

The inhibitory effect of natural antioxidants on formation of 2-amino-1-methyl-6-phenylimidazo
[4, 5-b] pyridine (PhIP) in a model system

by

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B.S., University of Baghdad, 2001
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Abstract

Heterocyclic amines (HCAs) are a class of mutagenic and carcinogenic compounds generated when muscle foods are cooked at high temperatures. Exposure to HCAs has been linked to human cancers, among them colon, prostate, breast, and pancreatic cancers. Research has focused recently on how HCAs form and how their formation can be inhibited. 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) is a common, potentially harmful HCA that forms via the Maillard reaction. The health consequences of consuming HCAs has caused the International Agency for Research on Cancer (IARC) to list PhIP as a “possible human carcinogen.” Antioxidant spices and flavonoid compounds have received considerable attention for their beneficial effect against HCA formation in our daily foods. Consumption of most these antioxidants has been found to protect against various chronic diseases such as cardiovascular diseases and cancers.

Chemical model systems help in assessing how HCA formation can be inhibited using different compounds. Chemical model systems are preferred because they limit side reactions that occur in meats, complicating analysis, and thus allow studying the chemical interactions among the precursors of HCAs and applied antioxidants. In this research a model system with 0.011 mmol glucose, 0.022 mmol creatinine, and 0.022 mmol phenylalanine in 90:10 diethylene glycol/water (v/v) was heat-treated at 180°C for 1 hour to test the formation of PhIP. Black and red pepper compounds such as pepper oil, piperine, D-limonene, P-cymene, and capsaicin and flavonoid compounds such as quercetin, apigenin, genistin, phlorizin, and catechin were added individually to the model system at three concentrations (125, 625, and 1250 ppm) to test their effect on PhIP formation. The PhIP contents were assessed using HPLC. The results indicate that four out of five antioxidant components of spices: black pepper oil, piperine, D-limonene, and

capsaicin significantly ($p < 0.05$) reduced PhIP formation, while P-cymene had no significant effect on PhIP formation. All flavonoid compounds also had a significant ($p < 0.05$) effect on PhIP formation. In addition, binary combinations of two antioxidant spices such as piperine and capsaicin and two flavonoid compounds such as genistin and catechin at 1:0.25, 1:0.5, and 1:1 ratios were also evaluated. Significant ($p < 0.05$) synergistic effects were observed among all combinations. Our results showed that when antioxidant spices and flavonoid compounds were added to model systems either individually or in combination, they reduced PhIP formation. These findings provide valuable information about antioxidant spices and flavonoid compounds as protective agents against HCA formation.

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Approved by:

Major Professor
J. Scott Smith

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Chemical model systems help in assessing how HCA formation can be inhibited using different compounds. Chemical model systems are preferred because they limit side reactions that occur in meats, complicating analysis, and thus allow studying the chemical interactions among the precursors of HCAs and applied antioxidants. In this research a model system with 0.011 mmol glucose, 0.022 mmol creatinine, and 0.022 mmol phenylalanine in 90:10 diethylene glycol/water (v/v) was heat-treated at 180°C for 1 hour to test the formation of PhIP. Black and red pepper compounds such as pepper oil, piperine, D-limonene, P-cymene, and capsaicin and flavonoid compounds such as quercetin, apigenin, genistin, phlorizin, and catechin were added individually to the model system at three concentrations (125, 625, and 1250 ppm) to test their effect on PhIP formation. The PhIP contents were assessed using HPLC. The results indicate that four out of five antioxidant components of spices: black pepper oil, piperine, D-limonene, and

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Dedication

To my parents who sacrificed everything for their children's education

and

My wonderful wife for her endless love

and

My children

Chapter 1 - Literature review

1.1 Introduction

Several studies report that diet is associated with different human diseases such as cancer (Sugimura 2002). Cancer is considered a significant public health problem worldwide, exceeded only by cardiovascular diseases (Oliveira et al. 2007). According to the estimates by the Centers for Disease Control and Prevention (CDC) in 2015, one of every four deaths in the United States is due to cancer (CDC 2015). Some of these deaths may be due to heterocyclic amines (HCAs) that form in the process of cooking meats. In 1912, French chemist Louis-Camille Maillard first described a non-enzymatic browning reaction that developed during food processing. Today, this reaction is called the Maillard browning reaction. Maillard browning reaction is caused by a combination of sugar, creatine or creatinine, and amino acids.

HCAs are among the most common and potentially harmful compounds that form during the Maillard reaction. HCAs are a group of mutagenic compounds that form when muscle rich food products are heated (Murkovic 2004). These compounds are a result of heating one or more of four precursors: glucose, amino acids, creatine, and creatinine (Jägerstad, Skog, Grivas, and Olsson 1991; Knize et al. 1985). Human exposure to HCAs is influenced by type of food and cooking practices, but portion size and intake frequency also are important (Skog 2004). Recently, the potential health risks of HCAs have been emphasized. Research indicates that consuming meats rich in HCAs may increase the risk of colon and other types of cancers in humans (Norell et al. 1986; Willett, Stampfer, Colditz, Rosner, and Speizer 1990). Therefore, finding ways to reduce HCA formation in foods is necessary. Research indicates that antioxidant spices used during food processing may inhibit the formation of HCAs (Tsen, Ameri, and Smith 2006; Puangsombat and Smith 2010).

Model systems have been developed to explain how mutagenic/carcinogenic HCAs form during food processing by observing the effect of time and temperature on the reaction (Bordas, Moyano, Puignou, and Glaceran 2004). Studies have, however, produced contradictory results; adding synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluence (BHT), propyl gallate (PG), tertiary butylhydroquinone (TBHQ), as well as natural antioxidants such as flavonoids and vitamins C and E, and other food components such as cherry, polyphenolic compounds from tea, spices, inulin, and olive oil, inhibit or promote formation of HCAs depending on the study (Pearson, Chen, Gray, and Aust 1992; Britt, Gomaa, Gray, and Booren 2002).

This literature review will provide an in-depth definition of HCAs, how HCAs form, and how they can be inhibited. A discussion of HCAs in chemical model systems is also included.

1.2 Heterocyclic Amines

Heterocyclic amines were first detected in 1970 (Takashi 1988), and subsequent studies studied their associated health risks for two decades (Sugimura 1995). HCAs are mutagenic and carcinogenic chemical compounds produced when high protein foods such as meat and fish are cooked at temperatures higher than 150°C (Knize, Salmon, Mehta, and Felton 1997); they are present at very low concentrations (Wakabayashi, Nagao, Esumi and Sugimura 1992). The International Agency for the Research of Cancer (IARC) has categorized some HCAs as possible carcinogens to humans (MeIQ, MeIQx, and PhIP, group 2B) and another as a probable carcinogen to humans (IQ, group 2A) (International Agency for Research on Cancer 1993). More than 25 HCAs have been identified as potent mutagens in cooked meat (Alaejos, Ayala, González, and Afonso 2008). In general, the most abundant HCAs, based on dietary intake, are 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3-methylimidazo[4,5-f]quinoxaline (IQx), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (DiMeIQx), and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (see Figure 1.1; Puangsombat, Gadgil, Houser, Hunt, and Smith 2012). Other HCAs include 2-amino-9H-dipyrido[2,3-b]indole (AαC), 2-amino-3-methyl-9H-pyrido[4,3-b]indole (MeAαC), 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), and 2-amino-dipyrido[1,2-a:3',2'-d]imidazole (Glu-P-2) (Cheng, Chen, and Wang 2006). According to Felton and Knize (1990), some HCAs are potent mutagens based on the Ames *Salmonella*/mutagenicity test.

HCAs produced in cooked foods depend on cooking time and temperature, meat type, cooking method (frying, broiling, and grilling), pH, water activity, carbohydrates, free amino

acids, and creatine (Oz, Kaban, and Kaya 2007; Oz, Kaban, and Kaya 2010a, 2010b, and 2010c). Heat, fat content, and lipid oxidation also increase HCA levels (Jägerstad, Skog, Arvidsson, and Solyakov 1998). Moreover, portion size and intake frequency also influence the onset of cancer due to HCAs formation (Skog 2004).

Table 1.1 summarizes the levels of major HCAs found in cooked meat and fish products. Cooking muscle foods produced more PhIP than MeIQx and DiMeIQx, the possible explanation for this because PhIP is produced by a different mechanism, so they produce faster than other polar HCAs (Knize and Felton 1995). IQ and MeIQ are rarely detected in meat samples (Knize and Felton 1995). Cooked offal products, like chicken liver, beef liver, beef tongue, and lamb kidney also have insignificant amounts of HCAs (Solyakov and Skog 2002, Khan, Bertus, Busquets, and Puignou 2009), probably because the creatine and creatinine levels present in these raw products are low (Khan, Bertus, Busquets, and Puignou 2009).

Table 1.1: Estimated MeIQx, DiMeIQx, and PhIP concentrations in cooked meat products by meat type and cooking method (Skog et al 1997, Solyakov and Skog 2002, Ni et al. 2008, and Iwasaki et al. 2010).

Muscle type	Cooking Method	HCA concentration (ng/g)		
		MeIQx	DiMeIQx	PhIP
Beef	Fried	nd-3.17	nd-2.06	nd-10.0
	Grilled/BBQ	nd-5.41	nd-2.35	nd-16.27
	Broiled	nd-1.80	nd-0.1	nd-18.40
Pork	Fried	nd-5.43	nd-3.30	nd-13.40
	Roasted	nd	nd	0.5-2.3
	Stewed	nd-0.7	nd-0.08	nd-0.1
	Smoked	nd	nd	0.7-7.4
Chicken	Fried	nd-2.34	nd-2.58	nd-48.54
	Grilled/BBQ	nd-7.70	nd-5.52	nd-304.71
	Roast/Broiled	nd-2.81	nd-1.98	nd-71.96
Fish	Fried	nd-6.44	nd-3.0	nd-17.0
	Grilled/BBQ	nd-4.00	nd-2.00	nd-50.3
	Steamed/Boiled	nd	nd	nd

nd = not detected

1.3 Classification, Chemical Structure, and Formation of Heterocyclic

Amines

HCAAs can be classified into two groups: polar and non-polar compounds. Most polar HCAAs are type's imidazoquinoline (IQ) and imidazoquinoxaline (IQx) as well as imidazopyridine while non-polar HCAAs are commonly pyridoindole or dipyridoimidazole (Murkovic 2004). HCAAs are categorized into polar and non-polar because of the order in which they elute from a column in reversed-phase chromatography as well as the fluorescence non-polar HCAAs (Murkovic 2007). Table 1.2 summarizes the chemical names and abbreviations of these HCAAs.

Table 1.2: Chemical names and abbreviations of the non-polar and polar heterocyclic amines (Sugimura et al. 2004).

Polar (Amino-imidazo-azaarenes)		
Quinolines	IQ	2-Amino-3-methylimidazo[4,5-f]quinoline
	MeIQ	2-Amino-3,4-dimethylimidazo[4,5-f]quinolone
Quinoxalines	IQx	2-Amino-3-methylimidazo[4,5-f]quinaxalines
	MeIQx	2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline
	4,8-DiMeIQx	2-Amino-3,4,8-trimethylimidazo[4,5-f]quinaxaline
	7,8- DiMeIQx	2-Amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline
	4,7,8-TriMeIQx	2-Amino-3,4,7,8-tetramethylimidazo[4,5-f]quinoxaline
Pyridines	PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
	DMIP	Dimethylimidazopyridine
	TMIP	Trimethylimidazopyridine
Quinolines	IQ	2-Amino-3-methylimidazo[4,5-f]quinoline
	MeIQ	2-Amino-3,4-dimethylimidazo[4,5-f]quinolone
Non-Polar (Amino-carbolines)		
α -amino-	A α C	2-Amino-9H-dipyrido[2,3-b]indole
carbolines	MeA α C	2-Amino-3-methyl-9H-dipyrido[2,3-b]indole
β -amino-	NorHarman	9H-pyrido[3,4-b]indole
carbolines	Harman	1-methyl-9H-pyrido[3,4-b]indole
γ -amino-	Trp-P-1	3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole
carbolines	Trp-P-2	3-Amino-1-methyl-5H-pyrido[4,3-b]indole

1.3.1 Polar heterocyclic amines

Polar heterocyclic amines are also known as aminoimidazo-azaarenes (AIAs). AIAs contain a 2-amino-imidazo moiety along with an N-methyl group attached to one nitrogen in the imidazo ring (see Figure 1.1; Cheng, Chen, and Wang 2006). The AIAs can be categorized into three subgroups: quinolines, which are called IQ-type (e.g., IQ and MeIQ); quinoxalines, which are called IQx-type (e.g., IQx, MeIQx and DiMeIQx); and pyridines, which include PhIP, DMIP, and TMIP (Cheng et al. 2006).

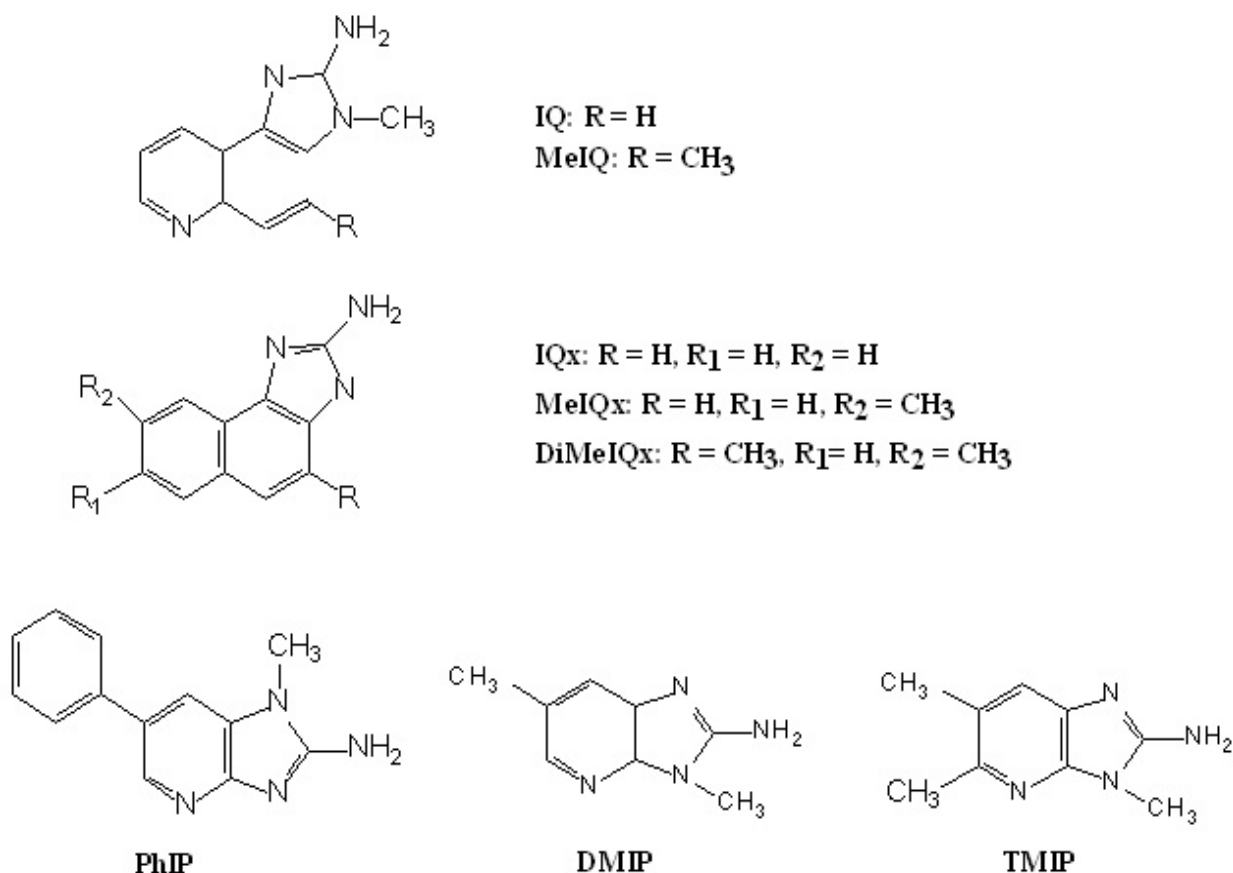


Figure 1.1: Chemical structure of polar heterocyclic amines (Adapted from Murkovic 2004).

In general, three main precursors are necessary to form polar HCAs: creatinine, amino acids, and carbohydrates found in muscle foods (Knize and Felton 2005); HCAs can form between 150 and 250°C (Murkovic 2004). The Maillard reaction occurs when food is cooked; the cooking produces free radicals that react with creatinine and enhance HCA formation (Pearson, Chen, Gray, and Aust 1992; Kikugawa 1999). Creatinine must be present for IQ and IQx- type HCAs to be produced (Murkovic 2007).

HCAs are often found in fried, grilled, baked, or roasted muscle meats and fish (Murkovic 2004); cooking processes like frying, grilling, barbequing most commonly produce more HCAs than other cooking methods (Cross and Sinha 2004). Puangsombat et al. (2012) reported that cooked meat products are more likely to form HCAs than well-done meat. Various physical and chemical properties promote HCA formation, including meat type, pH, water activity, amino acids, creatinine, and carbohydrates (Oz and Kaya 2011).

Extensive studies have suggested that quinolines and quinaxalines- type HCAs are produced via the Maillard reaction. Figure 1.2 illustrates how free radicals form along two different pathways in the Maillard reaction. The first pathway produces IQ-type HCAs when glycol-aldehyde alkylimine forms and is oxidized to produce glyoxal monoalkylimine, which can then be reduced to glyoxal. The molecules of glyoxal monoalkylimine condense, leading to production of pyridine radicals. The second pathway forms IQx-type HCAs through the biomolecular ring of glycol-aldehyde alkylimine to form dialkyl-dihydro pyrazine, and then after one electron loss, the dialkyl-pyrazine radical forms. The free radicals formed along either the pyridine or dialkyl-pyrazine pathways then react with the aldehyde and creatinine found in protein foods, which produces HCAs. Both the radicals and aldehyde in the reaction determine

which type of HCA forms (Pearson, Chen, Gray, and Aust 1992; Jägerstad, Skog, Arvidsson, and Solyakov 1998; Skog, Johansson, and Jägerstad 1998).

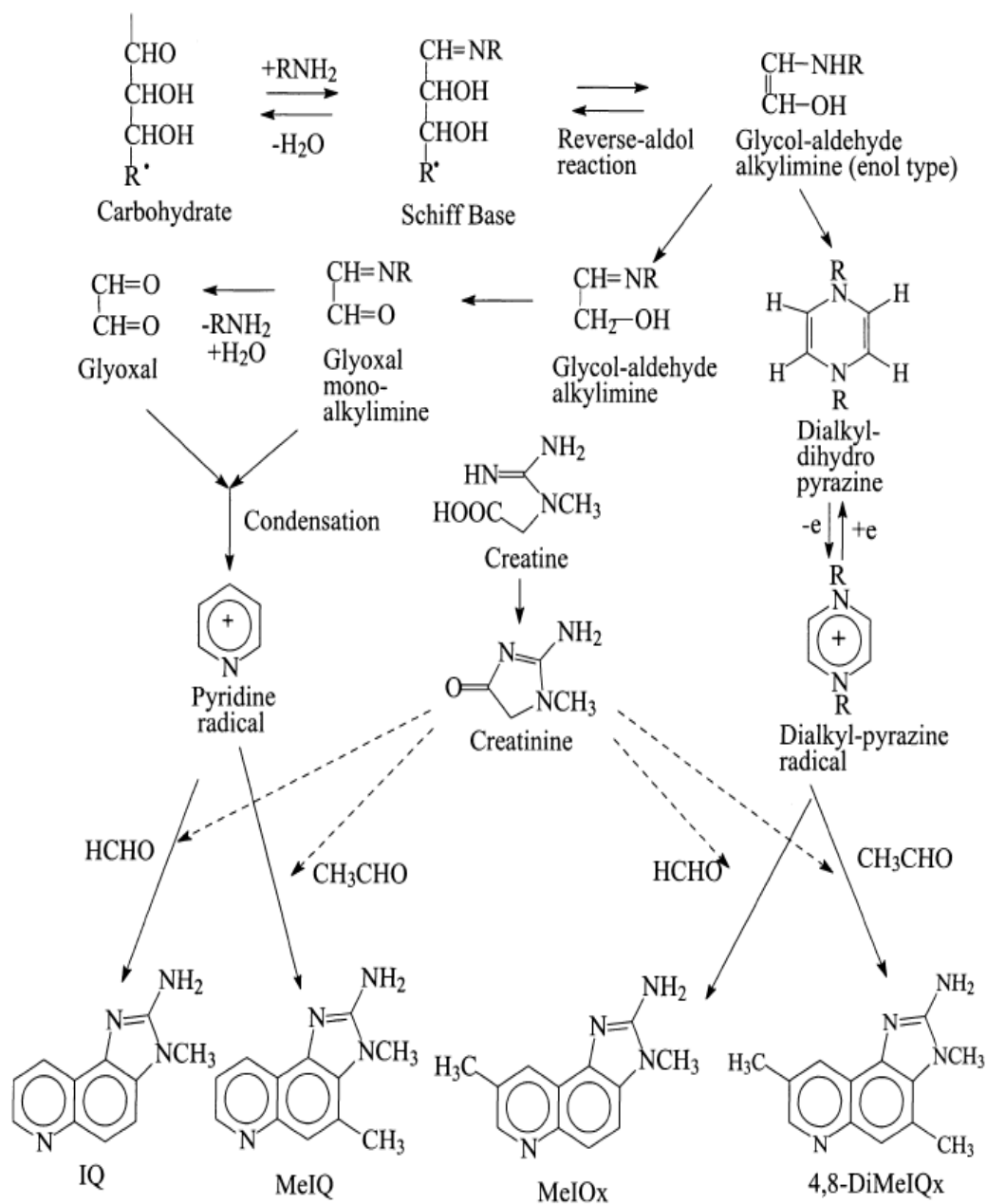


Figure 1.2: Formation of IQ-type and IQx-type heterocyclic amines involving pyridine and pyrazine intermediates (Adapted from Vitaglione and Fogliano 2004).

