FORMATION AND INHIBITION OF THE HETEROCYCLIC AMINE 2-AMINO-1-METHYL-6-PHENYLIMIDAZO[4,5-*B*]PYRIDINE (PHIP) IN A MODEL SYSTEM

by

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Abstract

Heterocyclic amines (HCAs) are a class of mutagenic and carcinogenic chemical compounds formed on the outside of meat and fish when cooked at high temperatures. 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is the most abundantly formed HCA. HCAs have been found to cause cancer in mice and rats; PhIP specifically has been found to cause breast, rectal, prostate, and colon cancers. Model systems are often used to replicate the HCA chemical reactions in meat products without causing the many side reactions when meat is cooked at high temperatures. Model systems are also a useful way to study the effects of different variables and compounds on the formation of HCAs without using meat. A model system using amounts of 0.2 mmol glucose, 0.4 mmol creatinine, and 0.4 mmol phenylalanine in 10:90 water/diethylene glycol (v/v) was used to study the formation of PhIP. Differing levels of black pepper oil, black pepper extract, and rosemary extract (36, 71, 142, 285, 550 μL), synthetic antioxidants BHT and TBHQ (0.05 mmol, 0.1 mmol, 0.2 mmol, 0.4 mmol), and piperine (4.02 mg, 8.04 mg, 16.14 mg, 31.14 mg) were added to the model system to study their effect on PhIP formation. PhIP formation with added BHT (0.2 and 0.4 mmol) and TBHQ (0.4 mmol) were not significantly different from the control. All other added compounds decreased PhIP formation significantly from the control at $p < 0.05$. Solid phase micro extraction (SPME) headspace analysis was conducted on ground black pepper, black pepper oil, and black pepper extract to determine possible components responsible for PhIP inhibition. Six volatile compounds were found in common between ground black pepper, black pepper oil, and black pepper extract: 1R-α-pinene, 3-carene, caryophyllene, α-caryophyllene, cyclohexene, and D-limonene. D-limonene and caryophyllene had the largest peak areas, suggesting those compounds may play a part in PhIP inhibition in model systems.

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Chapter 1 - Literature Review

Introduction

In 1912, Louis-Camille Maillard first described a non-enzymatic browning reaction that occurred in food products. This reaction was later named after him and today is known as the Maillard browning reaction. Maillard browning requires a reducing sugar (e.g. glucoe) and free amino acids (e.g. phenylalaine). Maillard browning is extremely common in the food industry and is responsible for many favorable and unfavorable colors and flavors in food products. Roughly three decades later, Widmark (1939) first discovered that extracts from roasted horse meat painted onto the backs of mice resulted in an increase of malignant tumor growth. He concluded that the roasted horse meat must contain multiple carcinogenic substances. This study shows one of the first examples of harmful compounds forming on meat products that are heated.

Some of the most common, potentially harmful, constituents of the Maillard browning reaction are heterocyclic amines (HCAs). The study of these compounds has been going on since the late 1970's/early 1980's. Many studies use model systems to mimic precursor concentrations and heating conditions of meat without having to deal with meat and the many side reactions and other compounds formed when meat is cooked. Much of the current data has been on HCA inhibition, although information about HCA promotion has been found in some studies.

The focus of this review will be to give a basic overview of HCAs. It will include a more in-depth definition of HCAs, how HCAs are formed, how HCAs are inhibited, and will finish with information about the formation, inhibition, and promotion of HCAs in chemical model systems.

Heterocyclic Amines

First discovered by Sugimura and others (1977), HCAs are a class of mutagenic and carcinogenic chemical compounds. They are formed on the outside of meat and fish, at part per billion (ppb) levels, when cooked at high temperatures greater than 150 ˚C. HCAs have been detected in airborne particles, diesel exhaust particles, cigarette smoke, cooking fumes, rain water, sewage water, incineration ash, and soil (Kataoka 1997).

Currently, there are more than 25 HCAs known to cause strong mutagenicity (Cheng and others 2006). In general, the most abundantly formed 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) (Figure 1-1). (NTP 2014; Puangsombat and others 2012). Other HCAs include 2-amino-9*H*-dipyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[4,3-*b*]indole (MeAαC), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), and 2-amino-dipyrido[1,2-*a*:3’,2’-*d*]imidazole (Glu-P-2) (Figure 1-1) (Cheng and others 2006).

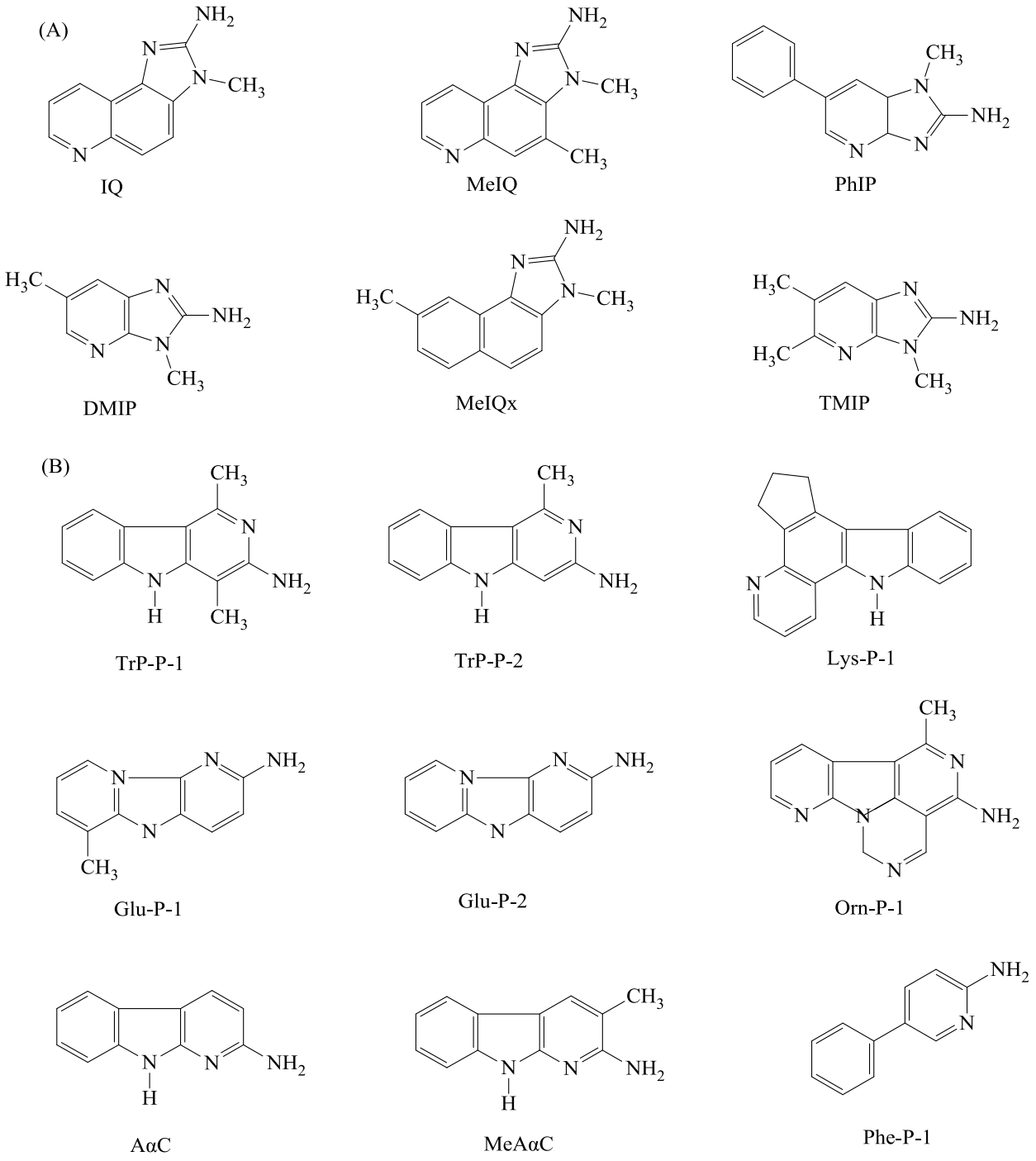


Figure 1-1: Select polar (A) and non-polar (B) HCAs.

Types of Heterocyclic Amines

HCAs can be separated into two major categories: polar (Table 1-1) and non-polar (Table 1-2) HCAs. These HCAs can then be further distinguished by type as quinoline, quinoxaline,

pyridine, pyridoindole, or dipyridoimidazole (Murkovic 2004). Each of the polar HCAs contain a 5-membered imidazole ring (two CH groups replaced by nitrogen) with an exocyclic amino group (a nitrogen atom bonded to one to three carbon atoms) and *N*-methyl group that scientists have determined come from creatine (Brown and Poon 2011; Knize and Felton 2008). The distinction of a HCA being polar or non-polar refers to the order in which they elute from a column in reversed-phase chromatography, and can indicate whether or not the HCA fluoresces (Murkovic 2007). The polar HCAs elute from a column first (i.e. towards the start of the run) and non-polar HCAs elute last (i.e. towards the end of the run). The non-polar HCAs typically fluoresce, while the only polar HCA known to fluoresce is PhIP (Murkovic 2007).

Table 1-1: List of polar heterocyclic amines (Adapted from Murkovic 2004)

HCA	**Moiety**	**Full Chemical Name**
IQ	Quinoline	2-amino-3-methylimidazo[4,5-*f*]quinoline
IQx	Quinoxaline	2-amino-3-methylimidazo[4,5-*f*]quinoxaline
MeIQ	Quinoline	2-amino-3,4-dimethylimidazo[4,5*f*]quinoline
MeIQx	Quinoxaline	2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
4,8-DiMeIQx	Quinoxaline	2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
7,8-DiMeIQx	Quinoxaline	2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline
4,7,8-TriMeIQx	Quinoxaline	2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline
PhIP	Pyridine	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
DMIP	Pyridine	2-amino-1,6-dimethylimidazo[4,5-*b*]pyridine
TMIP	Pyridine	2-amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine

HCAs can be classified as amino-carbolines (formed at temperatures above 300 ˚C) or as aminoimidazo-azaarenes (AIAs) (formed at temperatures below 300 ˚C). The amino-carbolines are further classified as pyridoindoles, pyridoimidazoles, phenylpyridines, tetraazafluoranthrenes, or benzimidazoles, while AIAs are further classified as IQ, IQx, or imidazopyridines (Cheng and others 2006; Kataoka 1997). Amino-carbolines contain two six-membered aromatic rings, one or both of which can be pyridine, and one five-membered ring in between the six-membered ring (Cheng and others 2006). The AIAs contain an imidazole ring with a *N*-methyl group attached (Cheng and others 2006).

Table 1-2: List of non-polar heterocyclic amines (Adapted from Murkovic 2004)

HCA	**Moiety**	**Full Chemical Name**
Trp-P-1	Pyridoindole	3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole
Trp-P-2	Pyridoindole	3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole
Glu-P-1	Dipyridoimidazole	2-amino-6-methyl-dipyrido[1,2-*a*:3',2'-*d*]imidazole
Glu-P-2	Dipyridoimidazole	2-amino-dipyrido[1,2-*a*:3',2'-*d*]imidazole
Lys-P-1	Other	3,4-cyclopentenopyrido[3,2-*a*]carbazole
Orn-P-1	Other	4-amino-6-methyl-1*H*-2,5,10,10*b*-tetraaza-fluoranthene
Phe-P-1	Other	2-amino-5-phenylpyridine
AαC	Pyridoindole	2-amino-9*H*-pyrido[2,3-*b*]indole
MeAαC	Pyridoindole	2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole
Norharman		β-carboline
Harman		9-methyl-β-carboline

Heterocyclic Amine Formation

The major precursors for the formation of amino-carbolines are amino acids (or proteins) and sugar. While formation has not been found to be dependent on creatin(in)e, some experiments show that the addition of creatin(in)e in model systems does increase the production of certain amino-carbolines (Cheng and others 2006; Jägerstad and others 1998). The mechanism of formation of the HCAs Trp-P-1, Trp-P-2, AαC, and MeAαC are unknown (Arvidsson and others 1999). It has been assumed that amino-carbolines are formed through amino acid and protein pyrolysis and via free radical reactions (Jägerstad and others 1998).

While the exact mechanisms for HCA formation are largely unknown and complicated, it is assumed by many that HCAs are formed via the Maillard browning reaction and by the formation of Strecker aldehydes (Arvidsson and others 1999; Cheng and others 2006). It has been suggested that the amino-imidazo portion of HCAs come from creatine, with the remaining portions formed via Strecker degradation products (NTP 2014; Skog and others 1998). There are multiple other factors that determine which types of HCAs are formed and how much of each HCA is formed, they include the type(s) of amino acid(s), precursor concentrations, heating temperature and time, and any compounds added to study inhibition.

Johansson and others (1995) tested different amino acids in model systems and found the type of HCA(s) produced depended on the type of amino acid. IQx was formed with the addition of arginine, glycine, or tyrosine to the model system. MeIQx was formed with the addition of threonine, lysine, tryptophan, and alanine. 4,8-DiMeIQx is also formed with alanine, while PhIP is formed with phenylalanine. Borgen and others (2001) studied the effect of different amino acids on HCA formation and found that different HCAs will form with different amino acids present. Similar studies were performed by Kataoka and others (2012) and Zamora and others (2013). Robbana-Barnat and others (1996) and Skog and others (1998) reviewed similar results,

where different amino acids could form the same HCAs. For example, threonine, glycine, lysine, alanine, and serine often formed both MeIQx and DiMeIQs within the system.

Time and temperature of model system and meat heating is another important component of HCA formation. In general, as either the amount of time and/or temperature a meat, or model system, is heated, the more HCAs form within the system (Bordas and others 2004; Cheng and others 2006; Robbana-Barnat and others 1996; Skog and others 1998; Skog and others 2000). Bordas and others (2004) conducted a study involving varying heating times and temperatures and found that all HCAs tested had an increase in formation as the temperature and time increased. The amount of HCAs formed ranged in varying amounts of 0.1-2.6 μg/g (Harman and Norharman), 3.5-10.7 ng/g (MeIQx), 10-43 ng/g (IQ), and up to 256 ng/g (PhIP). HCAs tend to form better at higher temperatures (> 150 ˚C), but there have been some studies that show HCAs can form at lower temperatures if given enough time (Cheng and others 2006; Robbana-Barnat and others 1996; Skog and others 1998). The method of cooking is also a factor in HCA formation, with many studies recommending a microwave pre-treatment of meats to lessen the HCAs formed (Cheng and others 2006; Robbana-Barnat and others 1996; Skog and others 1998).

Finally, the precursor concentrations play a major role in the formation of HCAs, especially with regard to the sugar(s) used within the model systems. In general, when the sugar concentration is half the molar concentration of creatin(in)e and the amino acid(s) within the system, an optimal amount of HCA formation occurs (Cheng and others 2006; Skog and others 1998). Skog and Jägerstad (1990) tested multiple mono- and disaccharides in HCA model systems. They found that a significant increase in the sugar concentration from the typical molar concentrations resulted in a decrease in HCA formation of up to half the original concentration of HCAs, as well as HCA mutagenicity. Kikugawa and others (2000) also found similar results

when testing different sugar concentrations. In general, there would be a slight increase, followed by a plateau, and a decrease in HCA concentrations as more sugar was added to the system. Understanding how these treatments affect HCA formation can help identify ways to decrease HCA formation in meats in order to prevent or lessen the health implications of HCAs.

Health Implications of Heterocyclic Amines

The International Agency for Research on Cancer (IARC 1997) has listed IQ as a *probable human carcinogen (Group 2A)* and PhIP, MeIQ, MeIQx, AαC, MeAαC, Trp-P-1, Trp-P-2, and Glu-P-1 as *possible human carcinogens (Group 2B)*. MeIQ, MeIQx, IQ, PhIP, AαC, MeAαC, Glu-P-1, Glu-P-2, Trp-P-1, and Trp-P-2 are also listed as *reasonably anticipated to be a human carcinogen* by the National Toxicology Program Report on Carcinogens (NTP 2014). HCAs are present in meat that has been cooked at high temperatures and they are present at different concentrations, resulting in differing amounts of HCAs in cooked foods (Table 1-3).

The main concerns of HCAs are their mutagenic and carcinogenic properties. HCAs have been shown to cause multiple cancers in mice and rat models, including liver, pancreatic, breast, stomach, lung, blood vessel, and colorectal cancers (Cheng and others 2006; Li and others 2007; Nowell and others 2002; Wakabayashi and others 1992). On their own, most HCAs are not mutagenic or carcinogenic in nature, however, their ability to form DNA adducts gives them their mutagenic capabilities (Cheng and others 2006).

Table 1-3: Amount of select HCAs in cooked foods (ng/g cooked food) (Adapted from Wakabayashi and others 1992).

HCA	Daily Intake (ng/g cooked food)
PhIP	0.56-69.2
IQ	0.16-0.19
MeIQ	0.03
MeIQx	0.64-6.44
4,8-DiMeIQx	0.10-0.81
Trp-P-1	0.12-0.21
Trp-P-2	0.15-0.25
AαC	0.21-2.50
MeAαC	0.19

2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)

In 1986, Felton and others were able to isolate and identify a new HCA from fried ground beef: PhIP (Figure 1-2). PhIP is one of the most abundantly formed HCAs with temperatures similar to common cooking practices. The dietary intake of PhIP is typically the highest, followed by MeIQx, IQ, and MeIQ (NTP 2014).

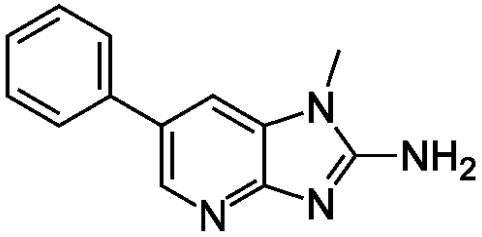


Figure 1-2: Chemical structure of PhIP.

PhIP Formation

The mechanism for the formation of PhIP has not been largely studied, although PhIP has been formed within model systems containing glucose, creatin(in)e, and phenylalanine (Arvidsson and others 1997; Johansson and others 1995; Shioya and others 1987). It is thought that PhIP formation begins with the Strecker aldehyde phenylacetaldehyde being formed, followed by the condensation of phenylacetaldehyde with creatinine and dehydration (Murkovic 2004). Zöchling and Murkovic (2002) found that when a mixture of phenylacetaldehyde and creatinine were heated in a model system, more PhIP was formed than in a standard model system of phenylalanine, creatinine, and glucose. They concluded that the degradation of phenylalanine to phenylacetaldehyde was a necessary step in the formation of PhIP. It has been suggested by Arvidsson and others (1997) that a mono-molecular reaction is the rate-limiting step in PhIP formation.

Health Implications of PhIP

The National Toxicology Program (NTP 2014) Report on Carcinogens lists PhIP as *reasonably anticipated to be a human carcinogen* and the International Agency for Research on Cancer (IARC 1997) lists PhIP as a *possible human carcinogen (Group 2B)*. While other HCAs have been shown to cause multiple types of cancer, especially in the liver, PhIP has been shown to cause breast, colon, rectal, and prostate cancers in mice and rat studies (Shirai and others 1997; Sinha and others 2000; Zhu and others 2003). Gooderham and others (2002) noted the site specificity of PhIP coincided with the most common sites associated with diet-induced cancers in humans. These results, combined with later results (Gooderham and others 2007), suggest that PhIP may have additional or alternative mechanisms of action, other than those found in other HCAs, which can cause cancer formation. Gooderham and others (2002, 2007) also determined

PhIP has some genotoxic effects in cells. Many of these studies have found that PhIP forms DNA adducts within the glands and tissues of mice and rats.

Inhibition of Heterocyclic Amines

HCAs have been shown, in multiple studies, to cause different types of cancer including breast, colon, prostate, skin, lymphoid tissue, ear duct, blood vessels, and liver (Sugimura 2000). For this reason, many scientists have strived to discover compounds and ingredients within the food industry that can decrease or prevent HCA formation in meat. Because the exact formation mechanisms of many HCAs have only been hypothesized, it is difficult to determine how certain compounds, such as antioxidants, inhibit their formation. Figure 1-3 shows a proposed mechanism for HCA formation and possible sites where antioxidants prevent formation.

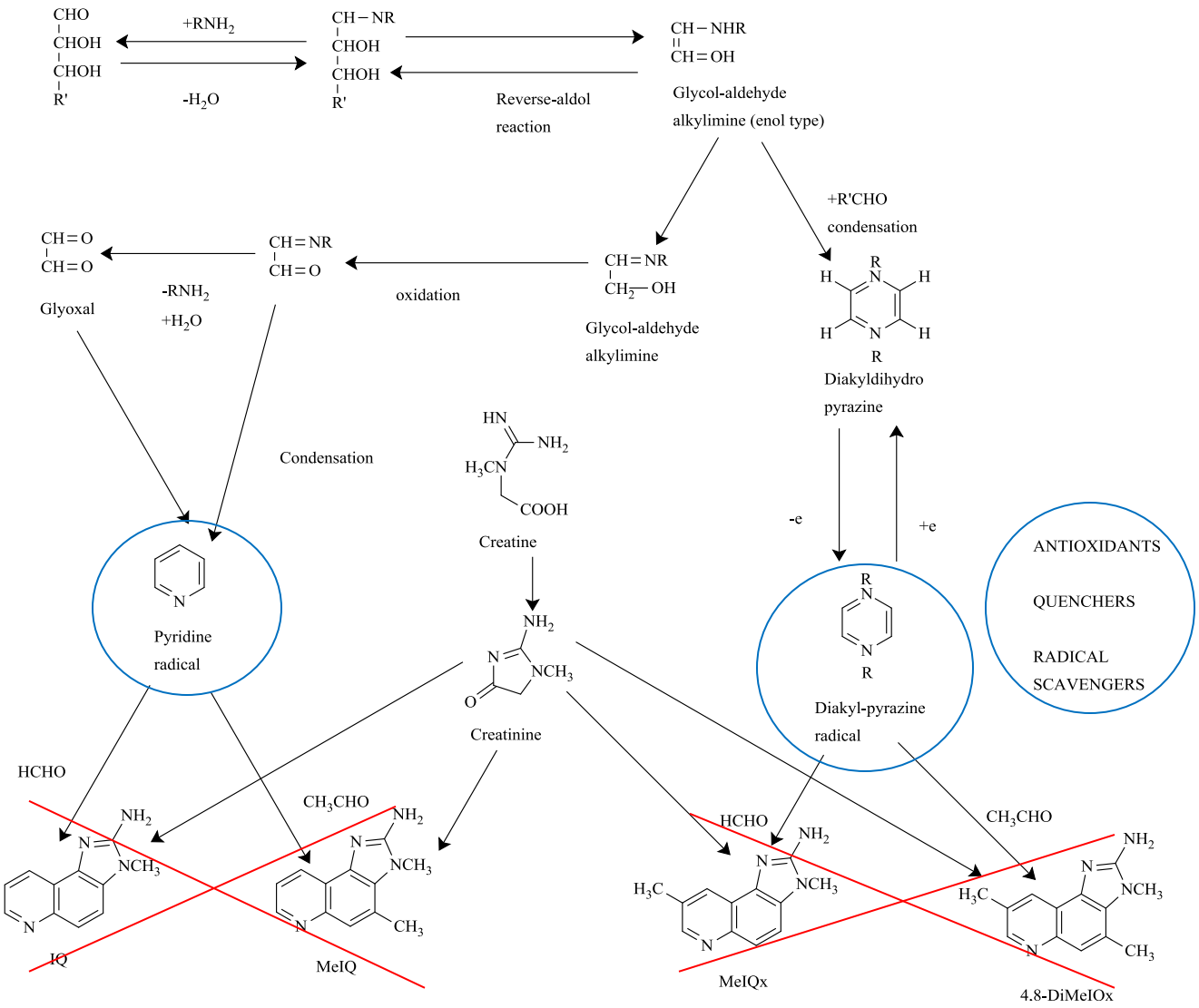


Figure 1-3: Proposed sites where antioxidants inhibit HCA formation (Adapted from Vitaglione and Fogliano 2004).

Antioxidants

Free radicals can be produced in the body as by-products of metabolism, or can be formed due to exposure to harmful compounds or radiation (Vijayakumar and others 2004). Antioxidants are a class of compounds used to prevent or slow lipid oxidation and scavenge free radicals and are often used in the food industry (Vijayan and Thampuran 2000). There are a number of synthetic and natural antioxidants, the natural ones being more readily accepted by consumers because they are seen as "better" than synthetic antioxidant compounds (Gülçin

2005). Antioxidants have been shown to inhibit HCA formation in a number of studies, both in meat and in chemical model systems.

Natural Antioxidants

Natural antioxidants are antioxidants found in nature, such as those in food or those formed during food processing. Common natural antioxidants include tocopherols (vitamin E), polyphenolics, flavonoids, anthocyanins, and phytochemicals. Tea, herbs, spices, and fruits and vegetables are foods often used because of their antioxidative properties (Shahidi and Wanasundara 1992; Vitaglione and Fogliano 2004). Natural antioxidants are often used to reduce lipid oxidation of food products (Grün 2009).

