  0.34  0.00  0.00  1.28  10.02 0.92  1.05  0.97
      1.00  1.28  1.02  0.92  1.02  0.91  0.97  1.07  0.96  1.20
      1.94  0.00  0.78  0.78  1.01  1.00  0.00  1.72  1.15  3.69
      0.92
64    2     9     2     69.10 15.10 0.87  0.30  0.17  5.87  0.92
      6.34  3.58  2.00  28.80395478 13.05 0.98  6.27  2.08  0.84
      31.26 10.42 10.78 11.74 0.00  2.89  20.80 7.29  0.00  0.00
      4.82  0.00  0.00  0.00  0.00  0.00  0.00  0.35  0.37  0.93
      0.58  0.00  1.68  0.97  0.81  0.90  0.00  1.32  1.00  1.10
      0.00  0.00  1.42  0.00  0.00  1.00  0.00  0.00  0.00  3.60
      0.90
65    6     9     2     82.30 15.45 0.91  0.28  0.12  6.60  0.97
      56.44 14.87 7.00  26.04440312 12.72 0.84  5.02  1.69  1.04
      20.11 6.63  8.68  20.48 1.81  3.52  8.09  10.73 0.30  0.66
      2.27  0.00  0.52  0.00  0.36  2.21  13.64 1.54  28.36 0.55
      21.26 3.28  1.08  0.62  0.65  1.58  5.30  1.61  0.39  1.63
      2.77  80.67 0.67  0.00  1.55  0.00  4.04  2.96  1.57  1.93
      0.48
66    6     9     2     96.40 16.20 0.93  0.27  0.19  8.40  1.07
      59.45 6.03  2.00  28.74267353 12.52 0.84  6.13  1.60  0.97
      21.82 6.66  10.34 15.09 0.00  3.18  8.50  13.27 0.00  0.18
      2.99  0.74  1.04  0.00  0.33  1.78  14.09 1.78  8.00  0.60
      5.86  1.34  1.17  0.62  0.77  1.16  0.00  1.46  0.41  2.01
      0.00  22.00 0.88  0.75  3.10  0.00  3.70  2.39  1.62  2.59
      0.64
67    7     9     2     96.60 16.65 1.20  0.21  0.18  8.30  1.02
      7.70  3.99  2.00  20.960114   13.03 0.59  4.93  1.89  0.68
      18.99 11.43 13.21 11.50 0.72  2.43  21.29 7.08  0.21  0.00
      3.46  0.88  0.26  0.00  0.11  0.78  7.64  0.84  1.00  1.03
      0.76  1.34  1.02  1.06  0.99  0.89  2.11  1.11  1.02  1.07
```

```
      1.94  0.00  1.02  0.90  0.78  0.00  1.23  1.05  0.88  5.45
      1.36
68    8     9     2     92.90 18.15 0.83  0.24  0.11  7.02  1.05
      38.12 21.37 8.00  27.45521556 12.42 0.68  5.24  1.32  0.72
      33.01 5.43  5.94  23.10 0.00  3.65  14.15 6.32  0.57  0.40
      3.10  0.70  0.00  0.00  0.00  0.00  3.62  0.29  18.37 0.54
      13.96 0.14  1.78  0.51  0.44  1.78  0.00  1.67  0.68  0.96
      5.27  48.89 0.91  0.71  0.00  0.00  0.00  0.00  0.42  2.52
      0.63
69    1     10    2     100.70      18.60 0.92  0.30  0.23  7.06
      1.17  6.57  4.42  2.00  21.06945297 12.54 1.09  4.53  2.45
      0.62  .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .
70    1     10    2     113.00      19.00 1.10  0.24  0.19  7.36
      1.00  7.50  3.63  2.00  24.87786237 13.09 1.02  6.22  1.84
      0.63  .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .
71    2     10    2     108.00      18.20 1.01  0.27  0.20  6.87
      1.13  5.87  3.39  2.00  29.5036804  8.35  0.59  4.58  1.24
      0.65  15.99 10.73 19.04 9.38  0.00  1.66  17.68 8.37  0.23
      0.00  2.91  0.87  0.27  0.00  0.17  0.83  11.86 1.13  1.13
      1.09  0.97  0.83  0.86  1.00  1.43  0.72  0.00  0.76  0.85
      1.27  2.13  0.00  0.86  0.89  0.80  1.00  1.91  1.11  1.36
      4.68  1.17
72    2     10    2     101.00      18.35 0.87  0.21  0.19  7.31
      0.97  10.57 6.05  7.00  22.82054803 12.07 1.20  6.93  2.53
      1.23  20.02 9.98  10.76 15.51 0.00  2.80  25.17 4.94  0.00
      0.00  2.83  0.92  0.00  0.00  0.00  0.75  6.31  0.64  0.25
      0.98  0.57  0.58  1.08  0.93  0.81  1.19  0.00  1.28  1.21
      0.75  0.00  0.00  0.83  0.94  0.00  1.00  0.00  1.01  0.72
      4.34  1.08
73    6     10    2     107.30      18.40 0.86  0.13  0.25  8.10
      1.19  65.87 16.68 7.00  27.66150005 12.64 1.22  5.07  1.72
      0.93  19.43 6.21  11.43 18.57 0.00  3.19  8.21  12.89 0.00
      0.44  2.80  0.00  0.68  0.00  0.00  0.00  16.15 0.67  18.58
      0.61  13.81 0.62  1.05  0.58  0.86  1.43  0.00  1.46  0.39
      1.95  0.00  53.78 0.82  0.00  2.03  0.00  0.00  0.00  1.86
      2.21  0.55
74    6     10    2     93.90 16.05 0.95  0.29  0.23  7.90  1.18
      85.89 18.84 8.00  18.27303248 12.55 1.20  6.93  1.29  0.70
      22.43 5.58  8.73  19.16 0.28  3.85  11.73 7.79  0.30  3.69
      1.66  0.00  0.51  0.00  1.49  0.00  12.80 2.89  151.65
      0.58  113.88      0.76  1.21  0.52  0.65  1.48  0.82  1.76
      0.56  1.18  2.77  451.00      0.49  0.00  1.52  0.00  16.72
      0.00  1.47  2.68  0.67
75    7     10    2     116.80      17.35 1.04  0.25  0.13  7.60
      1.05  11.12 7.25  3.00  23.42825912 10.46 1.38  4.89  4.27
      0.58  15.82 11.60 13.99 13.95 0.54  1.33  28.09 3.62  0.13
      0.00  1.93  0.00  0.33  0.00  0.00  0.89  7.78  0.52  0.58
      1.16  0.45  1.22  0.85  1.08  1.05  1.07  1.58  0.61  1.35
      0.55  1.20  0.00  0.57  0.00  0.98  0.00  0.00  1.19  0.89
      3.06  0.76
76    8     10    2     99.60 16.85 0.87  0.27  0.21  6.78  1.09
      29.75 10.43 8.00  19.89395349 12.52 1.36  6.00  1.60  0.97
      24.45 7.26  10.19 22.43 0.00  4.27  13.56 4.88  0.00  0.00
```