Without creatinine, IQ and IQx- type HCAs can't be produced. Dialkyl-pyrazine radicals form more quickly than pyridine radicals. This shows why more IQx-type HCAs are found in meat products than IQ-type HCAs (Murkovic 2004).

The heterocyclic aromatic amine PhIP (2-amino-1- methyl-6-phenylimidazo [4,5-b]pyridine) is one of the most abundant HCAs produced in cooked meat and fish during normal cooking (Zöchling, Murkovic, and Pfannhauser 2002); it is considered to be as a mutagenic compound (Sugimura 1997). Felton et al. (1986) isolated and identified PhIP in fried ground beef. The mechanism through which PhIP forms (Figure 1.3) is unlike the mechanism by which other HCAs form. Using model systems (Arvidsson, Van Boekel, Skog, and Jägerstad 1997) noticed that phenylalanine, creatinine, and glucose are the main precursors required for PhIP formation. PhIP formation begins with the formation of Strecker aldehyde phenylacetaldehyde, then the phenylacetaldehyde condenses to aldol and with creatinine produces PhIP as a final product (Murkovic 2004). Two explanations have been put forward on the origin of the nitrogen that forms the pyridine moiety in PhIP. The first is that nitrogen forms from the amino group when creatinine reacts with oxo groups of aldol intermediates. The second is that nitrogen originates from the amino group of phenylalanines (Murkovic 2004).

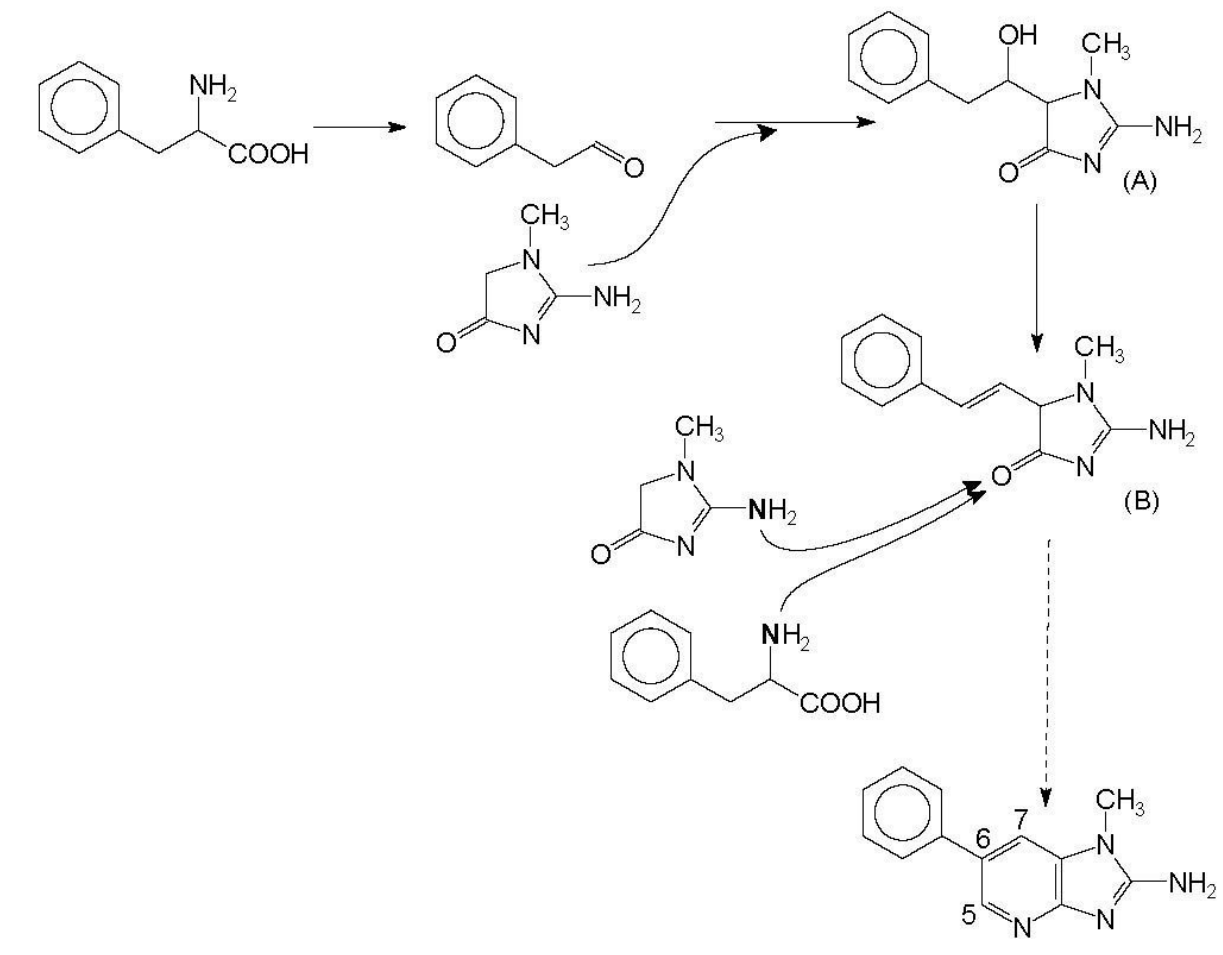
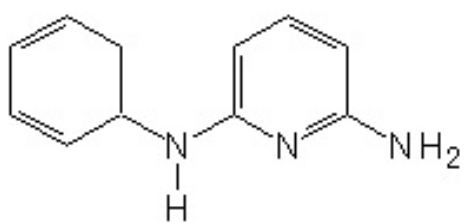


Figure 1.3: Formation of PhIP with identified intermediate reaction products (Adapted from Murkovic 2004).

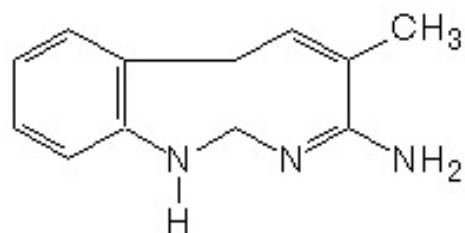
1.3.2 Non-polar heterocyclic amines

Non-polar heterocyclic amines are also called the amino-carbolines, have five-membered heterocyclic aromatic ring in two-six membered aromatic rings (Cheng et al. 2006) (Figure 1.4). The amino-carbolines include pyridoindoles, pyridoimidazoles, phenylpyridines, tetraazafluoranthrenes, and benzimidazoles. Non-polar HCAs can be further divided into an á-subgroup (e.g. AáC and MeAáC), â-subgroup (e.g., norharman, harman), ã-subgroup (e.g., Trp-P-1, Trp-P-2), and ä-subgroup (e.g., Glu-P-1, Glu-P-2).

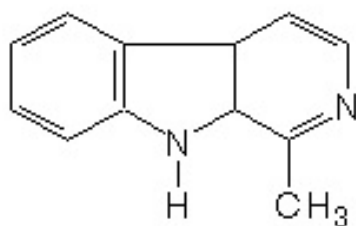
Non-polar HCAs are often called protein pyrolysates (Cheng et al. 2006), and are produced as pyrolysis products of amino acids via free radical reactions (Murkovic 2004; Murkovic 2007), generated at temperatures higher than 300°C (Cheng et al. 2006). However, harman, norharman, and Trp-P-1 HCAs form at lower temperatures (Arvidsson, Van Boekel, Skog, Solyakov, and Jägerstad 1999). Creatinine is not necessary to form non-polar HCAs (Murkovic 2004; Cheng et al. 2006). The amino acids and proteins included in this reaction are tryptophan, glutamic acid, lysine, phenylalanine, ornithine, creatine, or pyrolysed proteins (e.g., casein, albumin, gluten, or soybean globulin) (Jägerstad et al. 1998). Non-polar HCAs, including PhIP, do fluoresce very strongly in polar solvents (Murkovic 2007).



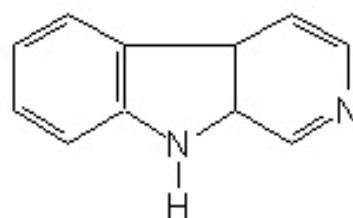
AaC



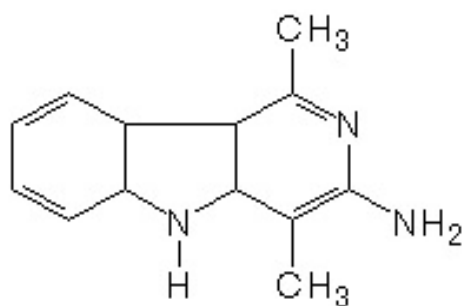
MeAaC



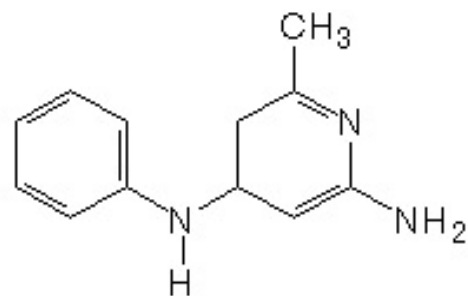
Harman



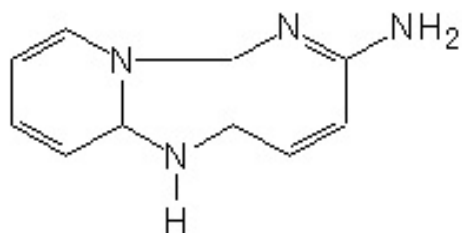
Norharman



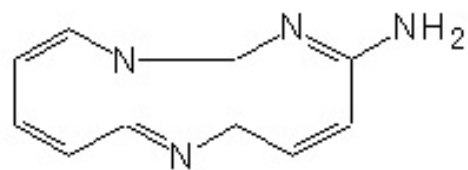
Trp-P-1



Trp-P-2



Glu-P-1



Glu-P-2

Figure 1.4: Chemical structure of non-polar heterocyclic amines (Adapted from Murkovic 2004).

1.4 Health Implications of Heterocyclic Amines

Higher intake of meat increases the risk of human cancer because eating more meat means more exposure to HCAs. Based on results from the Ames/ Salmonella test, HCAs are an important group of mutagens found in foods (Felton 2007). Animal studies show that HCAs can cause tumors in mammary glands, lung, colon, forestomach, and prostate (Ito et al. 1991; Shirai, Tamano, Sano, Masui, Hasegawa, and Ito 1995). Further studies have shown that HCA-DNA adducts could have a mutagenic effect in a variety of human tissues and organs including the breast, colorectum, and prostate, so human tissues may also be targeted by these carcinogens (Turesky and Vouros 2004; Malfatti et al. 2006; Zhu et al. 2006).

HCAs require metabolic activation to work as mutagens. They can form DNA adducts that may increase mutagenic activity (Sinha et al. 1999). Therefore, dietary intake and the level at which HCAs are metabolized increase the risk of cancer in humans (Cross and Sinha 2004). The most abundant HCAs formed in cooked meat are 2-amino-3,8-dimethylimidazo-[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethyl-imidazo[4,5-f]quinoxaline (4,8-DiMeIQx), and 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP), with PhIP being the most abundant (Zheng and Lee 2009). Previous studies estimated daily human exposure levels at approximately 250 to 300 ng per day, with levels of PhIP being the highest (~160 to 240 ng), followed by MeIQx (~70 to 90 ng) and 4,8-DiMeIQx (~4 to 6 ng) (Nowell et al. 2002; Li et al. 2007).

Many studies have detailed the correlation between consuming protein-rich foods and the development of various types of cancer. De Verdier et al. (1991) found a significant association among those who consumed well-done meat and colon, rectum, and colorectal cancers. Similarly, a case study from Uruguay found a strong association between meat consumption and those same cancers (DeStefani, DeneoPellegrini, Mendilaharsu, and Ronco 1997).

Many epidemiological studies have evaluated the association between HCAs genotoxicity and the incidence of cancers in humans. Over the past two decades, epidemiological studies have indicated that high exposure to well-done meat may significantly increase the risk of cancers in humans (Zheng and Lee 2009). Table 1.3 provides a list of epidemiological studies found a direct link between the exposure to HCAs and cancer risk. Among the HCAs known to date, PhIP was most often directly linked to cancer in a dose-response relationship (Zheng and Lee 2009).

Table 1.3: Summary of epidemiological studies evaluating the association of HCA exposure with cancer risk from 1996 to 2008 (Zheng and Lee 2009).

Exposure	Studies evaluated	Studies reporting positive associations (%)
Mutagenicity or total HCAs	10	7 (70.0%)
PhIP	13	8 (61.5%)
MeIQx	12	6 (50.0%)
DiMeIQx	11	6 (54.5%)

Several epidemiological studies have assessed the associations among eating well-done meat, HCA exposure, and colorectal cancer. Nowell et al. (2002) found a positive association between intake of well-done meat and specific HCAs, including MeIQx, DiMeIQx, and PhIP. This association was statistically significant for MeIQx. In another study (Butler et al. 2003) the risk of the association was strongest with DiMeIQx. For breast cancer, DeStefani et al. (1997) and Delfino et al. (2000) proposed that well-done meat intake and HCA exposure can significantly increase the risk of breast cancer incidence. A direct link has been observed between exposure to PhIP and the risk of breast cancer, but not for MeIQx and DiMeIQx (Zheng et al. 2002). Also, the link between the incidence of breast cancer and eating well-done meat may

be weakened by the genetic ability in some humans to metabolize enzymes that activate or deactivate HCAs (Zheng et al. 2002). Other studies have noted an association between well-done meat and the risk of prostate and pancreatic cancers. In a cohort study carried out in the U.S., Koutros et al. (2008) found that well-done meat intake increased the risk of prostate cancer. A study conducted in Minnesota involved 193 cases and 674 controls, found that eating grilled or barbequed red meat increases the risk of pancreatic cancer incidence (Anderson et al. 2002). Stolzenberg-Solomon et al. (2007) mentioned that high intakes of red meat, meat cooked at high temperatures, well-done meat, and very well-done meat were strongly associated with risk of pancreatic cancer in men but not in women.

The National Toxicology Program has categorized IQ, MeIQ, MeIQx, and PhIP as likely carcinogens in humans (U.S Department of Health and Human Services 2011). The International Agency for Research on Cancer (1993) agreed, classifying “IQ as probably carcinogenic to humans (Group 2A) and MeIQ, MeIQx, and PhIP as possibly carcinogenic to humans (Group 2B)”. Thus, high exposure to these genotoxic compounds can increase the incidence of cancer (Cheng, Chen, and Wang 2006). A better understanding of how the body reacts to HCAs may clarify why these compounds are mutagenic and potentially carcinogenic to humans.

1.5 Factors Affecting Heterocyclic Amine Formation

Before we can begin discussing the body’s reactions to HCAs, we first must detail what causes HCAs to form in the first place. Cooking time and temperature are important, as is the cooking method. Also the concentrations of precursors and lipids have an effect on which HCAs form.

1.5.1 Cooking time and temperature

Knize and Felton (2005) reported that both cooking time and temperature can influence formation of HCAs. In general, as the temperature and cooking time increased, more HCAs are generated (Skog, Augustsson, Steineck, Stenberg, and Jägerstad 1997; Johansson 1997). In a model system that includes glucose, creatinine, and phenylalanine as the main precursors, PhIP formation increased when the temperature rose from 180 to 225 °C (Skog and Jägerstad 1991), but HCAs didn't form in any significant amount when the cooking temperature was low (Johansson 1997). It is also reported that if the cooking temperature increases from 190 to 230 °C, HCA formation increases dramatically (Abdulkarim and Smith 1998). HCAs can, however, form at lower temperatures (100 °C) if given enough time (Jägerstad, Skog, Arvidsson, and Solyakov 1998). For instance, harman and norharman were noticeable at 100 °C after 150 min of cooking (Manabe, Kurihara, Wada, Tohyama, and Aramaki 1992). Bordas, Moyano, Puignou, and Galceran (2004) investigated the effect of different cooking times on HCA formation, and the results showed that as cooking time increased, HCA amounts increased, especially for PhIP, which increased from 9.3 ng/g to 19.5 ng/g. Figure 1.5 shows the relationship between different cooking temperatures and levels of total HCAs (ng/n) in fried beef. In Knize et al. (2005), the patties were heated until they reached the internal temperature of 70 °C. Knize and Felton (2005) found that in fried beef patties cooked at 140 °C, the amount of HCAs formed was very low

although increasing the cooking temperature to 250°C increased the amount of HCAs.

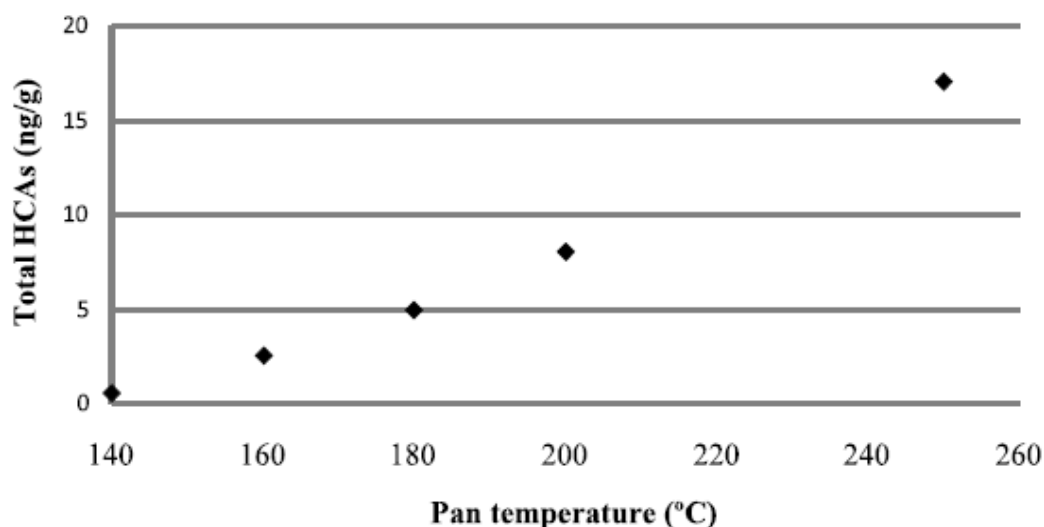


Figure 1.5: Formation of heterocyclic amines in beef patties after cooking to an internal temperature of 70°C at different frying pan temperatures (Adapted from Knize et al. 2005).

Another study assessed the effects of cooking time and temperature on HCA formation. The results showed that HCAs reached a maximum after 5 to 10 min of cooking. However, at 225°C, some HCAs (e.g., MeIQx and 7, 8-DiMeIQx) degraded, following a peak concentration. PhIP was not detected at 225°C (Arvidsson, Van Boekel, Skog, and Jägerstad 1997) (Figure 1.6). These results are consistent with results from other studies of a model system (Jackson and Hargraves 1995; Bordas, Moyano, Puignou, and Galceran 2004). Chiu and Chen (2000) demonstrated that when cooking time and temperature increased, HCAs are more susceptible to degradation. PhIP is less stable than IQx. Their findings are similar to previous studies, where PhIP was more likely to be destroyed at 225°C than 7,8-DiMeIQx, 4,8-DiMeIQx, and IQx (Arvidsson, Van Boekel, Skog, and Jägerstad 1997).