Tocopherols are monophenolic antioxidants found in nature, composed of two families of compounds (tocols and tocotrienols), each family containing four compounds designated with a prefix (α, β, γ, or δ) (Shahidi and Wanasundara 1992). Flavonoids are sometimes referred to as "primary antioxidants" due to their ability as free radical acceptors and chain breakers (Shahidi and Wanasundara 1992). Tea, herbs, and spices as antioxidants are discussed in the following pages. There are a number of methods that can be used to determine antioxidant activity of compounds.

Two of the most commonly used methods are the Folin-Ciocalteau total phenolic content (TPC) and 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability methods. TPC tests the total polyphenols of a compound by determining the amount of hydroxyl groups of a compound. The hydroxyl groups stabilize free radicals by donating hydrogen atoms. A high antioxidant activity is expected when more hydroxyl groups are present in a compound. The DPPH method is also used to test the amount of hydroxyl groups in a compound by determining a compound's ability to reduce the DPPH compound, changing its color from violet to yellow.

The more hydroxyl groups present, the greater color change. While some studies have found no correlation between TPC and DPPH (Hinneberg and others 2006; Su and others 2007), others have indicated a possible relationship (Wang and others 2008; Wojdylo and others 2007).

Synthetic Antioxidants

There are currently a number of synthetic antioxidants, shown in Figure 1-4, including butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), dodecyl gallate (DG), 1-O-hexyl-2,3,5,-trimethylhydroquinone (HTHQ), and tert-butylhydroquinone (TBHQ) (Shahidi and Wanasundara 1992; Vitaglione and Fogliano 2004). BHA and BHT are white, monohydric phenolic compounds used to prevent lipid oxidation in fats and oils. BHA is better than BHT at controlling short-chain fatty acid oxidation (Shahidi and Wanasundara 1992). TBHQ is an antioxidant used to stabilize and protect oils from oxidation during high temperature heating. PG, due to its relatively low melting point (148 ˚C), is not used to prevent oxidation in products heated to high temperatures (Shahidi and Wanasundara 1992). There is differing data with regards to antioxidants abilities to inhibit HCA formation due to the fact that some antioxidants show pro- and antioxidative effects in model systems depending on the concentration (Vitaglione and Fogliano 2004).

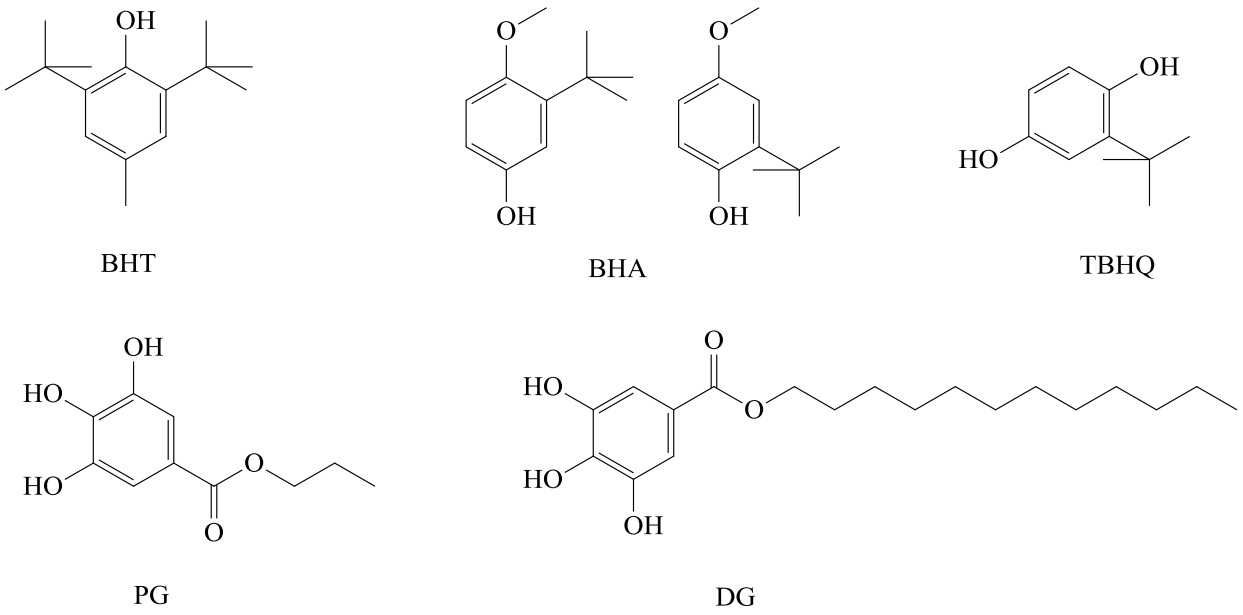


Figure 1-4: Chemical structures of the synthetic antioxidants BHT, BHA, TBHQ, PG, and DG.

The antioxidative effects of synthetic antioxidants being determined by concentration is supported by multiple studies, although there are some discrepancies. Moon and Shin (2013) conducted a study that found BHA inhibited PhIP formation by 62.9-99.2%, but only at a relatively high dose of 1000 ppm. Kikugawa (1999), however, found that as the concentration of BHA was increased in a model system, the HCA inhibition decreased. Ahn and Grün (2005) found that a combination of 0.02% BHT/BHA decreased IQ (17%), MeIQx (57%), and PhIP (22%) formation, but increased MeIQx (3.6%) and DiMeIQx (20%) formation in cooked beef. Another study (Johansson and Jägerstad 1996) found BHA, BHT, and PG significantly increased formation of MeIQx in model systems, while Oguri and others (1998) found no significant effects of BHA on MeIQx and PhIP formation in model systems.

Tea Polyphenols

Tea polyphenols have been shown to reduce HCA formation in meats. Polyphenols are known to have a large free radical scavenging ability, which is thought to inhibit HCA

formation. Weisburger and others (1994) found that black and green tea polyphenols (theaflavine gallate (TFG) and epigallocatechin gallate (EGCG)) reduced MeIQx and PhIP mutagenicity in chemical model systems. Apostolides and others (1996 and 1997) found similar results using black and green teas and their polyphenols to reduce the mutagenicity of only PhIP in model systems.

Tea polyphenols have been found to inhibit the mutagenic activity of HCAs in meat when applied to the outside of meat before frying (Weisburger and others 2002). Cheng and others (2009) researched the effect of EGCG on PhIP formation and the amount of the PhIP intermediate phenylacetaldehyde in a model system. They found that EGCG was effective at inhibiting PhIP formation, and that it reduced the amount of phenylacetaldehyde, up to 90% compared to the control, in the model system. The exact mechanism that allows tea polyphenols to inhibit HCA mutagenic activity and formation is unknown. However, there are a number of hypotheses as to why this is so, including that the polyphenols inhibit the NADPH cytochrome P450 reductase; that they inhibit the mutagenic activity of the *N*-hydroxylated HCAs in vitro; or by electrophile scavenging (Vitaglione and Fogliano 2004). A different study by Cheng and others (2007) showed four tea phenolic compounds (theaflavin 3,3'-digallate, EGCG, epicatechin gallate (ECG), and epigallocatechin (EGC)) effectively inhibit PhIP formation in chemical model systems.

Herbs and Spices

Herbs and spices are commonly used as food ingredients in industry to enhance and alter the flavor and aroma profiles and compositions of food products. Many spices and herbs have been shown to possess antioxidant and free radical scavenging abilities. Table 1-4 shows the TPC and DPPH results, from multiple studies, for some common spices and herbs used in the

food industry. Some of the most common "families" of herbs and spices are Lamiaceae (basil, mint, sage, rosemary, oregano, thyme, lavender), Apiaceae (anise, caraway, parsley, coriander, cumin, dill, fennel), Piperaceae (known as the pepper family), Lauraceae (sassafras, cinnamon, evergreen trees), and Myristicaceae (known as the nutmeg family).

Table 1-4: Total phenolic content (TPC) and DPPH results for a variety of herbs and spices.

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]

Adapted from [1]Damašius and others (2011); [2]Hinneberg and others (2006); [3]Su and others (2007); [4]Wang and others (2008); [5]Wojdylo and others (2007)

Black Pepper

Black pepper comes from the dried, mature fruit of the *Piper nigrum* L. plant, belonging to the Piperaceae family. Black pepper is mainly grown in Brazil, India, Indonesia, Malaysia, Sri Lanka, and Thailand and represents about 35% of the world trade in spices (Zachariah and Parthasarathy 2008). The characteristics of black pepper can be broadly separated into two

categories: compounds that give black pepper its pungency (i.e. piperine, piperanine, chavicine, piperettine, piperylin, and piperolein A and B), and compounds that contribute to the flavor and aroma of black pepper (Ravidran and Kallupurackal 2001; Zachariah and Parthasarathy 2008). The essential oil of black pepper contains compounds that contribute to the flavor and aroma profiles of black pepper. These compounds can be further separated into three categories: 1) monoterpene hydrocarbons and oxygenated compounds, 2) sesquiterpene hydrocarbons and oxygenated compounds, and 3) miscellaneous compounds (such as eugenol and safrole) (Narayanan 2000; Zachariah and Parthasarathy 2008). Black pepper has some phenolic compounds that are a mix of phenolic acid glycosides and flavonol glycosides, including isoquercetin, isorhamnetin, 3-0-β-D-rutinoside, kaempferol 3-0-arabinoside-7-rhamnoside, kaempferol-3-0-β-glucoside, quercetin 3-0-β-D-rutinoside, and sitostrol (Ravidran and Kallupurackal 2001).

While there has not been much research with black pepper and whether or not it has the ability to inhibit HCAs, multiple sources have cited black pepper's low radical scavenging and antioxidative abilities. Tipsrisukond and others (1998) found that piperine exhibited the most antioxidative ability of black pepper compounds. They also found that ground black pepper was a more effective antioxidant than extracted essential oils or oleoresins of black pepper. Vijayakumar and others (2004) discovered that when black pepper or piperine were given to rats on a high fat diet, the thiobarbituric acid reactive substances (TBARS) concentrations were similar to the levels found in the control rats. High TBARS indicate the failure of antioxidant defense mechanisms to prevent excess free radical formation. Another research group found results similar to those by Vijayakumar and others (2004): that essential oils and oleoresins of black pepper had antioxidative and radical scavenging effects within the thiobarbituric acid

(TBA) value, ferric thiocyanate (FTC), and DPPH assays (Kapoor and others 2009). They also found that as the concentration of oil or oleoresin increased, the scavenging effects increased.

One of the few research reports about black pepper's effect on HCA formation was performed by Oz and Kaya (2011). The article described the effect of black pepper on HCA formation in high fat meatballs. They found, overall, that black pepper decreased HCA formation in high fat meatballs, when applied to the meatballs before cooking, by 33% (175 ˚C), 12% (200 ˚C), and 100% (225 ˚C), and that the cooking temperature and the type of HCA affect how well the black pepper inhibits formation.

Rosemary

Rosemary (*Rosmarinus officinalis*) is a perennial herb commonly found in Mediterranean regions of the world. Rosemary is a member of the Lamiaceae family, which also includes herbs such as basil, mint, sage, oregano, lavender, and thyme. Rosemary is widely used in food processing and though it is not technically listed as a natural antioxidant or preservative, it is often used for this purpose in food products (Yanishlieva-Maslarova 2001). While rosemary has a high antioxidant ability, sometimes, due to its strong and unique flavor, strong odors and undesirable tastes can occur, making it unsuitable for use in certain food products (Schuler 1990). The three main chemical compounds thought to give rosemary its antioxidant abilities are rosmarinic acid, carnosol, and carnosic acid (Schuler 1990).

There are differing views on how well rosemary inhibits HCA formation. Multiple studies found that when testing HCA content in cooked beef and beef patties, rosemary as an extract (Puangsombat and Smith 2010), oleoresin (Ahn and Grün 2005), antioxidant powder (Tsen and others 2006), or as rosmarinic acid (Tsen and others 2006) effectively inhibited HCA formation. Other studies have found that rosemary, especially when used in chemical model

systems, does not inhibit PhIP. Damašius and others (2011) found when a dried rosemary extract was added to meat, the concentration of PhIP increased 1.15-1.21 times compared to the control, at two different concentrations. Zöchling and others (2002) found when extracted rosemary flavor was added to a PhIP model system, the PhIP concentration increased slightly compared to the control. A different study, conducted by Cheng and others (2007) used three phenolic acids (rosmarinic, carnosic, and chlorogenic) in a PhIP model system and found that all three enhanced PhIP formation. This same study, however, found that when rosmarinic and chlorogenic acids were added to beef patties, the formation of PhIP and other polar HCAs was significantly ($p < 0.05$) decreased compared to the control.

Chemical Model Systems

Chemical model systems are often used to study the formation, promotion, and inhibition of HCAs. One of the major advantages of using model systems to study HCAs is that many side reactions, which would be present if meat products were used, are reduced or eliminated (Jägerstad and others 1991; Murkovic 2004, 2007). Other components of meat are not present, resulting in a reduction of unwanted side reactions. Model systems are generally used to study the effects of precursors, precursor concentrations, reaction conditions (physical parameters), kinetics, and mechanisms of HCA formation (Jägerstad and others 1991; Skog and others 1998). Model systems can be made with meat juices, which are more relevant when comparing the amounts of HCAs formed in meat, however, these results are significantly more complicated to interpret due to the side reactions produced (Murkovic 2004).

Heterocyclic Amine and PhIP Formation in Model Systems

HCA model systems are composed of creatine or creatinine, amino acid(s), and reducing sugars (typically glucose) (Felton and Knize 1991; Knize and Felton 2008). The IQ type HCAs

(IQ, IQx, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx) have been formed in model systems containing creatine, glucose, and either glycine, threonine, lysine, serine, or alanine (Jägerstad and others 1991; Skog and others 1998). PhIP model systems typically contain glucose, creatin(in)e, and phenylalanine, although there have been some reports of PhIP forming with leucine, isoleucine, or tyrosine (Jägerstad and others 1991; Johansson and others 1995). Model systems are usually created in sealed vials and then heated at either one or multiple temperatures to form HCAs. Meat and meat juice model systems have also been used, however, due to the many side reactions and constituents created during the heating of meat and meat juices, chemical model systems are often used to lessen or eliminate those side reactions.

Heterocyclic Amine and PhIP Inhibition in Model Systems

Inhibiting or preventing the formation of HCAs is the focus of many research papers and studies. A more in-depth review of HCA inhibition is given in a previous section (pg. 10). Multiple studies have found a dose-dependent relationship between HCA inhibition and antioxidants (Johansson and Jägerstad 1996; Kikugawa and others 2000; Moon and Shin 2013) and phenolic compounds (Apostolides and others 1996,1997). Natural antioxidants and spices have been shown to inhibit HCA formation within model systems. Cheng and others (2009) tested the effects of EGCG and EGCG peracetate on PhIP and found both to significantly inhibit PhIP's formation in their model system. Kato and others (1996) also tested EGCG, along with BHA, PG, sesamol, and esculetin, as HCA inhibitors and found that all of them inhibited the formation of imidazoquinoxaline-type HCAs in a model system. The phenolic components of tea have been studied by Cheng and others (2007) and Weisburger and others (1994), both studies found that tea polyphenols were effective at inhibiting the formation of HCAs. Wong and others (2012) studied the effect of 11 different water-soluble vitamins on HCA formation. They found

that seven of the vitamins (B_1, B_3, pyridoxamine, pyridoxine, pyridoxal, B_7, and ascorbic acid) significantly inhibited the formation of HCAs in their model system.

Heterocyclic Amine and PhIP Promotion in Model Systems

There are very few studies that discussed the promotion of HCAs or PhIP in model systems. Of those that do discuss promotion, the experiments were set up to study inhibitory effects of certain compounds, instead of promoting effects. Johansson and Jägerstad (1996) studied the effects of pro- and antioxidants on heterocyclic amine formation in a model system. They found that many of the antioxidants increased the formation of the HCAs they were testing. They also discovered that the inhibitory effects of antioxidants were dose-dependent, meaning that antioxidants became pro-oxidants at higher concentrations. The antioxidants tested included BHA, BHT, TBHQ, tocopherols, PG, and ascorbic acid. Ascorbic acid was found to inhibit HCA formation, while the other antioxidants either increased formation or had no statistical significance. Vitaglione and Fogliano (2004) reviewed multiple papers and studies about the effects of antioxidants on HCA formation. While there are some conflicting results between studies, it has been made clear that the dose of antioxidant determines its ability to inhibit or promote formation.

Cheng and others (2007) found that three phenolic acids (rosmarinic, carnosic, and chlorogenic) increased the formation of PhIP. They found that rosmarinic and carnosic acid significantly increased PhIP formation, while chlorogenic acid caused no significant increase in PhIP. Wong and others (2012) studied the effect of 11 different water-soluble vitamins on HCA formation in a model system. They found that vitamins B_2, B_5, B_9, and B_{12} had no significant inhibitory effect on HCA formation. Even though none of the vitamins tested showed promotion, it is also important to determine which additives, spices, antioxidants, etc. have no significant

effect on HCAs because inhibiting or preventing formation is, usually, the overall goal when studying these effects.

Conclusion

While it has been hypothesized that an increase in HCA consumption increases a person's risk of cancer, much more research is needed in order to definitively prove a correlation between the two. There are a number of ways shown to reduce the formation of HCAs in meat, mainly consisting of adding compounds, such as spices or antioxidants, and lowering the cooking temperature of meat products.

Chemical model systems are useful for studying the effects of different treatments or conditions on HCA formation. Cooking time and temperature, type of precursors, precursor concentrations, and additives, such as spices and antioxidants are all variables that can be easily tested within a model system. While there have been numerous studies on some common antioxidants (BHT, BHA, TBHQ, EGCG), spices (rosemary, thyme, sage), and components of those spices (rosmarinic, carnosic, chlorogenic acids), there has been little research on compounds or common food ingredients, such as black pepper, that do not have high phenolic contents. The study of this and other common household spices and additives could, hopefully, lead to a better understanding of HCA inhibition.

References

Ahn J, Grün IU. 2005. Heterocyclic amines: 2. Inhibitory effects of natural extracts on the formation of polar and nonpolar heterocyclic amines in cooked beef. J. Food Sci. 70:C263-C268.

Arvidsson P, van Boekel MAJS, Skog K, Jägerstad M. 1997. Kinetics of formation of polar heterocyclic amines in a meat model system. J. Food Sci. 62:911-916.

Arvidsson P, van Boekel MAJS, Skog K, Solyakov A, Jägerstad M. 1999. Formation of heterocyclic amines in a meat juice model system. J. Food Sci. 64:216-221.

Apostolides Z, Balentine DA, Harbowy ME, Weisburger JH. 1996. Inhibition of 2-amno-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) mutagenicity by black and green tea extracts and polyphenols. Mutat. Res. 359:159-163.

Apostolides Z, Balentine DA, Harbowy ME, Hara Y, Weisburger JH. 1997. Inhibition of PhIP mutagenicity by catechins, and by theaflavins and gallate esters. Mutat. Res. 389:167-172.

Bordas M, Moyano E, Puignou L, Galceran MT. 2004. Formation and stability of heterocyclic amines in a meat flavour model system effect of temperature, time and precursors. J. Chrom. B. 802:11-17.

Borgen E, Solyakov A, Skog K. 2001. Effects of precursor composition and water on the formation of heterocyclic amines in meat model systems. Food Chem. 74:11-19.

Brown W, Poon T. 2011. Introduction to Organic Chemistry. 4th ed. Indianapolis, IN: John Wiley & Sons, Inc. 801p.

Cheng KW, Chen F, Wang M. 2006. Heterocyclic amines: Chemistry and health. Mol. Nutr. Food Res. 50:1150-1170.

Cheng KW, Chen F, Wang M. 2007. Inhibitory activities of dietary phenolic compounds on heterocyclic amine formation in both chemical model system and beef patties. Mol. Nutr. Food Res. 51:969-976.

Cheng KW, Wong CC, Chao J, Lo C, Chen F, Chu IK, Che CM, Ho CT, Wang M. 2009. Inhibition of the mutagenic PhIP formation by epigallocatechin gallate via scavenging of phenylacetaldehyde. Mol. Nutr. Food Res. 53:716-726.

Damašius J, Venskutonis PR, Ferracane R, Fogliano V. 2011. Assessment of the influence of some spice extracts on the formation of heterocyclic amines in meat. Food Chem. 126:149-156.

Felton JS, Knize MG. 1991. Occurrence, identification, and bacterial mutagenicity of heterocyclic amines in cooked food. Mutat. Res. 259:205-217.

Felton JS, Knize MG, Shen NH, Lewis PR, Andresen BD, Happe J, Hatch FT. 1986. The isolation and identification of a new mutagen from fried ground beef: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Carcinogenesis. 7:1081-1086.

Gooderham NJ, Creton S, Lauber SN, Zhu H. 2007. Mechanisms of action of the carcinogenic heterocyclic amine PhIP. Toxicol. Lett. 168:269-277.

Gooderham NJ, Zhu H, Lauber S, Boyce A, Creton S. 2002. Molecular and genetic toxicology of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Mutat. Res. 506-507:91-99.

Grün IU. 2009. Chapter 13: Antioxidants. In: Tarté R, editor. Ingredients in Meat Products: Properties, Functionality, and Applications. New York: Springer Science+Business Media, LLC. p 291-300.

Gülçin I. 2005. The antioxidant and radical scavenging activities of black pepper (*Piper nigrum*) seeds. Int. J. Food Sci. Nutr. 56:491-499.

Hinneburg I, Dorman HJD, Hiltunen R. 2006. Antioxidant activities of extracts from selected culinary herbs and spices. Food Chem. 97:122-129.

IARC. 1997. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. In: IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, vol. 56. Lyon: World Health Organization, International Agency for Research on Cancer. p 163-242.

Jägerstad M, Skog K, Arvidsson P, Solyakov A. 1998. Chemistry, formation and occurrence of genotoxic heterocyclic amines identified in model systems and cooked foods. Eur. Food Res. Technol. 207:419-427.

Jägerstad M, Skog K, Grivas S, Olsson K. 1991. Formation of heterocyclic amines using model systems. Mutat. Res. 259:219-233.

Johansson MAE, Fay LB, Gross GA, Olsson K, Jägerstad M. 1995. Influence of amino acids on the formation of mutagenic/carcinogenic heterocyclic amines in a model system. Carcinogenesis. 16: 2553-2560.

Johansson MAE, Jägerstad M. 1996. Influence of pro- and antioxidants on the formation of mutagenic-carcinogenic heterocyclic amines in a model system. Food Chem. 56:69-75.

Kapoor IPS, Singh B, Singh G, De Heluani CS, De Lampasona MP, Catalan CAN. 2009. Chemistry and in vitro antioxidant activity of volatile oil and oleoresins of black pepper (*Piper nigrum*). J. Agric. Food Chem. 57:5358-5364.

Kataoka H. 1997. Methods for the determination of mutagenic heterocyclic amines and their applications in environmental analysis. J. Chrom. A. 774:121-142.

Kataoka H, Miyake M, Saito K, Mitani K. 2012. Formation of heterocyclic amine-amino acid adducts by heating in a model system. Food Chem. 130:725-729.

Kato T, Harashima T, Moriya N, Kikugawa K, Hiramoto K. 1996. Formation of the mutagenic/carcinogenic imidazoquinoxaline-type heterocyclic amines through the unstable free radical Maillard intermediates and its inhibition by phenolic antioxidants. Carcinogenesis. 17:2469-2476.

Kikugawa K. 1999. Involvement of free radicals in the formation of heterocyclic amines and prevention by antioxidants. Cancer Lett. 143:123-126.

Kikugawa K, Hiramoto K, Kato T. 2000. Prevention of the formation of mutagenic and/or carcinogenic heterocyclic amines by food factors. BioFactors. 12:123-127.

Knize MG, Felton JS. 2008. Heterocyclic amines. In: Picó Y. Food Contaminants and Residue Analysis, vol. 51. Atlanta, GA: Elsevier. p 685-704.

Li D, Day RS, Bondy ML, Sinha R, Nguyen NT, Evans DB, Abbruzzese JL, Hassan MM. 2007. Dietary mutagen exposure and risk of pancreatic cancer. Cancer Epidemiol. Biomarkers Prev. 16:655-661.

Moon SE, Shin HS. 2013. Inhibition of mutagenic 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) formation using various food ingredients in a model system. Food Sci. Biotechnol. 22:323-329.

Murkovic M. 2004. Formation of heterocyclic aromatic amines in model systems. J. Chrom. B. 802:3-10.

Murkovic M. 2007. Analysis of heterocyclic aromatic amines. Anal. Bioanal. Chem. 389:139-146.

Narayanan CS. 2000. Chemistry of black pepper. In: Ravindran PN, editor. Black Pepper: *Piper nigrum*. Australia: Harwood Academic Publishers. p 143-162.

Nowell S, Coles B, Sinha R, MacLeod S, Ratnasinghe DL, Stotts C, Kadlubar FF, Ambrosone CB, Lang NP. 2002. Analysis of total meat intake and exposure to individual heterocyclic amines in a case-control study of colorectal cancer: contribution of metabolic variation to risk. Mutat. Res. 506-507:175-185.