```
      3.85  0.00  0.00  0.00  0.00  0.00  9.12  0.31  0.25  0.70
      0.49  0.35  1.32  0.68  0.76  1.73  0.00  1.95  0.65  0.74
      0.00  0.00  1.13  0.00  0.00  0.00  0.00  0.00  1.05  3.02
      0.75
77    7     11    2     111.30      15.75 0.83  0.42  0.20  7.45
      1.10  8.21  5.81  3.00  16.86987149 12.29 2.20  5.51  1.13
      0.55  18.38 10.11 9.85  16.52 0.91  1.28  28.76 3.36  0.12
      0.00  2.27  0.61  0.22  0.00  0.14  0.77  6.71  0.76  0.54
      1.02  0.42  1.49  0.99  0.94  0.74  1.27  2.66  0.59  1.38
      0.51  1.11  0.00  0.67  0.62  0.66  0.00  1.57  1.03  0.77
      3.70  0.92
78    7     11    2     105.70      17.55 0.96  0.32  0.27  7.49
      1.12  5.89  5.34  3.00  20.33548798 12.79 0.75  5.78  1.04
      0.48  19.32 10.58 16.68 11.15 0.70  1.96  20.38 5.51  0.21
      0.00  2.13  0.60  0.28  0.00  0.17  0.89  9.44  0.89  0.93
      1.07  0.71  1.44  1.04  0.98  1.25  0.86  2.05  0.90  0.98
      0.83  1.94  0.00  0.63  0.61  0.83  0.00  1.91  1.19  1.08
      4.18  1.04
79    7     11    2     111.10      17.50 0.99  0.15  0.23  8.24
      1.02  6.33  4.21  3.00  13.55896754 13.33 0.91  5.16  1.14
      0.56  15.66 12.18 15.30 10.78 1.62  1.64  26.65 3.55  0.05
      0.00  3.07  0.74  0.25  0.00  0.00  1.07  7.43  0.67  0.33
      1.19  0.30  2.34  0.84  1.13  1.15  0.83  4.74  0.75  1.28
      0.54  0.46  0.00  0.90  0.75  0.75  0.00  0.00  1.44  0.85
      3.74  0.93
80    8     11    2     90.20 16.95 1.07  0.33  0.20  6.68  0.97
      32.14 12.11 3.00  20.8360918  12.88 1.25  8.98  1.57  0.94
      11.59 6.90  9.51  16.19 2.37  3.71  10.99 10.60 0.35  0.21
      1.91  0.59  0.62  0.00  0.00  2.29  22.15 1.23  10.17 0.63
      7.65  4.18  0.62  0.64  0.71  1.25  6.93  1.70  0.53  1.61
      3.24  25.67 0.56  0.60  1.85  0.00  0.00  3.07  2.54  2.56
      0.64
81    8     11    2     115.40      16.85 1.22  0.34  0.20  7.41
      1.20  46.95 14.69 4.00  25.76455164 12.72 0.98  8.22  2.39
      0.57  30.19 4.94  5.80  23.10 0.00  4.40  10.74 9.51  0.19
      0.00  4.17  0.72  0.51  0.00  0.00  0.34  5.39  0.65  1.07
      0.47  0.94  0.36  1.62  0.46  0.43  1.78  0.00  2.01  0.52
      1.44  1.76  0.00  1.23  0.73  1.52  0.00  0.00  0.46  0.62
      2.51  0.63
82    8     11    2     106.70      17.40 1.08  0.31  0.16  7.81
      1.12  14.79 13.43 4.00  17.33590623 12.57 0.95  9.91  1.77
      0.95  43.31 7.28  11.77 21.49 0.00  4.34  0.00  11.80 0.00
      0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.60
      0.52  0.50  0.00  2.33  0.68  0.88  1.65  0.00  1.99  0.00
      1.79  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00  0.00
      3.09  0.77
83    1     11    2     107.40      16.70 1.15  0.25  0.15  6.86
      1.16  5.30  4.26  4.00  12.69516968 12.36 0.66  6.09  0.75
      0.23  .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .
84    2     11    2     100.70      18.40 1.17  0.25  0.20  7.87
      1.21  10.76 4.13  4.00  20.43807849 12.98 1.43  7.42  2.52
      0.68  12.90 12.33 17.98 10.82 1.25  1.48  27.78 3.47  0.13
      0.00  2.78  0.80  0.14  0.00  0.00  0.73  7.41  0.70  0.58
      1.28  0.72  1.83  0.69  1.15  1.35  0.83  3.66  0.68  1.33
      0.53  1.20  0.00  0.82  0.81  0.42  1.00  0.00  0.98  0.85
      3.74  0.93
```