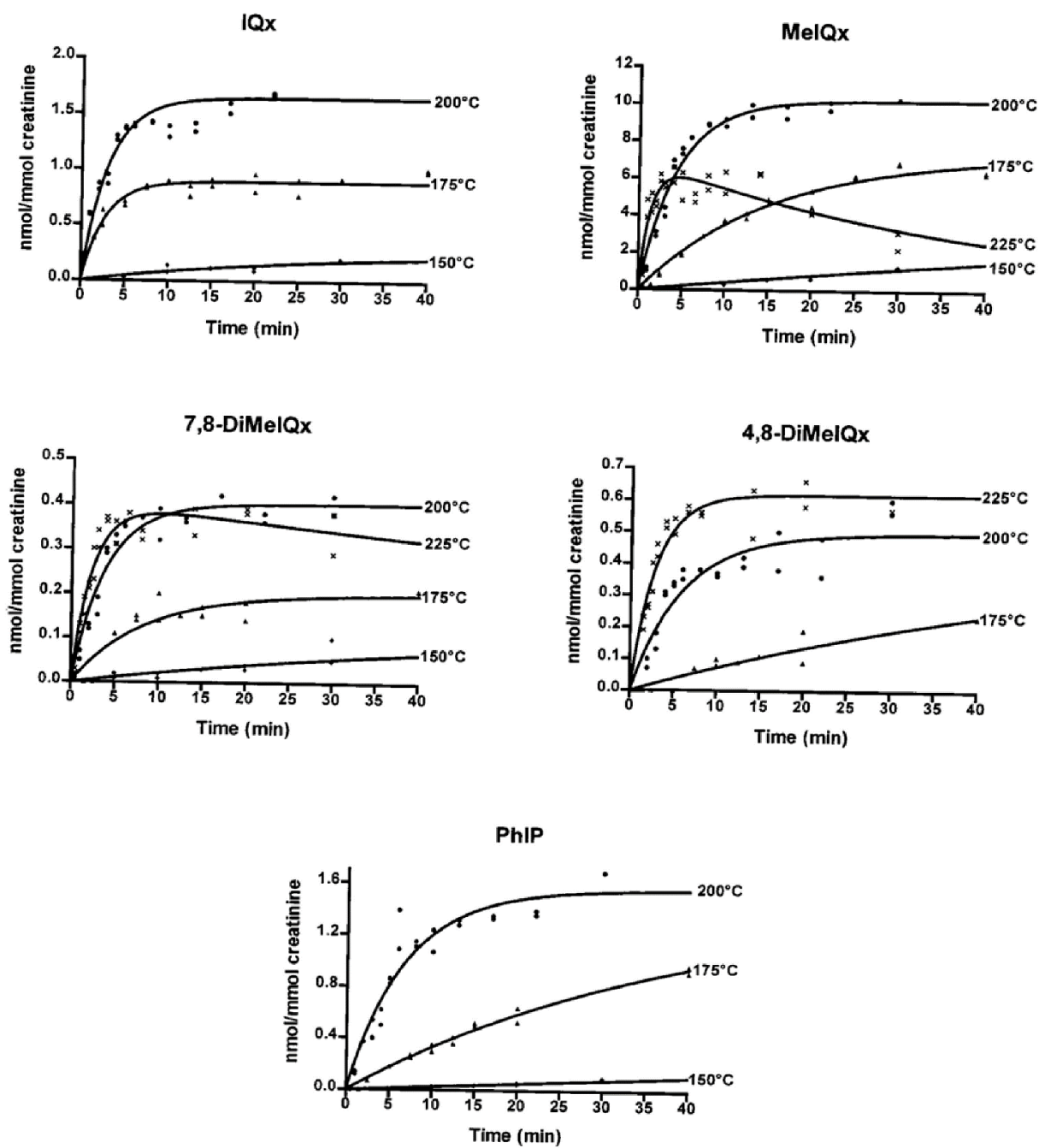


Figure 1.6: Formation of HCAs at 150, 175, 200, and 225°C (Adapted from Arvidsson et al. 1997).

1.5.2 Cooking methods

Cooking method also affects HCA formation and lead to heat transfer and water loss (Skog, Steineck, Augustsson, and Jägerstad 1995). Of the many high-temperature cooking methods, pan-frying, grilling, and broiling transfer temperature through radiative and conductive processes. These methods cause the highest concentrations of HCAs (Chen and Chiu 1998). Other cooking methods (oven-roasting and baking) produce low or intermediate levels of HCAs (Skog, Eneroth, and Svanberg 2003). Boiling and steaming normally occur below 100°C (212°F), so for these cooking methods, only low or undetectable levels of HCAs occur (Chen and Chiu 1998).

Comparing different methods of cooking meat patties to the same internal temperature of 88°C shows HCAs levels in pan-fried pork patties with 17.2 ng/g HCAs, oven-broiled pork patties with 5.0 ng/g, and boiled pork patties with 1.0 ng/g (Shin 2005). Liao, Wang, Xu, and Zhou (2010) compared chicken breasts cooked in different ways at a temperature of 180°C, finding that HCAs in charcoal-grilled chicken (35.77 ng/g) were significantly higher than pan-fried chicken (22.97 ng/g) and deep-fried chicken (3.31 ng/g). Oz, Kaban, and Kaya (2010) pointed out that the total HCA level in rainbow trout when cooked by microwave for 9 min (8.19 ng/g) was higher than barbecuing for 9 min (5.22 ng/g), panfrying at 200°C for 15 min (3.77 ng/g), cooking on a hot-plate at 200°C for 15 min (1.59 ng/g), and cooking in the oven at 200°C for 15 min (1.44 ng/g).

Microwave pretreatment can reduce HCA levels in meat products. Felton, Fultz, Dolbeare, and Knize (1994) investigated using microwave pretreatment to reduce HCA formation in fried beef patties. Beef patties that were pretreated in a microwave for 2 minutes before being fried at 250°C for 6 minutes per side showed decreased HCA levels compared to

untreated beef patties. Release of water-soluble precursors of HCAs (creatine, amino acid, and sugar) through microwave pretreatment reduces HCA generation (Felton, Fultz, Dolbeare, and Knize 1994).

1.5.3 Concentration of precursors

As we mentioned before the main precursors of HCAs in food are sugar, creatine, and amino acids. It has been documented that addition of reducing sugars (e.g., glucose, fructose, and lactose) above a certain concentration range might reduce HCA levels in beef patties. For instance, when reducing sugar (2.5-fold increase in glucose) was added to a dry lyophilized meat extract at 200°C for a total of 30 minutes, HCA production did not decrease, but when glucose was increased 5-fold in a model system, IQ was inhibited and the amounts of MeIQx, DiMeIQx, and PhIP decreased, indicating that glucose added at high concentrations inhibited mutagenic HCA production (Skog, Johansson, and Jägerstad 1998; Skog, Solyakov, and Jägerstad 2000). Tai, Lee, and Chen (2001) investigated the effect of added reducing sugar to fried fish fiber at different concentrations and found that HCA production increased to 14% when reducing sugars were added at 9% concentrations; however, HCA production decreased as the concentrations of reducing sugars increased, by 43% with a concentration of glucose at 19%.

When a model system was used to investigate HCA formation by heating creatinine, glucose and amino acids in proportions similar to what is found in bovine meat, after 15 minutes, the retention of creatinine and amino acids was more than 20% whereas glucose was eliminated after only 2.5 minutes (see Figure 1.7). This indicates that glucose was the limiting precursor. However, adding glucose to the system did not increase HCA production (Arvidsson, Van Boekel, Skog, and Jägerstad 1997).

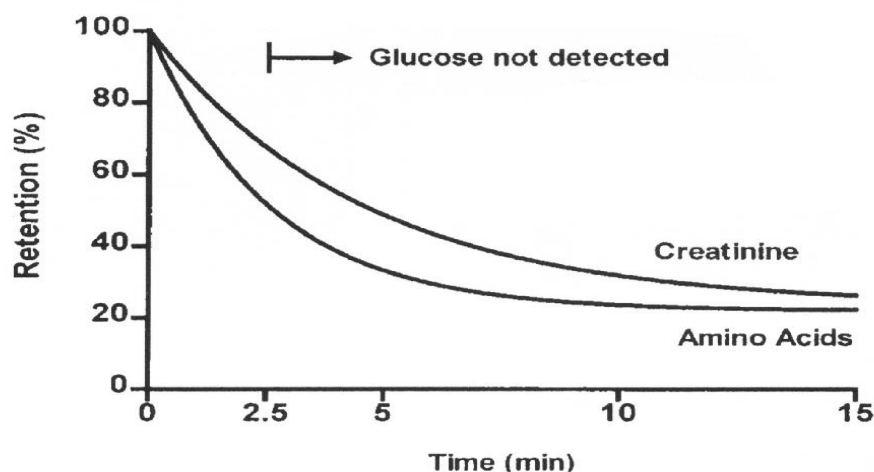


Figure 1.7: Decay of precursors (creatine, amino acids, and glucose) through heating at 200°C for 15 min (Adapted from Arvidsson, Van Boekel, Skog, and Jägerstad 1997).

Skog and Jägerstad (1990) had one explanation for why sugars inhibit HCA formation at high concentrations. Glucose reacts with creatine. These reactions resulted in a decrease in HCA formation. Previous studies show that carbohydrates dehydrate in Maillard reactions, producing other products. Some of these reactions can significantly inhibit the formation of HCAs (Persson, Sjöholm, Nyman, and Skog 2004). Understanding how these reactions affect HCA formation can help identify strategies for reducing the potential health risks of HCAs in foods eaten daily.

Bordas, Moyano, Puignou, and Galceran (2004) demonstrated that adding a 5-fold increase in creatinine led to the formation of IQ, MeIQx, and PhIP. However, increasing creatinine up to 25 fold resulted in an increase in the concentration of PhIP but a decrease in IQ and IQx. Skog, Johansson, and Jägerstad (1998) investigated the results of adding 50 times the amounts of glycine, alanine, and phenylalanine to lyophilized meat extract, showing that HCA (MeIQx and PhIP) formation increased. Amino acids, including phenylalanine, leucine, isoleucine, and tyrosine were found to be precursors in generating PhIP (Pais, Salmon, Knize, and Felton 1999).

1.5.4 Lipid

Despite the important role of lipid content in mutagenic activity, its exact effects of lipid are not well understood. Some studies noticed enhancing effects (Felton, Jägerstad, Knize, Skog, and Wakabayashi 2000); others found no effects (Johansson, Fredholm, Bjerne, and Jägerstad 1995) or even inhibition of HCA formation (Knize 1985). Hwang and Ngadi (2003) found that diluting precursors in meat can increase the effect of lipids on inhibition of HCAs. On the other hand, lipids, as an effective heat transfer agent, can enhance HCA formation (Hwang and Ngadi 2002). In a model system, lipid accelerates the formation of free radicals via oxidation, which effectively enhances the formation of HCAs (Felton, Jägerstad, Knize, Skog, and Wakabayashi 2000). Adding Fe^{2+} or Fe^{3+} to the model system, including glucose, creatine, and glycine, led to increasing the amount of MeIQx and PhIP not being detected (Skog, Solyakov, and Jägerstad 2000), probably due to iron accelerate lipid peroxidation, and thus, the formation of free radicals (Felton, Jägerstad, Knize, Skog, and Wakabayashi 2000). Lipid content and its effects must still be studied because they both increase and decrease HCA formation.

1.6 Inhibiting Formation of Heterocyclic Amines

As mentioned before, consuming meat high in HCAs may increase the incidence of various cancer in human. Thus, finding ways to minimize the potential risk of HCAs is important.

1.6.1 Modifying cooking methods

Because time and temperature are significant in HCA formation and as time and temperature increase, HCAs generally increase, to minimize mutagenic activity, cooking at a lower temperature and avoiding prolonged cooking times will reduce formation of heterocyclic amines in food (Skog, Eneroth, and Svanberg 2003). Knize and Felton (2005) have also noted

that flipping foods every minute during cooking until the proper internal temperature is reached can also reduce HCAs in food.

Microwave pretreatment is another way to reduce HCAs in meat products. Felton, Fultz, Dolbeare, and Knize (1994) conducted a study to assess how microwave pretreatment affected HCAs in fried beef patties, finding that microwave pretreatment did reduce HCAs in meat. Three minutes of microwave pretreatment of beef patties before frying the patties resulted in 3- to 9-fold decrease in HCAs, including MeIQx, IQ, DiMeIQx, and PhIP. The reduction of HCA levels was probably due to releasing water-soluble precursors of HCAs (creatine, sugar, and amino acids) by approximately 30% during microwaving.

1.6.2 Improving water-holding capacity

Water is a significant part of the transport of water-soluble precursors of HCAs to the surface of meat during cooking. Adding water-binding substances (soy protein, starch, and polysaccharides) to the meat can help reduce surface temperature and prevent transport of water-soluble precursors, leading to a decrease in HCAs in cooked meat (Kikugawa 2004). Persson, Graziani, Ferracane, Fogliano, and Skog (2003) found adding a solution of 1.5% sodium chloride and 0.3% sodium tripolyphosphate to beef burgers had a significant effect on HCAs in cooked meat as well. Using such way to increase water-holding capacity in beef patties decreases HCAs significantly.

1.6.3 Marination

Marinating meats before cooking can tenderize the meat, enhance flavor, and increase the water-binding capacity of meats, therefore juiciness of the meat will increase (Baczwski and Mandigo 2003; Alvarado and McKee 2007). A variety of ingredients can be used to marinate foods at home: spices, vinegar, lemon juice, wine, soy sauce, salt, and sugar. People also often

buy marinade in ready-to-use forms like powders or liquid marinades that require little home preparation (Yusop, O'Sullivan, and Kerry 2010). Many commercial liquid-based marinades have water, oil, phosphate, salt, spices, functional additives (e.g., xanthan gum or guar gum), antimicrobial agents (e.g., sorbate or benzoate), and organic acids (e.g., acetic, lactic, or citric acid) (Miller 1998).

Many studies have reported how marinades influence HCA formation in meat products. Smith, Ameri, and Gadgil (2008) evaluated the effects of three different commercially available marinades, Caribbean, Southwest, and herb, on HCA content in beef steak. The results showed the Caribbean mixture significantly reduced HCA content (88%), followed by the herb (72%), and Southwest (57%). Gibis (2007) also investigated marinades that used garlic, onion, and lemon on HCA formation in fried beef patties. The optimum amount of onion, garlic, and lemon juice reduced HCAs by 31%, 29%, and 15%. The findings showed garlic had the most significant effect in reducing MeIQx and PhIP formation. More recently, Quelhas et al. (2010) found that marinating beef in green tea reduced PhIP levels by 74% in pan-fried beef although MeIQx and DiMeIQx were not reduced. Busquets, Puignou, Galceran, and Skog (2006) investigated the effects of red wine marinades on fried chicken breast. The results show that PhIP levels were reduced by 88%. Nerurkar, Marchand, and Cooney (1999) found that commercial teriyaki sauce and turmeric-garlic sauce significantly reduced PhIP and MeIQx levels in barbecued beef steaks, but marinating with barbecue sauce elevated PhIP and MeIQx levels up to 4-fold. The authors explained the increases in HCA levels were due to the high fructose corn syrup and honey in the sauce.

1.6.4 Adding Antioxidants

Previous studies have noted that antioxidants scavenge free radicals at various points along the HCA pathways, thus preventing HCA formation (Figure 1.8). Vijayan and Thampuran (2000) classified antioxidants as a group of compounds that prevent lipid oxidation and scavenge free radicals. Strong evidence suggests that various synthetic and natural antioxidants do scavenge pyridine and dialkyl-free radicals along the IQ and IQx-type pathways, thus inhibiting IQ and IQx moieties (Vitaglione and Fogliano 2004).

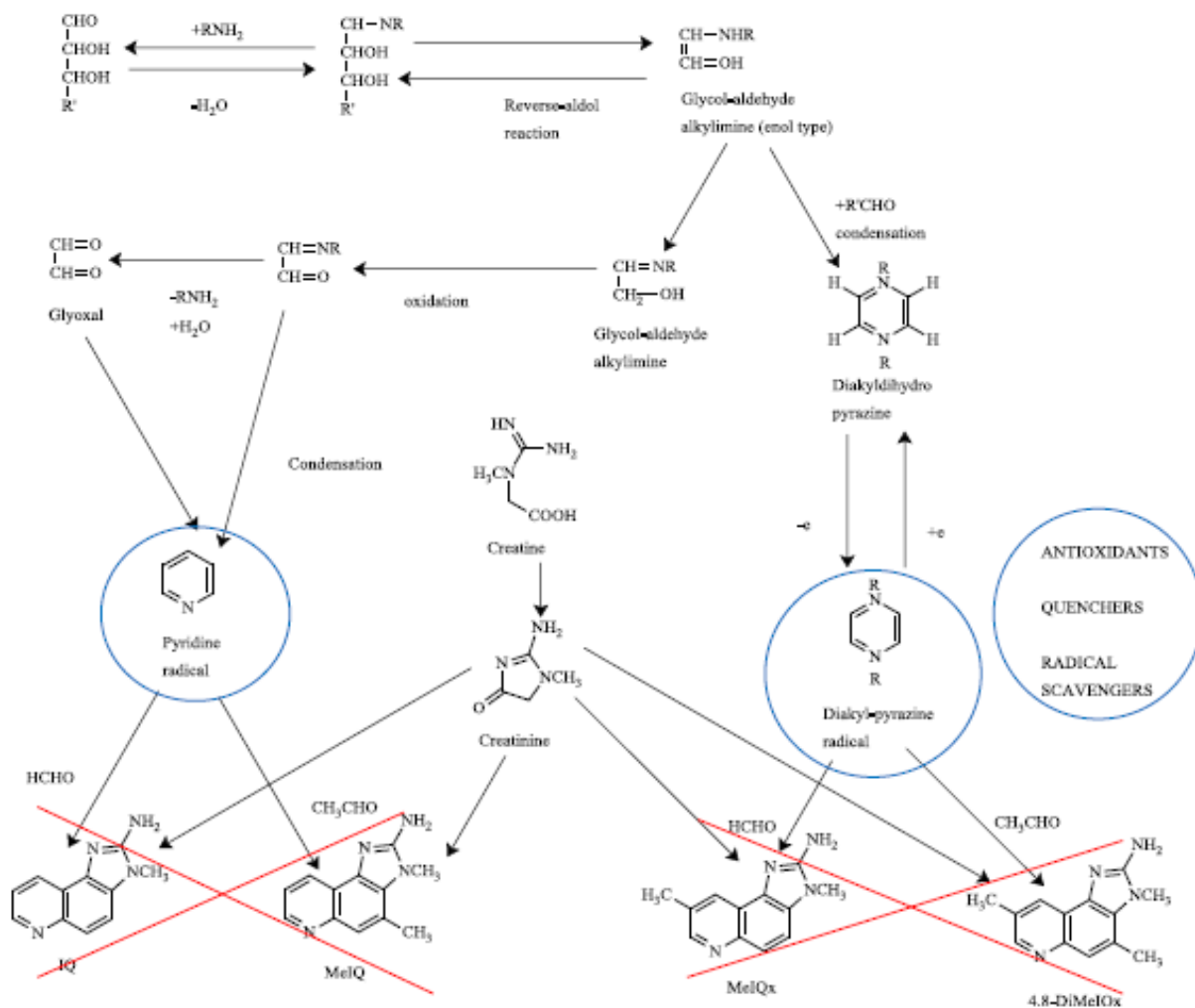


Figure 1.8: Pathway of HCA formation and suggested points where antioxidants impede reactions (Adapted from Vitaglione and Fogliano 2004).

1.6.4.1 Synthetic Antioxidants

Various synthetic antioxidants appear to limit mutagenic activity, including butylated hydroxyanisole (BHA), propyl gallate (PG), tert-butylhydroquinone (TBHQ), and 1-Ohexyl-2, 3, 5-trimethylhydroquinone (HTHQ) (Vitaglione and Fogliano 2004). Moon and Shin (2013) noted significantly reduced PhIP levels (63% -99%) at relatively high doses of BHA (1000 ppm). However, Kikugawa (1999) investigated BHA in a model system and showed that as the concentration of BHA increased, that effect was reduced. Evaluating the effects of synthetic antioxidant agents such as BHA, BHT, and PG in a model system, showed that all these antioxidants increased the formation of MeIQx (Johansson and Jägerstad 1996). In contrast, Oguri, Suda, Totsuka, Sugimura, and Wakabayashi (1998) noted BHA had no significant effects on MeIQx and PhIP formation in a model system. In terms of toxicological safety, synthetic antioxidants may have carcinogenic effects, so consumers are increasingly interested in natural antioxidants in the meat and poultry industry (Karre, Lopez, and Getty 2013).