NTP (National Toxicology Program). 2014. Heterocyclic amines. In: Report on Carcinogens. 13th ed. Research Triangle Park, NC: U.S. Department of Health and Human Services. Available from: http://ntp.niehs.nih.gov/pubhealth/roc/roc13/.

Oguri A, Suda M, Totsuka Y, Sugimura T, Wakabayashi K. 1998. Inhibitory effects of antioxidants on formation of heterocyclic amines. Mutat. Res. 402:237-245.

Oz F, Kaya M. 2011. The inhibitory effect of black pepper on formation of heterocyclic aromatic amines in high-fat meatball. Food Control. 22:596-600.

Puangsombat K, Gadgil P, Houser TA, Hunt MC, Smith JS. 2012. Occurrence of heterocyclic amines in cooked meat products. Meat Sci. 90:739-746.

Puangsombat K, Smith JS. 2010. Inhibition of heterocyclic amine formation in beef patties by ethanolic extracts of rosemary. J. Food Sci. 75:T40-T47.

Ravindran PN, Kallupurackal JA. 2001. Black Pepper. In: Peter KV, editor. Handbook of Herbs and Spices, vol. 1. Cambridge, England: Woodhead Publishing Limited. p 62-110.

Robbana-Barnat S, Rabache M, Rialland E, Fradin J. 1996. Heterocyclic amines: occurrence and prevention in cooked food. Environmental Health Perspectives. 104:280-288.

Schuler P. 1990. Natural antioxidants exploited commercially. In: Hudson BJF, editor. Food Antioxidants. London: Elsevier Applied Science. p 99-170.

Shahidi F, Wanasundara PK. 1992. Phenolic Antioxidants. CRC Crit. Rev. Food Nutr. 32:67-103.

Shioya M, Wakabayashi K, Sato S, Nagao M, Sugimura T. 1987. Formation of a mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in cooked beef, by heating a mixture containing creatinine, phenylalanine, and glucose. Mutat. Res. 191:133-138.

Shirai T, Sano M, Tamano S, Takahashi S, Hirose M, Futakuchi M, Hasegawa R, Imaida K, Matsumoto KI, Wakabayashi K, Sugimura T, Ito N. 1997. The prostate: a target for carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5*b*]pyridine (PhIP) derived from cooked foods. Cancer Res. 57:195-198.

Sinha R, Gustafson DR, Kulldorf M, Wen WQ, Cerhan JR, Zheng W. 2000. 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, a carcinogen in high-temperature-cooked meat, and breast cancer risk. J. Nat. Cancer Institute. 92:1352-1354.

Skog K, Jägerstad M. 1990. Effects of monosaccharides and disaccharides on the formation of food mutagens in model systems. Mutat. Res. 230:263-272.

Skog KI, Johansson MAE, Jägerstad MI. 1998. Carcinogenic heterocyclic amines in model systems and cooked foods: a review on formation, occurrence and intake. Food and Chem. Toxicol. 36:879-896.

Skog K, Solyakov A, Jägerstad M. 2000. Effects of heating conditions and additives on the formation of heterocyclic amines with reference to amino-carbolines in a meat juice model system. Food Chem. 68:299-308.

Sugimura T. 2000. Nutrition and dietary carcinogens. Carcinogenesis. 21:387-395.

Sugimura T, Nagao M, Kawachi T, Honda M, Yahagi T, Seino Y, Sato S, Matsukura N, Matsushima T, Shirai A, Sawamura A, Matsumoto H. 1977. Mutagen-carcinogens in foods with special reference to highly mutagenic pyrolytic products in broiled foods. In: Hiatt HH, Watson JD, Winsten JA, editors. Origins of Human Cancer. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory. p 1561-1577.

Su L, Yin JJ, Charles D, Zhou K, Moore J, Yu L. 2007. Total phenolic contents, chelating capacities, and radical-scavenging properties of black peppercorn, nutmeg, rosehip, cinnamon and oregano leaf. Food Chem. 100:990-997.

Tipsrisukond N, Fernando LN, Clarke AD. 1998. Antioxidant effects of essential oil and oleoresin of black pepper from supercritical carbon dioxide extractions in ground pork. J. Agric. Food Chem. 46:4329-4333.

Tsen SY, Ameri F, Smith JS. 2006. Effects of rosemary extracts on the reduction of heterocyclic amines in beef patties. J. Food Sci. 71:C469-C473.

Vijayakumar RS, Surya D, Nalini N. 2004. Antioxidant efficacy of black pepper (*Piper nigrum* L.) and piperine in rats with high fat diet induced oxidative stress. Redox Rep. 9:105-110.

Vijayan KK, Thampuran RVA. 2000. Pharmacology, toxicology and clinical applications of black pepper. In: Ravindran PN, editor. Black Pepper: *Piper nigrum*. Australia: Harwood Academic Publishers. p 455-466.

Vitaglione P, Fogliano V. 2004. Use of antioxidants to minimize the human health risks associated to mutagenic/carcinogenic heterocyclic amines in food. J. Chrom. B. 802:189-199.

Wakabayashi K, Nagao M, Esumi H, Sugimura T. 1992. Food-derived mutagens and carcinogens. 1992. Cancer Res. 52:2092s-2098s.

Wang HF, Wang YK, Yih KH. 2008. DPPH free-radical scavenging ability, total phenolic content, and chemical composition analysis of forty-five kinds of essential oils. J. Cosmet. Sci. 59:509-522.

Weisburger JH, Nagao M, Wakabayashi K, Oguri A. 1994. Prevention of heterocyclic amine formation by tea and tea polyphenols. Cancer Lett. 83:143-147.

Weisburger JH, Veliath E, Larios E, Pittman B, Zang E, Hara Y. 2002. Tea polyphenols inhibit the formation of mutagens during the cooking of meat. Mutat. Res. 516:19-22.

Widmark EMP. 1939. Presence of cancer-producing substances in roasted food. Nature. 143:984.

Wojdylo A, Oszmiański J, Czemerys R. 2007. Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chem. 105:940-949.

Wong D, Cheng KW, Wang M. 2012. Inhibition of heterocyclic amine formation by water-soluble vitamins in Maillard reaction model systems and beef patties. Food Chem. 133:760-766.

Yanishlieva-Maslarova NV. 2001. Rosemary and sage as antioxidants. In: Peter KV, editor. Handbook of herbs and spices, vol. 1. Cambridge, England: Woodhead Publishing Limited. p 269-275.

Zachariah TJ, Parthasarathy VA. 2008. Black Pepper. In: Parthasarathy VA, Chempakam B, Zachariah TJ, editors. Chemistry of Spices. United Kingdom: Biddles Ltd, King's Lynn. p 21-40.

Zamora R, Alcón E, Hidalgo FJ. 2013. Effect of amino acids on the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in creatinine/phenylalanine and creatinine/phenylalanine/4-oxo-2-nonenal reaction mixtures. Food Chem. 141:4240-4245.

Zhu J, Chang P, Bondy ML, Sahin AA, Singletary SE, Takahashi S, Shirai T, Li D. 2003. Detection of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-DNA adducts in normal breast tissues and risk of breast cancer. Cancer Epide., Biomarkers, Prevent. 12:830-837.

Zöchling S, Murkovic M. 2002. Formation of the heterocyclic aromatic amine PhIP: identification of precursors and intermediates. Food Chem. 79:125-134.

Zöchling S, Murkovic M, Pfannhauser W. 2002. Effects of industrially produced flavours with pro- and antioxidative properties on the formation of the heterocyclic amine PhIP in a model system. J. Biochem. Biophys. Methods. 53:37-44.

Chapter 2 - Solvent concentration comparisons in model systems, total phenolic content (TPC), and DPPH radical scavenging activity of synthetic antioxidants, spices, and spice extracts

Abstract

Heterocyclic amines (HCAs) are chemical compounds formed as by-products of heating meat and fish. PhIP, specifically, is the most abundantly formed HCA. Antioxidants and spices have been shown to inhibit the formation of HCAs in model systems. The effect of solvent concentrations on PhIP formation was performed. Each model system contained amounts of 0.22 mmol glucose, 0.44 mmol creatinine, and 0.44 mmol phenylalanine and was heated at 180 ˚C for one hour. The ratios of 20:80, 15:85, 10:90, 5:95, and 0:100 water:diethylene glycol (v/v) were tested. The ratio of 10:90 was chosen as the best option for PhIP formation in the model system. The total phenolic content (TPC) and DPPH free radical scavenging ability of two synthetic antioxidants (butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ)) and two spices (black pepper and rosemary) were also determined. The TPC results, in mg gallic acid equivalents, were 0.420 for ground black pepper, 2.433 for the black pepper oil, 0.318 for the black pepper extract, 1.983 for rosemary, 1.07 for the rosemary extract, 63.53 for BHT, and 93.10 for TBHQ. The DPPH results were 2.74% for ground black pepper, 76.74% for the black pepper oil, 9.20% for the black pepper extract, 46.32% for rosemary, 91.13% for the rosemary extract, 24.75% for BHT, and 12.44% for TBHQ.

Introduction

In order to study the formation, promotion, and inhibition of HCAs, scientists often use chemical model systems. Model systems are used to mimic precursor concentrations, types of precursors, and time and heating conditions of meat. They are also used to lessen or eliminate complex, and many times unwanted, side reactions that occur when cooking meat (Jägerstad and others 1991; Murkovic 2004, 2007). Model systems are also used to determine ideal cooking conditions and food sources for HCA formation (Knize and Felton 2005). In general, model systems are defined as containing a reducing sugar, one or more amino acids, and creatin(in)e in an aqueous system (Cheng and others 2006; Knize and Felton 2005). In order to form PhIP in a model system, glucose, creatin(in)e, and phenylalanine are needed.

Two common amounts of these three precursors are 0.2 mmol glucose, 0.4 mmol creatin(in)e, and 0.4 mmol phenylalanine (Cheng and others 2007, 2009; Oguri and others 1998; Wong and others 2012), and 0.45 mmol glucose, 0.9 mmol creatin(in)e, and 0.9 mmol phenylalanine (Johansson and Jägerstad 1996; Johansson and others 1995; Skog and Jägerstad 1991). Many times, these model systems use diethylene glycol, either instead of water or 1-20% water, to promote heat transfer throughout the system (Cheng and others 2006). These model systems are then heated in a sealed glass vial to maximize formation and minimize any evaporation of water that may occur. Chemical model systems allow scientists to easily study the effects of precursors, precursor concentrations, reaction conditions (e.g. time, temperature), kinetics, and added inhibitors, such as antioxidants, herbs, and spices, on the formation and inhibition of HCAs (Jägerstad and others 1991; Skog and others 1998).

Antioxidants and spices are often used in an attempt to inhibit HCA formation in both meat and chemical model systems. Antioxidants are used in the food industry to prevent or slow lipid oxidation and to scavenge free radicals. Free radicals are produced in the body as by-

products of metabolism or are formed from exposure to harmful compounds or radiation (Vijayakumar and others 2004). Many antioxidants, herbs, and spices have shown high phenolic contents and radical scavenging abilities (Hinneburg and others 2006; Salazar and others 2014; Shahidi and Wanasundara 1992; Su and others 2007; Wang and others 2008). Polyphenolics and free radical scavengers have been thought to be effective HCA inhibitors (Murkovic 2004).

For this reason, it is important to be able to quantify the antioxidative abilities of compounds in order to compare their effectiveness as free radical scavengers. TPC and DPPH free radical scavenging ability assays are two ways to predict how effective an antioxidant, herb, or spice may be at inhibiting HCAs by measuring their hydroxyl groups. The purpose of this study was to determine an effective chemical model system to study PhIP formation and inhibition and to determine the TPC and DPPH values for ground black pepper, black pepper oil, black pepper extract, rosemary, rosemary extract, BHT, and TBHQ. Some of these compounds will be used in a later study to determine their ability to inhibit PhIP formation in the chemical model system.

Materials and Methods

Materials

McCormick Science Institute (MSI) (Hunt Valley, MD, U.S.A.) provided standard samples of ground black pepper blend, and rosemary powder. The 100% pure *Piper nigrum* oil (steam distilled black pepper) was purchased from Nature's Kiss Essential Oils (Moreno Valley, CA, U.S.A.). A PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) standard was purchased from Toronto Research Chemicals, Inc (Ontario, Canada). Sodium bicarbonate, methanol (*Optima*), acetonitrile (certified ACS), syringe filters (nylon, 0.2 μm), and acetic acid was purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Triethylamine (BioUltra), Folin

& Ciocalteu's phenol reagent, 2,2-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), D-(+)-Glucose, gallic acid, L-phenylalanine, creatinine, and diethylene glycol (BioUltra) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A). Ethanol (200 proof) was purchased from Decon Laboratories, Inc (King of Prussia, PA, U.S.A.). Deionized water was prepared using a Sybron/Barnstead PCS unit (Barnstead/Thermolyne, Inc, Dubuque, IA, U.S.A.).

Model System Preparation

The model systems were prepared as described by Cheng and others (2007) with modifications. Solutions of 1 mmol of glucose (0.18016 g), creatinine (0.11312 g), and phenylalanine (0.16519 g) were made in 10 mL volumetric flasks. The final precursor amounts were 0.2 mmol glucose, 0.4 mmol creatinine, and 0.4 mmol phenylalanine, made by mixing 110 μL of the 1 mmol glucose solution, and 220 μL of the 1 mmol creatinine and phenylalanine solutions in a 1 mL reaction vial, for a total volume of 550 μL. The effect of deionized water:diethylene glycol (v/v) was tested by preparing solutions of 20:80, 15:85, 10:90, 5:95, and 0:100 water:diethylene glycol for each 1 mmol concentration of precursor amounts. For example, a solution of 1 mmol glucose was made with a 20:80 water:diethylene glycol ratio, a separate solution of 1 mmol glucose was made with a 15:85 water:deithylene glycol ratio, etc. for each precursor.

The reaction vials were then placed inside brass vessels, as described in Hussain (2015), and tightly closed to prevent water loss during heating. The brass vessels were placed inside a forced air oven set at 180 ˚C and heated for 1 hour.After 1 hour, the brass vessels were removed and cooled on ice for 5 minutes before removing the reaction vials and storing at room

temperature for further analysis. All model systems were diluted 1:10 with methanol and syringe filtered before HPLC analysis.

Analysis of PhIP

Analysis of PhIP was achieved with an HP 1050 series HPLC with auto-sampler, coupled with and HP 1050 series UV detector and an HP 1046A programmable fluorescence detector according to the methods described by Gross and others (1992) and Gross and Grüter (1992) with slight modifications. PhIP separation was obtained by reversed-phase chromatography using a TSKgel ODS-80T_M (4.6 mm x 25 cm x 5 μm) column fitted with a TSKgel guardgel ODS-80T_M (3.2 mm x 1.5 cm) guard column (TOSOH Biosciences; Tokyo, Japan). The mobile phases used were 0.01 M triethylamine, buffer adjusted to pH 3.6 with acetic acid (A) and acetonitrile (B).

A mobile phase system gradient was used, as described by Puangsombat and others (2012) with slight modifications. The linear gradient began with 95% A and 5% B, changing to 75% A and 25% B over 30 minutes. The ratio of 75% A and 25% B was held constant for 5 minutes (30-35 minutes), before returning to the original ratio of 95% A and 5% B for a total run time of 45 minutes. A 10 minute equilibration of the column was added after each sample with a mobile phase ration of 95% A and 5% B. The mobile phase was sent through the system at a rate of 1.0 mL/minute, with the column temperature set at 40 ˚C. For PhIP detection, the UV detector was set at 315 nm and the fluorescence detector had an emission of 229 nm and an excitation of 437 nm.

Total Phenolic Content (TPC) of Antioxidants and Spices

Total phenolic content (TPC) was performed as described by Chang and others (2006) with slight modifications. Black pepper and rosemary were prepared for TPC by mixing 1.00 g

of each spice with 10 mL of 95% ethanol. The solutions were then shaken for 2 hours on a wrist-action shaker (Burrell Corporation; Pittsburg, PA, U.S.A.). After 2 hours, the solutions were filtered using Whatman #4 filter paper (Whatman International Ltd.; Maidstone, United Kingdom) and analyzed for TPC.

The synthetic antioxidants (BHT and TBHQ) were prepared by dissolving 0.25 g of each in 10 mL of 95% ethanol. Both the antioxidant and spice solutions were diluted 1/5 (v/v) with 95% ethanol. An aliquot of 0.1 mL of the antioxidant or spice solutions were mixed with 2 mL of deionized water and 0.2 mL of Folin-Ciocalteu's reagent and was held at room temperature for 6 minutes. One mL of 7.5% sodium bicarbonate solution was added to each sample and the samples were held in the dark, at room temperature, for 2 hours.

A blank was prepared by mixing 2 mL of water with 0.2 mL of Folin-Ciocalteu's reagent, holding for 6 minutes, adding 1 mL of 7.5% sodium bicarbonate, and storing in the dark, at room temperature, for 2 hours. A stock solution of gallic acid (500 μg/mL) was prepared in a volumetric flask with deionized water. To make standards, starting with 250 μg/mL, the stock solution was serial diluted with deionized water to make 5 total standards for a calibration curve. A 0.1 mL aliquot of each standard solution was mixed with 2 mL of deionized water and 0.2 mL of Folin-Ciocalteu's reagent and held at room temperature for 6 minutes. One mL of 7.5% sodium bicarbonate was added and stored in the dark, at room temperature, for 2 hours.

A Genesys 10vis spectrophotometer (Thermo Scientific; Waltham, MA, U.S.A.) was set to 765 nm. The blank was measured and subtracted to make the absorbance zero. Each standard or sample was then read and the absorbance recorded. The results were reported as μg gallic acid equivalents per 1.0 g dried spice, extract, oil, or antioxidant using a standard curve (absorbance

vs. μ/mL). The coefficient of determination (R^2) for the standard curve was 0.9998 (Appendix A, Figure A-1).

DPPH Radical Scavenging Activity of Antioxidants and Spices

DPPH radical scavenging activity was performed as described by Singh and others (2002) with slight modifications. Samples of spices or antioxidants were prepared by mixing 0.2 g of the sample with 100 mL of 95% ethanol and shaking at room temperature with a wrist-action shaker (Burrell Corporation; Pittsburg, PA, U.S.A.). After 2 hours, the samples were filtered through Whatman #4 filter paper (Whatman International Ltd.; Maidstone, United Kingdom). An aliquot of 0.1 mL of each spice extract, antioxidant extract, oil, or, for the control, 95% ethanol, was mixed with 2.9 mL of freshly prepared 0.1 mM DPPH methanolic solution, and stored in the dark, at room temperature, for 30 minutes.

A Genesys 10vis spectrophotometer (Thermo Scientific; Waltham, MA, U.S.A.) was set to 517 nm. A blank of ethanol was read and the absorbance set to zero. The control, spice, or antioxidant sample absorbance was read and recorded. The percent radical scavenging activity was calculated using the equation:

$$DPPH\ scavenging\ activity\ (\%) = [\frac{(Abs_{control} - Abs_{sample})}{Abs_{control}}]\ x\ 100$$

Quantification and Statistical Analysis

A 1000 ppb PhIP standard stock solution was made by dissolving 0.1 mg of PhIP in 100 mL of methanol. A standard curve was made by preparing and analyzing, using fluorescence detection, PhIP standards with concentrations of 31.25, 62.5, 125, 250, and 500 ppb (Appendix A, Figure A-2). The coefficient of determination (R^2) was 0.9999.

Results

Solvent Concentration Comparisons

PhIP was separated by the HPLC method described above and eluted at 30 minutes. PhIP was quantified by calculating the amount of PhIP (μg/L) using a standard curve (Appendix A, Figure A-2). The results of five different water:diethylene glycol ratios are shown in Table 2-1. No PhIP was formed in a model system of 100:0 water:diethylene glycol (v/v) at heating times of 30, 60, 90, or 120 minutes at 180 ˚C.

Table 2-1: Effect of water:diethylene glycol ratio on PhIP formation in a model system

Water (%)	Diethylene glycol (%)	Average PhIP Concentration (mg/L)	Coefficient of Variation (%)	Water Activity
20	80	1.357 ± 0.185	13.65	UR*
15	85	1.304 ± 0.156	11.93	0.7002
10	90	1.469 ± 0.137	9.310	0.6251
5	95	0.763 ± 0.479	62.71	UR*
0	100	0.736 ± 0.327	44.47	UR*

*UR=unable to read due to diethylene glycol interference with the water activity meter

Figure 2-1 shows the effect of the amount of water in the model system on the formation of PhIP. The ratio of 10:90 water:diethylene glycol (v/v) was determined to be the best choice for continuing model system experiments because it produced the most PhIP, had the smallest standard deviation, and had the lowest coefficient of variation (CV) of 9.310.

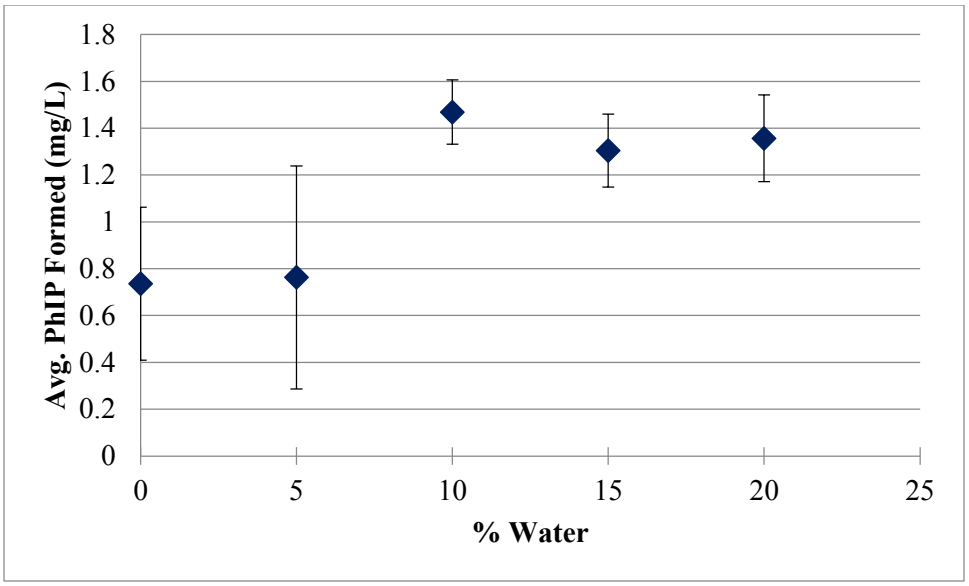


Figure 2-1: Relationship between amount of water in the chemical model system and average PhIP formation. The bars represent one standard deviation for each ratio.

Total Phenolic Content

Total phenolic content (TPC) is used to measure the number of hydroxyl groups in a compound. As the number of hydroxyl groups increases, the antioxidant activity of a compound should increase. The TPC results, in mg of gallic acid equivalents, were 0.420 mg for ground black pepper, 2.433 mg for the black pepper oil, 0.318 mg the for black pepper extract, 1.983 mg for rosemary, 1.070 mg for the rosemary extract, 254.12 mg for BHT, and 372.4 mg for TBHQ per 1.0 g of each compound. Figure 2-2 shows a visual representation of TPC for ground black pepper, black pepper oil, black pepper extract, rosemary, and rosemary extract.

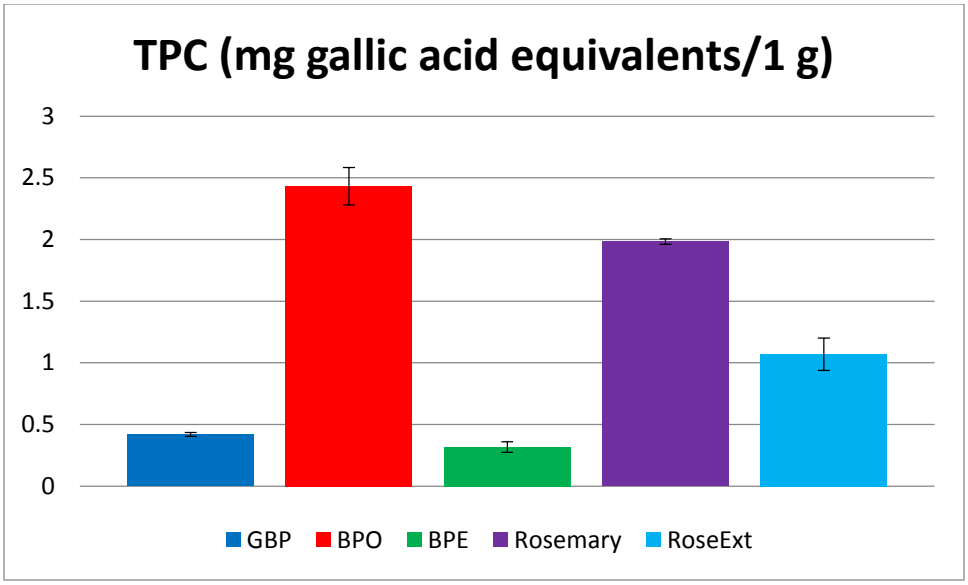


Figure 2-2: Total phenolic contents of ground black pepper (GBP), black pepper oil (BPO), black pepper extract (BPE), rosemary, and rosemary extract (RoseExt) in mg gallic acid equivalents/1 g of compound (n=3). The bars represent one standard deviation for each spice or extract.