```
85    1     12    2     89.80 18.45 1.05  0.18  0.11  6.36  0.97
      5.80  3.59  4.00  19.30915879 11.96 1.09  9.47  1.66  1.02  .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
86    1     12    2     100.10      22.10 1.03  0.13  0.16  7.08
      1.05  5.64  4.88  4.00  21.7052629  12.06 1.22  3.64  2.37
      1.64  .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .
87    2     12    2     89.50 20.20 1.09  0.23  0.14  7.15  1.00
      4.34  2.88  4.00  18.12835565 11.31 1.20  7.29  0.66  0.86
      21.98 10.26 11.96 13.22 0.00  1.59  19.93 7.09  0.00  0.00
      4.37  1.43  0.43  0.00  0.00  0.31  7.42  0.90  0.36  0.94
      0.43  0.42  1.18  0.95  0.90  1.02  0.00  0.73  0.96  1.07
      0.00  0.00  1.29  1.46  1.28  1.00  0.00  0.42  0.85  4.14
      1.03
88    2     12    2     95.20 20.30 1.07  0.23  0.18  7.90  1.15
      7.29  5.33  4.00  16.06890241 9.28  1.90  8.36  1.59  0.42
      17.02 11.49 13.83 13.95 0.00  2.08  20.72 5.77  0.00  0.00
      3.98  1.68  0.56  0.00  0.00  0.00  8.93  0.94  0.29  1.03
      0.49  0.34  0.92  1.07  1.04  1.07  0.00  0.95  1.00  0.87
      0.00  0.00  1.17  1.71  1.67  1.00  0.00  0.00  1.03  3.31
      0.82
89    6     12    2     96.80 20.75 1.01  0.28  0.18  7.12  1.28
      77.29 17.35 8.00  21.83412816 12.36 1.52  6.76  3.09  0.67
      27.89 6.90  10.64 17.75 1.18  4.11  8.21  10.80 0.00  0.32
      2.75  0.68  0.39  0.00  0.40  1.54  6.44  1.42  13.58 0.61
      10.25 2.09  1.50  0.64  0.80  1.37  3.45  1.88  0.39  1.64
      0.00  39.11 0.81  0.69  1.16  0.00  4.49  2.07  0.74  2.11
      0.53
90    6     12    2     95.40 19.60 1.00  0.30  0.14  8.19  1.20
      64.05 23.15 8.00  16.61477044 9.47  1.56  4.67  2.64  1.33
      25.31 6.81  11.98 14.45 0.00  2.37  10.30 9.98  0.00  0.30
      2.98  0.00  0.44  0.00  0.43  0.93  13.70 1.41  12.73 0.68
      9.44  0.94  1.36  0.63  0.90  1.11  0.00  1.08  0.49  1.51
      0.00  36.67 0.88  0.00  1.31  0.00  4.83  1.25  1.57  2.24
      0.56
91    7     12    2     91.10 18.85 0.94  0.22  0.16  6.59  1.01
      9.33  6.34  4.00  18.77412673 10.14 1.43  5.42  1.65  1.01
      25.65 10.78 15.33 10.99 0.00  1.51  17.69 6.76  0.00  0.00
      4.46  1.18  0.41  0.00  0.00  0.00  5.25  0.62  0.34  1.00
      0.17  0.20  1.38  1.00  1.15  0.85  0.00  0.69  0.85  1.02
      0.00  0.00  1.31  1.20  1.22  0.00  0.00  0.00  0.60  2.63
      0.66
92    8     12    2     96.10 22.50 1.06  0.21  0.16  7.21  1.05
      33.89 7.80  4.00  25.89364389 12.75 1.02  4.44  0.51  0.40
      22.87 5.90  7.94  22.70 0.00  4.37  13.02 5.22  0.00  0.12
      2.39  0.49  0.00  0.00  0.00  1.80  13.20 0.73  5.15  0.59
      4.17  1.31  1.23  0.55  0.59  1.75  0.00  2.00  0.63  0.79
      0.00  14.67 0.70  0.50  0.00  0.00  0.00  2.41  1.52  2.06
      0.51
93    1     13    2     111.50      21.10 0.96  0.24  0.17  7.11
      1.19  8.82  6.65  5.00  20.54937919 12.58 1.09  5.38  0.86
      0.32  .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
```

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      .     .     .     .     .     .     .     .     .
94    1     13    2     95.00 19.40 1.18  0.29  0.15  7.55  0.96
      7.07  6.23  5.00  17.43178804 12.08 0.86  6.13  2.18  0.93  .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .
95    2     13    2     115.30      23.15 0.99  0.27  0.19  7.81
      1.10  7.76  5.20  5.00  25.60582946 10.48 1.16  4.31  0.96
      0.41  13.13 11.56 14.43 12.44 0.61  2.35  21.70 6.50  0.19
      0.00  2.59  0.80  0.32  0.00  0.25  1.39  11.75 1.36  0.91
      1.07  0.96  1.67  0.71  1.08  1.08  0.96  1.78  1.08  1.04
      0.98  1.76  0.00  0.76  0.81  0.95  1.00  2.81  1.86  1.35
      2.72  0.68
96    2     13    2     108.70      20.10 0.97  0.29  0.21  7.94
      1.16  7.46  4.62  5.00  26.41064747 10.72 1.13  6.80  2.03
      1.35  14.73 13.94 14.32 9.76  0.73  1.69  24.76 5.05  0.00
      0.00  3.73  1.24  0.38  0.00  0.00  0.92  8.73  0.96  0.26
      1.19  0.44  1.46  0.79  1.30  1.07  0.75  2.14  0.77  1.19
      0.77  0.00  0.00  1.10  1.26  1.13  1.00  0.00  1.23  1.00
      2.96  0.74
97    6     13    2     103.80      18.45 0.94  0.20  0.14  9.92
      1.10  57.49 29.35 8.00  21.05752014 12.80 0.91  3.07  1.69
      1.05  10.76 5.78  8.69  19.73 0.00  3.87  7.84  10.61 0.00
      0.44  0.00  1.24  0.58  0.00  0.00  3.22  27.25 1.49  18.46
      0.52  13.89 2.48  0.58  0.54  0.65  1.52  0.00  1.77  0.38
      1.61  0.00  53.78 0.00  1.26  1.73  0.00  0.00  4.32  3.13
      2.67  0.66
98    6     13    2     98.40 18.70 1.11  0.30  0.17  8.09  1.22
      85.38 12.77 5.00  20.63903943 12.86 0.75  7.56  1.39  0.79
      13.69 6.20  9.58  15.03 4.34  3.56  8.88  9.72  0.00  0.18
      2.17  0.63  0.76  0.00  0.36  2.98  21.89 2.01  7.82  0.57
      5.91  6.40  0.74  0.58  0.72  1.16  12.70 1.63  0.43  1.47
      0.00  22.00 0.64  0.64  2.27  0.00  4.04  4.00  2.51  2.13
      0.53
99    7     13    2     100.30      17.80 0.90  0.29  0.13  8.46
      1.06  8.95  7.42  5.00  15.89648946 12.67 0.77  8.49  1.60
      0.54  14.54 11.39 17.25 9.76  3.70  2.59  20.18 8.39  0.29
      0.14  1.85  0.00  0.15  0.00  0.14  1.47  8.16  0.78  7.02
      1.11  5.24  4.58  0.78  1.06  1.29  0.75  10.82 1.19  0.97
      1.27  2.68  17.11 0.54  0.00  0.45  0.00  1.57  1.97  0.94
      2.78  0.69
100   8     13    2     99.40 19.10 0.89  0.31  0.14  8.31  1.18
      61.85 9.61  5.00  22.95365171 12.72 0.61  6.62  1.09  0.53
      27.40 6.45  13.56 22.69 0.00  4.46  9.15  4.87  0.00  0.00
      4.19  0.00  0.00  0.00  0.00  0.00  7.23  0.29  0.25  0.69
      0.51  0.28  1.47  0.60  1.02  1.75  0.00  2.04  0.44  0.74
      0.00  0.00  1.23  0.00  0.00  0.00  0.00  0.00  0.83  3.09
      0.77
101   7     14    2     115.00      19.75 0.86  0.37  0.22  7.16
      1.04  13.82 9.65  6.00  17.79044721 13.15 0.59  6.31  1.32
      0.72  17.24 11.07 13.09 10.94 0.69  2.31  24.28 6.00  0.20
      0.00  2.56  0.77  0.19  0.00  0.00  0.96  9.71  0.64  0.92
      1.06  0.73  1.47  0.93  1.03  0.98  0.84  2.02  1.06  1.17
      0.91  1.85  0.00  0.75  0.78  0.57  0.00  0.00  1.29  1.12
      3.38  0.84
102   7     14    2     107.80      20.20 1.03  0.29  0.20  7.66
      1.41  7.48  4.44  6.00  16.41844379 12.41 0.59  5.29  1.06
      0.49  10.67 12.67 12.48 12.52 1.41  2.01  24.76 4.11  0.09
```