1.6.4.2 Natural Antioxidants

Several studies have been reported that natural antioxidants, such as phenolic, polyphenol, and flavonoids from plants such as tea, herbs, and spices, can inhibit mutagen formation (Vitaglione and Fogliano 2004).

1.6.4.3 Tea Polyphenols

Tea polyphenols can scavenge a large number of free radicals, which reduces HCAs in foods. For instance, black and green tea polyphenols (theaflavin gallate (TFG) and epigallocatechin gallate (EGCG) in a model system significantly reduced MeIQx and PhIP levels (Weisburger, Nagao, Wakabayashi, and Oguri 1994). Apostolides, Balentine, Harbowy, Hara, and Weisburger (1997) had similar results, showing that black and green teas and their

polyphenols significantly inhibited PhIP formation in a model system. Tea polyphenols appear to lower HCA formation when applied to the meat surface before frying (Weisburger, Veliath, Larios, Pittman, Zang, and Hara 2002). In a study investigating the effect of theaflavin 3, 3'-digallate and epigallocatechin (EGC) on mutagenic HCAs in fried beef patties before frying, Cheng, Chen, and Wang (2007) found that theaflavin 3, 3'-digallate inhibited PhIP, MeIQx, and 4, 8-DiMeIQx by close to 50%, but EGC had less effect on PhIP while the effects on MeIQx and DiMeIQx remained much the same. In a similar experiment, Zhang, Yu, Mei, and Wang (2013) tested the effect of tea polyphenols on fried pork, finding that they suppressed the formation of PhIP, MeIQx, and DiMeIQx. Tea polyphenols seem to have the greatest effect on PhIP formation.

1.6.4.4 Natural Flavonoids

Flavonoids are a group of polyphenolic compounds widely found in plants (Kumar and Pandey 2013). Several studies have reported that flavonoids are anti-mutagenic, anti-carcinogenic, anti-inflammatory, and antiviral (Kampa, Nifli, Notas, and Castanas 2007). Structural class is not the only identifying feature; degree of hydroxylation, substitution and degree of polymerization can also determine the chemical nature of flavonoids (Heim, Tagliaferro, and Bobilya 2002). The potential health benefits of flavonoid compounds arise because of their ability to prevent oxidative stress. One possible mechanism to explain how flavonoids can work as antioxidants is their ability to scavenge radicals and their direct reactions with enzyme functions (Mishra, Kumar, and Pandey 2013; Halliwell and Gutteridge 2015). In general, flavonoids have a fifteen-carbon skeleton in two benzene rings (Figure 1.9 A and B) that connect to each other through a heterocyclic pyrene ring (Figure 1.9 C) (Kumar and Pandey 2013).

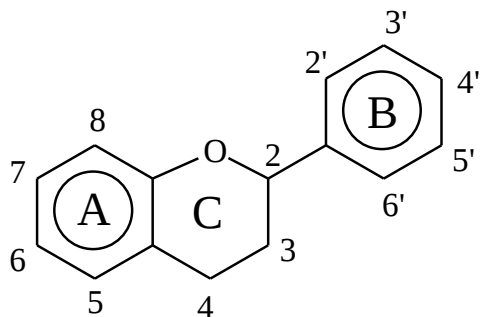


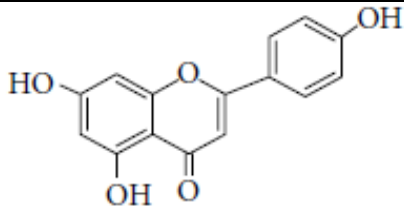
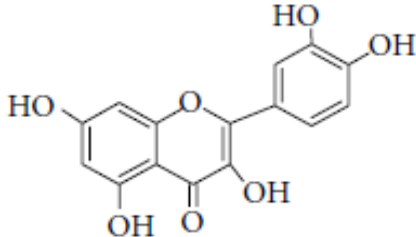
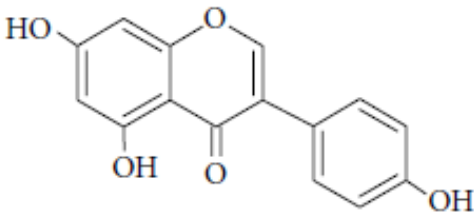
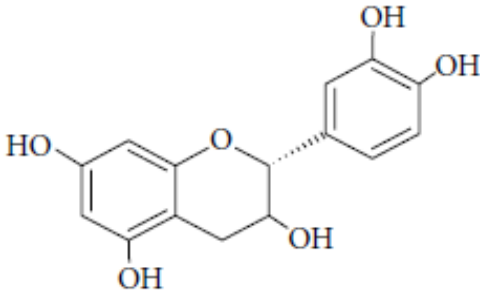
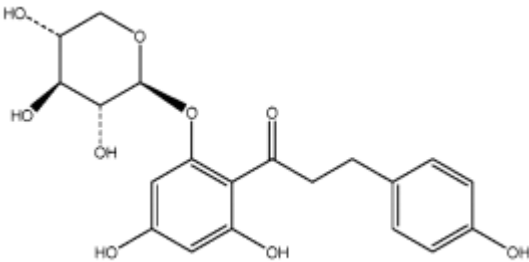
Figure 1.9: Basic flavonoid structure (Adapted from Kumar and Pandey 2013).

Flavonoids can be classified using their chemical structures: flavanol, flavone, flavonol, flavanone, isoflavone, and anthocyanidin (Kumar and Pandey 2013). Table 1.4 provides the classification, structure, and dietary sources of some flavonoids. Many studies have proved that diets rich in flavonoids are associated with reduced risk of cancer. For instance, one study conducted among 34,708 post-menopausal women investigated the effect of flavonoid consumption on the incidence of different types of lung, colon, and breast cancers. The results showed that regular flavonoid consumption protected against lung cancer. However, flavonoids had no protective effect against other cancers (Cutler et al. 2008). A meta-analysis of 12 studies conducted among post-menopausal women investigated the effect of flavonoid consumption on breast cancer. The results showed that participants who consumed large amounts of flavonols and flavones significantly reduced their risk of breast cancer (Hui, Qi, Qianrong, Xiaoli, Jundong, and Mantian 2013). A number of flavonoids such as quercetin, apigenin, genistin, phlorizin, and catechin proved to have antioxidant activity. Table 1.5 shows the chemical structure of flavonoid compounds considered in the study.

Table 1.4: Classification, structure, and food sources of some dietary flavonoids (Kumar and Pandey 2013).

Class	Flavonoid	Dietary source
Flavanol	(+)-Catechin, (–)-Epicatechin, and Epigallocatechin	Tea
Flavone	Chrysin, apigenin, Rutin, luteolin, and luteolin glucosides	Fruit skins, red wine, buckwheat, red pepper, and tomato skin
Flavonol	Kaempferol, quercetin, myricetin, and tamarixetin	Onion, red wine, olive oil, berries, and grapefruit.
Flavanone	Naringin, naringenin, taxifolin, and hesperidin	Citrus fruits, grapefruits, lemons, and oranges
Isoflavone	Genistin and daidzin	Soyabean
Anthocyanidin	Apigenidin and cyaniding	Cherry, easberry, and strawberry

Table 1.5: The chemical structure of the 5 flavonoids considered in this study (Adapted from Cheng et al. 2007; Kumar and Pandey 2013).

Flavonoids	Structure
Apigenin	
Quercetin	
Genistein	
Catechin	
Phloridzin	

1.6.4.5 Herbs and spices

Many herbs and spices also inhibit HCA formation. Some families of herbs and spices used most commonly used to inhibit HCA formation are Lamiaceae (basil, mint, sage, rosemary, oregano, thyme, and lavender), Apiaceae (anise, caraway, parsley, coriander, cumin, dill, and fennel), Piperaceae (the pepper family), Lauraceae (sassafras, cinnamon, and evergreen trees), and Myristicaceae (the nutmeg family). Table 1.6 shows the TPC and DPPH results from multiple studies for some common spices and herbs used in the food industry.

Table 1.6: Total phenolic content (TPC) and DPPH results for a variety of herbs and spices. Adapted from ¹Damašius et al. (2011); ²Hinneberg et al. (2006); ³Su et al. (2007); ⁴Wang et al. (2008); ⁵Wojdylo et al. (2007).

Herb/Spice	TPC (mg GAE/g)	DPPH (%)
Basil	147 ± 1.60 ³	19.82 ± 0.002 ⁴
Black Pepper	1.32 ± 0.00 ²	5.13 ± 0.011 ⁴
Cinnamon	18.56 ± 0.31 ³	96.74 ± 0.004 ⁴
Nutmeg	2.26 ± 0.01 ³	78.69 ± 0.043 ⁴
Oregano	136 ± 0.82 ¹	79.6 ± 2.04 ⁵
Parsley	29.2 ± 0.44 ²	39.9 ± 1.34 ⁵
Rosemary	142 ± 3.58 ¹	10.28 ± 0.006 ⁴
Sage	1.6 ± 0.094 ⁴	8.70 ± 0.009 ⁴
Thyme	77.8 ± 1.71 ¹	92.29 ± 0.002 ⁴

In a study testing the antioxidants activity of three Lamiaceae spices, including oregano, peppermint and lemon balm, Capecka, Mareczek, and Leja (2005) observed that fresh and dried oregano had more total antioxidant activity than peppermint and lemon balm. The researchers also tested the total phenolics, finding that dried peppermint had the highest (2580 mg 100/g

fresh matter), followed by fresh lemon balm (2253 mg 100/g fresh matter) and dried oregano (2221 mg 100/g fresh matter).

Previous studies have investigated the spices in Lamiaceae family for their ability to inhibit HCA formation in meat products. Damašius, Venskutonis, Ferracane, and Fogliano (2011) found that oregano extract inhibited PhIP formation the most. Thyme also inhibited PhIP formation but not as much as oregano. Marjoram and rosemary had little effect on PhIP formation, and basil could also inhibit PhIP formation in meat.

1.6.4.5.1 Black pepper

Black pepper is the dried, mature fruit of the *Piper nigrum* L. plant, belonging to the family Piperaceae. It originated in Brazil, India, Indonesia, Malaysia, Sri Lanka, and Thailand and represents about 35% of the world trade in spices (Zachariah and Parthasarathy 2008). It has been reported that black pepper has the ability to improve blood circulation, increases the flow of saliva, and stimulates the appetite (Pruthi 1993).

Black pepper has two main groups of compounds: pungent compounds (e.g., piperine, piperanine, chavicine, piperettine, piperylin, and piperolein A and B) and compounds responsible for the flavor and aroma of black pepper (Zachariah and Parthasarathy 2008). The volatile essential oil of black pepper includes compounds responsible for the flavor and aroma of black pepper. Black pepper has about 2 to 2.6% volatile oils and 6 to 13% oleoresin (Zachariah and Parthasarathy 2008). One major bioactive compound of black pepper is piperine (Singletary 2010). The pungency of black pepper is due to piperine and the volatile oil (Zachariah and Parthasarathy 2008). Black pepper has approximately 3.5% piperine, but levels increase as pepper matures and among different cultivars (Zachariah and Parthasarathy 2008). In recent studies, researchers have found that piperine has chemopreventive and antioxidants abilities. It

also has immunomodulatory, anti-carcinogenic, hepatoprotective, anti-inflammatory (Darshan and Doreswamy 2004), antimicrobial (Yang et al. 2002), and antiulcer effects (Bai and Xu 2000). Further studies have shown that piperine is bio-transformative and can enhance the bioavailability of drugs such as rifampicin, sulfadiazine, tetracycline, and phenytoin either through increasing their absorption or slowing down how quickly a patient metabolizes the drug (Wu 2007). Strong evidence suggests that piperine can reduce blood cholesterol, triglycerides, and glucose (Mueller and Hingst 2013). Consuming piperine orally can significantly enhance the digestive enzymes of pancreas and intestines in addition to increasing secretion of bile acid (Ulbricht, Chao, Costa, Rusie-Seamon, Weissner, and Woods 2008). Despite the beneficial health effects of piperine, it is not very water soluble. Therefore, using piperine at high concentrations can be toxic to the central nervous and reproductive systems (Veerareddy, Vobalaboina, Nahid 2004; Pachauri, Gupta, and Ghosh 2015).

Singletary (2010) reported that black pepper has antioxidants and can scavenge free radicals. Tipsrisukond, Fernando, and Clarke (1998) postulated that piperine had more antioxidants properties than other herbs and spices. These researchers also demonstrated that ground black pepper significantly inhibited HCA formation, better than black pepper essential oils or oleoresins.

Other volatile compounds in black pepper that could inhibit PhIP formation are D-limonene and P-cymene. D-limonene is a colorless liquid obtained from the oils of oranges, grapefruit, and lemon (Vergheze 1968). D-limonene is heavily used in food industry as flavor additives in soap, food, and beverages. It is used also in non-alcoholic beverages, ice cream and ices, sweets, baked goods, puddings, and chewing gum (Sun 2007). D-limonene has no toxicity in human. Previous studies of D-limonene in mice and rats showed that D-limonene induces

renal tumor in male rats while insignificant effect observed in female rats and both genders of mice. Subsequently, human studies found that D-limonene has a significant protective effect against mutagenic and carcinogenic activity in humans (Sun 2007). Medicinally, it has found a wonderful ability to help with different health issues including dissolve cholesterol-containing gallstones, relieves acid reflux, and improving digestion (Sun 2007). It has been stated that D-limonene has a significant effect against various types of cancers such as breast and colorectal cancers (Vigushin et al. 1998). D-limonene, however, can convert into p-cymene via dehydrogenation (Nguyen, Duus, and Le 2013). P-cymene it is a natural compound found in aromatic plants such as thyme and oregano (Marchese et al. 2017). It is oil nutrient called “terpenes” which are obtained from the essential oils (Quintans-Júnior et al. 2013; Quintans et al. 2013). This compound has been proven to have potent antioxidants, anti-inflammatory, anxiolytic, anticancer, and antimicrobial activities (Marchese et al. 2017). In animal studies, p-cymene found that has the ability to prevent oxidative stress and improves antioxidants enzymes activity (Quintans-Júnior et al. 2013; de Oliveira et al. 2015). As a health compound, p-cymene used for treatment coughing and helps in removing phlegm (Joglekar, Panaskar, and Arvindekar 2014), it can be used also as fungicides and pesticides (Bonjardim et al. 2012). According to the U.S. Food and Drug Administration, both D-limonene and P-cymene are generally recognized as safe (GRAS) (Food and Drug Administration 1991).

1.6.4.5.2 Hot Red Pepper (*Capsicum annum* L.)

Another compound of pepper is hot red pepper (*Capsicum annum* L.). *Capsicum* belongs to the family of Solanaceae and goes by different names depending on location and pepper type. The most common pepper names are chili, bell, red, green, or simply pepper (Faustino, Barroca, and Guiné 2007). Unlike black pepper, it is in the fruit-vegetable family and is eaten daily by many humans. It contains high amounts of vitamin C and total soluble phenolics (KumAr and TATA 2009). *Capsicum* is the only compound that contains an alkaloid named capsaicinoids which produces the spicy hot effect (Hayman and Kam 2008). Red peppers are known to reduce the risk of cancer (Nishino, Murakoshi, Tokuda, and Satomi 2009). Table 1.7 shows the content of selected compounds of the hot red pepper.

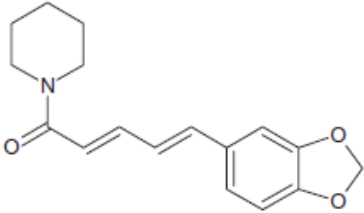
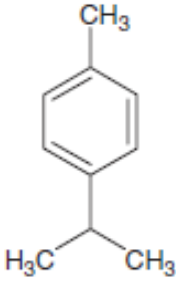
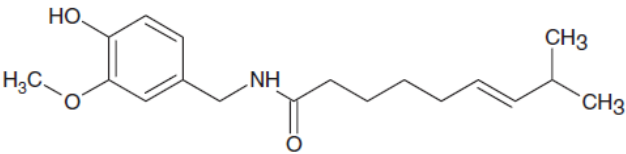
Table 1.7: Content of selected compounds of hot red pepper (Puvača et al. 2014).

Compounds	Amount
Capsaicin	309.30 µg/g
Dihydrocapsaicin	238.20 µg/g
Vitamin C	120.25 mg/100
Carotenoids	1060.24 µg/g
Total phenols	2150.25 µg/g
Total flavonoids	1.60 µmol/g
Antioxidant capacity	150.25 µmol/g

Some studies have investigated the effects of capsaicin on HCA formation. Zeng et al. (2017) evaluated the impact of chilli pepper and capsaicin on the formation of HCAs in roast beef patties. Their results showed that capsaicin reduced total HCAs and PhIP concentrations by 80% to 98% at 2 mg of capsaicin. In addition, the capsaicin itself was more effective than chili pepper in inhibiting HCA formation, possibly because other components of chili peppers can enhance HCA formation. Oz and Kaya (2011) also assessed the effects of red pepper on HCA formation in fried beef, finding that when applied to the surface of the meat before cooking, red

pepper reduced HCA levels between 75% and 100%. Table 1.8 shows the chemical structure of black and red pepper compounds considered in the study.

Table 1.8: The chemical structure of black and red pepper compounds considered in this study (Adapted from Parthasarathy, Chempakam, and Zachariah 2008).

Black and red pepper compounds	Structure
Piperine	
D-limonene	
P-cymene	
Capsaicin	

1.7 Chemical Model Systems

Formation, inhibition, and promotion of HCAs can be studied through chemical model systems. The purpose of using chemical model systems to investigate HCAs is to reduce complex side reactions that normally occur in protein-rich foods (Murkovic 2007). These model systems allow studying the effects of reaction compositions and precursor concentrations at a certain temperature (Bordas, Moyano, Puignou, and Glaceran 2004). Controlling model system precursors (creatine/creatinine, carbohydrate, and free amino acids) allows researchers to identify the complex reactions of HCAs (Messner and Murkovic 2004).

1.7.1 Formation of Heterocyclic Amines in Model Systems

The main precursors of HCAs in a model system are like glucose, creatine, and amino acids (Felton and Knize 1991). Polar HCAs can form between 150 and 250°C while non-polar HCAs are generated at a higher temperature, 300°C or more (Cheng et al. 2006). A mixture of creatine, glucose, and either glycine, lysine, serine, or alanine generated different types of HCA in a model system, including IQ, IQx, MeIQ, MeIQx, 4, 8-DiMeIQx, and 7, 8-DiMeIQx (Jägerstad, Skog, Grivas, and Olsson 1991; Skog, Johansson, and Jägerstad 1998). PhIP model systems typically contain glucose, creatine, and amino acids like phenylalanine, although other studies have reported that other amino acids, including leucine, isoleucine, and tyrosine, were precursors of PhIP (Pais, Salmon, Knize, and Felton 1999). Usually, model systems are diluted in diethylene glycol-water because it has a high boiling point and allows steady heat transfer (Messner and Murkovic 2004). Model systems are developed in sealed vials and heated under temperature and time limits. Cheng et al. (2006) incorporated meat and meat juice into a model system to investigate all HCAs in meat samples. However, the complex reactions formed during

the heating of the meat means the preferred method for a model system is to exclude meat and meat juices to avoid these reactions (Murkovic 2004).

1.7.2 Inhibition of Heterocyclic Amines

Many studies have investigated the effects of antioxidants and phenolic compounds on HCA inhibition in a model system (Johansson and Jägerstad 1996; Apostolides, Balentine, Harbowy, Hara, and Weisburger 1997; Moon and Shin 2013). Cheng et al. (2009) investigated the effect of epigallocatechin gallate (EGCG) and EGCG peracetate on PhIP formation in model systems. The results showed that these antioxidants significantly inhibit PhIP formation. Oguri, Suda, Totsuka, Sugimura, and Wakabayashi (1998) also found that EGCG, luteolin, and quercetin significantly reduced PhIP, MeIQx, and IQ in a model system. Tea polyphenols also inhibit HCA formation. Weisburger, Nagao, Wakabayashi, and Oguri (1994) and Cheng, Chen, and Wang (2007) investigated the effect of tea polyphenols on the HCA formation in a model system. Their findings indicated that tea polyphenols suppress HCA formation. Wong, Cheng, and Wang (2012) evaluated the effect of 11 water-soluble vitamins on HCA formation. The results showed that six of these vitamins (pyridoxiamine, pyridoxine, nicotinic acid, biotin, thiamine, and l-ascorbic acid) effectively inhibit HCA formation.

1.8 Conclusion

The significant association between HCA consumption and a number of health risks requires more research. Many studies suggest that reducing HCA formation occurs not only through reducing the cooking temperature but also through adding natural antioxidants such as tea polyphenols, flavonoids, and spices, which can reduce the mutagenic compound levels.