DPPH

The DPPH free radical scavenging activity assay is used to determine the free radical scavenging ability of antioxidants and spices. The DPPH results were 2.74% for ground black pepper, 76.74% for the black pepper oil, 9.20% for the black pepper extract, 46.32% for rosemary, 91.13% for the rosemary extract, 24.75% for BHT, and 12.44% for TBHQ. Figure 2-3 shows a visual representation of DPPH radical scavenging ability of ground black pepper, black pepper oil, black pepper extract, rosemary, rosemary extract, BHT, and TBHQ.

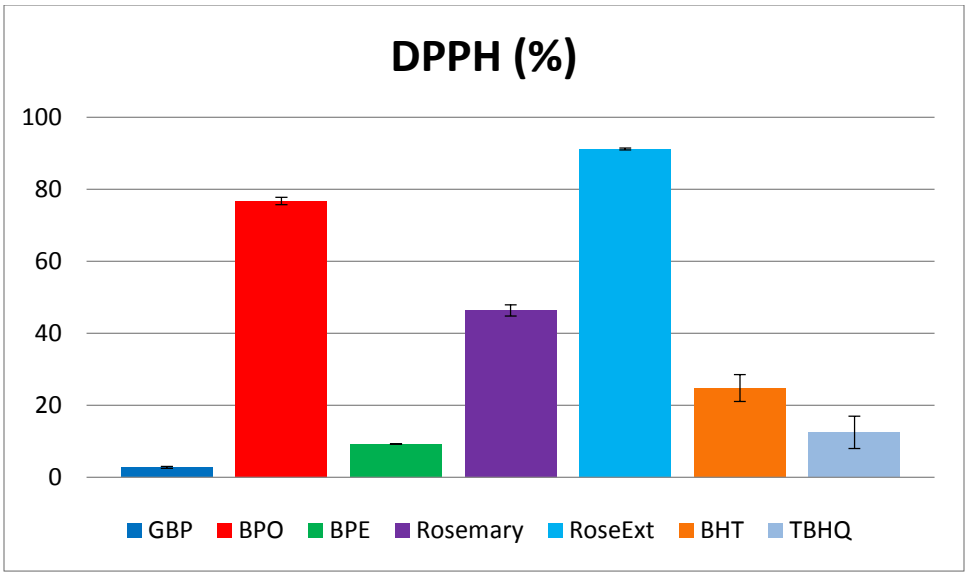


Figure 2-3: DPPH radical scavenging abilities (%) of ground black pepper (GBP), black pepper oil (BPO), black pepper extract (BPE) , rosemary, rosemary extract (RoseExt), BHT, and TBHQ. The bars represent one standard deviation for each spice, extract, or antioxidant (n=3).

Discussion

When creating a chemical model system to study the formation and inhibition of HCAs, it is important to determine the best parameters in order to effectively study formation. The precursor amounts of 0.2 mmol glucose, 0.4 mmol creatinine, and 0.4 mmol phenylalanine are similar, if not identical, to concentrations already determined to give an optimum amount of PhIP for study in chemical model systems (Cheng and others 2007, 2009; Oguri and others 1998; Wong and others 2012). Other precursor concentrations have been used, but it is typical for the creatinine and phenylalanine concentrations to be the same and the glucose concentration to be half the concentration of creatinine and phenylalanine.

The preliminary results for this study, pertaining to water:diethylene glycol ratios within a chemical model system, determined a ratio of 10:90 water:diethylene glycol (v/v) to be the best for this model system based on the amount of PhIP formed and variability. The higher water ratios remained about the same, indicating that it should not matter if 10, 15, or 20% water is

used within the model system. However, the lower water ratios of 0 and 5% water showed large decreases in PhIP formation, and large increases in both standard deviations and CV percentages, indicating that at these ratios the formation of PhIP are not as consistent as the higher water ratios (Appendix A, Table A-1). When a 100% water model system was tested, no PhIP was formed, indicating that the use of diethylene glycol to promote heat transfer is needed to form PhIP in chemical model systems. The ratio at which PhIP starts to decrease in formation, after increasing the amount of water, was not determined in this study.

TPC and DPPH are two common methods used to determine the antioxidant activity of compounds. TPC measures the total polyphenols of a compound by determining the amount of hydroxyl groups of a compound. These hydroxyl groups stabilize free radicals by donating hydrogen atoms. A high antioxidant activity and higher TPC value are expected when more hydroxyl groups are present in a compound. DPPH also measures the number of hydroxyl groups in a compound by determining a compound's ability to reduce DPPH, changing the solution's color from dark violet to yellow. The more hydroxyl groups present, the greater color change of the solution.

The results of this study show ground black pepper and the black pepper extract had low TPC and DPPH, indicating a low amount of hydroxyls present. The black pepper oil showed higher TPC and DPPH values, indicating a decent amount of hydroxyls present. The rosemary and rosemary extract both had higher TPC and DPPH than the ground black pepper and black pepper extract, and a lower TPC than the black pepper oil. The oil had a higher DPPH than the rosemary spice, but a lower DPPH than the rosemary extract. The trends of the spices and extracts suggest a correlation between TPC and DPPH. The synthetic antioxidant results, however, do not support this hypothesis. BHT and TBHQ had much higher TPC than the spices,

extracts, and oil, but the DPPH values were lower than all other compounds except for the ground black pepper and the black pepper oil. These results are contradictory and can support neither previous studies that have indicated a possible relationship between TPC and DPPH (Wang and others 2008; Wojodylo and others 2007), or other studies that have found no correlation between the two (Hinneberg and others 2006; Su and others 2007).

The high DPPH of 76.74 ± 1.000% and higher TPC value of 2.433 ± 0.152 mg GA/g for the black pepper oil, compared to the ground black pepper and the black pepper extract, suggests black pepper oil contains more hydroxyls than the ground black pepper and the black pepper extract or higher amounts of other compounds that could account for high TPC or DPPH results. Though few studies have been performed in regards to the phenolic content and radical scavenging ability of black pepper, a few have indicated a small amount of phenolic content and radical scavenging ability of black pepper. Kapoor and others (2009) studied the radical scavenging effects of volatile oil and oleoresins of black pepper. They reported that as the concentration of oil or oleoresin increased, the DPPH radical scavenging (%) increased. Su and others (2007) measured TPC for black peppercorns with both acetone (50%) and methanol (80%) extracts. The acetone extract had a TPC of 1.32 mg GA/g and the methanol extract had a TPC of 0.91 mg GA/g of black peppercorn.

Wang and others (2008) and Gülçin (2005) measured DPPH and either TPC or total antioxidant activity of black pepper, but had different results. Wang and others (2008) used black pepper essential oil and found it to have a TPC of 17 ± 3.325 10 mg GA/g of oil, and low DPPH, 5.13 ± 0.011%. Gülçin (2005) tested water and ethanol extracts of black pepper. The total antioxidant activity results were 95.5% for the water extract and 93.3% for the ethanol extract. The DPPH results were 55 ± 4.16% for the water extract and 48 ± 5.18% for the ethanol extract.

Wojdylo and others (2007) tested freeze-dried, ground rosemary for TPC and DPPH. The TPC result was 1.71 ± 0.02 mg GA/100g (dry weight). The DPPH result was 513 ± 5.99 μM trolox/100 g (dry weight). Damašius and others (2011) tested one extract of rosemary. The TPC result was 142 ± 3.58 mg GA/g of extract. The DPPH IC_{50} result was 0.25 ± 0.02 mg/mL. Puangsombat and Smith (2010) measured TPC and DPPH for five different extracts of rosemary (0, 10, 20, 30, and 40% ethanol). The TPC results ranged from 33.45-38.93 mg GA/g. The antioxidant activity IC_{50} on DPPH radical results ranged from 0.52-0.69 mg/mL. The TPC rosemary results of our study were 1.983 ± 0.021 mg GA/g of spice and 1.070 ± 0.130 mg GA/g of extract. The DPPH rosemary results were 46.32 ± 1.518% for the spice and 91.13 ± 0.263% for the extract. The rosemary results of our study, both with TPC and DPPH, were obtained using similar methods to previous studies, but different extraction procedures and, therefore, cannot be accurately compared to these previous studies.

Conclusion

This study tested the effect of water:diethylene glycol ratio on PhIP formation in a chemical model system, and the TPC and DPPH values of ground black pepper, black pepper oil, black pepper extract, rosemary, rosemary extract, BHT, and TBHQ. The water:diethylene glycol ratio chosen to be used in further study of PhIP formation and inhibition was the 10:90 water:diethylene glycol (v/v) ratio because it had the highest average PhIP formation, lowest CV, and the smallest standard deviation. Further study involving PhIP inhibition within the model system is described in a later chapter and should be continued with other compounds to determine which antioxidants, herbs, spices, or other food components are effective at inhibiting PhIP formation in model systems.

The TPC and DPPH results are different than previous studies, especially for the black pepper oil. Compared to the ground black pepper and the black pepper extract, the black pepper oil had much higher TPC and DPPH values. These higher values suggest black pepper oil may contain one or more compounds, not present in ground black pepper or the black pepper extract, which could be the cause of these higher values. High TPC and DPPH values indicate possible phenolic activity, suggesting black pepper oil may be effective at inhibiting PhIP or other HCA formation since high total phenolics has been hypothesized to cause inhibition. Further studies to determine which compounds are responsible for the high DPPH and TPC values could aid in determining which compounds can inhibit PhIP formation. This information could also lead to the study of other compounds that may not have high TPC and DPPH values as possible inhibitors, and may help determine the exact mechanism of inhibition, or formation, of PhIP in chemical model systems.

References

Chang CH, Lin HY, Chang CY, Liu YC. 2006. Comparisons on the antioxidant properties of fresh, freeze-dried and hot-air-dried tomatoes. J. Agric. Food Chem. 77:478-485.

Cheng KW, Chen F, Wang M. 2007. Inhibitory activities of dietary phenolic compounds on heterocyclic amine formation in both chemical model system and beef patties. Mol. Nutr. Food Res. 51:969-976.

Cheng KW, Chen F, Wang M. 2006. Heterocyclic amines: chemistry and health. Mol. Nutr. Food Res. 50:1150-1170.

Cheng KW, Wong CC, Chao J, Lo C, Chen F, Chu IK, Che CM, Ho CT, Wang M. 2009. Inhibition of mutagenic PhIP formation by epigallocatechin gallate *via* scavenging of phenylacetaldehyde. Mol. Nutr. Food Res. 53:716-725.

Damašius J, Venskutonis PR, Ferracane R, Fogliano V. 2011. Assessment of the influence of some spice extracts on the formation of heterocyclic amines in meat. Food Chem. 126:149-156.

Gross GA, Grüter A. 1992. Quantitation of mutagenic/carcinogenic heterocyclic aromatic amines in food products. J. Chrom. A. 592:271-278.

Gross GA, Grüter A, Heyland S. 1992. Optimization of the sensitivity of high-performance liquid chromatography in the detection of heterocyclic aromatic amine mutagens. Food and Chem. Toxicol. 30:491-498.

Gülçin I. 2005. The antioxidant and radical scavenging activities of black pepper (*Piper nigrum*) seeds. Int. J. Food Sci. Nutr. 56:491-499.

Hinneberg I, Dorman HJD, Hiltunen R. 2006. Antioxidant activities of extracts from selected culinary herbs and spices. Food Chem. 97:122-129.

Hussain FA. 2015. Formation of 4(5)-methylimidazole in processed meat and in glucose amino acid model system [DPhil dissertation]. Manhattan, KS: Kansas State Univ. 183 p. Available from: http://hdl.handle.net/2097/18975.

Jägerstad M, Skog K, Grivas S, Olsson K. 1991. Formation of heterocyclic amines using model systems. Mutat. Res. 259:219-233.

Johansson MAE, Fay LB, Gross GA, Olsson K, Jägerstad M. 1995. Influence of amino acids on the formation of mutagenic/carcinogenic heterocyclic amines in a model system. Carcinogenesis. 16:2553-2560.

Johansson MAE, Jägerstad M. 1996. Influence of pro- and antioxidants on the formation of mutagenic-carcinogenic heterocyclic amines in a model system. Food Chem. 56:69-75.

Kapoor IPS, Singh B, Singh G, de Heluani CS, de Lampasona MP, Catalan CAN. 2009. Chemistry and in vitro antioxidant activity of volatile oil and oleoresins of black pepper (*Piper nigrum*). J. Agric. Food Chem. 57:5358-5364.

Knize MG, Felton JS. 2005. Formation and human risk of carcinogenic heterocyclic amines formed from natural precursors in meat. Nutr. Reviews. 63:158-165.

Mittal R, Gupta RL. 2000. *In Vitro* antioxidant activity of piperine. Methods Find. Exp. Clin. Pharmacol. 22:271-274.

Murkovic M. 2004. Formation of heterocyclic aromatic amines in model systems. J. Chrom. B. 802:3-10.

Murkovic M. 2007. Analysis of heterocyclic aromatic amines. Anal. Bioanal. Chem. 389:139-146.

Oguri A, Suda M, Totsuka Y, Sugimura T, Wakabayashi K. 1998. Inhibitory effects of antioxidants on formation of heterocyclic amines. Mutat. Res. 402:237-245.

Puangsombat K, Gadgil P, Houser TA, Hunt MC, Smith JS. 2012. Occurrence of heterocyclic amines in cooked meat products. Meat Sci. 90:739-746.

Puangsombat K, Smith JS. 2010. Inhibition of heterocyclic amine formation in beef patties by ethanolic extracts of rosemary. J. Food Sci. 75:T40-T47.

Salazar R, Arámbula-Villa G, Hidalgo FJ, Zamora R. 2014. Structural characteristics that determine the inhibitory role of phenolic compounds on 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) formation. Food Chem. 151:480-486.

Shahidi F, Wanasundara PK. 1992. Phenolic antioxidants. CRC Crit. Rev. Food Nutr. 32:67-103.

Singh RP, Chidambara-Murthy KN, Jayaprakasha GK. 2002. Studies on the antioxidant activity of pomegranate (*Punica granatum*) and seed extracts using *in vitro* models. J. Agric. Food Chem. 50:81-86.

Skog K, Jägerstad M. 1991. Effects of glucose on the formation of PhIP in a model system. Carcinogenesis. 12:2297-2300.

Skog K, Johansson MAE, Jägerstad MI. 1998. Carcinogenic heterocyclic amines in model systems and cooked foods: a review on formation, occurrence and intake. Food and Chem. Toxicol. 36:879-896.

Su L, Yin JJ, Charles D, Zhou K, Moore J, Yu L. 2007. Total phenolic contents, chelating capacities, and radical-scavenging properties of black peppercorn, nutmeg, rosehip, cinnamon and oregano leaf. Food Chem. 100:990-997.

Vijayakumar RS, Surya D, Nalini N. 2004. Antioxidant efficacy of black pepper (*Piper nigrum* L.) and piperine in rats with high fat diet induced oxidative stress. Redox Report. 9:105-110.

Wang HF, Wang YK, Yih KH. 2008. DPPH free-radical scavenging ability, total phenolic content, and chemical composition analysis of forty-five kinds of essential oils. J. Cosmet. Sci. 59:509-522.

Wojdylo A, Oszmiański J, Czemerys R. 2007. Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chem. 105:940-949.

Wong D, Cheng KW, Wang M. 2012. Inhibition of heterocyclic amine formation by water-soluble vitamins in Maillard reaction model systems and beef patties. Food Chem. 133:760-766.

Chapter 3 - Effects of black pepper (oil and extract), synthetic antioxidants (BHT and TBHQ), rosemary extract, and piperine on PhIP formation in model systems

Abstract

PhIP is the most abundantly formed heterocyclic amine (HCA). HCA formation in other model systems has been shown to be inhibited by natural and synthetic antioxidants. The effects of a black pepper volatile oil, black pepper ethanolic extract, rosemary ethanolic extract, butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and piperine on PhIP formation in model systems were evaluated. The black pepper volatile oil inhibited PhIP formation, on average, 31.40% with 36 μL, 30.78% with 71 μL, 25.73% with 142 μL, 22.69% with 285 μL, and 43.49% with 550 μL. The black pepper ethanolic extract inhibited PhIP formation, on average, 25.26% with 36 μL, 24.00% with 71 μL, 30.51% with 142 μL, 38.26% with 285 μL, and 40.56% with 550 μL. The rosemary ethanolic extract inhibited the formation of PhIP, on average, 27.66% with 36 μL, 22.11% with 71 μL, 24.88% with 142 μL, 43.93% with 285 μL, and 31.14% with 550 μL. BHT inhibited the formation of PhIP, on average, 13.96% with 0.05 mmol, 55.62% with 0.1 mmol, 6.299% with 0.2 mmol, and 8.867% with 0.4 mmol. TBHQ inhibited the formation of PhIP, on average, 18.97% with 0.05 mmol, 24.08% with 0.1 mmol, and 21.96% with 0.2 mmol, and promoted the formation of PhIP, on average, 5.27% with 0.4 mmol. Piperine inhibited the formation of PhIP, on average, 23.89% with 4.02 mg, 20.08% with 8.04 mg, 23.47% with 16.14 mg, and 28.75% with 31.14 mg. These data suggest that black pepper inhibits the formation of PhIP in model systems even though its antioxidant activity is low.

Introduction

Heterocyclic amines (HCAs) are a class of mutagenic and carcinogenic chemical compounds formed on the outside of meat and fish that have been cooked at high temperatures. Of the more than 25 HCAs currently identified, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most abundantly formed (Cheng and others 2006; Puangsombat and others 2012). PhIP was first identified and isolated in 1986 by Felton and others. PhIP is formed in model systems containing glucose, creatin(in)e, and phenylalanine (Arvidsson and others 1997; Johansson and others 1995, Shioya and others 1987; Zöchling and Murkovic 2002). Due to its highly mutagenic and carcinogenic properties, PhIP has been listed as a *possible human carcinogen* by the International Agency for Research on Cancer (1997) and as *reasonably anticipated to be a human carcinogen* by the National Toxicology Program (2014). HCAs have been found to cause different cancers in mice and rats, and PhIP, specifically, has been shown to cause breast, colon, rectal, and prostate cancers in mice and rat studies (Choudhary and others 2012; Ito and others 1991; Shirai and others 1997).

Previous studies have tested the effects of synthetic antioxidants and natural spices on HCA and PhIP formation, both in meat and chemical model systems. Several studies found the effects of BHA (Johansson and Jägerstad 1996; Moon and Shin 2013; Vitaglione and Fogliano 2004), BHT (Ahn and Grün 2005; Vitaglione and Fogliano 2004), and epigallocatechin gallate (EGCG) (Cheng and others 2007, 2009; Kikugawa 1999) on HCA formation was dose-dependent. Some studies have tested the common spice rosemary for HCA inhibition (Damašius and others 2011; Puangsombat and Smith 2010; Tsen and others 2006). These studies all found rosemary to be an effective inhibitor of HCAs.

Black pepper (*Piper nigrum*) is a spice often used in industry and individual households that comes from the dried, mature fruit of the *Piper nigrum* L. plant, belonging to the Piperaceae

family. The characteristics of black pepper can be broadly separated into two categories: compounds that give black pepper its pungency (i.e. piperine, piperanine, chavicine, piperettine, piperylin, and piperolein A and B), and compounds that contribute to the flavor and aroma of black pepper (Ravidran and Kallupurackal 2001; Zachariah and Parthasarathy 2008). While there has not been much research on black pepper and its ability to inhibit HCAs, several sources have cited black pepper's radical scavenging and antioxidative abilities. Tipsrisukond and others (1998) found that piperine exhibited the most antioxidative activity of black pepper compounds. They also found that ground black pepper was a more effective antioxidant than extracted essential oils or the oleoresins of black pepper. Vijayakumar and others (2004) showed that when black pepper or piperine were given to rats on a high fat diet, the thiobarbituric acid reactive substances (TBARS) concentrations from liver, heart, kidney, intestine, and aorta tissues were similar to the levels found in the control rats. High TBARS indicate the failure of antioxidant defense mechanisms to prevent excess free radical formation. Kapoor and others (2009) found similar results, that essential oils and oleoresins of black pepper had high antioxidative and radical scavenging effects using the thiobarbituric acid (TBA) and DPPH assays. They also found that as the concentration of oil or oleoresin increased from 5μL to 20μL, the scavenging effects increased.

One of the few research reports about black pepper's effect on HCA formation was performed by Oz and Kaya (2011). They found, overall, that black pepper decreased HCA formation in high fat meatballs when added before cooking, by 33% at 175 ˚C, 12% at 200 ˚C, and 100% at 225 ˚C, and that the cooking temperature and the type of HCA affects how well the black pepper inhibits formation. The purpose of this study was to determine the effects of a black

pepper oil, a black pepper extract, a rosemary extract, BHT, TBHQ, and piperine on PhIP formation in a model system.

Materials and Methods

Materials

McCormick Science Institute (Hunt Valley, MD, U.S.A.) provided standard samples of finely powdered, ground black pepper blend, and finely powdered rosemary. A 100% pure *Piper nigrum* oil (steam distilled black pepper) was purchased from Nature's Kiss Essential Oils (Moreno Valley, CA, U.S.A.). The PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) standard was purchased from Toronto Research Chemicals, Inc (Ontario, Canada). A piperine standard was purchased from Acros Organics (New Jersey, U.S. A.). Methanol (*Optima*), acetonitrile (certified ACS), syringe filters (nylon, 0.2 μm), and acetic acid was purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Triethylamine (BioUltra), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), D-(+)-Glucose, L-phenylalanine, creatinine, and diethylene glycol (BioUltra) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A). Deionized water was prepared using a Sybron/Barnstead PCS unit (Barnstead/Thermolyne, Inc, Dubuque, IA, U.S.A.).

Model System Preparation

A black pepper extract was made by shaking 1.0 g of ground black pepper in 100 mL of 80:20 ethanol:water (v/v) solution for two hours on a wrist action shaker (Burrell Corporation; Pittsburg, PA, U.S.A.). The solution was filtered through Whatman #4 filter paper (Whatman International Ltd., Maidstone, United Kingdom). A 5 mL aliquot of the solution was dried down with nitrogen and reconstituted with 5 mL of diethylene glycol. The final concentration of the black pepper extract was 1.0 g/100 mL.

A rosemary extract was made by shaking 1.0 g of rosemary in 100 mL of 95% ethanol for two hours on a wrist action shaker (Burrell Corporation; Pittsburg, PA, U.S.A.). The solution was filtered through Whatman #4 filter paper (Whatman International Ltd., Maidstone, United Kingdom). A 5 mL aliquot of the solution was dried down with nitrogen and reconstituted with 5mL of diethylene glycol. The final concentration of the rosemary was 1.0 g/100 mL.

The model systems were prepared as described by Cheng and others (2007) with modifications. Solutions of 1mmol glucose (0.18016 g), creatinine (0.11312 g), and phenylalanine (0.16519 g), all in 10% water, 90% diethylene glycol, were made in 10 mL volumetric flasks. The precursor molar concentrations were 0.2 mmol glucose, 0.4 mmol creatinine, and 0.4 mmol phenylalanine, made by mixing 110 μL of the 1 mmol glucose solution, and 220 μL of the 1 mmol creatinine and phenylalanine solutions in a 1 mL reaction vial, for a total volume of 550 μL. The uncapped reaction vials were them placed inside brass vessels, as described in Hussain (2015), and tightly closed to prevent volume loss during heating. The brass vessels were placed inside an oven set at 180 ˚C and allowed to heat for 1 hour.

After 1 hour, the brass vessels were immediately removed and cooled on ice for 5 minutes before removing the reaction vials and storing at room temperature for further analysis. The effects of black pepper and rosemary were tested by adding differing levels of the extracts or oil (36, 71, 142, 285, and 550 μL; n=10) to the model systems containing the precursors before heating. The synthetic antioxidants (BHT and TBHQ) were added in levels of 0.05, 0.1, 0.2 and 0.4 mmol (n=5) amounts to the model systems. Piperine was added in levels of 4.02, 8.04, 16.14, and 31.14 mg (n=10) amounts. All model systems were diluted 1:10 with methanol and syringe filtered before HPLC analysis.

Analysis of PhIP

Analysis of PhIP was achieved with an HP 1050 series HPLC with auto-sampler, coupled with and HP 1050 series UV detector and an HP 1046A programmable fluorescence detector according to the methods described by Gross and others (1992) and Gross and Grüter (1992) with slight modifications. PhIP separation was obtained by reversed-phase chromatography using a TSKgel ODS-80T_M (4.6 mm x 25 cm x 5 µm) column fitted with a TSKgel guardgel ODS-80T_M (3.2 mm x 1.5 cm) guard column (TOSOH Biosciences; Tokyo, Japan). The mobile phases used were 0.01 M triethylamine, buffer adjusted to pH 3.6 with acetic acid (A) and acetonitrile (B).