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      0.00  2.96  0.71  0.25  0.00  0.12  1.61  13.63 1.06  0.48
      1.10  0.44  2.62  0.57  1.18  0.93  0.96  4.12  0.92  1.19
      0.62  0.83  0.00  0.87  0.72  0.75  0.00  1.35  2.16  1.57
      2.34  0.58
103   8     14    2     117.60      21.35 1.04  0.26  0.15  7.53
      1.17  23.38 9.58  6.00  17.66936596 13.07 0.66  5.96  1.11
      0.54  22.43 6.91  9.03  25.31 0.00  4.50  15.94 5.33  0.00
      0.64  0.00  0.00  0.60  0.00  0.00  1.28  8.02  0.63  26.34
      0.69  20.07 0.88  1.21  0.64  0.68  1.95  0.00  2.06  0.77
      0.81  0.00  78.22 0.00  0.00  1.79  0.00  0.00  1.72  0.92
      2.77  0.69
104   8     14    2     108.30      19.05 1.03  0.34  0.15  7.73
      1.17  23.13 21.39 6.00  16.33296551 13.28 0.82  14.27 2.17
      0.92  15.73 6.30  11.01 16.47 1.94  4.15  9.24  10.42 0.00
      0.41  3.05  1.25  0.44  0.00  0.48  2.35  16.76 1.99  17.23
      0.62  13.00 3.58  0.85  0.59  0.82  1.27  5.68  1.90  0.44
      1.58  0.00  50.11 0.90  1.27  1.31  0.00  5.39  3.15  1.93
      3.47  0.87
105   1     14    2     107.00      19.90 1.06  0.33  0.17  7.27
      0.95  10.53 4.38  6.00  16.01275748 12.15 0.70  6.09  1.06
      0.50  .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .
;