Chemical model systems are helpful in studying the effects of different variables on HCA formation (e.g., cooking time and temperature, types of precursors, precursor concentrations, and antioxidants). Previous studies have noted that natural antioxidant such as spices and flavonoid compounds have significant effects on HCA formation, but information is still lacking on the effects of these antioxidants. Therefore, developing an effective chemical model system could lead to improved studies of the effects of antioxidants on the formation of HCAs.

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Chapter 2 - The Effect of Black and Red Pepper Compounds on PhIP Formation in Model Systems

2.1 Abstract

This study investigated the inhibitory effect of black and red pepper compounds on the formation of 2-amino-1-methyl-6-phenylimidazo [4,5-b]-pyridine (PhIP) in chemical model systems; PhIP is the most abundant heterocyclic amine (HCA) formed in cooked meat. Model systems explain how the mutagenic/carcinogenic HCAs form during food processing using the effect of time and temperature, and applied antioxidants on the reaction. PhIP generates through chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour. The model systems were dissolved in 90:10 diethylene glycol/water. Using the model systems, individual effects of black pepper oil, piperine, D-limonene, P-cymene, and capsaicin on the formation of PhIP has been evaluated. In this study, the inhibitory effect of black and red pepper compounds was studied at three different concentrations. Black pepper oil, piperine, D-limonene, and capsaicin significantly reduced ($P < 0.05$) PhIP formation. However, P-cymene, when added to model systems, did not inhibit PhIP formation.

2.2 Introduction

Heterocyclic amines (HCAs) are a group of mutagenic and carcinogenic chemical compounds that are generated when muscle-rich foods are cooked at temperatures higher than 150°C (Murkovic 2004). The first discovery of carcinogenic compounds in heated meat was in 1939; Widmark, a Swedish chemist, applied extracts of fried horse meat to mice and found that they induced cancer (Widmark 1939). HCA levels are detected in meat products depending on kind of meat product, cooking temperature, and cooking time (Gibis, Kruwinnus, and Weiss 2015). Several epidemiological studies have reported that consuming fried meat products increases the risk of several kinds of cancers in humans (Norell et al. 1986; Willett, Stampfer, Colditz, Rosner, and Speizer 1990). Because of the health consequences of HCA, the International Agency for Research on Cancer (IARC) has listed 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) as “possible human carcinogens,” and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) has been categorized as “a probable human carcinogen” (IARC 1993). These same HCAs have been classified as “reasonably anticipated to be a human carcinogen” by the National Toxicology Program (NTP) (National Toxicology Program 2014). PhIP has been reported to have more effect on DNA-cell damage than MeIQX and DiMeIQX (Lynch, Knize, Boobis, Gooderham, Davies, and Murray 1992). It is also one of the most abundant HCAs produced in cooked meat and fish during normal cooking (Zöchling, Murkovic, and Pfannhauser 2002). Therefore, researchers are interested in studying the effect of PhIP on foods eaten daily. Some studies of HCA use chemical model systems because it limits side reactions that occur in meats (Murkovic 2004). Model systems also allow researching the chemical interactions among the precursors and applied antioxidants (Bordas, Moyano, Puignou,

and Glaceran 2004). Meat and chemical model systems are used extensively to investigate the effect of antioxidants on HCA formation (Cheng, Chen, and Wang 2007; Kikugawa 2004). Many studies have suggested that antioxidants scavenge free radicals at various points along the HCA pathways, thus preventing HCA formation (Vitaglione and Fogliano 2004).

Black pepper is the dried, mature fruit of the *Piper nigrum* L. plant, belonging to the family Piperaceae and representing about 35% of the world trade in spices (Zachariah and Parthasarathy 2008). One of the important bioactive compounds of black pepper is piperine (Singletary 2010). Black pepper has approximately 3.5% piperine, but levels increase based on pepper maturity and cultivar (Zachariah and Parthasarathy 2008). Previous studies have reported that piperine can be chemopreventive, antioxidants, immunomodulatory, anti-carcinogenic, and anti-inflammatory (Darshan and Doreswamy 2004). Oz and Kaya (2011) studied piperine for its ability to inhibit HCA formation in a high-fat meatball, finding that PhIP levels decreased by 100% over control group. D-limonene and P-cymene are volatile compounds found in black pepper. Previous studies have reported that they have a beneficial health effect, working against mutagenic and carcinogenic formation (Sun 2007; Marchese et al. 2017).

Another type of pepper is hot red pepper (*Capsicum annum* L.). *Capsicum* belongs to the family of Solanaceae and goes by different names depending on location and pepper type (Faustino, Barroca, and Guiné 2007). Capsaicin has high amounts of vitamin C and total soluble phenolics (KumAr and TATA 2009) and is reported to reduce the risk of cancer (Nishino, Murakoshi, Tokuda, and Satomi 2009). In a study evaluating the effects of capsaicin on mutagenic activity in roast beef patties, Zeng et al. (2017) found that capsaicin inhibited PhIP by 80% to 98%. Despite the antioxidant proprieties of black and red pepper compounds, few studies have investigated whether these antioxidants inhibit HCA formation in chemical model systems.

Therefore, the objective of this study was to investigate the effect of black and red pepper compounds in chemical model systems containing glucose, creatinine, and phenylalanine as the main precursors.

2.3 Materials

All standards of black and red pepper compounds (black pepper oil, piperine, D-limonene, p-cymene, and capsaicin) were purchased from Sigma-Aldrich Chemical, Co. (St. Louis, MO, USA). The PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine) standard was obtained from Toronto Research Chemicals, Inc. (Ontario, Canada). D-glucose (99.5%), L-phenylalanine (98%), creatinine, diethylene glycol, and trimethylamine were provided by the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Acetic acid (HPLC grade), acetonitrile (HPLC grade), methanol (HPLC grade), and syringe filters (nylon, 0.2 μm) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was prepared using a Sybron/Barnstead PCS unit (Barnstead/Thermolyne, Inc., Dubuque, IA, USA).

2.4 Method

2.4.1 Model System Preparation

The model systems were prepared similarly to Cheng, Chen, and Wang (2007) with slight modifications. Solutions of 1 mmol Glucose (0.18016 g), creatine (0.11312 g), and phenylalanine (0.16519 g) were dissolved in 90% diethylene glycol, 10% deionized water (v/v) mixture in 10 ml volumetric flasks. The precursor molar concentrations were 0.011 mmol glucose, 0.022 mmol creatinine, and 0.022 mmol phenylalanine. The effect of the black and red pepper compounds on PhIP formation was tested after individual addition (10 mg) of black pepper oil, piperine, D-limonene, P-cymene, and capsaicin to the model systems.

All model systems were run in 1 mL reaction vial and inserted into brass vessels with 2 screw caps on the top and bottom, and 4 holes (1cm × 1 cm) on the body to ensure that heat is transferred effectively throughout the model systems. Before heat treatment, all reactions of model systems were vortexed vigorously to ensure that all solutions were thoroughly mixed. The brass vessels were tightly closed and placed into an oven (HP 5890; Agilent Technologies, Inc., Santa Clara, CA, USA). Where they were heated at 180°C for one hour. After heating, the brass vessels were removed, and the vials were cooled on ice for five min before further examination. No antioxidant treatment was added to the control group. The total assay volume was brought to 800 µL, and all treatments prepared in triplicate. All model systems were syringe filtered and diluted 1:10 with methanol before submitting reaction solutions to HPLC analysis.



Figure 2.1: Picture showing the reaction vials before heating.



Figure 2.2: Picture showing the reaction vials after heating.



Figure 2.3: Picture indicating how reaction vials were placed into brass vessels for heating.

2.4.2 Analysis of PhIP

PhIP analysis was performed on HP 1050 series HPLC (Agilent Technologies) coupled with an HP 1050 series diode array UV-visible detector and an HP 1046 fluorescence detector. PhIP separation was performed using reversed-phase chromatography with a TSKgel ODS-80TM (4.6 mm x 25 cm x 5 μ m) column and a TSK guardgel ODS-80TM (3.2 mm x 1.5 cm) guard column (TOSOH Biosciences; Tokyo, Japan). The column temperature was set at 40°C. Each treatment (20 μ L) was injected onto a reverse-phase column and eluted with a mobile phase containing 0.01 M trimethylamine in deionized water (pH was adjusted to 3.6 with acetic acid) and acetonitrile at a flow rate of 1 ml/min. Mobile phase gradients were used following Puangsombat, Gadgil, Houser, Hunt, and Smith (2012) with slight modifications. A linear HPLC gradient started with 95% of 0.01 M trimethylamine and 5% acetonitrile and then decreased to

75% 0.01 M trimethylamine and 25% acetonitrile over 30 minutes. After 35 min, the initial ratio of 95% 0.01 M trimethylamine and 5% acetonitrile was maintained for 4 min to equilibrate the column. PhIP detecting was obtained by setting fluorescence detector at 437 nm emission and excitation of 229 nm.

2.4.3 Quantification and Statistical Analysis

A 250 ppm PhIP standard solution was prepared by dissolving 1mg of PhIP in 4 ml of methanol. A standard curve was prepared by analyzing PhIP standards at concentrations of 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0.007813 ppm (Appendix A). The coefficient of determination (R^2) was 0.9976. Limit of detection (LOD) of PhIP was 0.201 ppm and the limit of quantification (LOQ) was 0.669 ppm (Appendix B). Statistical analysis was performed using SAS version 9.4 to analyze the data obtained. The data were analyzed using a one-way ANOVA test to determine significant differences between control group and treatments at $p < 0.05$.

2.5 Results and Discussion

In this study, black pepper compounds (black pepper oil, piperine, D-limonene, and P-cymene) and red pepper compound (capsaicin) were investigated to determine their effect on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour. Results showing the effect of black and red pepper compounds on PhIP formation (tables 1-5) are represented as means $\pm$ standard error for each level of the treatment. Other than P-cymene, all black and red pepper compounds showed a significant $p < 0.05$ effect on PhIP formation. Figure 2.4 shows the PhIP standard peak was achieved by setting the fluorescence detector at 229 nm excitation and emission at 437 nm. Figure 2.4 shows PhIP eluted from the column at approximately 16.4 minutes. Figure 2.5 shows the PhIP model system

(control), which included the main precursors 0.011 mmol glucose, 0.022 mmol creatinine, and 0.022 mmol phenylalanine. No treatments were added to the control group.

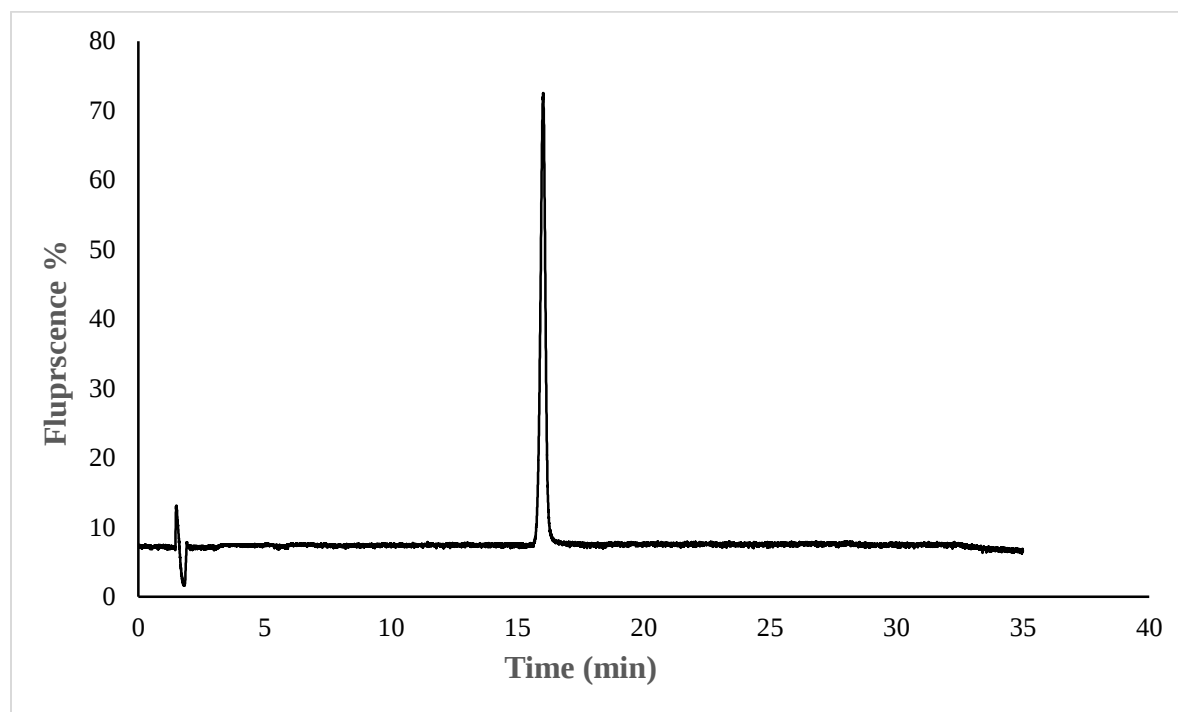


Figure 2.4: HPLC chromatograph of 200 ppb PhIP standard, separated with a TSKgel ODS-80TM (4.6 mm x 25 cm x 5 μ m) column and a TSKgel guardgel ODS-80TM (3.2 mm x 1.5 cm) guard column. Fluorescence detector was set at 437 nm emission and excitation at 229 nm.

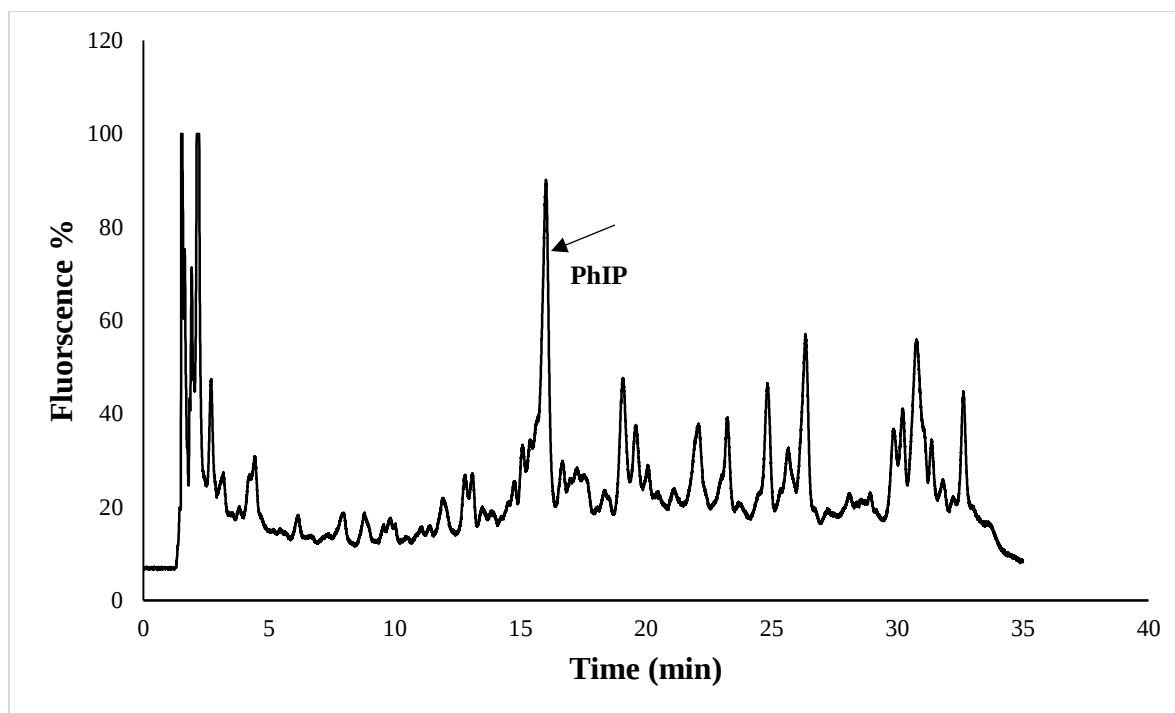


Figure 2.5: HPLC chromatograph of PhIP model system control (0.011 mmol glucose, 0.022 mmol creatinine, 0.022 mmol phenylalanine in 10% water, 90% diethylene glycol solution), separated using a TSKgel ODS-80TM (4.6 mm x 25 cm x 5 μ m) column and a TSKgel guardgel ODS-80TM (3.2 mm x 1.5 cm) guard column. Fluorescence detector was set at 229 nm excitation and emission at 437 nm.

Effects of black and red pepper compounds on PhIP formation

Although some studies have evaluated the effect of black and red pepper compounds on PhIP formation in meat products, few studies have evaluated the effect of these antioxidants on PhIP formation in chemical model systems. The first antioxidant investigated in this study was black pepper oil. Table 2.1 shows the effect of black pepper oil on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour. The inhibitory effect of black pepper oil was studied at three levels: 125, 625, and 1250 ppm. On average, the reduction percentage of PhIP was 24%, 35%, and 34% for each respective level. Black pepper oil at all three levels had a significant ($p < 0.05$) effect on PhIP formation. It

appears that higher concentrations of black pepper oil reduced PhIP formation more than the lower concentration. Kelly and Smith (2015) stated that when black pepper oil was added to chemical model system containing (glucose, creatinine, and phenylalanine) at different concentrations of 36 μ L, 71 μ L, 142 μ L, 285 μ L, and 550 μ L, PhIP levels were reduced by 31.4%, 30.8%, 25.7%, 23%, and 43.5% at each respective level. As postulated by Tipsrisukond, Fernando, and Clarke (1998); Gulcin (2005); and Su, Yin, Charles, Zhou, Moore, and Yu (2007), black pepper has antioxidant and iron chelating abilities that lead to lower mutagenic activity. Other studies reported that black pepper added to the surface of meat reduced PhIP levels by 100%, while the inhibitory effect of black pepper on other HCAs, including IQ, MeIQ, and 4, 8-DiMeIQx, ranged from 12 to 100% (Oz and Kaya 2011).

Table 2.1: Effect of black pepper oil on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

Black pepper oil (ppm)	PhIP (μg/ml)*	Inhibition (%)
Control	1.84 $\pm$ 0.083	
125	1.40 $\pm$ 0.083	24
625	1.19 $\pm$ 0.083	35
1250	1.21 $\pm$ 0.083	34

Significant differences were observed between control and treatments at $p < 0.05$.

*Means $\pm$ standard error for each level of the treatment (n=3).

Piperine was the second antioxidant tested in the study. Table 2.2 provides the results of adding piperine on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour. The inhibitory effect of piperine was studied at three levels: 125, 625, and 1250 ppm. On average, the percentage of PhIP was reduced by 15%, 15%, and 20% for each respective level. Piperine added at all three levels significantly ($p < 0.05$) reduced PhIP formation.

Table 2.2: Effect of piperine on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

Piperine (ppm)	PhIP (µg/ml) *	Inhibition (%)
Control	1.90 ± 0.049	
125	1.61 ± 0.049	15
625	1.62 ± 0.049	15
1250	1.53 ± 0.049	20

Significant differences were observed between control and treatments at $p < 0.05$.

*Means ± standard error for each level of the treatment (n=3).

As shown in Table 2.2, the higher concentrations of piperine reduced PhIP formation more than lower concentrations. Kelly and Smith (2015) reported similar results, stating when piperine was added to model systems at different concentrations (4.02 mg, 8.04 mg, 16.14 mg, and 31.14 mg), PhIP content was reduced by 24%, 20%, 23.5%, and 29% at each respective level. Also, piperine inhibits PhIP formation in beef patties. Zeng et al. (2017) evaluated the effect of piperine on PhIP formation in roast beef patties and found that adding 0.005%, 0.010%, and 0.015% piperine to the surface of meat patties reduced PhIP levels by 62%, 60%, and 56% at each respective level.

Another compound evaluated in this study was D-limonene. It is a chemical compound found in the peels of citrus fruits. Table 2.3 provides the results on the effect of D-limonene on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour. The inhibitory effect of D- Limonene was studied at three levels: 125, 625, 1250 ppm. On average, PhIP formation was reduced by 19%, 16%, and 15% at each respective level. D-limonene at all three levels had a significant ($p < 0.05$) effect on PhIP formation. Table 2.3 shows the lower concentration of D- limonene reduced PhIP more than the higher concentration.

Table 2.3: Effect of D-limonene on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

D-limonene (ppm)	PhIP (µg/ml) *	Inhibition (%)
Control	1.60 ± 0.043	
125	1.29 ± 0.043	19
625	1.34 ± 0.043	16
1250	1.36 ± 0.043	15

Significant differences were observed between control and treatments at $p < 0.05$.

*Means ± standard error for each level of the treatment (n=3).