A mobile phase system gradient was used, as described by Puangsombat and others (2012) with slight modifications. The linear gradient began with 95% A and 5% B, changing to 75% A and 25% B over 30 minutes. The ratio of 75% A and 25% B was held constant for 5 minutes (30-35 minutes), before returning to the original ratio of 95% A and 5% B for a total run time of 45 minutes. A 10 minute equilibration of the column was added after each sample. The mobile phase was sent through the system at a rate of 1.0 mL/minute, with the column temperature set at 40 ˚C. For PhIP detection the fluorescence detector had an emission of 229 nm and an excitation of 437 nm.

Analysis of Black Pepper Oil and Extract

Black pepper extract and black pepper oil were analyzed according to the method described by Scott and others (2005), with modifications, using a HP 1050 series HPLC with a HP 1050 series UV detector to determine piperine concentration. Piperine separation was obtained with reversed-phase chromatography using a TSKgel ODS-80T_M (4.6 mm x 25 cm x 5

μm) column with a TSKgel guardgel ODS-80T_M (3.2 mm x 1.5 cm) guard column (TOSOH Biosciences; Tokyo, Japan). The mobile phases were deionized water (A) and acetonitrile (B).

A mobile phase system gradient was used to separate piperine. The linear gradient began with 70% A and 30% B, changing to 40% A and 60% B over 20 minutes, increasing to 10% A and 90% B by 30 minutes, then returning to the original ratio for a total run time of 35 minutes. A 10 minute equilibration of the column was added after each sample with a mobile phase ratio of 70% A and 30% B. The flow rate was 1.0 mL/minute and the column temperature was set to 40˚C. For piperine detection, the UV detector was set to record data at 340 nm and 210 nm. The black pepper oil was diluted 1:2 in ethanol and the black pepper extract was diluted 1:2 in methanol. Piperine eluted at approximately 20.5 minutes.

SPME Headspace of Black Pepper

Solid phase micro extraction (SPME) was performed on ground black pepper, the black pepper volatile oil, and black pepper ethanolic extract according to the method described by Puangsombat and Smith (2010). One mL of the extract, oil (diluted 1:50), or 1.0 g of ground black pepper were placed in a 5-mL glass vial and placed in a heat block. A preconditioned SPME fiber was inserted into the vial and the fiber was exposed to the headspace at 60 ˚C for 15 minutes. The SPME fiber was then withdrawn and immediately placed into the gas chromatography-mass spectrometry (GC-MS) injector port and desorbed for 15 minutes at 260 ˚C.

GC-MS was performed using an HP5890 GC series II Plus fitted with a HP-5MS column (cross-linked 5% PhMe siloxane, 30 m x 0.22 mm x 0.25 μm) and an HP MSD 5972 detector. An oven temperature program was used with an initial temperature of 50˚C held for 3 minutes, followed by a temperature increase of 7 ˚C/minute until 180 ˚C was reached, and then held at

180 ˚C for 5 minutes. The injector temperature was 260 ˚C and the detector temperature was 300 ˚C. The flow rate was 1 mL/minute with helium as the carrier gas. The volatile compounds were identified by comparing the spectra to known spectra in the NIST08 database.

Quantification and Statistical Analysis

A 1000 ppm piperine standard stock solution was made by dissolving 100 mg of piperine in 100 mL of ethanol. A standard curve was made for each wavelength (340 nm and 210 nm) by preparing and analyzing piperine standards with concentrations of 31.25, 62.5, 125, 250, and 500 ppm (Appendix A, Figures A-3 and A-4). The coefficients of determination (R^2) were 0.9994 at 340 nm and 1 at 210 nm. A 1000 ppb PhIP standard stock solution was made by dissolving 0.1 mg of PhIP in 100 mL of methanol. A standard curve was made by preparing and analyzing, using fluorescence detection, PhIP standards with concentrations of 31.25, 62.5, 125, 250, and 500 ppb (Appendix A, Figure A-2). The coefficient of determination (R^2) was 0.9999. SAS (Version 9.4, 2012, SAS Institute Inc., Cary, NC, U.S.A.) was used to analyze the obtained HPLC data. The experiment was a matched pairs statistical design. Analysis of variance (ANOVA), with SAS, was used to determine significant differences between the treatments (black pepper oil, black pepper extract, rosemary extract, BHT, TBHQ, and piperine) and the controls at $p < 0.05$.

Results

Black Pepper Oil

The black pepper oil was analyzed on the HPLC with UV detection to determine the piperine concentration of the oil. The oil was diluted 1:2 in ethanol. Piperine eluted at approximately 20.5 minutes. Figure 3-1 identifies the piperine peak at 340 nm. Black pepper oil contain approximately 1.83 ppm piperine.

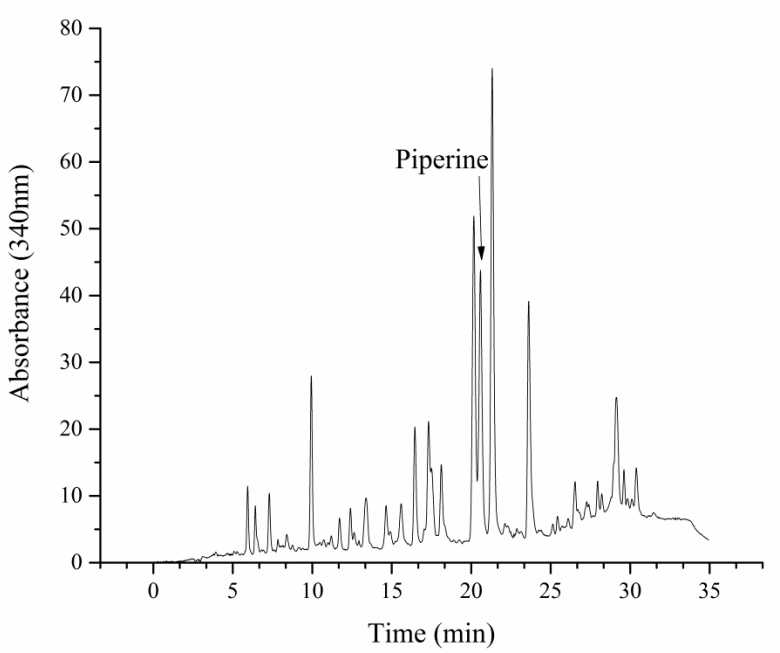


Figure 3-1: HPLC chromatogram of black pepper oil, separated with a TSKgel ODS-80$_{TM}$ column. The UV detector was set at 340 nm.

An overlaid chromatogram of the PhIP control model system with a PhIP standard is shown in Figure 3-2. The model system control contained amounts of 0.2 mmol glucose, 0.4 mmol creatinine, and 0.4 mmol phenylalanine in 10:90 water:diethylene glycol (v/v). The analysis of PhIP was performed as described on page 51. PhIP eluted from the column at approximately 31.4 minutes. A control model system was run each day a set of treatment model systems was run. The number of controls for each treatment level varied based on how many models for each treatment level was run. Each treatment was black pepper oil, black pepper extract, rosemary extract, BHT, TBHQ, or piperine added at a different amount.

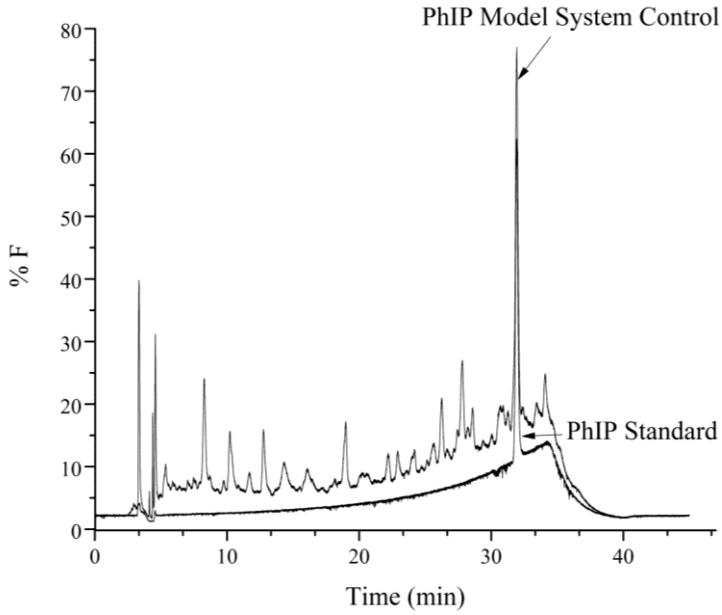


Figure 3-2: HPLC chromatogram of 125 ppb PhIP standard, overlaid with a PhIP model system control, separated with a TSKgel ODS-80$_{TM}$ column. The fluorescence detector was set at Ex=229, Em=437.

Black pepper oil was added to the model systems in five different levels and, on average, inhibited the amount of PhIP formed by 31.40% with 36 µL, 30.78% with 71 µL, 25.73% with 142 µL, 22.69% with 285 µL, and 43.49% with 550 µL. Figure 3-3 illustrates the relationship between level of black pepper oil and the average decrease in PhIP formation. At all levels, changes in PhIP concentration were significantly different from the control at p-value < 0.05 (Appendix A, Table A-4).

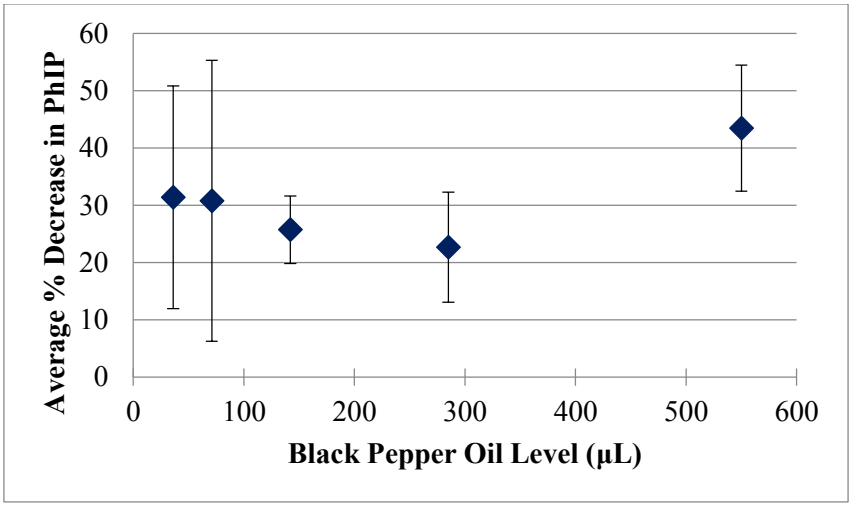


Figure 3-3: Relationship between level of black pepper oil and the average decrease in PhIP formation (n=10). The bars represent one standard deviation for each treatment.

Black Pepper Extract

The black pepper extract was analyzed on the HPLC with UV detection to determine the piperine concentration of the extract. The extract was diluted 1:2 with methanol. Piperine eluted at approximately 20.5 minutes. Figure 3-4 identifies the piperine peak at 340 nm. Black pepper extract contain approximately 366.9 ppm piperine.

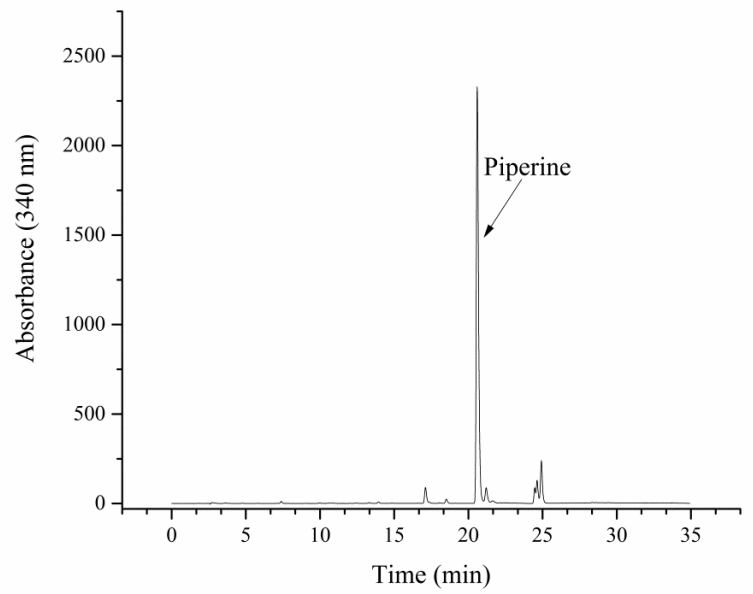


Figure 3-4: HPLC chromatogram of black pepper extract, separated with a TSKgel ODS-80$_{TM}$ column. The UV detector was set at 340 nm.

Black pepper extract was added to the model system in five different levels and, on average, inhibited the formation of PhIP by 25.26% with 36 µL, 24.00% with 71 µL, 30.51% with 142 µL, 38.26% with 285 µL, and 40.56% with 550 µL. Figure 3-5 illustrates the relationship between black pepper extract level and the average decrease in PhIP formation (n=10). At all levels, changes in PhIP concentration were significantly different from the control at p-value < 0.05 (Appendix A, Table A-4).

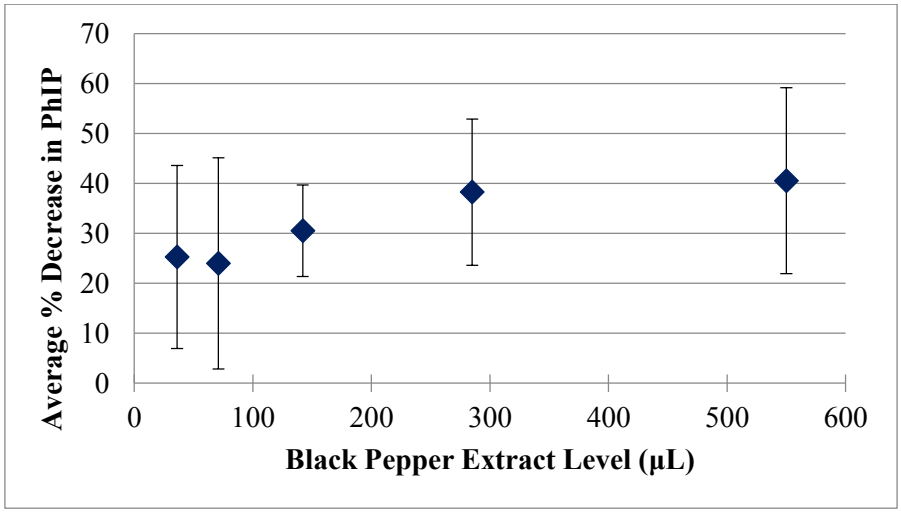


Figure 3-5: Relationship between level of black pepper extract and the average decrease in PhIP formation (n=10). The bars represent one standard deviation for each treatment.

BHT

BHT was added to the model systems at levels of 0.05, 0.1, 0.2, and 0.4 mmol. BHT inhibited the formation of PhIP, on average, by 13.96% with 0.05 mmol, 55.62% with 0.1 mmol, 6.299% with 0.2 mmol, and 8.867% with 0.4 mmol. Figure 3-6 illustrates the relationship between level of BHT and average decrease in PhIP formation (n=5). The two lowest levels of 0.05 and 0.1 mmol were significantly different from the control at p-value < 0.05 (Appendix A, Table A-5). The two highest levels of 0.2 and 0.4 mmol were not significantly different from the control at p-value < 0.05.

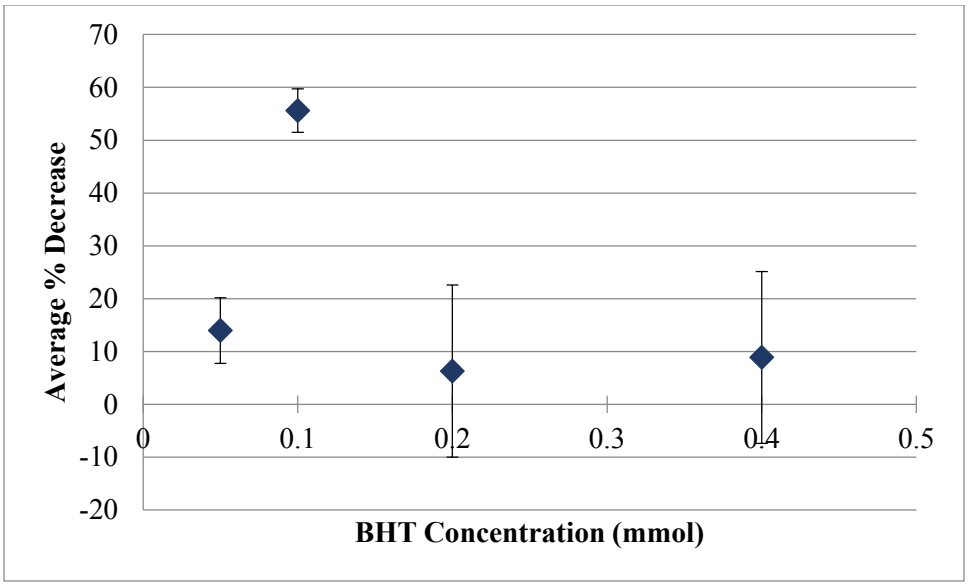


Figure 3-6: Relationship between level of BHT and average decrease in PhIP formation (n=5). The bars represent one standard deviation for each treatment.

TBHQ

TBHQ was added to the model systems at levels of 0.05, 0.1, 0.2, and 0.4 mmol. TBHQ inhibited the formation of PhIP, on average, by 18.97% with 0.05 mmol, 24.08% with 0.1 mmol, and 21.96% with 0.2 mmol, and promoted the formation of PhIP, on average, by 5.27% with 0.4 mmol. Figure 3-7 illustrates the relationship between level of TBHQ and the average decrease in PhIP formation (n=5). The three highest levels of 0.05, 0.1, and 0.2 mmol were significantly different from the control at p-value < 0.05 (Appendix A, Table A-5). The level of 0.4 mmol was not significantly different from the control at p-value < 0.05.

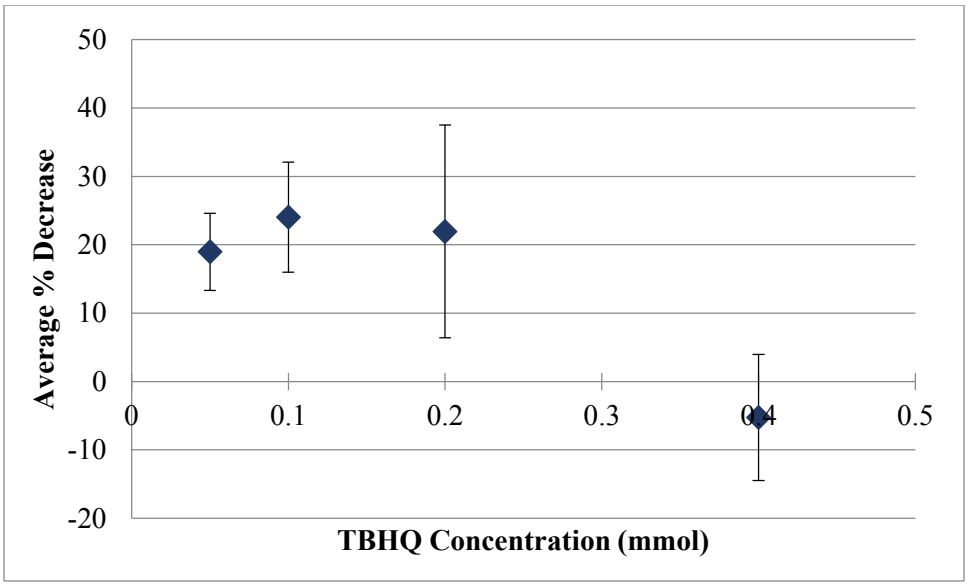


Figure 3-7: Relationship between the level of TBHQ and average decrease in PhIP formation (n=5). The bars represent one standard deviation for each treatment.

Rosemary

Rosemary was added to the model system in five different levels and, on average, inhibited the formation of PhIP by 27.66% with 36 µL, 22.11% with 71 µL, 24.88% with 142 µL, 43.93% with 285 µL, and 31.14% with 550 µL. Figure 3-8 illustrates the relationship between the level of rosemary and average decrease in PhIP formation (n=10). At all levels, decreases were significantly different from the control at p-value < 0.05 (Appendix A, Table A-4).

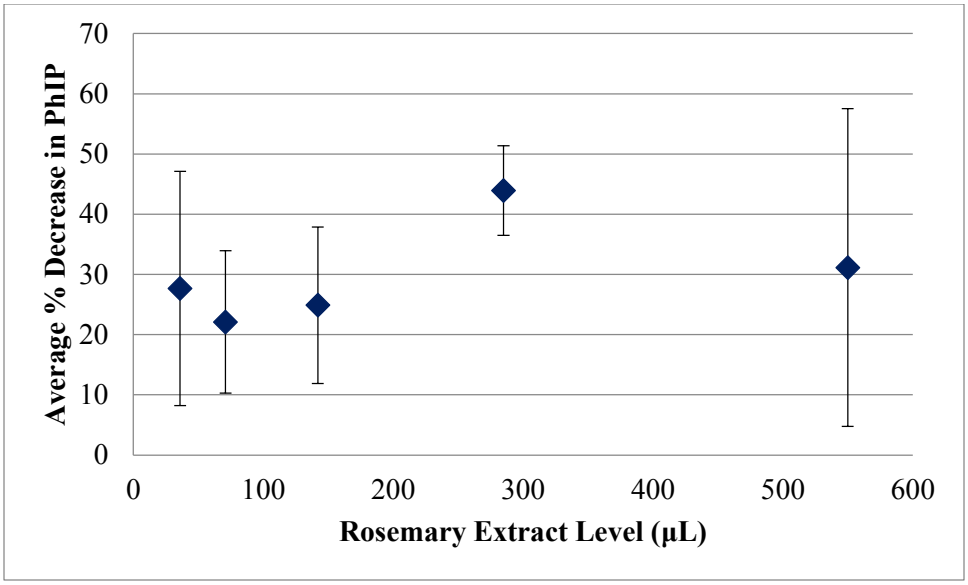


Figure 3-8: Relationship between the level of rosemary and average decrease in PhIP formation (n=10). The bars represent one standard deviation for each treatment.

Piperine and SPME Headspace Analysis

Piperine, a major component of black pepper, was added to the model system to determine if it is capable of inhibiting PhIP formation on its own. In previous studies, piperine has been shown to have some antioxidant activity (Mittal and Gupta 2000; Vijayakumar and others 2004). Piperine makes up about 2.0-7.4% of black pepper, depending on the cultivar (Ravindran and Kallupurackal 2001). Piperine inhibited PhIP formation, on average, 23.89% with 4.02 mg, 20.08% with 8.04 mg, 23.47% with 16.14 mg, and 28.75% with 31.14 mg (n=10) (Figure 3-9). Decreases using piperine were all statistically significant from the control at p-value < 0.05 (Appendix A, Table A-6).

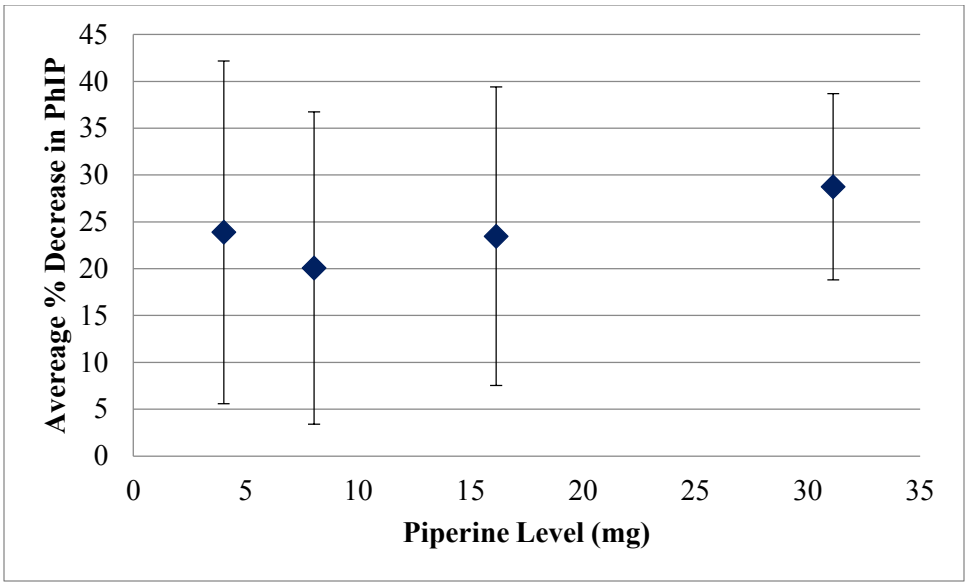


Figure 3-9: Relationship between level of piperine and average decrease in PhIP formation (n=10). The bars represent one standard deviation for each treatment.

SPME headspace analysis was conducted using GC-MS on ground black pepper (Figure 3-10), black pepper oil (Figure 3-11), and black pepper extract (Figure 3-12) to determine compounds, other than piperine, in common with the three. Six chemical compounds were found in all three: 1R-α-pinene, 3-carene, D-limonene, cyclohexene 4-ethenyl-4-methyl-3-(1-methylethenyl), caryophyllene, and α-caryophyllene (Table 3-1). Caryophyllene and D-limonene had the largest peak areas in all three spectra, suggesting they could be alternate sources of PhIP inhibition.