proc sort data = HFHL_PhIP_Study; by diet;
proc sort data = Exp1; by diet;
proc sort data = Exp2; by diet;

libname Store1 'c:\users\public';
proc format;
value Dietfmt
1='Basal diet without PhIP'
2='Basal diet with PhIP'
3='21% Cruciferous diet'
4='21% Apiaceous diet'
5='Combination diet'
6='ITC diet'
7='Furanocoumarin diet'
8='Combination of phytochem diet'
;

Value blockfmt
1='first day of microsome isolation from rats'
2='second day of microsome isolation from rats'
3='third day of microsome isolation from rats'
4='fourth day of microsome isolation from rats'
5='fifth day of microsome isolation from rats'
6='sixth day of microsome isolation from rats'
7='seventh day of microsome isolation from rats'
;

Value Experimentfmt
1='vegetable diet experiment'
2='phytochemical diet experiment'
;
```

```
Value BasalPhIPfmt
1='Experiment I Basal with PhIP'
2='Experiment II Basal with PhIP'
;

Value BasalnoPhIPfmt
1='Experiment I Basal no PhIP'
2='Experiment II Basal no PhIP'
;
proc print data=HFHL_PhIP_Study; by Diet experiment ; format Diet
Dietfmt. Experiment Experimentfmt.
;
proc print data=Exp1; by Diet experiment ; format Diet Dietfmt.
Experiment Experimentfmt.
;
proc print data=Exp2; by Diet experiment ; format Diet Dietfmt.
Experiment Experimentfmt.
;

proc means data=Exp1 n mean stderr std min max; by diet experiment;
TITLE 'ANOVA descriptive statistics for Experiment 1';
format Diet dietfmt. Experiment experimentfmt.;
var Weight_gain--DNA_adduct_P DNA_adduct_t CYP1A_metabolites
CYP1A1_metabolites_P CYP1A2_metabolites_P CYP1A1_metabolites
CYP1A2_metabolites Glucuronidated Sulfated Methylated
Five_hydroxylated N2_hydroxylated;
run;
proc means data=Exp2 n mean stderr std min max; by diet experiment;
TITLE 'ANOVA descriptive statistics for Experiment 2';
format Diet dietfmt. Experiment experimentfmt.;
var Weight_gain--DNA_adduct_P DNA_adduct_t CYP1A_metabolites
CYP1A1_metabolites_P CYP1A2_metabolites_P CYP1A1_metabolites
CYP1A2_metabolites Glucuronidated Sulfated Methylated
Five_hydroxylated N2_hydroxylated;
run;
*Proc glm will provide Least square means (under matrix for p-values),
Means (under multiple comparison for letter grouping);