P-cymene, an aromatic organic compound, was the fourth antioxidant tested in the study. Table 2.4 provides the results of the effect P-cymene had on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour. The inhibitory effect of P-cymene was studied at three levels: 125, 625, 1250 ppm. On average, PhIP formation was reduced by 13%, 15%, and -7 % for each respective level. Adding P-cymene to the model system had no significant effect on PhIP formation. The data on P-cymene shows the higher concentrations of P-cymene actually enhanced PhIP formation. These results agree with previous research, where high concentrations of synthetic antioxidants enhanced HCA formation although low concentrations had some inhibitory effect (Johansson and Jägerstad 1996; Oguri, Suda, Totsuka, Sugimura, and Wakabayashi 1998; Ahn and Grün 2005).

Table 2.4: Effect of P-cymene on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

P-cymene (ppm)	PhIP (µg/ml) *	Inhibition (%)
Control	1.34 ± 0.049	
125	1.16 ± 0.049	13
625	1.14 ± 0.049	15
1250	1.43 ± 0.049	-7

No significant differences were observed between control and treatments at $p < 0.05$.

*Means ± standard error for each level of the treatment (n=3).

Capsaicin, hot red pepper compound, was the final antioxidant tested in this study. Table 2.5 shows the effect of capsaicin on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour. The inhibitory effect of capsaicin was studied at three levels: 125, 625, and 1250 ppm. On average, PhIP formation was reduced by 16%, 12%, and 12% at each respective level. Capsaicin did have a significant ($p < 0.05$) effect on PhIP formation. Table 5 shows the lower concentration of capsaicin reduced PhIP more than the higher concentration.

Table 2.5: Effect of capsaicin on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

Capsaicin (ppm)	PhIP (µg/ml) *	Inhibition (%)
Control	2.06 ± 0.039	
125	1.73 ± 0.039	16
625	1.81 ± 0.039	12
1250	1.81 ± 0.039	12

Significant differences were observed between control and treatments at $p < 0.05$.

*Means ± standard error for each level of the treatment (n=3).

Adding capsaicin at the rate of 1% to the surface of meat before frying has been reported to reduce HCAs (IQ, MeIQ, 4,8 –DiMeIQx, and PhIP) by 75 to 100% (Oz and Kaya 2011). However, Zeng et al. (2014) investigated the effect of 6 Chinese spices (pricklyash peel, star anise, fennel, cumin, capsaicin, and black pepper) on the HCA formation in roast beef patties. Their findings indicated that the only spice inhibiting HCA formation was pricklyash peel while the other spices enhanced HCA formation.

In summary, although black and red pepper compounds lack sufficient hydroxyl groups, our results showed both black and red pepper compounds reduced PhIP formation in a model system at different percentages. Sichel, Corsaro, Scalia, Di Bilio, and Bonomo (1991) and Wang, Jin, and Ho (1999) reported that with more hydroxyl groups, a higher inhibitory effect can be

obtained. We did not see more reduction in compounds that had more hydroxyl groups. The mechanism of PhIP formation is not completely understood. Cheng, Chen, and Wang (2007) indicated that the formation of free radicals might not be the main step in PhIP formation. Therefore, antioxidants may not prevent PhIP formation. Because the mechanism of PhIP formation is not yet clear, we can postulate a mechanism explaining how black pepper protects against PhIP formation: the chemical structure of piperine has a carbonyl group that can be categorized as a ketone. Ketones can react with primary aromatic amines, creating a product with a carbon-nitrogen double bond if an acid catalyst is present (Brown and Poon 2011). Piperine may thus react with PhIP or one of its precursors, preventing formation. Even though black and red pepper compounds did reduce PhIP formation, more research is needed to more thoroughly investigate these compounds and how they work.

2.6 Conclusion

This study used chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour to inhibit the mutagenic compound, PhIP, which is classified by IARC as a potential carcinogen in protein-rich foods. The findings show that four out of five antioxidants (black pepper oil, piperine, D-limonene, and capsaicin) did significantly ($p < 0.05$) reduce PhIP formation while P-cymene did not. The effect of black and red pepper compounds on HCA formation in chemical model systems have not been studied extensively. Our results provide some insight into the effect of black and red pepper compounds on HCA formation, but we must still seek a better understanding of how black and red pepper compounds actually inhibit HCA formation, thus allowing us to minimize carcinogenic compounds in our daily foods.

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Chapter 3 - Effects of Natural Flavonoid Compounds on PhIP

Formation in Model Systems

3.1 Abstract

Natural flavonoid compounds have received much attention because they have the ability to inhibit some formation of genotoxic heterocyclic amines (HCAs) in our daily food. In this study, five natural flavonoid compounds (quercetin, apigenin, genistin, phlorizin, and catechin) were investigated for their inhibitory effects on PhIP formation in chemical model systems. These compounds were individually added into chemical model systems containing glucose, creatinine, and phenylalanine and heat-treated at 180°C for 1 hour. The PhIP content was determined using HPLC. All investigated natural flavonoid compounds significantly ($p < 0.05$) reduced PhIP formation. Among the five flavonoids, genistin had the highest effect, followed by catechin, quercetin, and apigenin. Phlorizin had the least effect on PhIP formation. These findings could help in selecting natural products that might help reduce the potential risk of HCA formation in our foods.

3.2 Introduction

PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine) is one type of heterocyclic aromatic amine (HCA) classified as mutagenic and carcinogenic. It is produced during normal cooking of protein-rich foods such as meat, fish, and chicken (Zöchling, Murkovic, and Pfannhauser 2002). Felton et al. (1986) were the first to discover the carcinogenic PhIP compound in fried beef. PhIP is produced when glucose, creatinine, and certain amino acids like phenylalanine, isoleucine, or tyrosine interact (Johansson and Jägerstad 1993). Skog (1993) reported that precursor concentrations, type of amino acid, and cooking time and temperature also affect HCA formation in foods. Animal studies have showed that PhIP is mutagenic in a variety of tissues and organs, including lung, small/large intestine, liver, mammary gland, colon, prostate, and lymphoid tissue (Sugimura, Wakabayashi, Nakagama, and Nagao 2004). Epidemiological studies also have found that high exposure to PhIP, which is mainly present in muscle foods, may induce colon and breast cancer (Willett, Stampfer, Colditz, Rosner, and Speizer 1990). Therefore, the National Toxicology Program of The U.S. Department of Health and Human Services (2011) has classified PhIP as “reasonably anticipated to be a human carcinogen”.

The literature also reports that free radicals contribute to HCA formation (Kikugawa 1999). Thus, adding natural antioxidants has been extensively used because they are highly effective in reducing HCA formation (Hasaniya, Youn, Xu, Hernaez, and Dashwood 1997). For instance, antioxidants such as spices (Murkovic, Steinberger, and Pfannhauser 1998; Tsen, Ameri, and Smith 2006) and phenolic compounds (Oguri, Suda, Totsuka, Sugimura, and Wakabayashi 1998; Cheng, Chen, and Wang 2007; Vitaglione, Monti, Ambrosino, Skog, and Fogliano 2002), added to meats before cooking, minimize PhIP formation although other studies

have shown the opposite, that antioxidants from natural herbs and spices can significantly enhance PhIP formation. For instance, adding 1% of 5 Chinese spices (star anise, fennel, cumin, chili pepper, and black pepper) to roast beef patties significantly increased PhIP formation (Zeng et al. 2014). Zöchling, Murkovic, and Pfannhauser (2002) also investigated the effect of flavorings such as Monascus red and flavorings extracted from thyme, marjoram, and rosemary in model systems. Their findings showed increased PhIP formation, possibly because adding high concentrations of antioxidants can work as a pro-oxidant. A study by Cheng et al. (2007) reported on an investigation of four fruit extracts such as apple, elderberry, grape seed, and pineapple, to see if they inhibited HCA formation in beef patties. Their findings revealed that apple and grape seed extract significantly reduced MeIQx, 4, 8-DiMeIQx, and PhIP levels by 70% over the control group. Many other natural flavonoid compounds such as apigenin, epigallocatechin gallate, genistein, kaempferol, luteolin, phlorizin, and quercetin have also been investigated for their inhibitory effect on PhIP and total HCA formation. The results showed that most of these compounds significantly ($p < 0.05$) inhibit formation of PhIP and total HCAs. Of all of these compounds, epigallocatechin gallate inhibited 60 to 80% of PhIP and HCA formation and quercetin inhibited 55 to 70% of PhIP and HCA formation (Teng, Hu, Tao, and Wang 2018). Using model systems, Cheng et al. (2007) demonstrated that proanthocyanidins effectively reduced PhIP and MeIQx formation while phloridzin only reduced PhIP formation. However, chlorogenic acid was a potent inhibitor of MeIQx formation but significantly encouraged PhIP formation (Zeng, Li, He, Qin, and Chen 2016).

Flavonoids are a group of polyphenolics largely found in fruits, vegetables, and beverages like tea and wine (Haza, Coto, and Morales 2011). Many flavonoid compounds are natural antioxidants, and most have been found to have antioxidant properties (Butković,

Klasinc, and Bors 2004). In fact, Kozłowska and Szostak-Wegierek (2014) reported that consuming high amounts of flavonoid compounds helped protect against chronic diseases such as cardiovascular disease, cancer, and neurodegenerative diseases.

Previous studies have demonstrated that natural flavonoids reduce PhIP formation in meat products. Because of the complex reactions that occur during heating of meat (Murkovic 2004), model systems were developed to avoid these reactions, so the effect of natural flavonoids could be studied. Therefore, the objective of this study was to investigate the antioxidant effects of different types of natural flavonoid compounds such as quercetin, apigenin, genistin, phlorizin, and catechin which may inhibit PhIP formation in chemical model systems. Because free radicals are produced as a result of the Maillard reaction enhance HCA formation, we hypothesized that adding natural flavonoids into the chemical model system could significantly reduce PhIP formation.

3.3 Materials

All chemicals and solvents were of HPLC or analytical grade. Solvents like acetonitrile, acetic acid, and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was prepared using a Sybron/Barnstead PCS unit (Barnstead/Thermolyne, Inc., Dubuque, IA, USA). PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine) standard was purchased from Toronto Research Chemicals, Inc. (Ontario, Canada). Quercetin, apigenin, genistin, phlorizin, catechin, D-glucose (99.5%), L- phenylalanine (98%), creatinine, diethylene glycol, and trimethylamine were purchased from Sigma-Aldrich Chemical, Co. (St. Louis, MO, USA). Syringe filters (nylon, 0.2 μm) were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

3.4 Method

3.4.1 Model System Preparation

The effect of natural flavonoid antioxidants on PhIP formation was investigated using a modified method developed by Cheng, Chen, and Wang (2007). The precursors, 0.011 mmol glucose, 0.022 mmol creatinine, and 0.022 mmol phenylalanine were dissolved in 10% deionized water, 90% diethylene glycol (v/v) mixture. The effect of the natural flavonoids on PhIP formation was evaluated after individual addition (10 mg) of quercetin, apigenin, genistin, phlorizin, and catechin to the model systems containing the precursors mentioned above. Control group had no treatment. The reactants were added to 1 mL reaction vial and then placed into brass vessels with 2 screw caps on the top/bottom, and 4 holes (1cm × 1 cm) on the body of brass vessels to facilitate heat transfer from heating block to the reaction vials. Before heat treatment, all model systems contents were vortexed vigorously to make sure that all reactants were thoroughly mixed. The brass vessels were tightly closed and placed into a heating block (HP 5890; Agilent Technologies, Inc., Santa Clara, CA, USA). The heating temperature was set at 180°C for 1 hour, and the vessels were then immediately cooled down on ice for five min before further analysis. All model system samples were syringe filtered and diluted 1:10 with methanol before HPLC separation.

3.4.2 Analysis of PhIP

PhIP samples were analyzed on an HP 1050 series HPLC (Agilent Technologies) coupled with an HP 1050 series diode array UV-visible detector and an HP 1046 fluorescence detector. Separation of PhIP was carried out on a reversed-phase column using a TSKgel ODS-80TM (4.6 mm x 25 cm x 5µm) column and a TSK guard gel ODS-80TM (3.2 mm x 1.5 cm) guard column (TOSOH Biosciences; Tokyo, Japan), with a mobile phase of acetonitrile (solvent B) and 0.01 M

trimethylamine (pH was adjusted to 3.6 with acetic acid) (solvent C). Mobile phase gradients followed a modified method suggested by (Puangsombat, Gadgil, Houser, Hunt, and Smith 2012). The initial ratio of a linear HPLC gradient started with 5% B and 95% C and then decreased to 25% B and 75% C over 30 minutes. After 35 min, the initial ratio of 5% B and 95% C was maintained for 4 min to equilibrate the column before the next injection. The flow rate of the mobile phases was 1 ml/min, the injection volume was 20 μ L for each treatment, and the column temperature was set at 40°C. For PhIP detecting, fluorescence detector was setting at 437 nm emission and excitation of 229 nm.

3.4.3 Quantification Statistical Analysis

A standard solution of PhIP was prepared by dissolving 1 mg in 4 ml of methanol. Quantitative determination was performed using a standard curve by analyzing PhIP standards at concentrations of 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0.007813 ppm (Appendix A). The coefficient of determination (R^2) was 0.9976. Limit of detection (LOD) of PhIP was 0.201 ppm, and the limit of quantification (LOQ) was 0.669 (Appendix B). The results were analyzed using SAS version 9.4. Paired samples t-test was performed to determine significant differences between control group and the treatment groups. The significance level (P-value) was set at 0.05. All treatments were done in triplicate.

3.5 Results and Discussion

The main dietary sources of natural flavonoids are fruits and vegetables; they have been identified as powerful inhibitors of carcinogenic compounds (Haza, Coto, and Morales 2011; Kozłowska and Szostak-Wegierek 2014). In this study, five natural flavonoid compounds were investigated for their protective effect against PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour. HPLC analysis was performed to determine PhIP contents generated through the chemical model system. Figure 3.1 shows the peak of the PhIP standard, and PhIP control model system eluted from the column at approximately 16.4 minutes. Results for the effect of flavonoid compounds on PhIP formation (tables 1-5) are represented as means $\pm$ standard error for each treatment level. All flavonoid compounds reduced PhIP content to varying degrees compared to control group. All flavonoids tested in this study significantly ($p < 0.05$) reduced PhIP formation. All antioxidant compounds were investigated individually at 3 levels: 125, 625, and 1250 ppm. Of all five flavonoids, genistin showed the highest inhibition effect on PhIP formation. On average, PhIP formation was reduced by 35% for the 125 ppm level, 42% for 625 ppm, and 29% for 1250 ppm. This Indicates that genistin might be a powerful inhibitor against PhIP formation. Catechin ranked second. On average, PhIP formation was reduced by 22, 23, and 22%. Quercetin was third with, on average, reduction rates of 15, 15, and 30%; apigenin, on average showed reduction rates of 25, 16, and 15%. Phlorizin was lowest, showing, on average, reducing PhIP formation by 14, 13, and 18%.

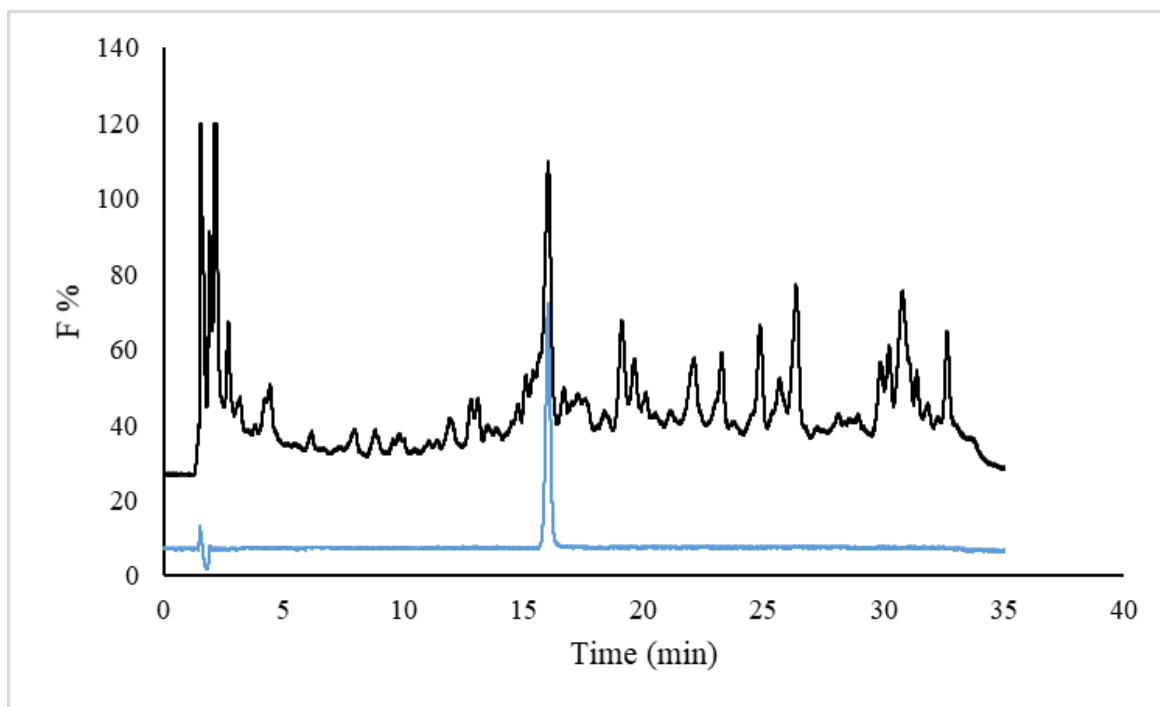


Figure 3-1. HPLC chromatograph of 200 ppb PhIP standard and model system control (0.011 mmol glucose, 0.022 mmol creatinine, 0.022 mmol phenylalanine in 10% water, 90% diethylene glycol solution) separated with a TSKgel ODS-80TM (4.6 mm x 25 cm x 5 μ m) column and a TSK guard gel ODS-80TM (3.2 mm x 1.5 cm) guard column. Fluorescence detector was set at 229 nm of excitation and at 437 nm of emission.

Table 3.1: Effect of quercetin on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

Quercetin (ppm)	PhIP (μ g/ml) *	Inhibition (%)
Control	1.69 $\pm$ 0.065	
125	1.43 $\pm$ 0.065	15
625	1.43 $\pm$ 0.065	15
1250	1.19 $\pm$ 0.065	30

Significant differences were observed between control and treatments at $p < 0.05$.

*Means $\pm$ standard error for each level of the treatment (n=3).

Table 3.2: Effect of apigenin on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

Apigenin (ppm)	PhIP (µg/ml) *	Inhibition (%)
Control	1.90 ± 0.058	
125	1.43 ± 0.058	25
625	1.60 ± 0.058	16
1250	1.62 ± 0.058	15

Significant differences were observed between control and treatments at $p < 0.05$.

*Means ± standard error for each level of the treatment (n=3).

Table 3.3: Effect of genistin on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

Genistin (ppm)	PhIP (µg/ml) *	Inhibition (%)
Control	1.79 ± 0.091	
125	1.17 ± 0.091	35
625	1.03 ± 0.091	42
1250	1.27 ± 0.091	29

Significant differences were observed between control and treatments at $p < 0.05$.

*Means ± standard error for each level of the treatment (n=3).

Table 3.4: Effect of phlorizin, on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

Phlorizin (ppm)	PhIP (µg/ml) *	Inhibition (%)
Control	1.95 ± 0.048	
125	1.67 ± 0.048	14
625	1.70 ± 0.048	13
1250	1.60 ± 0.048	18

Significant differences were observed between control and treatments at $p < 0.05$.

*Means ± standard error for each level of the treatment (n=3).

Table 3.5: Effect of catechin on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

Catechin (ppm)	PhIP (µg/ml) *	Inhibition (%)
Control	2.23 ± 0.067	
125	1.74 ± 0.067	22
625	1.72 ± 0.067	23
1250	1.74 ± 0.067	22

Significant differences were observed between control and treatments at $p < 0.05$.

*Means ± standard error for each level of the treatment (n=3).