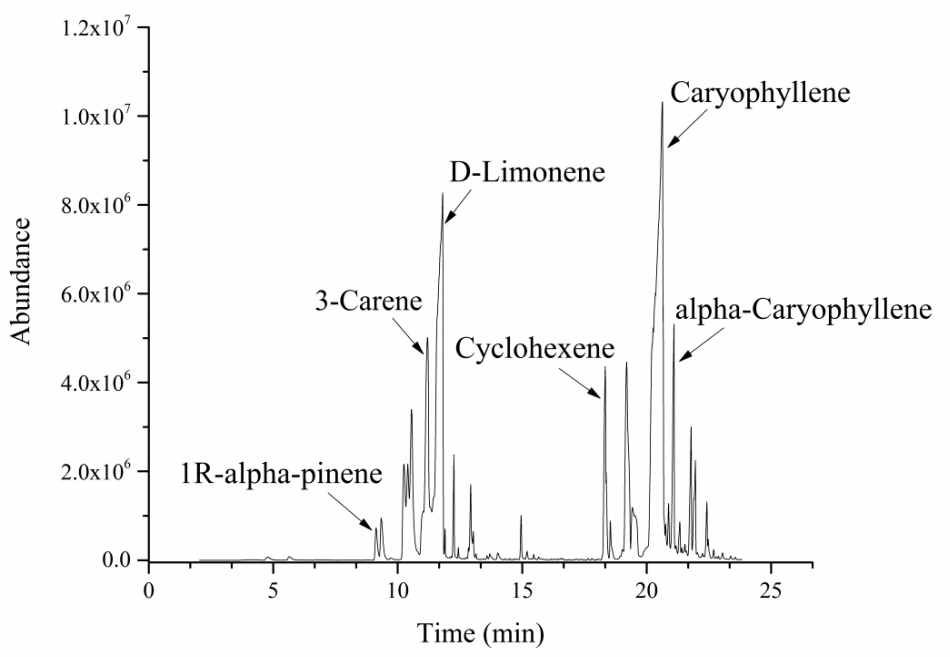


Figure 3-10: GC-MS spectra of ground black pepper SPME headspace analysis; separated on an HP5890 GC fitted with an HP-5 MS column (cross-linked 5% PhMe siloxane, 30 m x 0.22 mm x 0.25 µm film thickness) and an HP MSD 5972 detector. Flow rate of 1.0 mL/minute with helium as the carrier gas. Injector temperature was 260 ˚C and detector temperature was 300 ˚C.

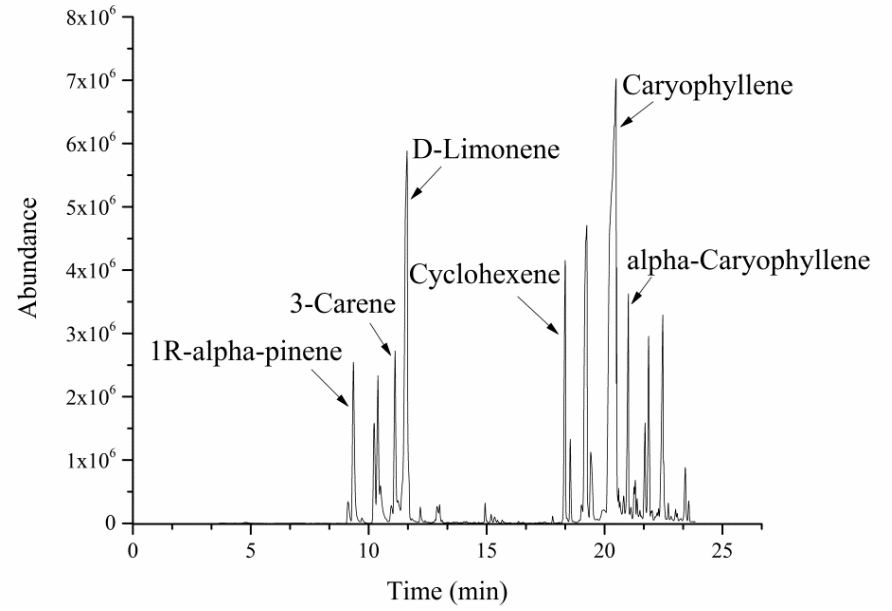


Figure 3-11: GC-MS spectra of black pepper oil SPME headspace analysis; separated on an HP5890 GC fitted with an HP-5 MS column (cross-linked 5% PhMe siloxane, 30 m x 0.22 mm x 0.25 µm film thickness) and an HP MSD 5972 detector. Flow rate of 1.0 mL/minute with helium as the carrier gas. Injector temperature was 260 ˚C and detector temperature was 300 ˚C.

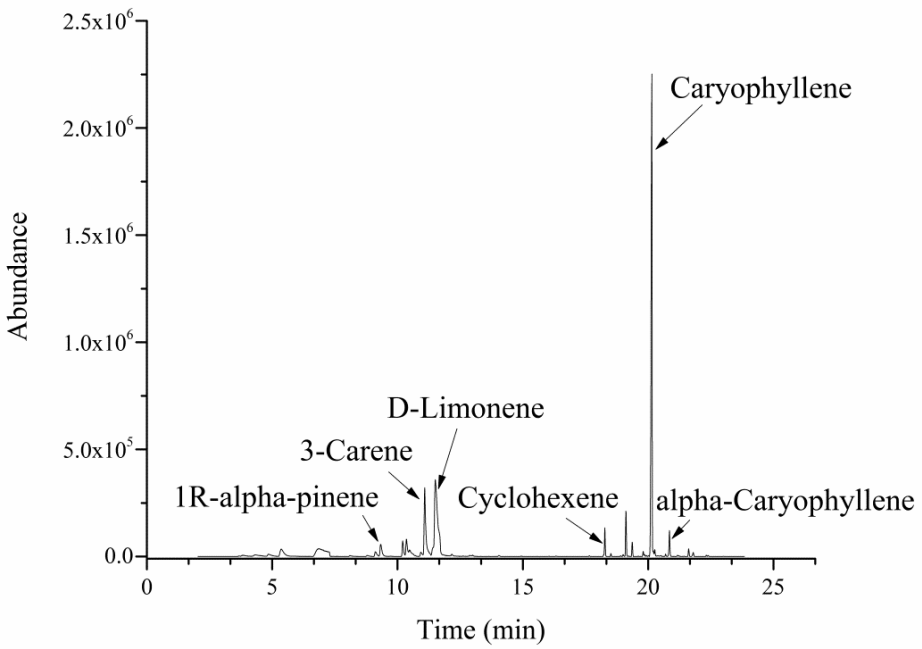
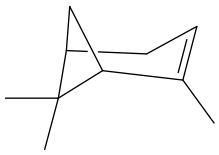
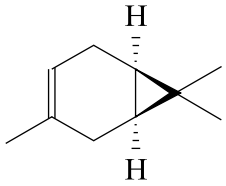
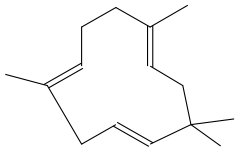
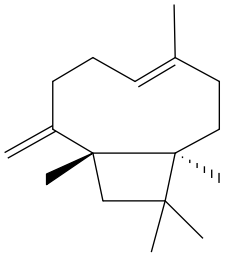
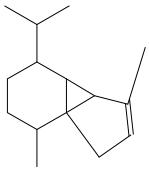
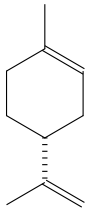


Figure 3-12: GC-MS spectra of black pepper extract SPME headspace analysis; separated on an HP5890 GC fitted with an HP-5 MS column (cross-linked 5% PhMe siloxane, 30 m x 0.22 mm x 0.25 μm film thickness) and an HP MSD 5972 detector. Flow rate of 1.0 mL/minute with helium as the carrier gas. Injector temperature was 260 ˚C and detector temperature was 300 ˚C.

Table 3-1: Table of the six chemical compounds in common between ground black pepper (GBP), black pepper oil (BPO), and black pepper extract (BPE), their chemical structures, and retention times (RT).

Compound	Chemical Structure	GBP-RT	BPO-RT	BPE-RT
1R-α-pinene		9.35	9.35	9.32
3-carene		11.20	11.12	11.09
α-caryophyllene		21.09	21.00	20.85
Caryophyllene		20.62	20.47	20.15
Cyclohexene,4-ethenyl-4-methyl-3-(1-methylethenyl)		18.33	18.33	18.27
D-limonene		11.79	11.62	11.51

Discussion

The results of this study show that black pepper can be used to decrease PhIP formation in chemical model systems, even though it has low antioxidant activity. The differences in PhIP formation were all significantly different from the control. Piperine was tested because it is one of the main components of black pepper, and since black pepper shows only very low phenolic content, it was thought that piperine might play a role in PhIP inhibition. Piperine's smaller decreases of the concentration of PhIP, compared to the higher amounts of black pepper oil and extract, suggest piperine is not the only compound in black pepper which has inhibitory abilities for PhIP. This is supported by the analysis of black pepper oil (pg. 44) and extract (pg. 46) for piperine content. The black pepper extract contained a considerable amount of piperine, while the black pepper oil contained very little, suggesting piperine may not be the main compound responsible for black pepper's inhibitory effects on PhIP.

The mechanism for the formation of PhIP has not been extensively studied, although PhIP has been formed within model systems containing glucose, creatin(in)e, and phenylalanine (Arvidsson and others 1997; Johansson and others 1995; Shioya and others 1987). It has been hypothesized that PhIP formation starts with the formation of the Strecker aldehyde phenylacetaldehyde, followed by the condensation of phenylacetaldehyde with creatinine and dehydration (Murkovic 2004). Zöchling and Murkovic (2002) found that when a mixture of phenylacetaldhyde and creatinine were heating in a model system, more PhIP was formed than in a standard model system containing only phenylalanine, creatinine, and glucose. They concluded that the degradation of phenylalanine to phenylacetaldehyde was a necessary step in the formation of PhIP.

Since the exact mechanism of formation of PhIP is unknown, the mechanism(s) of inhibition of PhIP using black pepper can only be speculated. Piperine contains a carbonyl group

within its chemical structure and can be classified as a ketone. Ketones have the ability to react with primary aromatic amines to make a product containing a carbon-nitrogen double bond in the presence of an acid catalyst (Brown and Poon 2011). It may be possible for piperine to react with PhIP, or a precursor of PhIP, preventing formation.

Other compounds in black pepper, including volatiles, may play a role in PhIP inhibition. Based on our SPME analysis, D-limonene and caryophyllene appear to be the most likely compounds that could cause PhIP inhibition. Caryophyllene does not seem to be reactive when looking at the structure. D-limonene, however, can form another compound, p-cymene, when it is hydroxylated and then dehydrated. Phenylalanine contains a hydroxyl group, making it possible for D-limonene to react with the PhIP precursors and preventing formation.

A study by Oz and Kaya (2011) indicated that black pepper had inhibitory effects on HCAs in high fat meatballs. They found, overall, that black pepper decreased HCA formation in high fat meatballs, when added to the meatballs before cooking, by 33% (175 ˚C), 12% (200 ˚C), and 100% (225 ˚C), and that the cooking temperature and the type of HCA affects how well the black pepper inhibits formation. However, this study was conducted at very high temperatures (200˚C and 225˚C). Arvidsson and others (1997) found that when the heating temperature of HCAs reaches higher than 200˚C the HCAs start to thermally degrade and become non-existent within the model system, indicating that the black pepper used in Oz and Kaya's study may not have been the reason the HCAs were not found in the meatballs.

Rosemary was used as an internal standard for comparison, as other studies have shown rosemary's inhibitory effects on HCA formation in meat products and chemical model systems (Ahn and Grün 2005; Cheng and others 2007; Puangsombat and Smith 2010; Tsen and others 2006). BHT and TBHQ were tested because of the conflicting results within the literature about

whether or not these synthetic antioxidants inhibit HCA formation and most of these studies used BHA, while some used BHT and TBHQ. These results supported previous studies with synthetic antioxidants that indicate HCA and PhIP inhibition is dependent on concentration, with low concentrations showing some inhibition, and high concentrations showing promotion or no effect (Ahn and Grün 2005; Johansson and Jägerstad 1996; Kikugawa 1999; Oguri and others 1998).

Conclusion

In this study, the mutagenic and carcinogenic heterocyclic amine PhIP is decreased with the addition of black pepper (as an oil or as an extract), rosemary (as an extract), piperine, BHT (at low concentrations), and TBHQ (at low concentrations) in chemical model systems. The trend for the black pepper, rosemary, and piperine shows as the amount of the additive is increased and the concentration of PhIP (mg/L) is decreased, with a linear relationship, followed by a slight positive trend. The trend for the synthetic antioxidants (BHT and TBHQ) shows that there is first a decrease of PhIP formed (at low concentrations), then the amount of PhIP formed increases (at high concentrations). Further studies into what compounds, besides piperine, in black pepper, may cause PhIP inhibition could possibly give an idea of how, exactly, PhIP is formed. Further studies into other herbs, spices, and natural and synthetic antioxidants would also be useful to better understand the mechanisms of formation of this HCA.

References

Ahn J, Grün IU. 2005. Heterocyclic amines:2. Inhibitory effects of natural extracts on the formation of polar and nonpolar heterocyclic amines in cooked beef. J. Food Sci. 70:C263-C268.

Arvidsson P, van Boekel MAJS, Skog K, Jägerstad M. 1997. Kinetics of formation of polar heterocyclic amines in a meat model system. J. Food Sci. 62:911-916.

Brown W, Poon T. 2011. Introduction to Organic Chemistry. 4th ed. Indianapolis, IN: John Wiley & Sons, Inc. 801p.

Cheng KW, Chen F, Wang M. 2006. Heterocyclic amines: chemistry and health. Mol. Nutr. Food Res. 50:1150-1170.

Cheng KW, Chen F, Wang M. 2007. Inhibitory activities of dietary phenolic compounds on heterocyclic amine formation in both chemical model systems and beef patties. Mol. Nutr. Food Res. 51:969-976.

Cheng KW, Wong CC, Chao J, Lo C, Chen F, Chu IK, Che CM, Ho CT, Wang M. 2009. Inhibition of mutagenic PhIP formation by epigallocatechin gallate *via* scavenging of phenylacetaldehyde. Mol. Nutr. Food Res. 53:716-725.

Choudhary S, Sood S, Donnell RL, Wang HCR. 2012. Intervention of human breast cell carcinogenesis chronically induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. Carcinogenesis. 33:876-885.

Damašius J, Venskutonis PR, Ferracane R, Fogliano V. 2011. Assessment of the influence of some spice extracts o the formation of heterocyclic amines in meat. Food Chem. 126:149-156.

Felton JS, Knize MG, Shen NH, Lewis PR, Andresen BD, Happe J, Hatch FT. 1986. The isolation and identification of a new mutagen from fried ground beef: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Carcinogenesis. 7:1081-1086.

Gross GA, Grüter A. 1992. Quantitation of mutagenic/carcinogenic heterocyclic aromatic amines in food products. J. Chrom. A. 592:271-278.

Gross GA, Grüter A, Heyland S. 1992. Optimization of the sensitivity of high-performance liquid chromatography in the detection of heterocyclic aromatic amine mutagens. Food and Chem. Toxicol. 30:491-498.

Hussain FA. 2015. Formation of 4(5)-methylimidazole in processed meat and in glucose amino acid model system [DPhil dissertation]. Manhattan, KS: Kansas State Univ. 183 p. Available from: http://hdl.handle.net/2097/18975.

[IARC] International Agency for Research on Cancer. 1997. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. In: IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, vol. 56. Lyon: World Health Organization, International Agency for Research on Cancer. p 163-242.

Ito N, Hasegawa R, Sano M, Tamano S, Esumi H, Takayama S, Sugimura T. 1991. A new colon and mammary carcinogen in cooked food, 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP). Carcinogenesis. 12:1503-1506.

Johansson MAE, Fay LB, Gross GA, Olsson K, Jägerstad M. 1995. Influence of amino acids on the formation of mutagenic/carcinogenic heterocyclic amines in a model system. Carcinogenesis. 16:2553-2560.

Johansson MAE, Jägerstad M. 1996. Influence of pro- and antioxidants on the formation of mutagenic-carcinogenic heterocyclic amines in a model system. Food Chem. 56:69-75.

Kapoor IPS, Singh B, Singh G, de Heluani CS, de Lampasona MP, Catalan CAN. 2009. Chemistry and in vitro antioxidant activity of volatile oil and oleoresins of black pepper (*Piper nigrum*). J. Agric. Food Chem. 57:5358-5364.

Kikugawa K. 1999. Involvement of free radicals in the formation of heterocyclic amines and prevention by antioxidants. Cancer Lett. 143:123-126.

Mittal R, Gupta RL. 2000. *In vitro* antioxidant activity of piperine. Methods Find. Exp. Clin. Pharmacol. 22:271-274.

Moon SE, Shin HS. 2013. Inhibition of mutagenic 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) formation using various food ingredients in a model systems. Food Sci. Biotechnol. 22:323-329.

Murkovic M. 2004. Formation of heterocyclic aromatic amines in model systems. J. Chrom. B. 802:3-10.

National Toxicology Program (NTP). 2014. Heterocyclic amines. In: Report on Carcinogens. 13th ed. Research Triangle Park, NC: U.S. Department of Health and Human Services. Available from: http://ntp.niehs.nih.gov/pubhealth/roc/roc13/.

Oguri A, Suda M, Totsuka Y, Sugimura T, Wakabayashi K. 1998. Inhibitory effects of antioxidants on formation of heterocyclic amines. Mutat. Res. 402:237-245.

Oz F, Kaya M. 2011. The inhibitory effect of black pepper on formation of heterocyclic aromatic amines in high-fat meatball. Food Control. 22:596-600.

Puangsombat K, Gadgil P, Houser TA, Hunt MC, Smith JS. 2012. Occurrence of heterocyclic amines in cooked meat products. Meat Sci. 90:739-746.

Puangsombat K, Smith JS. 2010. Inhibition of heterocyclic amine formation in beef patties by ethanolic extracts of rosemary. J. Food Sci. 75:T40-T47.

Ravindran PN, Kallupurackal JA. 2001. Black Pepper. In: Peter KV, editor. Handbook of Herbs and Spices, vol. 1. Cambridge, England: Woodhead Publishing Limited. p 62-110.

Scott IM, Puniani E, Jensen H, Livesey JF, Poveda L, Sánchez-Vindas P, Durst T, Arnason JT. 2005. Analysis of piperaceae germplasm by HPLC and LCMS: a method for isolating and identifying unsaturated amides from *Piper* spp extracts. J. Agric. Food Chem. 53:1907-1913.

Shioya M, Wakabayashi K, Sato S, Nagao M, Sugimura T. 1987. Formation of a mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in cooked beef, by heating a mixture containing creatinine, phenylalanine and glucose. Mutat. Res. 191:133-138.

Shirai T, Sano M, Tamano S, Takahashi S, Hirose M, Futakuchi M, Hasegawa R, Imaida K, Matsumoto, Wakabayashi K, Sugimura T, Ito N. 1997. The prostate: a target for carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) derived from cooked foods. Cancer Res. 57:195-198.

Tipsrisukond N, Fernando LN, Clarke AD. 1998. Antioxidant effects of essential oil and oleoresin of black pepper from supercritical carbon dioxide extractions in ground pork. J. Agric. Food Chem. 46:4329-4333.

Tsen SY, Ameri F, Smith JS. 2006. Effects of rosemary extracts on the reduction of heterocyclic amines in beef patties. J. Food Sci. 71:C469-C473.

Vijayakumar RS, Surya D, Nalini N. 2004. Antioxidant efficacy of black pepper (*Piper nigrum* L.) and piperine in rats with high fat diet induced oxidative stress. Redox Rep. 9:105-110.

Vitaglione P, Fogliano V. 2004. Use of antioxidants to minimize the human health risk associated to mutagenic/carcinogenic heterocyclic amines in food. J. Chrom. B. 802:189-199.

Zachariah TJ, Parthasarathy VA. 2008. Black Pepper. In: Parthasarathy VA, Chempakam B, Zachariah TJ, editors. Chemistry of Spices. United Kingdom: Biddles Ltd, King's Lynn. p 21-40.

Zöchling S, Murkovic M. 2002. Formation of the heterocyclic aromatic amine PhIP: identification of precursors and intermediates. Food Chem. 79:125-134.

Appendix A - Supplementary Data

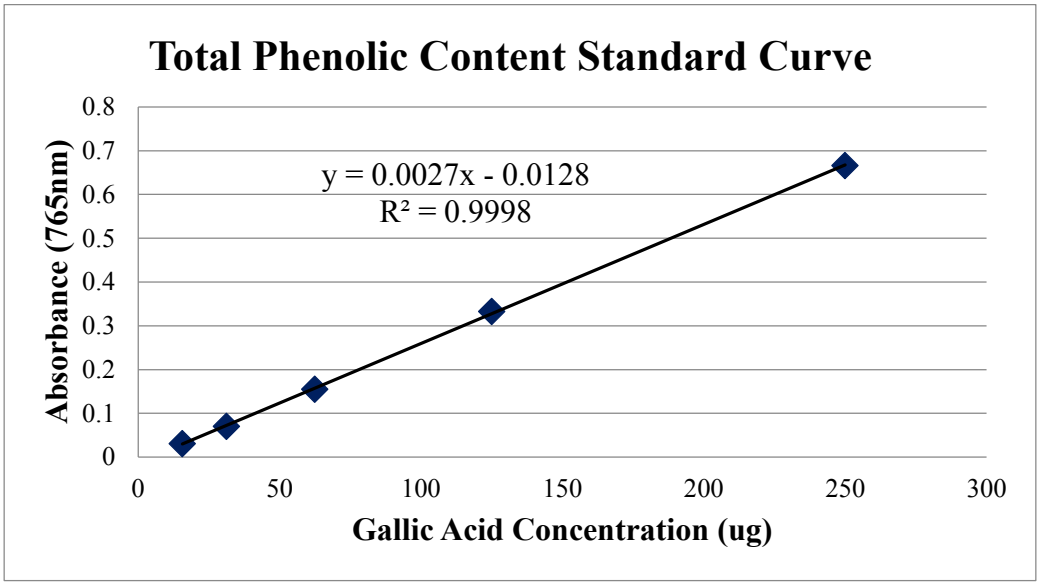


Figure A-1: Total phenolic content standard curve for Gallic acid concentration (µg)

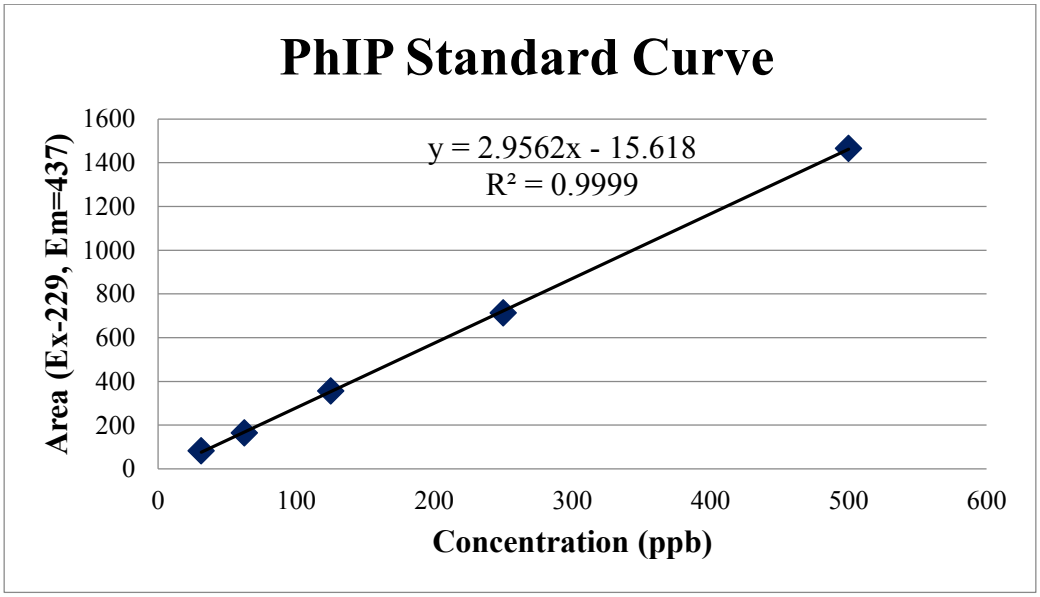


Figure A-2: PhIP standard curve measured using HPLC fluorescence at Ex=229, Em=347