proc glm data=Exp1;
title 'ANOVA for Experiment 1';
format Diet Dietfmt.;
class Diet block;
model Weight_gain--DNA_adduct_P DNA_adduct_t CYP1A_metabolites
CYP1A1_metabolites_P CYP1A2_metabolites_P CYP1A1_metabolites
CYP1A2_metabolites Glucuronidated Sulfated Methylated
Five_hydroxylated N2_hydroxylated=diet block;
Means Diet / duncan scheffe;
LSMeans Diet /stderr  pdiff;
run;

proc glm data=Exp2;
title 'ANOVA for Experiment 2';
format Diet Dietfmt.;
class Diet block;
model Weight_gain--DNA_adduct_P DNA_adduct_t CYP1A_metabolites
CYP1A1_metabolites_P CYP1A2_metabolites_P CYP1A1_metabolites
CYP1A2_metabolites Glucuronidated Sulfated Methylated
Five_hydroxylated N2_hydroxylated=diet block;
Means Diet / duncan scheffe;
```

```
LSMeans Diet /stderr  pdiff;
run;

proc glm data=HFHL_PhIP_Study;
title 'Basal groups with PhIP (comparison of basal groups with PhIP
of project I and II)';
format BasalPhIP BasalPhIPfmt.;
class BasalPhIP;
model  Weight_gain--DNA_adduct_P DNA_adduct_t CYP1A_metabolites
CYP1A1_metabolites_P CYP1A2_metabolites_P CYP1A1_metabolites
CYP1A2_metabolites Glucuronidated Sulfated Methylated
Five_hydroxylated N2_hydroxylated=BasalPhIP;
Means BasalPhIP / duncan scheffe;
LSMeans BasalPhIP /stderr  pdiff;
run;

proc glm data=HFHL_PhIP_Study;
title 'Basal groups with no PhIP (comparison of basal groups no PhIP
of project I and II)';
format BasalnoPhIP BasalnoPhIPfmt.;
class BasalnoPhIP;
model  Weight_gain--DNA_adduct_P DNA_adduct_t CYP1A_metabolites
CYP1A1_metabolites_P CYP1A2_metabolites_P CYP1A1_metabolites
CYP1A2_metabolites Glucuronidated Sulfated Methylated
Five_hydroxylated N2_hydroxylated=BasalnoPhIP;
Means BasalnoPhIP / duncan scheffe;
LSMeans BasalnoPhIP /stderr  pdiff;
run;
ods graphics on;

proc factor data=Exp1 out=store1 nfactors=4

PLOT
method=principal
simple
scree
priors=one
mineigen=1
round
rotate=varimax
flag=.4
;
title 'PCA analysis Exp 1';
var I--XVII;
ods graphics off;
run;
proc plot data=store1;
title 'Plot of PC# vs DNA adducts - Exp 1';
plot (FACTOR1 FACTOR2 FACTOR3 FACTOR4)*DNA_adduct;
run;
PROC CORR DATA=store1;
TITLE 'Correlation with PCs - Exp 1';
var FACTOR1 FACTOR2 FACTOR3 FACTOR4;
with DNA_adduct;
run;
PROC CORR DATA=Exp1;
TITLE 'Correlation with metabolites - Exp 1';
var I--XVII;
with DNA_adduct;
```

```
run;
PROC CORR DATA=Exp1;
TITLE 'Correlation with grouped metabolites - Exp 1';
var CYP1A_metabolites CYP1A1_metabolites CYP1A2_metabolites
Glucuronidated Sulfated Methylated Five_hydroxylated N2_hydroxylated;
with DNA_adduct;
run;

PROC CORR DATA=control1;
TITLE 'Correlation with metabolites and grouped metabolites vs. DNA
adduct in control group - Exp 1';
var I--XVII CYP1A_metabolites CYP1A1_metabolites CYP1A2_metabolites
Glucuronidated Sulfated Methylated Five_hydroxylated N2_hydroxylated;
with DNA_adduct;
run;
proc corr data = cruciferousdiet;
TITLE 'Correlation with metabolites and grouped metabolites vs. DNA
adduct in cruciferous group - Exp 1';
var I--XVII CYP1A_metabolites CYP1A1_metabolites CYP1A2_metabolites
Glucuronidated Sulfated Methylated Five_hydroxylated N2_hydroxylated;
with DNA_adduct;
run;
proc corr data = apiaceousdiet;
TITLE 'Correlation with metabolites and grouped metabolites vs. DNA
adduct in apiaceous group - Exp 1';
var I--XVII CYP1A_metabolites CYP1A1_metabolites CYP1A2_metabolites
Glucuronidated Sulfated Methylated Five_hydroxylated N2_hydroxylated;
with DNA_adduct;
run;
proc corr data = Vegetables_combination;
TITLE 'Correlation with metabolites and grouped metabolites vs. DNA
adduct in vegetables combination - Exp 1';
var I--XVII CYP1A_metabolites CYP1A1_metabolites CYP1A2_metabolites
Glucuronidated Sulfated Methylated Five_hydroxylated N2_hydroxylated;
with DNA_adduct;
run;
ods graphics on;
proc factor data=Exp1 out=store2 nfactors = 4
priors=smc
MSA
Scree
preplot
rotate=varimax
reorder
plot
flag= .4
;
title 'Factor analysis of PhIP metabolites - Exp 1';
var I--XVII;
ods graphics off;
proc plot data=store2;
title 'Plot of factors vs DNA adducts - Exp 1';
plot (FACTOR1 FACTOR2 FACTOR3 FACTOR4)*DNA_adduct;
run;
PROC CORR DATA=STORE2;
TITLE 'Correlation with factors - Exp 1';
var FACTOR1 FACTOR2 FACTOR3 FACTOR4;
with DNA_adduct;
run;
```