The results of this study were consistent with previous research showing that these natural flavonoid compounds could effectively reduce PhIP formation in a chemical model system. In the literature, natural flavonoid compounds have been noted to protect against different types of cancer including lung, hepatocellular, and colorectal cancers (Khanduja, Gandhi, Pathania, and Syal 1999; Yang et al. 2000; Seufi, Ibrahim, Elmaghraby, and Hafez 2009). Previous studies have also shown that flavonoid compounds protect against HCA formation. For instance, bamboo leaves (AOB) and flavonoids such as apigenin, luteolin, orientin, homoorientin, vitexin, isovitex, isorhamnetin, fisetin, and hesperetin all inhibited PhIP formation in model systems (Zhang, Luo, Shao, Yu, and Wang 2014). Many studies have evaluated the effects of natural antioxidants on HCA formation in both chemical model systems and beef patties. Oguri, Suda, Totsuka, Sugimura, and Wakabayashi (1998) investigated how luteolin, quercetin, catechins, epigallocatechin gallate, and caffeic acid affected HCA formation in a chemical model system. The results showed reduced levels of PhIP and MeIQx by 3 to 75% compared to the control group. Previous studies have also reported that some flavonoids differ in how they inhibit PhIP formation than other types of HCAs. For instance, although quercetin inhibited PhIP formation the most, baicalein did not. In contrast, baicalein was more effective on

MeIQx and 7, 8-diMeIQx formation than quercetin (Yu, Shao, Liu, Zhang, and Wang 2016). Cheng et al. (2007) reported on three different phenolic compounds (proanthocyanidins, phloridzin, and chlorogenic acid), which when added to model systems to reduce PhIP and MeIQx formation showed that proanthocyanidins inhibited both PhIP and MeIQx the most, and phloridzin significantly ($p < 0.05$) inhibited PhIP formation. On the other hand, chlorogenic acid was a potent inhibitor of MeIQx but enhanced PhIP formation. Zhu, Zhang, Wang, Chen, and Zheng (2016) studied the effect of eight different flavonoids (apigenin, luteolin, kaempferol, quercetin, genistein, naringenin, phlorizin, and EGCG) on HCA profiles in roast beef patties cooked at 230°C for 20 min. Their findings show that most of these antioxidants significantly ($p < 0.05$) inhibited all HCAs investigated in the study (Harman, Norharman, 4, 8-MeIQx, MeIQx, DMIP, 1, 5, 6-TMIP, and PhIP). However, apigenin did not inhibit MeIQx formation, EGCG did not inhibit 1, 5, 6-TMIP formation, and luteolin did not inhibit PhIP formation. Cheng, Chen, and Wang (2007) showed that adding phenolic antioxidants such as quercetin, theaflavin 3, 3'-digallate, epicatechin gallate, rosmarinic acid, and naringenin to a chemical model system had no effect on PhIP, MeIQx, and 4, 8- DiMeIQx formation. However, when these antioxidants added to the surface of beef patties, the levels of PhIP, MeIQx, and 4, 8- DiMeIQx were significantly inhibited. Moreover, among these antioxidants, naringenin was the strongest inhibitor in both the model system and beef patties. Other research also found that adding phenolic compounds such as rutin and chlorogenic acid to roast beef patties significantly ($p < 0.05$) enhanced PhIP formation. One possible explanation for these differences may be that in model systems PhIP is produced using phenylalanine while MeIQx and 7, 8-diMeIQx model systems are produced using glycine (Shao, Han, Zhang, Zhang, and Wang 2018). Formation of PhIP and MeIQx involves

different interaction pathways (Pearson, Chen, Gray, and Aust 1992; Murkovic, Weber, Geiszler, Fröhlich, and Pfannhauser 1999; Zöchling and Murkovic 2002).

3.6 Conclusion

In conclusion, this study proved that natural flavonoid compounds can reduce PhIP formation in chemical model systems. The findings are consistent with previous results that natural flavonoid compounds can effectively reduce PhIP formation at different concentrations with a varying percentages of reduction. All flavonoid compounds investigated in the study significantly ($p < 0.05$) reduced PhIP formation although genistin was the strongest inhibitor while phlorizin had the least effect on PhIP formation. Thus, consuming such these natural antioxidants in our foods may significantly reduce the potential risk of ingesting carcinogenic compounds.

3.7 References

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Chapter 4 - Investigating the Synergetic Effect of Various Natural Antioxidants to Inhibit PhIP Formation in Model Systems

4.1 Abstract

Natural antioxidants have many biological functions and serve as antioxidant and anti-inflammatory agents. Although the antioxidant effects of many spices and flavonoid compounds on PhIP formation have been evaluated, research related to the synergistic antioxidant effect of various spices and flavonoids on PhIP formation is not well studied. In addition, at least some research shows a combination of compounds inhibits HCAs more strongly than a single antioxidant (Cheng et al. 2007). Therefore, in this study, binary combinations of two antioxidant spices like piperine and capsaicin and two flavonoid compounds like genistin and catechin were investigated using a chemical model system that contained glucose, creatinine, and phenylalanine. All ratios of mixed spices and corresponding flavonoid compounds were as follows: 1:0.25, 1:0.5, and 1:1. The synergistic effect was assessed by identifying the reduction percentage of PhIP formation. Significant ($p < 0.05$) synergistic antioxidant effects were found for all combinations. The combination of piperine and genistin had a potent synergistic effect for all combinations (26, 27, and 41% reduction in PhIP). The combination of catechin and capsaicin had less synergistic effect (14, 13, and 15%). Knowing the antioxidants with the best synergistic effects could be useful in developing dietary antioxidants, leading to lower HCA formation.

4.2 Introduction

Several studies have defined heterocyclic amines (HCAs) as mutagenic and carcinogenic compounds produced when high protein foods are cooked at high temperatures (Knize, Salmon, Mehta, Felton 1997; Abdulkarim and Smith 1998). Based on *Salmonella*/mutagenicity test, more than 25 HCAs have been identified in cooked foods (Alaejos and Afonso 2011; Gibis 2016). The International Agency for Research on Cancer (IARC) lists some of these as probably human carcinogens and some as possible human carcinogens (IRAC 1993). One of the most abundant HCAs formed in cooked meat and fish during normal cooking is PhIP (2-amino-1- methyl-6-phenylimidazo [4,5-b]pyridine) (Zöchling, Murkovic, and Pfannhauser 2002). Animal studies have found that food rich in PhIP increases the risk of prostate, breast, intestine, and liver cancers. It is also linked with an increase in cancer risk in humans (Carthew, DiNovi, and Setzer 2010). Many strategies have been identified for reducing the potential health risk of HCAs in our foods, including reducing cooking times and temperatures and food marinades. Adding antioxidants is another effective treatment to limit HCA formation because of antioxidants have free radical scavenging properties (Knize, Dolbeare, Carroll, Moore, and Felton 1994).

In recent years, natural products that promote health and fitness have received much attention especially because of their antioxidant proprieties (Craig 1999). Antioxidant spices are widely used because they enhance flavor, color, and aroma in our daily foods. Spices do have, moreover, beneficial health effects (Souza, Stamford, Lima, Trajano, and Barbosa 2005). Black and red pepper spices protect against liver and kidney diseases (Srinivasan 2007). Free radicals normally produced in our bodies as a result of oxidation have a correlation with chronic diseases such as cancer, cardiovascular disease, diabetes, pulmonary and neurological diseases (Reuter, Gupta, Chaturvedi, and Aggarwal 2010; Sosa, Moliné, Somoza, Paciucci, Kondoh, and LLeonart

2013). Black and red pepper spices, however, have free radical scavenging abilities and immunomodulatory properties which lead to protect the body from infectious diseases (Hadeel, Zaid, and Al Tae'e 2010).

Flavonoids are a group of phenolic compounds widely found in nature with many biological functions; they are antioxidants, anti-inflammatory agents, and antimicrobial agents (Han 2007; Liu et al. 2010). They are now receiving much attention from consumers and in food industries because they can protect against oxidative stress (Ghosh and Khan 2015). Various dietary flavonoid compounds such as apigenin, epigallocatechin gallate, genistein, kaempferol, luteolin, phlorizin, and quercetin have been investigated for their ability to inhibit HCA formation, especially PhIP. Other studies have revealed that these compounds significantly reduce HCA formation (Zhu, Zhang, Wang, Chen, and Zheng 2016).

The synergistic effect among antioxidants has become increasingly important because of consumer interest in preserving nutritional value and positive effects on health (Liang, Tian, Yang, Zhang, and Skibsted 2009). Synergistic effects occur when two compounds interact, producing more inhibitory effect than the individual compounds (Ghosh and Khan 2015; Uri 1961). Synergistic antioxidant effects have been reported in previous studies (Sharma, Perez, and Erhan 2007; Becker, Ntouma, Skibsted 2007; Marinova, Toneva, and Yanishlieva 2008). For instance, Hajimehdipoor, Shahrestani, and Shekarchi (2014) investigated the synergistic antioxidant effects between phenolic compounds such as caffeic acid, gallic acid, and chlorogenic acid, and flavonoids such as rutin and quercetin. The results showed that caffeic acid and gallic acid had the most potent synergistic effect among all combinations of the compounds. The synergistic antioxidant effect was also found in the presence of iron (Akhavan, Kalaei, Alavi, Ghiasi, and Esfandiar 2012), quercetin 3- β -glucoside (Hidalgo, Sánchez-Moreno, and de

Pascual-Teresa 2010), and some herbs (Jain, Pancholi, and Patel 2011). However, interaction among antioxidants in addition to other additives can also lead to antagonistic effect (Soleimani, Dehabadi, Wilson, and Tabil 2018). Becker, Ntouma, and Skibsted (2007) evaluated α -T, astaxanthin, quercetin, and rutin for their synergistic antioxidant effects. The results showed the factors that affect synergism and antagonism of antioxidants: solubility, polarity, and the hydrophilic nature of the antioxidants. Despite research into the individual effects of several spices such as piperine and capsaicin and natural flavonoids such as quercetin, apigenin, genistin, phlorizin, and catechin on PhIP formation (Oz and Kaya 2011; Zeng et al. 2014; Zhu, Zhang, Wang, Chen, and Zheng 2016), no study has investigated the synergistic effect among these antioxidants. Moreover, a combination of antioxidants would perhaps help reduce HCAs in our daily foods and positively affect health. Therefore, the aim of this study was to uncover the synergistic effects of two spices, piperine and capsaicin, when they interacted with two natural flavonoids, genistin and catechin, in binary combinations in a chemical model system.

4.3 Materials

Pure PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine) standard was purchased from Toronto Research Chemicals, Inc. (Ontario, Canada). Antioxidants flavonoids (genistin and catechin) and spices (piperine and capsaicin) standards were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). D-glucose (99.5%), L- phenylalanine (98%), creatinine, diethylene glycol, and trimethylamine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Solvents and chemicals such as acetic acid, acetonitrile (high-performance liquid chromatography [HPLC] grade), methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was processed by a Sybron/Barnstead PCS unit (Barnstead/Thermolyne, Inc, Dubuque, IA, USA). 0.2 μm syringe filters were provided by Fisher Scientific (Fair Lawn, NJ, USA).

4.4 Method

4.4.1 Model System Preparation

The effects of antioxidant flavonoids and spices on PhIP formation were evaluated using a model system with slight modifications (Cheng, Chen, and Wang 2007). The precursors, 0.011 mmol glucose, 0.022 mmol creatinine, and 0.022 mmol phenylalanine were dissolved in 10% deionized water, 90% diethylene glycol (v/v) mixture and mixed by vortexing. 10 mg of flavonoids and spices (genistin, catechin, piperine, and capsaicin) were added to the model systems. Samples without flavonoids and spices were used as control. All ratios of mixed spice compounds as they corresponded to flavonoids were as follows: 1:0.25, 1:0.5, and 1:1. The reaction substances were added to a 1 ml reaction vial, which were then inserted into brass vessels with 2 screw caps on the top and bottom and 4 holes (1cm $\times$ 1 cm) on the body. The brass vessels were then inserted into a heating block (HP 5890; Agilent Technologies, Inc., Santa

Clara, CA, USA), and heated at 180°C for 1 hour and then immediately cooled by placing the reaction vials on ice for 5 min. All model system samples were syringe filtered and diluted 1:10 with methanol before HPLC analysis.

4.4.2 Analysis of PhIP

HPLC separation was carried out on an HP 1050 series HPLC (Agilent Technologies) coupled with an HP 1050 series diode array UV-visible detector and an HP 1046 fluorescence detector. Separation of PhIP in the model system samples was performed using reversed-phase chromatography using a TSKgel ODS-80TM (4.6 mm x 25 cm x 5µm) column and a TSK guardgel ODS-80TM (3.2 mm x 1.5 cm) guard column (TOSOH Biosciences; Tokyo, Japan). The injection volume for each sample and the mobile phase rate were 20 µL and 1 ml/min, respectively. The mobile phase was composed of acetonitrile (solvent B) and 0.01 M trimethylamine (pH was adjusted to 3.6 with acetic acid) (solvent C). The mobile phase gradients were used as described previously (Puangsombat, Gadgil, Houser, Hunt, and Smith 2012) with minor modifications. The initial ratio of a linear HPLC gradient started with 95% C and 5% B and then decreased to 75% C and 25% B over 30 minutes. After 35 min, the initial ratio of 95% C and 5% B was maintained for 4 min to equilibrate of the column before the next injection. For PhIP detecting, fluorescence detector was setting at emission/ excitation wavelengths of 437 nm and 229 nm.

4.4.3 Quantification and Statistical Analysis

A standard method was performed to quantify PhIP in the model system. 1 mg of PhIP dissolved in 4 ml of methanol and gradually diluted to 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0.007813 ppm (Appendix A). A standard curve was processed after determining dilutions and peak areas. To determine linearity using a standard curve, the correlation

coefficient (R^2) was calculated and it was 0.9976. Limit of detection (LOD) of PhIP was 0.201 ppm and the limit of quantification (LOQ) was 0.0669 ppm (Appendix B). One-way ANOVA test was used to determine significant differences between control group the treatments. Results were analyzed using SAS 9.4. All samples were prepared in triplicate and statistical significant was considered at $p < 0.05$.

4.5 Results and Discussion

Antioxidant spices and flavonoids interact in several different ways to inhibit HCA formation. Combinations of these antioxidants can produce higher reductions of HCAs in our daily foods than single antioxidants (Cheng et al. 2007). In this study, a binary combination of antioxidant spices (piperine and capsaicin) and the flavonoid compounds that produced the highest inhibitory effect (catechin and genistein) were evaluated for their ability to reduce PhIP formation in a chemical model system heated at 180°C for 1 hour. HPLC analysis was performed to determine how much PhIP was generated in the model system. The combined ratio of antioxidant spices and flavonoids were as follows: 1:0.25, 1:0.5, and 1:1%. As expected, all combinations of antioxidants spices and flavonoids evaluated in this study significantly ($p < 0.05$) reduced PhIP formation, indicating their various antioxidant properties. The results indicated that the combined effect of piperine and catechin (with piperine at higher levels than catechin) was the strongest, ranging from 26% to 41% reduction (see Table 4.1) compared to control group. The synergistic effect increased as the concentrations of the combined compounds increased, indicating that piperine and catechin combined might be the most potent inhibitor of PhIP formation. The combination with higher levels of catechin and lower levels of piperine showed a lower synergistic effect with reduction ranging from 17% to 22% (see Table 4.1).

Table 4.1: Combined effect of piperine and catechin on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

Piperine + Catechin	PhIP (µg/ml) *	Inhibition (%)
Control	2.03 ± 0.091	
1:0.25	1.51 ± 0.091	26
1:0.5	1.49 ± 0.091	27
1:1	1.20 ± 0.091	41
Catechin + Piperine		
Control	2.00 ± 0.061	
1:0.25	1.67 ± 0.061	17
1:0.5	1.58 ± 0.061	21
1:1	1.56 ± 0.061	22

Significant differences were observed between control and treatments at $p < 0.0$.

*Means ± standard error for each level of the treatment (n=3).

Table 4.2 shows piperine and genistein (with piperine at higher levels than genistein) was the strongest. In contrast, combining higher levels of genistein and lower levels of piperine showed less effect. In fact, the effect of the synergistic interactions decreased as the concentrations of the combined compounds increased, although these results do show that piperine, when combined with other compounds, exhibits a high percentage of inhibition (see Table 4.2). Our results agree with previous results that demonstrated piperine, when combined with spices like curcumin (a bioactive compound of turmeric), a potent synergistic effect occurs. This is probably because piperine is one of the most active antioxidants reported to inhibit P-glycoprotein. Which is a protein responsible for transporting substances outside the cell membrane. P-glycoprotein is present in brain circulation, which might change the beneficial effects of other antioxidants such as like curcumin. Thus, piperine has a potent synergistic antioxidant effect through inhibiting the P-glycoprotein molecule. Piperine can also help other compounds by increasing their absorption (Shoba, Joy, Joseph, Majeed, Rajendran, and Srinivas

1998). Nimkar and Smith (2013) investigated the antioxidant interactions between black pepper and other spices such as rosemary, cinnamon, oregano, turmeric, thyme, and ginger on the inhibition of PhIP formation in beef patties. Significant synergistic effects against PhIP formation were observed, with the highest synergistic effect between black pepper and turmeric (94.7% inhibition).

Table 4.2: Combined effect of piperine and genistein on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

Piperine + Genistein	PhIP (µg/ml) *	Inhibition (%)
Control	2.64 ± 0.065	
1:0.25	2.08 ± 0.065	21
1:0.5	2.27 ± 0.065	14
1:1	2.27 ± 0.065	14
Genistein + Piperine		
Control	1.97 ± 0.044	
1:0.25	1.61 ± 0.044	18
1:0.5	1.75 ± 0.044	11
1:1	1.67 ± 0.044	15

Significant differences were observed between control and treatments at $p < 0.05$.

*Means ± standard error for each level of the treatment (n=3).

Table 4.3 shows the combined effects of capsaicin and catechin (with capsaicin at higher levels than catechin). The synergistic effect was equal among all levels of the combination. Combinations of higher levels of catechin and lower levels of capsaicin, however, exhibited less reduction against PhIP formation.

Table 4.3: Combined effect of capsaicin and catechin on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

Capsaicin + Catechin	PhIP (µg/ml) *	Inhibition (%)
Control	2.09 ± 0.053	
1:0.25	1.69 ± 0.053	19
1:0.5	1.69 ± 0.053	19
1:1	1.69 ± 0.053	19
Catechin + Capsaicin		
Control	2.55 ± 0.051	
1:0.25	2.20 ± 0.051	14
1:0.5	2.21 ± 0.051	13
1:1	2.18 ± 0.051	15

Significant differences were observed between control and treatments at $p < 0.05$.

*Means ± standard error for each level of the treatment (n=3).

Table 4.4: Combined effect of capsaicin and genistin on PhIP formation in chemical model systems containing glucose, creatinine, and phenylalanine heated at 180°C for 1 hour.

Capsaicin + Genistein	PhIP (µg/ml) *	Inhibition (%)
Control	2.07 ± 0.052	
1:0.25	1.66 ± 0.052	20
1:0.5	1.71 ± 0.052	17
1:1	1.72 ± 0.052	17
Genistein + Capsaicin		
Control	2.07 ± 0.042	
1:0.25	1.71 ± 0.042	17
1:0.5	1.77 ± 0.042	15
1:1	1.79 ± 0.042	15

Significant differences were observed between control and treatments at $p < 0.05$.

*Means ± standard error for each level of the treatment (n=3).

Synergistic effect of four antioxidants, including capsaicin, vitamin E, quercetin, and ascorbic acid have also been evaluated. The results have shown that capsaicin has a potent synergist effect (Maksimova, Mirceski, Gulaboski, Koleva, and Arsova-Sarafinovska 2016), On

the other hand, a combinations of different types of antioxidants can lead to antagonist effects, where individual effects overpower the combined effects (Hsieh, Hsieh, Wu, Peng, and Hsieh 2018). For instance, ternary combinations of rutin, caffeic acid, rosmarinic acid; chlorogenic acid, caffeic acid, rosmarinic acid; rutin, rosmarinic acid, gallic acid; and rutin, chlorogenic acid, caffeic acid showed significant antagonist effects, reducing their ability to inhibit HCA formation by approximately 16 to 22% (Hajimehdipoor, Shahrestani, and Shekarchi 2014). Zeng, Li, He, Qin, and Chen (2016) investigated the synergistic or antagonistic effect of phenolic compounds such as rutin and protocatechuic acid on HCA profiles in roast beef patties. Their findings indicated that combinations of these compounds had significant ($p < 0.05$) synergistic effects against harman and norharman-type HCAs, but significant ($p < 0.05$) antagonistic effects were observed for DMIP and 4, 8-DiMeIQx-type HCAs.

Finally, Table 4.4 shows the combined effect of capsaicin and genistin (with capsaicin at higher levels than genistein) was the strongest. In contrast, combinations with genistin at higher levels and capsaicin at lower levels showed less reduction of PhIP formation. These results show that lower concentrations of capsaicin and genistin combined reduced PhIP formation more than higher concentrations (see Table 4.4).