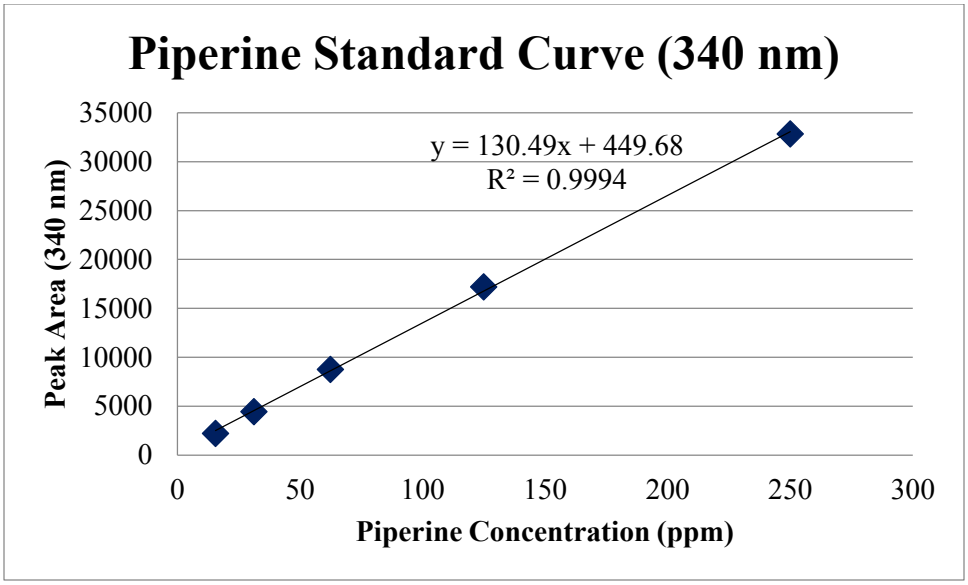


Figure A-3: Piperine standard curve measured using HPLC UV at 340 nm

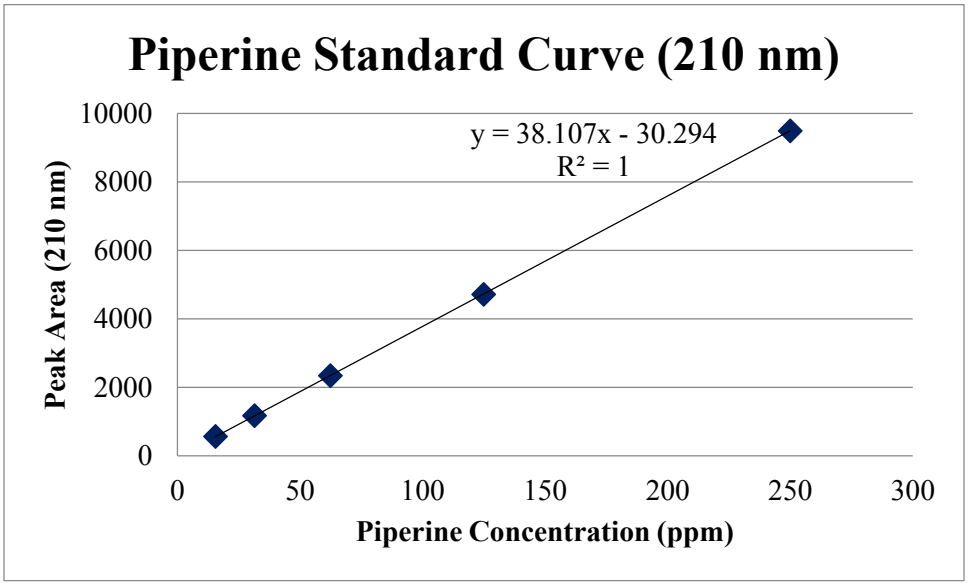


Figure A-4: Piperine standard curve measured using HPLC UV at 210 nm

Excel Data for Solvent Concentration Comparisons, TPC, DPPH, and Inhibition Data

Table A-1: Effect of water:diethylene glycol ratio on PhIP formation in a model system

Water Percentage	Diethylene Glycol Percentage	Avg. PhIP Conc. (mg/L)	% CV
20	80	1.357 ± 0.185	13.65
15	85	1.304 ± 0.156	11.93
10	90	1.469 ± 0.137	9.310
5	95	0.763 ± 0.479	62.71
0	100	0.736 ± 0.327	44.47

Table A-2: Total phenolic content for spices and antioxidants

Sample	Replicate	Absorbance (765nm)	mg Gallic acid	Avg mg Gallic Acid/g
Black Pepper	1	0.211	0.414	
Black Pepper	2	0.208	0.409	0.420 ± 0.016
Black Pepper	3	0.224	0.438	
Rosemary	1	1.045	1.959	
Rosemary	2	1.064	1.994	1.983 ± 0.021
Rosemary	3	1.065	1.996	
BHT[1]	1	0.365	279.8	
BHT[1]	2	0.280	216.8	254.13 ± 33.08
BHT[1]	3	0.346	265.8	
TBHQ[1]	1	0.473	359.8	
TBHQ[1]	2	0.496	376.8	372.4 ± 11.08
TBHQ[1]	3	0.501	380.6	
Blk Pep Oil	1	0.958	2.275	
Blk Pep Oil	2	1.033	2.446	2.433 ± 0.152
Blk Pep Oil	3	1.091	2.578	
Blk Pep Ext	1	0.127	0.357	
Blk Pep Ext	2	0.111	0.326	0.318 ± 0.043
Blk Pep Ext	3	0.083	0.272	
Rose Ext	1	0.505	1.083	
Rose Ext	2	0.429	0.937	1.070 ± 0.130
Rose Ext	3	0.561	1.191	

[1]Samples were diluted an additional 1:10

Table A-3: Radical scavenging activity (%) for spices and antioxidants

Sample	Replicate	Absorbance	Radical Scavenging Activity (%)	Avg. Radical Scavenging Activity (%)
Black Pepper	1	1.050	3.047	
Black Pepper	2	1.056	2.493	2.74 ± 0.282
Black Pepper	3	1.054	2.678	
Rosemary	1	0.568	47.55	
Rosemary	2	0.596	44.97	46.32 ± 1.518
Rosemary	3	0.580	46.45	
BHT	1	0.769	28.99	
BHT	2	0.832	23.18	24.75 ± 3.717
BHT	3	0.844	22.07	
TBHQ	1	1.003	7.387	
TBHQ	2	0.909	16.07	12.44 ± 4.511
TBHQ	3	0.933	13.85	
Blk. Pep. Oil	1	0.278	77.23	
Blk. Pep. Oil	2	0.276	77.40	76.74 ± 1.000
Blk. Pep. Oil	3	0.298	75.59	
Blk. Pep. Ext	1	1.107	9.34	
Blk. Pep. Ext	2	1.110	9.09	9.20 ± 0.128
Blk. Pep. Ext	3	1.109	9.17	
Rose. Ext.	1	0.112	90.83	
Rose. Ext.	2	0.107	91.24	91.13 ± 0.263
Rose. Ext.	3	0.106	91.32	

Table A-4: Effects of black pepper and rosemary on PhIP formation in model systems

Extract	Level (μL)	Dilution Factor	PhIP Control Avgs. (mg/L)	Avg. PhIP Conc. (mg/L)	Avg. % Decrease	p-Value
Black Pepper Oil	36	1.065	1.958 ± 0.433	1.343 ± 0.380	31.40 ± 19.44	0.0006
	71	1.129	1.965 ± 0.723	1.360 ± 0.482	30.78 ± 24.54	0.0033
	142	1.258	2.553 ± 0.188	1.896 ± 0.151	25.73 ± 5.910	< 0.0001
	285	1.518	2.961 ± 0.179	2.289 ± 0.284	22.69 ± 9.600	< 0.0001
	550	2.000	1.330 ± 0.127	0.751 ± 0.147	43.49 ± 11.02	< 0.0001
Black Pepper Extract	36	1.065	2.116 ± 0.356	1.582 ± 0.388	25.26 ± 18.33	0.0018
	71	1.129	1.965 ± 0.723	1.494 ± 0.415	24.00 ± 21.14	0.0059
	142	1.258	2.553 ± 0.188	1.774 ± 0.234	30.51 ± 9.163	< 0.0001
	285	1.518	2.961 ± 0.179	1.828 ± 0.433	38.26 ± 14.63	< 0.0001
	550	2.000	1.330 ± 0.127	0.790 ± 0.248	40.56 ± 18.62	< 0.0001
Rosemary Extract	36	1.065	1.847 ± 0.165	1.336 ± 0.359	27.66 ± 19.47	0.0015
	71	1.129	1.470 ± 0.200	1.145 ± 0.174	22.11 ± 11.85	0.0002
	142	1.258	1.536 ± 0.103	1.154 ± 0.200	24.88 ± 13.00	0.0002
	285	1.518	1.693 ± 0.197	0.949 ± 0.126	43.93 ± 7.455	< 0.0001
	550	2.000	1.495 ± 0.657	1.029 ± 0.394	31.14 ± 26.37	0.0046

Table A-5: Effects of BHT and TBHQ on PhIP formation in model systems

Antioxidant	Concentration (mmol)	PhIP Control (mg/L)	Avg. PhIP Conc. (mg/L)	Avg. % Decrease	p-Value
BHT	0.05	1.771	1.524 ± 0.110	13.96 ± 6.19	0.0073
	0.1	2.569	1.140 ± 0.106	55.62 ± 4.12	< 0.0001
	0.2	1.125	1.054 ± 0.183	6.299 ± 16.30	0.4364
	0.4	1.153	1.051 ± 0.188	8.867 ± 16.26	0.2897
TBHQ	0.05	1.682	1.363 ± 0.095	18.97 ± 5.64	0.0017
	0.1	2.086	1.585 ± 0.168	24.08 ± 8.04	0.0026
	0.2	2.009	1.568 ± 0.313	21.96 ± 15.56	0.0343
	0.4	0.872	0.918 ± 0.080	-5.27 ± 9.215	0.2703

Table A-6: Effect of piperine on PhIP formation in model systems

Level (mg)	PhIP Control Avg. (mg/L)	Avg. PhIP Conc. (mg/L)	Avg. % Decrease	p-Value
4.02	1.973 ± 0.142	1.501 ± 0.361	23.89 ± 76.60	0.0025
8.04	1.530 ± 0.511	1.222 ± 0.255	20.08 ± 83.08	0.0041
16.14	1.854 ± 74.25	1.418 ± 0.296	23.47 ± 67.95	0.0012
31.14	1.909 ± 0.144	1.360 ± 0.190	28.75 ± 34.56	< 0.0001

HPLC Chromatograms

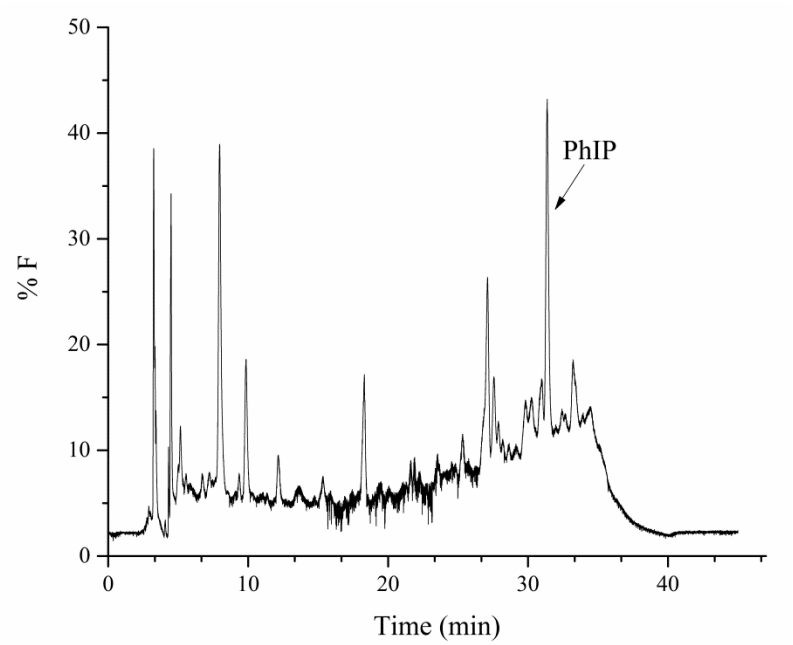


Figure A-5: HPLC chromatogram of PhIP model system control (0.22 mmol glucose, 0.44 mmol creatinine, 0.44 mmol phenylalanine in 10% water, 90% diethylene glycol solution), separated with a TSKgel ODS-80$_{TM}$ column as described in Chapter 2 (pg 33). The fluorescence detector was set at Ex=229, Em=437. PhIP eluted at 31.4 minutes.

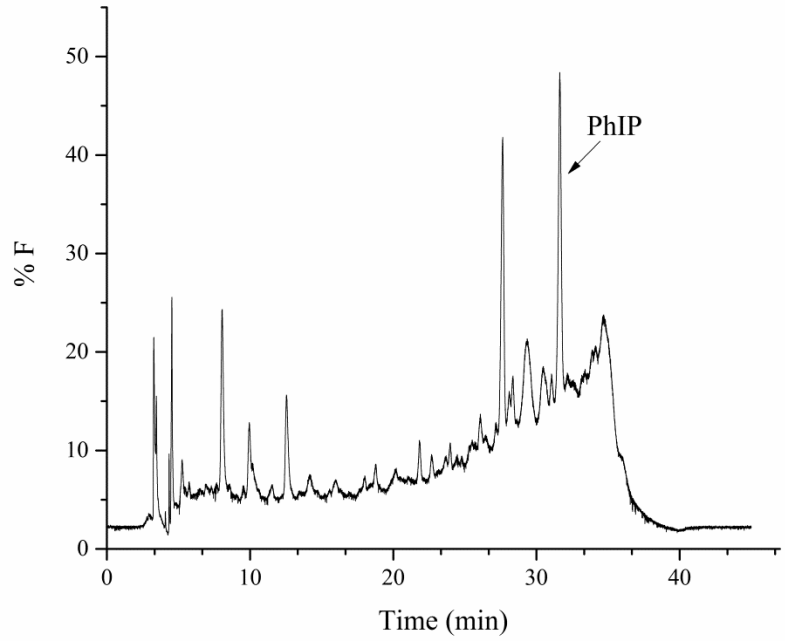


Figure A-6: HPLC chromatogram of a PhIP model system with 142 µL black pepper oil added; separated with a TSKgel ODS-80TM column. The fluorescence detector was set at Ex=229, Em=437.

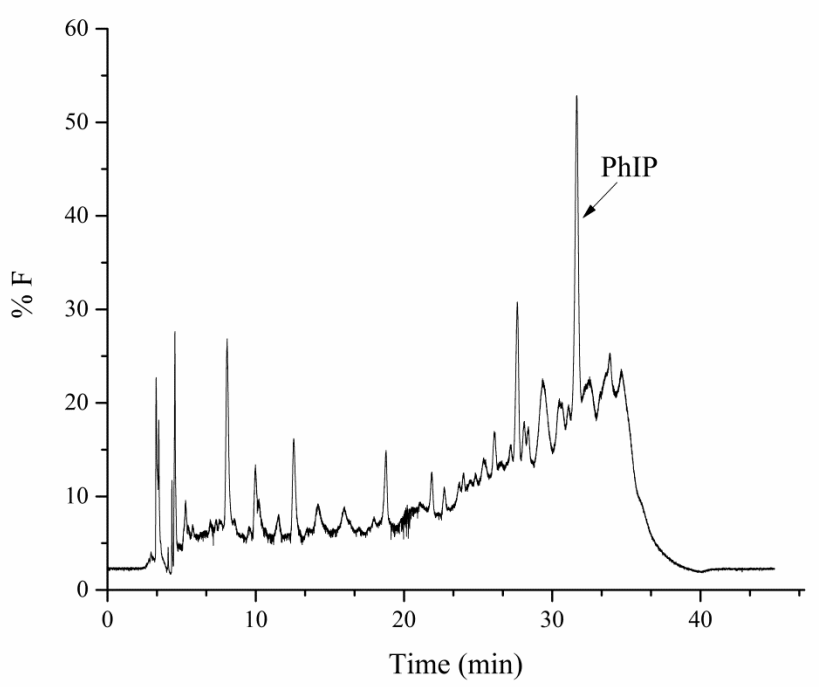


Figure A-7: HPLC chromatogram of a PhIP model system with 142 µL black pepper extract added; separated with a TSKgel ODS-80TM column. The fluorescence detector was set at Ex=229, Em=437.

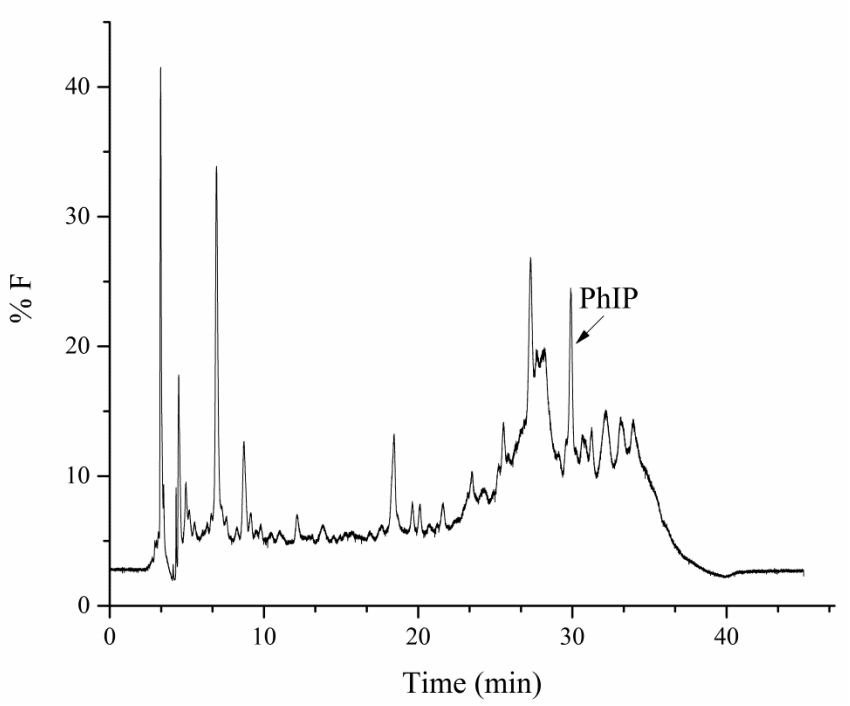


Figure A-8: HPLC chromatogram of a PhIP model system with 0.2mmol BHT added; separated with a TSKgel ODS-80$_{TM}$ column. The fluorescence detector was set at Ex=229, Em=437.

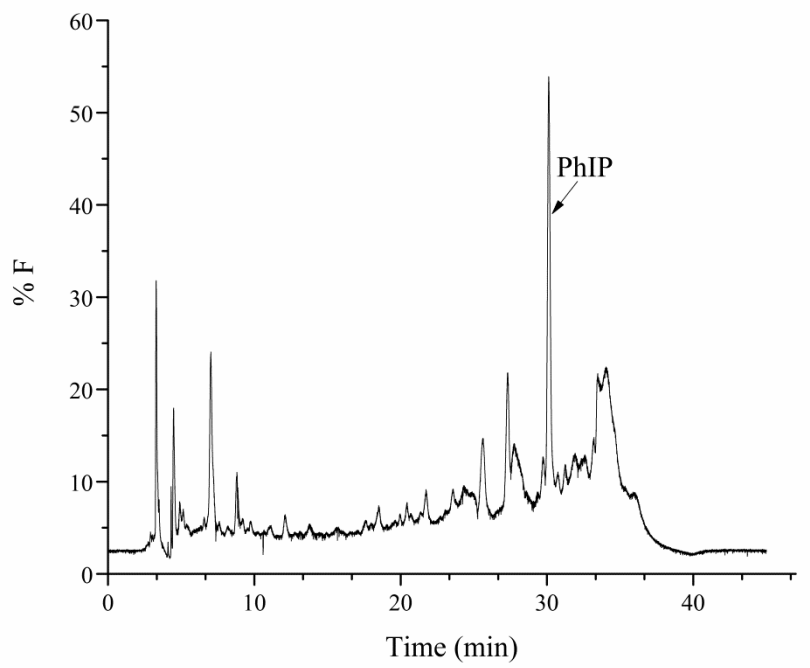


Figure A-9: HPLC chromatogram of a PhIP model system with 0.2mmol TBHQ added; separated with a TSKgel ODS-80$_{TM}$ column. The fluorescence detector was set at Ex=229, Em=437.

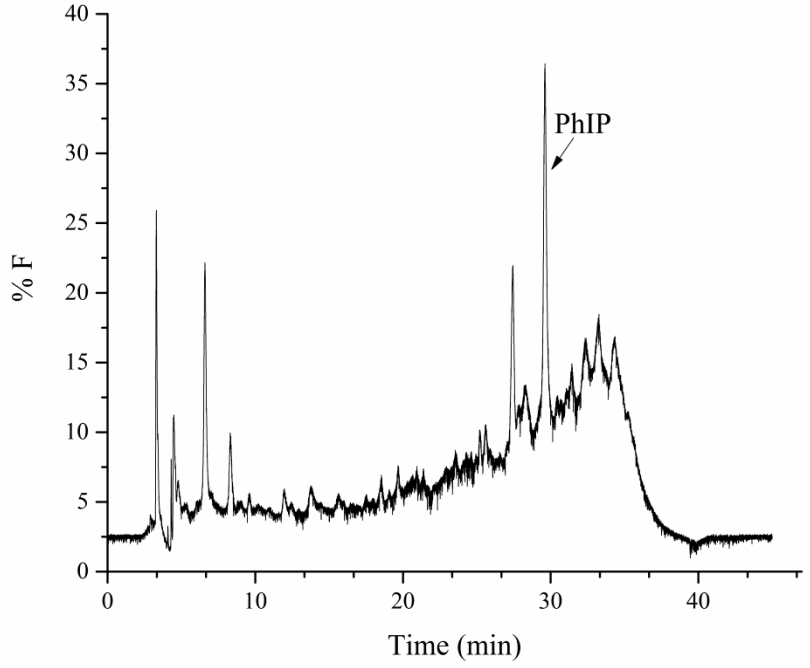


Figure A-10: HPLC chromatogram of a PhIP model system with 142µL rosemary extract added; separated with a TSKgel ODS-80$_{TM}$ column. The fluorescence detector was set at Ex=229, Em=437

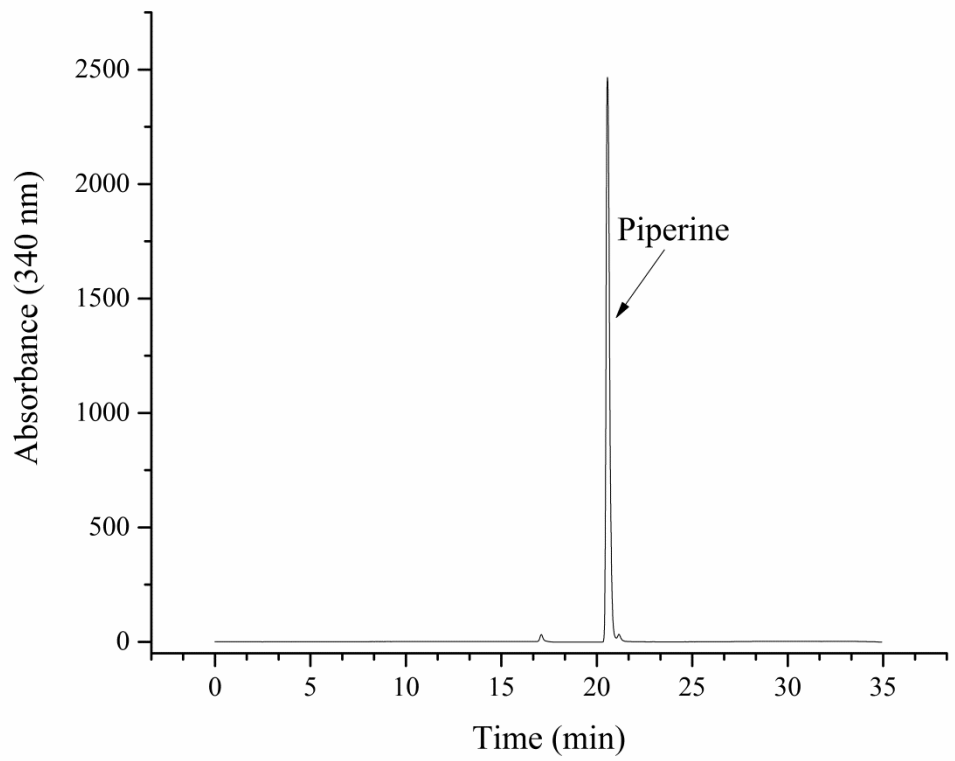
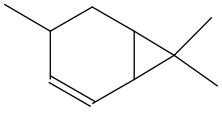
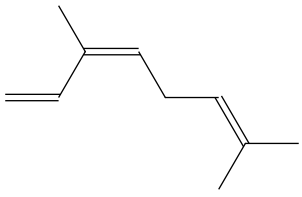
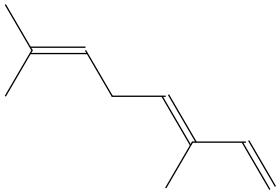
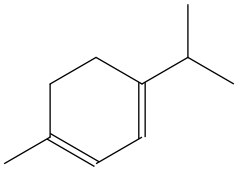
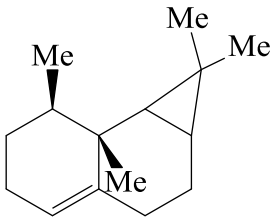
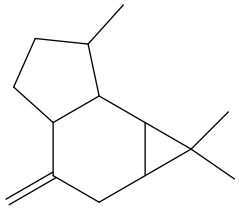
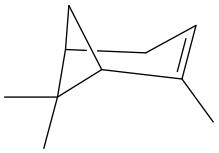
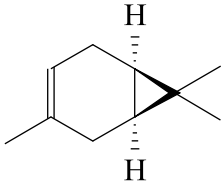
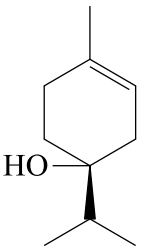


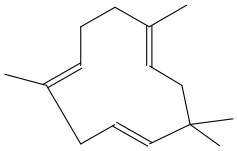
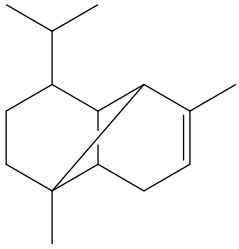
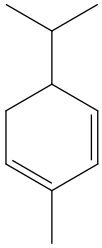
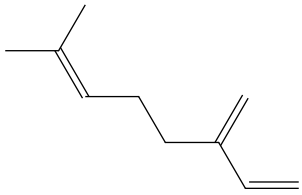
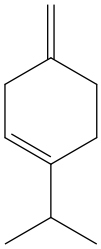
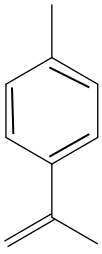
Figure A-11: HPLC chromatogram of 250 ppm piperine standard, separated with a TSKgel ODS-80$_{TM}$ column as described in Chapter 3 (pg 52). The UV detector was set at 340 nm. Piperine eluted at approximately 20.5 minutes.