```
proc factor data=Exp2 out=store3 nfactors=4

PLOT
method=principal
simple
scree
priors=one
mineigen=1
round
rotate=varimax
flag=.4
;
title 'PCA analysis Exp 2';
var I--XVII;
ods graphics off;
run;
proc plot data=store3;
title 'Plot of PC# vs DNA adducts - Exp 2';
plot (FACTOR1 FACTOR2 FACTOR3 FACTOR4)*DNA_adduct;
run;
PROC CORR data=store3;
TITLE 'Correlation with PCs - Exp 2';
var FACTOR1 FACTOR2 FACTOR3 FACTOR4;
with DNA_adduct;
run;
ods graphics on;
proc factor data=Exp2 out=store4 nfactors = 4
priors=smc
MSA
Scree
preplot
rotate=varimax
reorder
plot
flag= .4
;
title 'Factor analysis of PhIP metabolites - Exp 2';
var I--XVII;
ods graphics off;
proc plot data=store4;
title 'Plot of factors vs DNA adducts - Exp 2';
plot (FACTOR1 FACTOR2 FACTOR3 FACTOR4)*DNA_adduct;
run;
PROC CORR DATA=store4;
TITLE 'Correlation with factors - Exp 2';
var FACTOR1 FACTOR2 FACTOR3 FACTOR4;
with DNA_adduct;
run;
PROC CORR DATA=Exp2;
TITLE 'Correlation with metabolites - Exp 2';
var I--XVII;
with DNA_adduct;
run;
PROC CORR DATA=Exp2;
TITLE 'Correlation with grouped metabolites - Exp 2';
var CYP1A_metabolites CYP1A1_metabolites CYP1A2_metabolites
Glucuronidated Sulfated Methylated Five_hydroxylated N2_hydroxylated;
with DNA_adduct;
run;
```

```
PROC CORR DATA=control2;
TITLE 'Correlation with metabolites and grouped metabolites vs. DNA
adduct in control group - Exp 2';
var I--XVII CYP1A_metabolites CYP1A1_metabolites CYP1A2_metabolites
Glucuronidated Sulfated Methylated Five_hydroxylated N2_hydroxylated;
with DNA_adduct;
run;
proc corr data = ITCdiet;
TITLE 'Correlation with metabolites and grouped metabolites vs. DNA
adduct in PEITC and I3C diet - Exp 2';
var I--XVII CYP1A_metabolites CYP1A1_metabolites CYP1A2_metabolites
Glucuronidated Sulfated Methylated Five_hydroxylated N2_hydroxylated;
with DNA_adduct;
run;
proc corr data = FCdiet;
TITLE 'Correlation with metabolites and grouped metabolites vs. DNA
adduct in FCs diet - Exp 2';
var I--XVII CYP1A_metabolites CYP1A1_metabolites CYP1A2_metabolites
Glucuronidated Sulfated Methylated Five_hydroxylated N2_hydroxylated;
with DNA_adduct;
run;
proc corr data = Phytochem_combination;
TITLE 'Correlation with metabolites and grouped metabolites vs. DNA
adduct in Phytochem combination - Exp 2';
var I--XVII CYP1A_metabolites CYP1A1_metabolites CYP1A2_metabolites
Glucuronidated Sulfated Methylated Five_hydroxylated N2_hydroxylated;
with DNA_adduct;
run;

proc reg data=Exp1;
title 'Exp 1 Multiple regression of Phip metabolites against DNA
adducts';
var I--XVII DNA_adduct;
model DNA_adduct= I--XVII/selection=stepwise;
run;

proc reg data=Exp1;
title 'Exp 1 Multiple regression of enzyme expression against DNA
adducts ';
var CYP1A1_E      CYP1A2_E    UGT1A1_E    SULT1A1_E DNA_adduct;
model DNA_adduct=CYP1A1_E     CYP1A2_E    UGT1A1_E
      SULT1A1_E/selection=stepwise;
run;
proc reg data=Exp1;
title 'Exp 1 Multiple regression of enzyme activity against DNA
adduct';
var CYP1A1  CYP1A2 UGT1A1     SULT1A1 DNA_adduct;
model DNA_adduct=CYP1A1 CYP1A2 UGT1A1 SULT1A1/selection=stepwise;
run;
proc reg data=Exp1;
title 'Exp 1 Multiple regression of grouped metabolites against DNA
adduct';
var CYP1A_metabolites CYP1A1_metabolites CYP1A2_metabolites
Glucuronidated Sulfated Methylated Five_hydroxylated N2_hydroxylated
DNA_adduct;
model DNA_adduct=CYP1A_metabolites CYP1A1_metabolites
CYP1A2_metabolites Glucuronidated Sulfated Methylated
Five_hydroxylated N2_hydroxylated/selection=stepwise;
run;
```