4.6 Conclusion

All combinations of antioxidant spices (piperine and capsaicin) and flavonoids (genistin and catechin) had significant synergistic effects, with combinations of piperine and genistin having the highest synergistic effect. Combinations of capsaicin and catechin had the least synergistic effect. The results of this study show all tested antioxidants have a synergistic effect in reducing PhIP formation in a chemical model system, but future studies could determine how applying antioxidant spices and flavonoid compounds to beef patties would be helpful to promote human consumption of meat products with fewer HCAs.

4.7 References

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Appendix A - PhIP Standard Curve

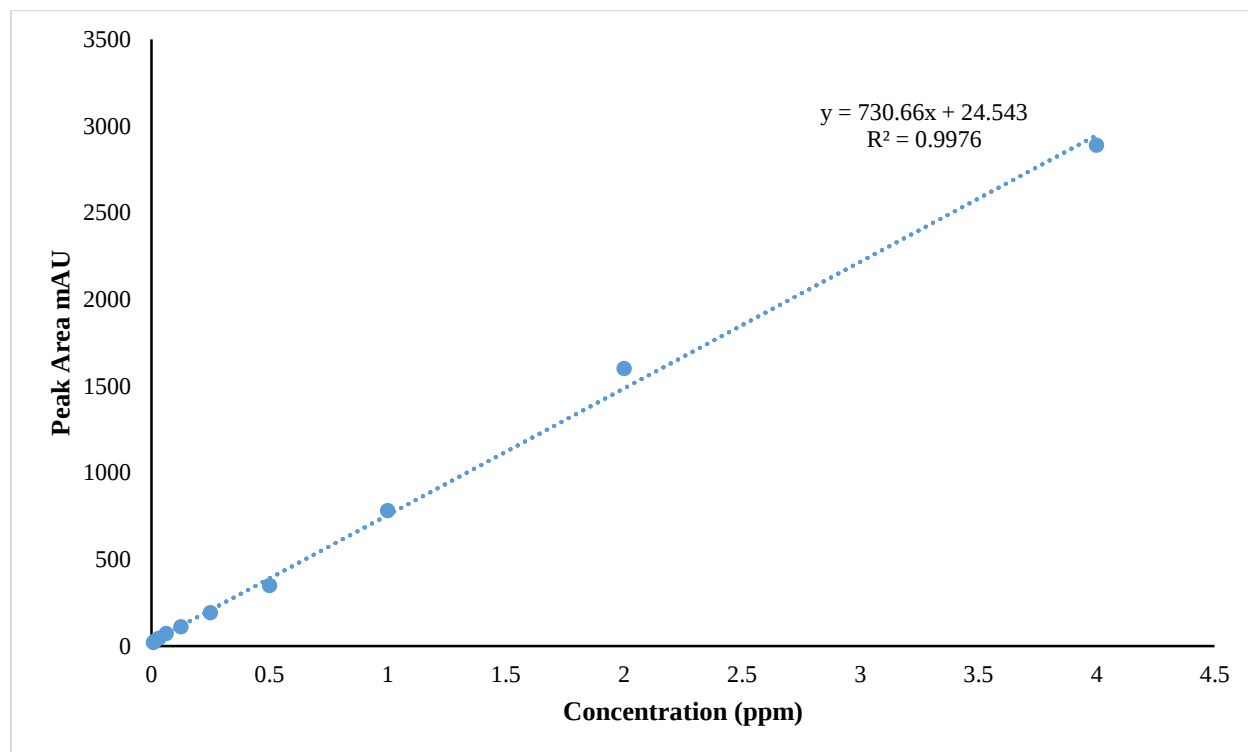


Figure A.1: PhIP standard curve obtained by HPLC fluorescence at Ex = 229 nm, Em = 437 nm.

Appendix B - Limit of detection (LOD) and limit of quantification (LOQ) calculations

Table B.1: LOD and LOQ for PhIP using the HP 1090 HPLC fluorescence at Ex = 229 nm, Em = 437 nm.

Concentration (ppm)	Peak Area
0.007813	19
0.015625	29
0.03125	44
0.0625	71
0.125	111
0.25	191
0.5	349
1	781
2	1600
4	2890
LOD	0.201 ppm
LOQ	0.669 ppm

Appendix C - Chromatograph

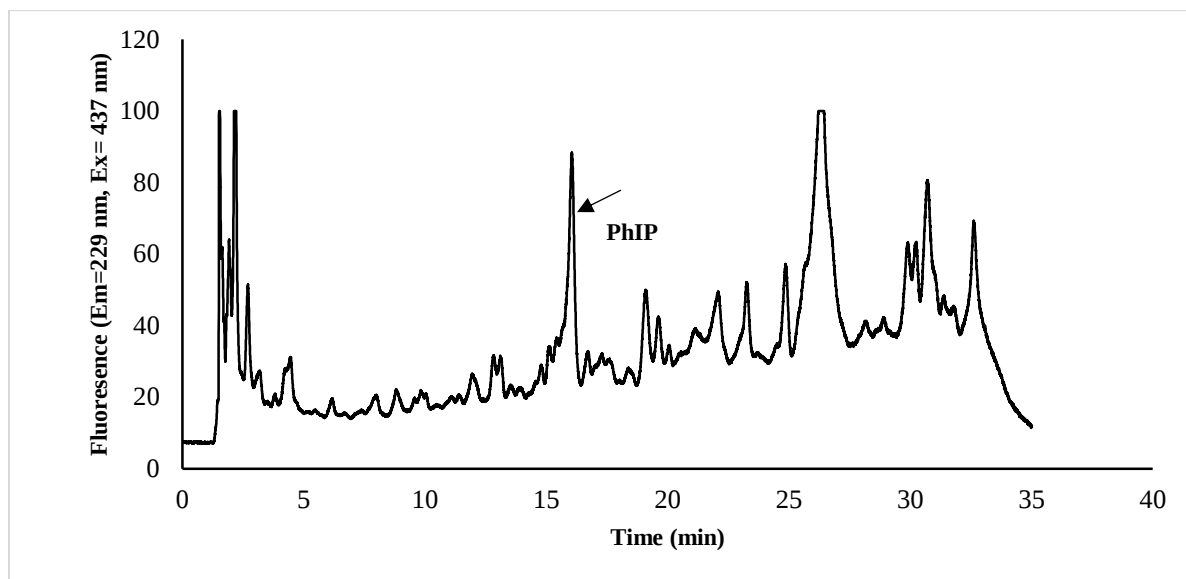


Figure C.1: HPLC chromatogram of a PhIP model system with 1250 ppm of piperine added; separated with a TSKgel ODS-80TM column. The fluorescence detector was set at Ex=229 nm, Em=437 nm.

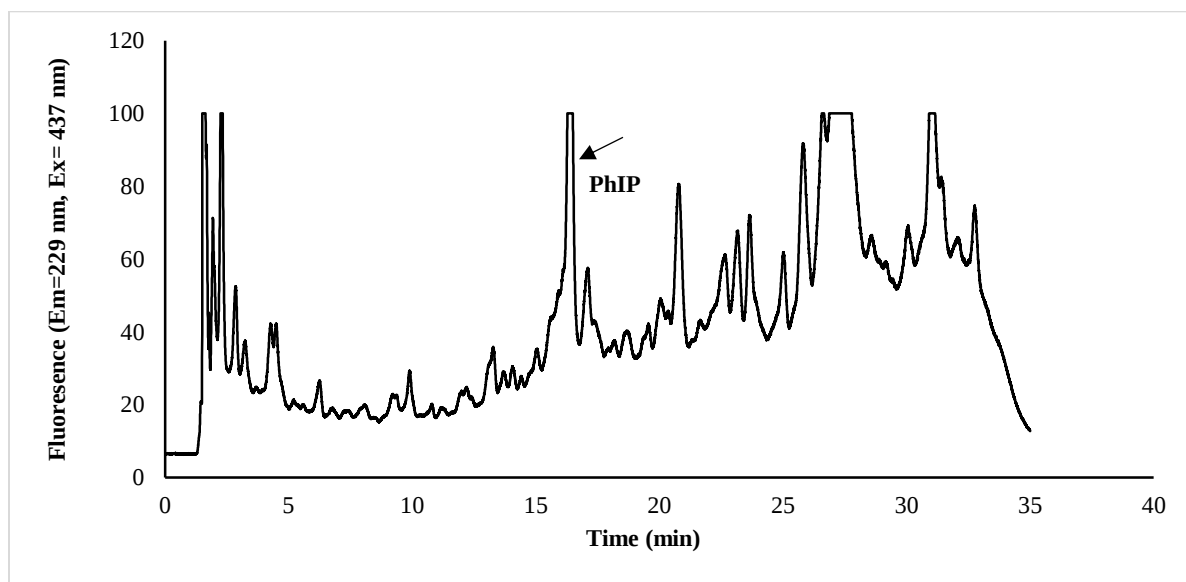


Figure C.2: HPLC chromatogram of a PhIP model system with 125 ppm of capsaicin added; separated with a TSKgel ODS-80TM column. The fluorescence detector was set at Ex=229 nm, Em=437 nm.

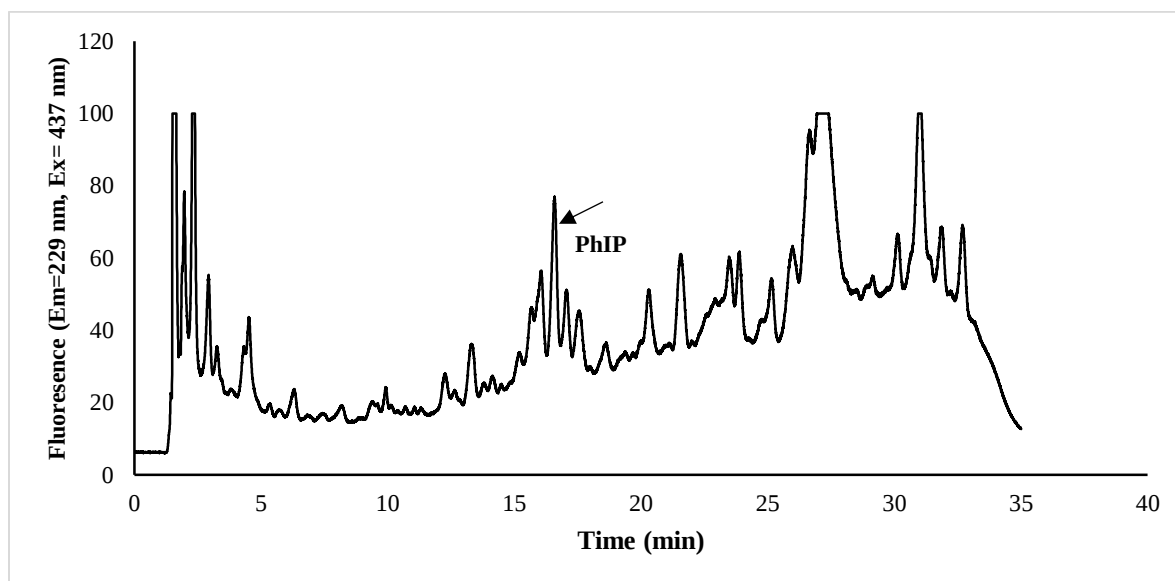


Figure C.3: HPLC chromatogram of a PhIP model system with 625 ppm of genistin added; separated with a TSKgel ODS-80TM column. The fluorescence detector was set at Ex=229 nm, Em=437 nm.

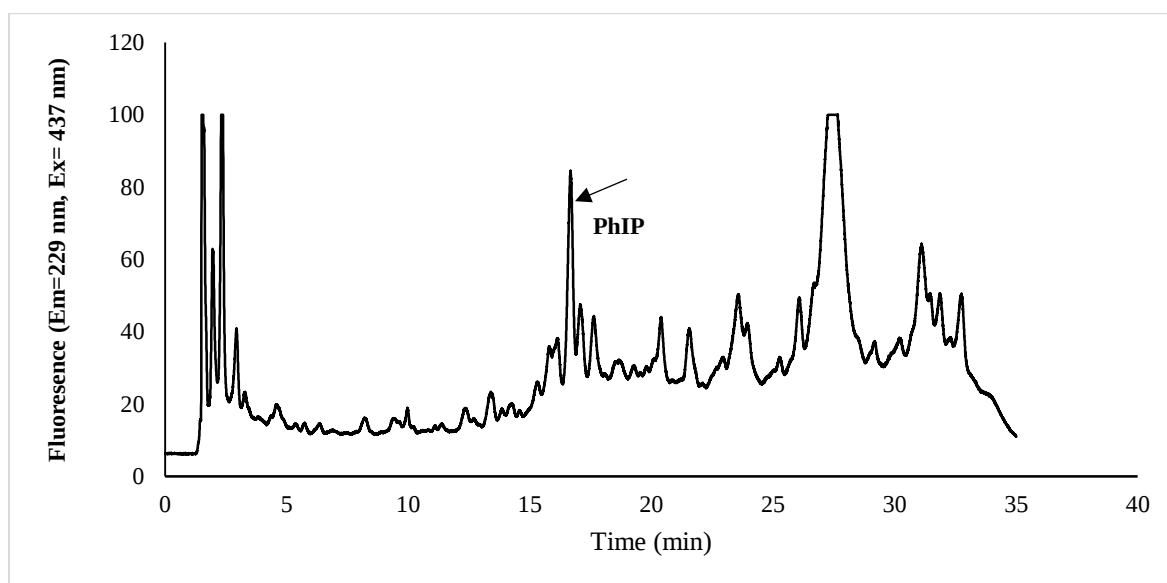


Figure C.4: HPLC chromatogram of a PhIP model system with 1250 ppm of catechin added; separated with a TSKgel ODS-80TM column. The fluorescence detector was set at Ex=229 nm, Em=437 nm.

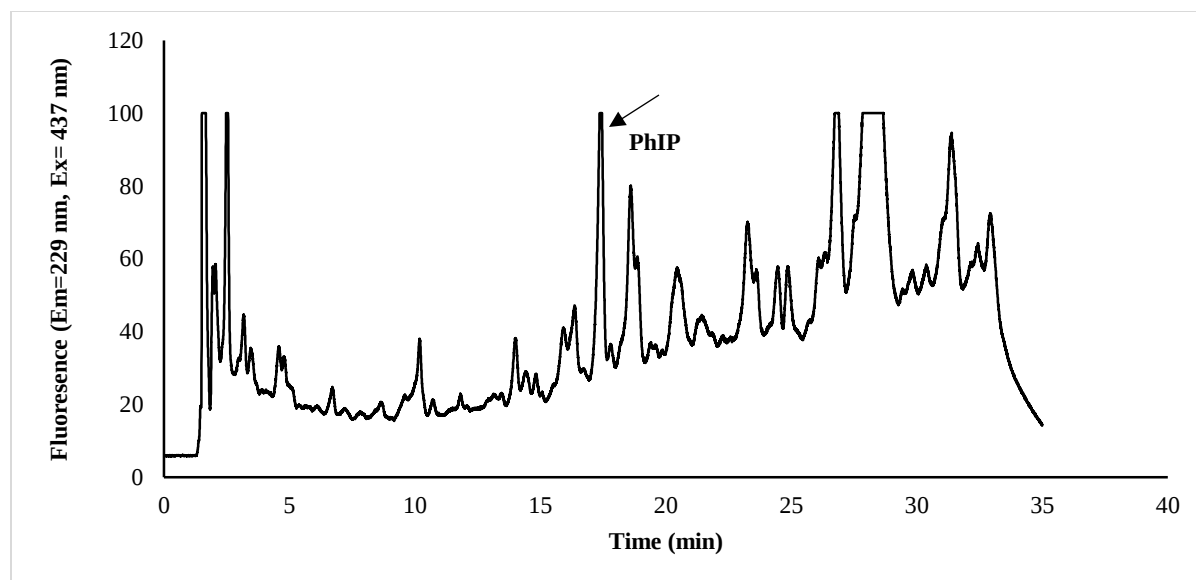


Figure C.5: HPLC chromatogram of a PhIP model system with 1250 ppm of capsaicin and catechin added; separated with a TSKgel ODS-80TM column. The fluorescence detector was set at Ex=229 nm, Em=437 nm.

Appendix D - SAS Codes

Black pepper oil

```
Data BlackPepperOil ;
input  PHIP_Control PHIP_after;
datalines ;
1454 896
1319 878
1338 945

1454 936
1319 970
1338 768

1454 1049
1319 1007
1338 1068
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

D-limonene

```
Option ls=120;
Data Lamenone ;
input  PHIP_Control PHIP_after;
datalines ;
1202 945
1164 1000
1114 1098

1202 976
1164 920
1114 1110

1202 906
1164 889
1114 1101
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

Piperine

```
Option ls=120;
Data Piperine ;
input  PHIP_Control PHIP_after;
datalines ;
1481 1189
1410 1093
1344 1135

1481 1294
1410 1259
1344 1071

1481 1230
1410 1233
1344 1149
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

P-cymene

```
Option ls=120;
Data Cymene ;
input  PHIP_Control PHIP_after;
datalines ;
1103 1100
995 1072
912 1042

1103 980
995 841
912 752

1103 788
995 833
912 993
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

Capsaicin

```
Option ls=120;
Data Capsaicin ;
input  PHIP_Control PHIP_after;
datalines ;
1584 1320
1473 1368
1525 1346

1584 1365
1473 1309
1525 1377

1584 1270
1473 1304
1525 1300
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

Apigenin

```
Option ls=120;
Data Apigen ;
input  PHIP_Control PHIP_after;
datalines ;
1307 1281
1572 1219
1349 1134

1307 1243
1572 1138
1349 1192

1307 1054
1572 1048
1349 1117
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

Quercetin

```
Option ls=120;
Data Quercetin;
input  PHIP_Control PHIP_after;
datalines ;
1650 1239
1652 1250
1680 1409

1650 1245
1652 1241
1680 1361

1650 1242
1652 1278
1680 1372
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

Genistein

```
Option ls=120;
Data Genistein;
input  PHIP_Control PHIP_after;
datalines ;
1388 918
1263 980
1349 967

1388 919
1263 743
1349 660

1388 873
1263 935
1349 834
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

Catechin

```
Option ls=120;
Data Catechin ;
input  PHIP_Control PHIP_after;
datalines ;
1360 887
1238 770
1189 1045

1360 1040
1238 1223
1189 945

1360 1094
1238 1002
1189 1124
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

Capsaicin and catchin

```
Option ls=120;
Data capsaicin and catchin ;
input  PHIP_Control PHIP_after;
datalines ;
1533 1264
1500 1271
1616 1250

1533 1260
1500 1266
1616 1260

1533 1253
1500 1254
1616 1261
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

Phlorizin

```
Option ls=120;
Data Phlorizin ;
input  PHIP_Control PHIP_after;
datalines ;
1599 1215
1360 1211
1393 1156

1599 1288
1360 1220
1393 1281

1599 1262
1360 1307
1393 1157
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

Catchin and capsaicin

```
Option ls=120;
Data catchin and capsaicin ;
input  PHIP_Control PHIP_after;
datalines ;
1888 1572
1891 1620
1874 1665

1888 1722
1891 1632
1874 1574

1888 1650
1891 1712
1874 1526
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

Piperine and genistin

```
Option ls=120;
Data Piperine and genistin ;
input  PHIP_Control PHIP_after;
datalines ;
1901 1591
1964 1689
2000 1766

1901 1667
1964 1678
1579 1699

1901 1588
1964 1470
2000 1583
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

Piperine and catchin

```
Option ls=120;
Data Piperine and catchin ;
input  PHIP_Control PHIP_after;
datalines ;
1439 884
1496 857
1579 968

1439 1090
1496 1109
1579 1135

1439 1191
1496 1101
1579 1102
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

genistin and piperine

```
Option ls=120;
Data genistin and piperine ;
input  PHIP_Control PHIP_after;
datalines ;
1492 1256
1407 1230
1489 1250

1901 1366
1964 1251
1579 1290

1901 1171
1964 1252
2000 1175
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

Catchin and Piperine

```
Option ls=120;
Data Catchin and Piperine ;
input  PHIP_Control PHIP_after;
datalines ;
1505 968
1453 1261
1493 1267

1505 1149
1453 1218
1493 1170

1505 1291
1453 1258
1493 1189
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

Capsaicin and catchin

```
Option ls=120;
Data capsaicin and catchin ;
input  PHIP_Control PHIP_after;
datalines ;
1533 1264
1500 1271
1616 1250

1533 1260
1500 1266
1616 1260

1533 1253
1500 1254
1616 1261
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```

Catchin and capsaicin

```
Option ls=120;
Data catchin and capsaicin ;
input  PHIP_Control PHIP_after;
datalines ;
1888 1572
1891 1620
1874 1665

1888 1722
1891 1632
1874 1574

1888 1650
1891 1712
1874 1526
;

run;

ods graphics on;
proc ttest;
paired PHIP_Control*PHIP_after;
run;
```