```
proc reg data=Exp2;
title 'Exp 2 Multiple regression of Phip metabolites against DNA
adducts';
var I--XVII DNA_adduct;
model DNA_adduct=I--XVII/selection=stepwise;
run;

proc reg data=Exp2;
title 'Exp 2 Multiple regression of enzyme expression against DNA
adducts';
var CYP1A1_E      CYP1A2_E    UGT1A1_E    SULT1A1_E DNA_adduct;
model DNA_adduct=CYP1A1_E     CYP1A2_E    UGT1A1_E
      SULT1A1_E/selection=stepwise;
run;
proc reg data=Exp2;
title 'Exp 2 Multiple regression of enzyme activity against DNA
adduct';
var CYP1A1  CYP1A2 UGT1A1     SULT1A1 DNA_adduct;
model DNA_adduct=CYP1A1 CYP1A2 UGT1A1     SULT1A1/selection=stepwise;
run;
proc reg data=Exp2;
title 'Exp 2 Multiple regression of grouped metabolites against DNA
adduct';
var CYP1A_metabolites CYP1A1_metabolites CYP1A2_metabolites
Glucuronidated Sulfated Methylated Five_hydroxylated N2_hydroxylated
DNA_adduct;
model DNA_adduct=CYP1A_metabolites CYP1A1_metabolites
CYP1A2_metabolites Glucuronidated Sulfated Methylated
Five_hydroxylated N2_hydroxylated/selection=stepwise;
run;


proc corr data = HFHL_PhIP_Study;
TITLE 'Correlation with methylated metabolites vs. CYP1A mediated
metabolites in both projects';
var CYP1A_metabolites CYP1A1_metabolites_P CYP1A2_metabolites_P;
with Methylated_P;
run;

proc corr data = Exp1;
TITLE 'Correlation with methylated metabolites vs. CYP1A mediated
metabolites in Exp1';
var CYP1A_metabolites CYP1A1_metabolites CYP1A2_metabolites;
with Methylated;
run;


proc corr data = Exp2;
TITLE 'Correlation with methylated metabolites vs. CYP1A mediated
metabolites in Exp2';
var CYP1A_metabolites CYP1A1_metabolites CYP1A2_metabolites;
with Methylated;
run;


proc corr data = Exp1;
TITLE 'Correlation with CYP1A1 activity vs. CYP1A1 expression in
Exp1';
var CYP1A1;
```

```
with CYP1A1_E;
run;

proc corr data = Exp1;
TITLE 'Correlation with CYP1A2 activity vs. CYP1A2 expression in
Exp1';
var CYP1A2;
with CYP1A2_E;
run;

proc corr data = Exp1;
TITLE 'Correlation with UGT1A1 activity vs. UGT1A1 expression in
Exp1';
var UGT1A1;
with UGT1A1_E;
run;

proc corr data = Exp1;
TITLE 'Correlation with SULT1A1 activity vs. SULT1A1 expression in
Exp1';
var SULT1A1;
with SULT1A1_E;
run;

proc corr data = Exp2;
TITLE 'Correlation with CYP1A1 activity vs. CYP1A1 expression in
Exp2';
var CYP1A1;
with CYP1A1_E;
run;

proc corr data = Exp2;
TITLE 'Correlation with CYP1A2 activity vs. CYP1A2 expression in
Exp2';
var CYP1A2;
with CYP1A2_E;
run;

proc corr data = Exp2;
TITLE 'Correlation with UGT1A1 activity vs. UGT1A1 expression in
Exp2';
var UGT1A1;
with UGT1A1_E;
run;

proc corr data = Exp2;
TITLE 'Correlation with SULT1A1 activity vs. SULT1A1 expression in
Exp2';
var SULT1A1;
with SULT1A1_E;
run;


proc corr data = HFHL_PhIP_Study;
TITLE 'Correlation with CYP1A1 activity vs. CYP1A1 expression in
HFHL_PhIP_Study';
var CYP1A1;
with CYP1A1_E;
run;
```

```
proc corr data = HFHL_PhIP_Study;
TITLE 'Correlation with CYP1A2 activity vs. CYP1A2 expression in
HFHL_PhIP_Study';
var CYP1A2;
with CYP1A2_E;
run;

proc corr data = HFHL_PhIP_Study;
TITLE 'Correlation with UGT1A1 activity vs. UGT1A1 expression in
Exp2';
var UGT1A1;
with UGT1A1_E;
run;

proc corr data = HFHL_PhIP_Study;
TITLE 'Correlation with SULT1A1 activity vs. SULT1A1 expression in
HFHL_PhIP_Study';
var SULT1A1;
with SULT1A1_E;
run;

quit;
```