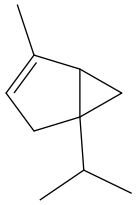
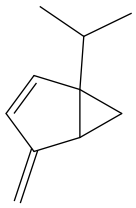
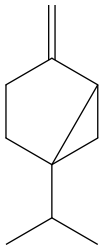
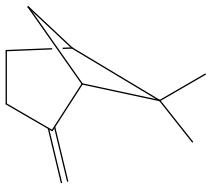
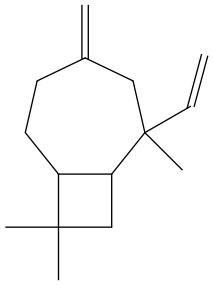
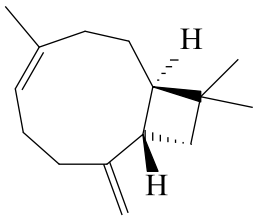
SPME Headspace Analysis of Black Pepper

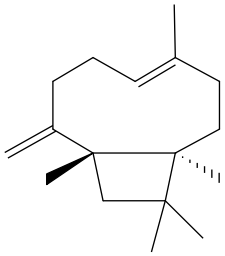
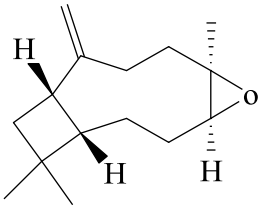
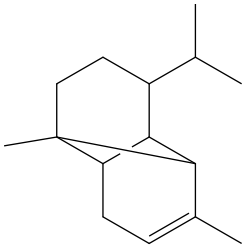
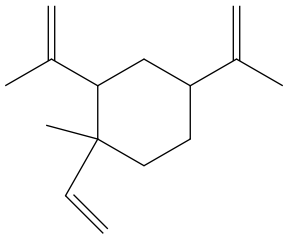
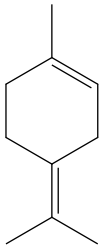
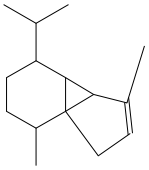
Table A-7: SPME headspace data summary for ground black pepper, black pepper oil, and black pepper extract. (GBP=ground black pepper; BPO=black pepper oil; BPE=black pepper extract). Values indicate retention times, blanks indicate the compound was not found in the corresponding additive.

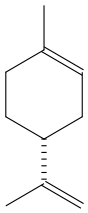
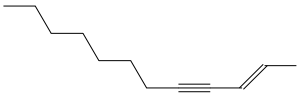
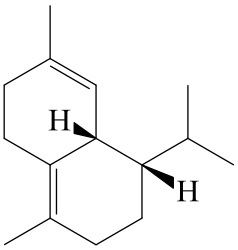
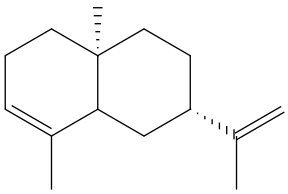
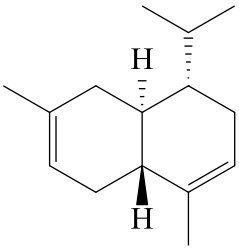
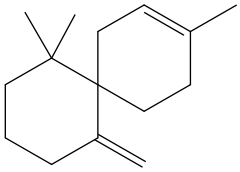
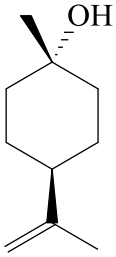
Chemical	Structure	GBP	BPO	BPE
(+)-4-carene			12.91	
1,3,6-octatriene,3,7-dimethyl-		10.51		

1,3,6-octatriene,3,7-dimethyl-(Z)-		11.20		
1,4-cyclohexadiene,1-methyl-4-(1-methylethyl)		12.25	12.19	
1H-cyclopropa(a)naphthalene,1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-,(1aR,7R,7aR,7bS)-		21.33		
1H-cycloprop(e)azulene,decahydro-1,1,7-trimethyl-4-methylene-,[1aR-(1aα,4aα,7α,7aβ,7bα)]-			21.72	
1R-α-pinene		9.35	9.35	9.32
3-carene		11.20	11.12	11.09
3-cyclohexen-1-ol,4-methyl-1-(1-methylethyl)-(R)-		14.96	14.94	

α-caryophyllene		21.09	21.00	20.85
α-cubebene		18.55	18.55	
α-phellandrene		9.14	10.97	
β-myrcene		10.56		
β-phellandrene		10.24		
Benzene,1-methyl-4-(1-methylethenyl)-			13.01	

Bicyclo(3.1.0)-hex-2-ene,2-methyl-5-(1-methylethyl)		9.14	9.13	
Bicyclo(3.1.0)hex-2-ene,4-methyl-1-(1-methylethyl)			10.24	
Bicyclo(3.1.0)hexane,4-methylene-1-(1-methylethyl)-				10.21
Bicyclo(3.1.1)heptane,6,6-dimethyl-2-methylene-, (1S)-			10.39	10.36
Bicyclo(5.2.0)nonane,2-methylene-4,8,8-trimethyl-4-vinyl		20.88		
Bicyclo(7.2.0)undec-4-ene,4,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-			20.81	

Caryophyllene		20.62	20.47	20.15
Caryophyllene oxide			23.57	
Copaene		19.20		19.12
Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-,(1α,2β,4β)-			19.43	19.36
Cyclohexene,1-methyl-4-(1-methylethylidene)		12.93		
Cyclohexene,4-ethenyl-4-methyl-3-(1-methylethenyl)		18.33	18.33	18.27

D-limonene		11.79	11.62	11.51
(E)-2-Dodecen-4-yne			23.42	
Naphthalene,1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,(1S-cis)-		22.42	22.47	
Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-,[2R-(2α,4aα,8aβ)]		21.78		21.62
Naphthalene,1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,[1S-(1α,4aβ,8aα)]-			21.30	
Spiro(5.5)undec-2-ene,3,7,7-trimethyl-11-methylene-,(-)-		21.95		
Terpineol, cis-β		12.44		

3D Plots of Piperine

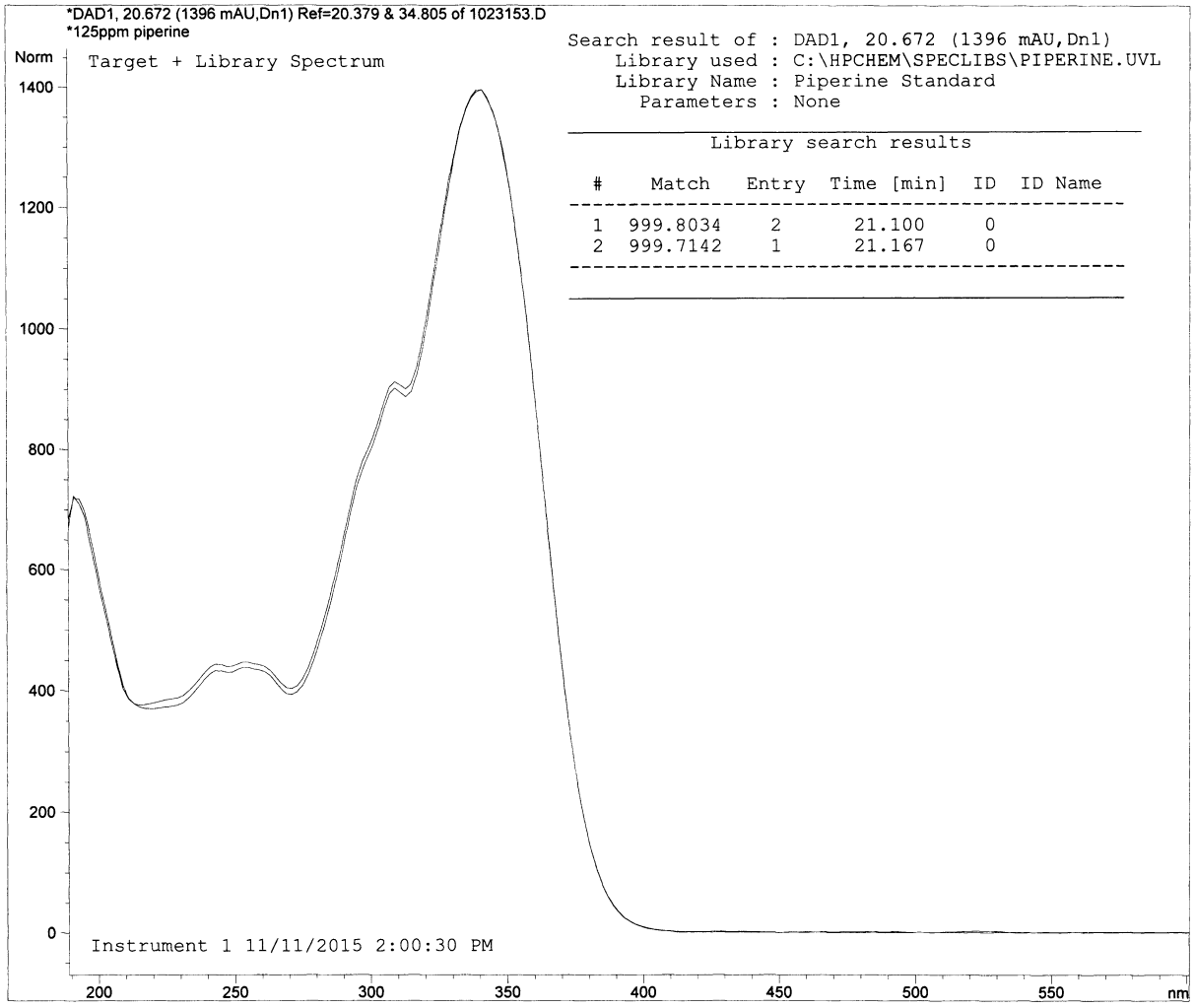


Figure A-12: Spectral match of piperine (bottom line) with a compound in black pepper extract (top line) peak at 21.1 minutes.

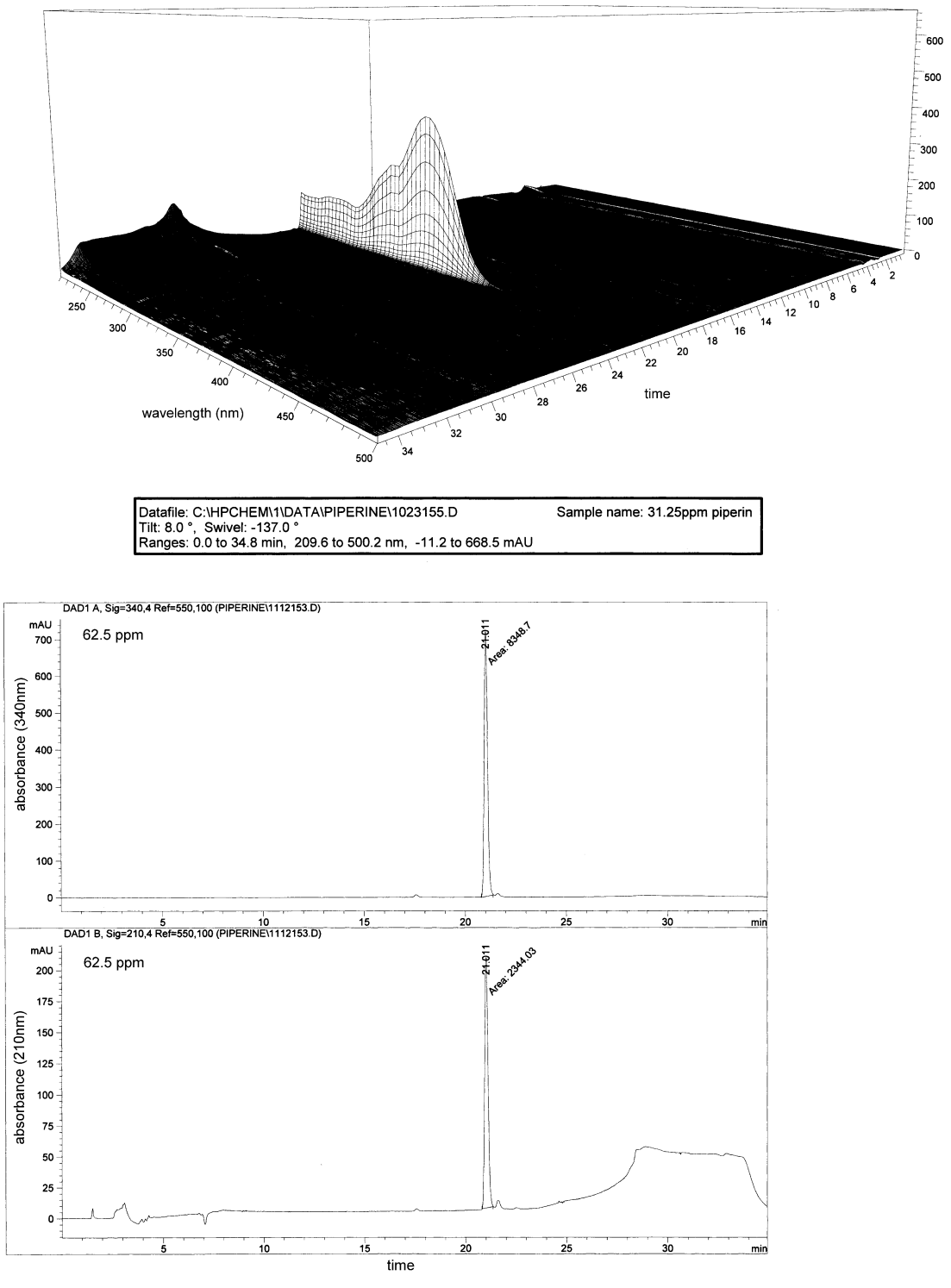


Figure A-13: 3D plot and 2D plots of 62.5 ppm piperine standard.

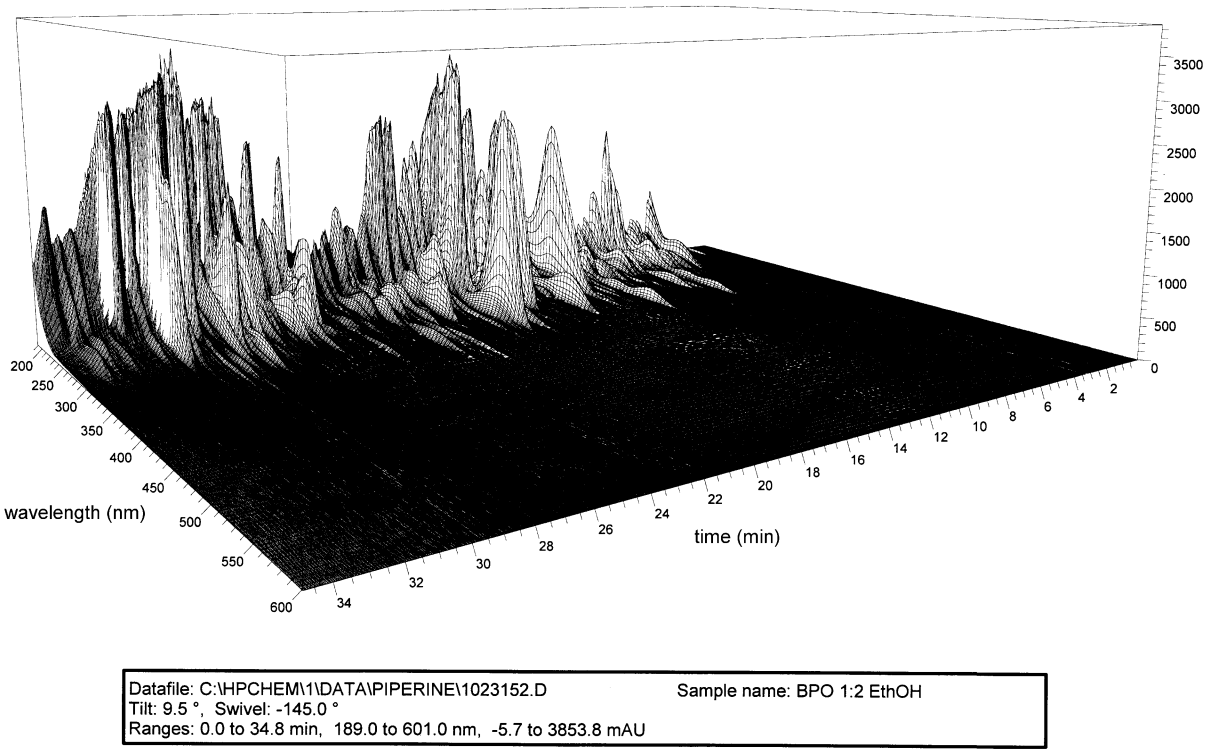


Figure A-14: 3D plot of black pepper oil diluted 1:2 with ethanol; 2D plot shown in Chapter 3 (pg. 56).

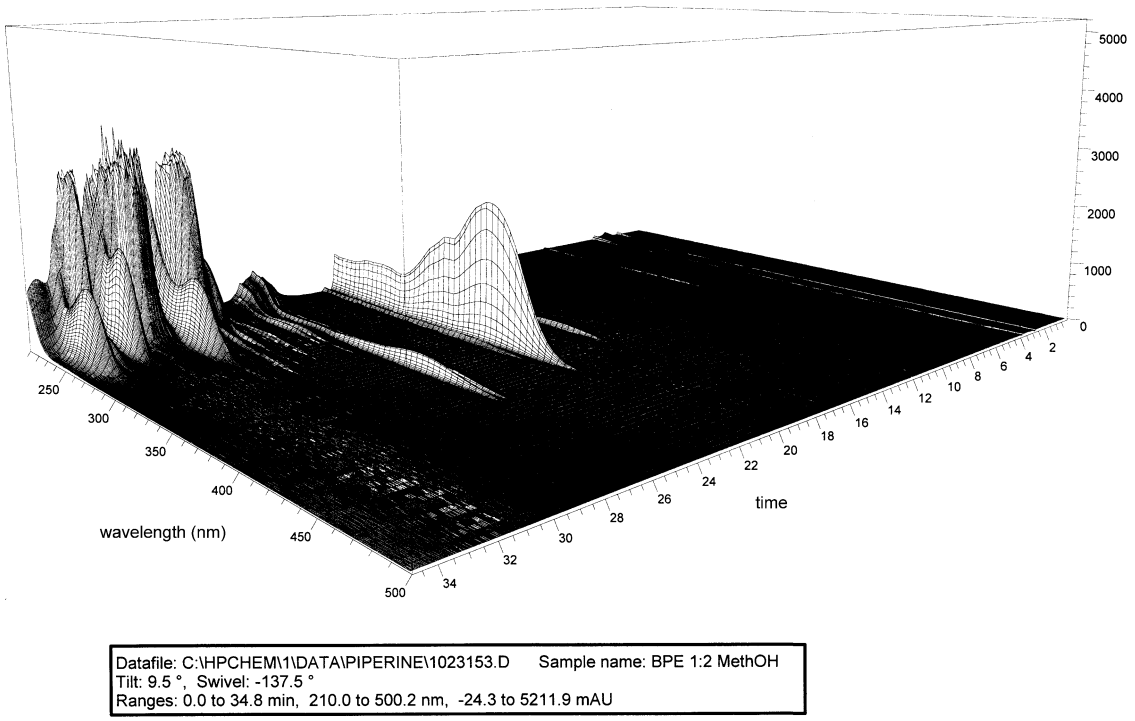


Figure A-15: 3D plot of black pepper extract diluted 1:2 in methanol; 2D plot shown in Chapter 3 (pg. 58).

SAS Codes

```
data bht1;
  input Trt1 Cont1;
datalines;
1607      1771
1584      1771
1377      1771
1615      1771
1436      1771
;
run;
  ods graphics on;
   proc ttest;
     paired Cont1*Trt1;
   run;

   data bht2;
  input Trt2 Cont2;
datalines;
1075      2569
1288      2569
1206      2569
1025      2569
1107      2569
;
run;
  proc ttest;
     paired Cont2*Trt2;
   run;

   data bht3;
  input Trt3 Cont3;
datalines;
808.8     1125
1126      1125
1142      1125
1267      1125
926.9     1125
;
run;
   proc ttest;
     paired Cont3*Trt3;
   run;

    data bht4;
  input Trt4 Cont4;
datalines;
798.1     1153
1122      1153
1157      1153
1258      1153
918.7     1153
;
run;
   proc ttest;
     paired Cont4*Trt4;
   run;

data blackpepperextract1;
  input Trt1 Cont1;
datalines;
1353      2116
1136      2116
1298      2116
1114      2116
2000      2116
1989      2116
1377      2116
1530      2116
2172      2116
1847      2116
;
run;
  ods graphics on;
   proc ttest;
     paired Cont1*Trt1;
   run;

data blackpepperextract2;
  input Trt2 Cont2;
datalines;
1127      1965
1105      1965
1183      1965
924.8     1965
1529      1965
1397      1965
1688      1965
1985      1965
2152      1965
1846      1965
;
run;
   proc ttest;
     paired Cont2*Trt2;
   run;

   data blackpepperextract3;
  input Trt3 Cont3;
datalines;
2095      2553
1944      2553
1690      2553
1284      2553
2014      2553
1753      2553
1546      2553
1791      2553
```

```
1799    2553
1827    2553
;
run;
  proc ttest;
    paired Cont3*Trt3;
  run;

   data blackpepperextract4;
input Trt4 Cont4;
datalines;
1758    2961
1180    2961
2169    2961
2096    2961
2022    2961
1966    2961
1583    2961
1638    2961
1262    2961
2605    2961
;
run;
  proc ttest;
    paired Cont4*Trt4;
  run;

 data blackpepperextract5;
 input Trt5 Cont5;
datalines;
688.6   1330
565.0   1330
928.8   1330
442.0   1330
741.0   1330
561.8   1330
766.6   1330
869.4   1330
1157    1330
1183    1330
;
run;
  proc ttest;
    paired Cont5*Trt5;
  run;

data blackpepperoil1;
 input Trt1 Cont1;
datalines;
826.2   1958
1093    1958
1118    1958
1034    1958
1170    1958
1565    1958
1360    1958
1371    1958
1847    1958
2045    1958
;
run;
 ods graphics on;
  proc ttest;
    paired Cont1*Trt1;
  run;

  data blackpepperoil2;
input Trt2 Cont2;
datalines;
1134    1965
1219    1965
959.6   1965
819.5   1965
1038    1965
858.2   1965
2130    1965
1824    1965
1813    1965
1809    1965
;
run;
proc ttest;
    paired Cont2*Trt2;
  run;

  data blackpepperoil3;
 input Trt3 Cont3;
datalines;
1858    2553
2231    2553
1906    2553
1707    2553
1809    2553
1757    2553
1790    2553
1980    2553
1930    2553
1993    2553
;
run;
  proc ttest;
    paired Cont3*Trt3;
  run;

   data blackpepperoil4;
 input Trt4 Cont4;
datalines;
1926    2961
2751    2961
2338    2961
2553    2961
2020    2961
```

```
2537    2961
1910    2961
2238    2961
2430    2961
2187    2961
;
run;
  proc ttest;
    paired Cont4*Trt4;
  run;

 data blackpepperoil5;
 input Trt5 Cont5;
datalines;
613.0   1330
710.2   1330
576.2   1330
540.4   1330
971.6   1330
832.0   1330
809.4   1330
884.4   1330
692.4   1330
884.2   1330
;
run;
  proc ttest;
    paired Cont5*Trt5;
  run;

data piperine1;
 input Trt1 Cont1;
datalines;
2133    1973
1521    1973
1213    1973
1047    1973
1020    1973
1931    1973
1645    1973
1663    1973
1480    1973
1360    1973
;
run;
 ods graphics on;
  proc ttest;
    paired Cont1*Trt1;
  run;

data piperine2;
 input Trt2 Cont2;
datalines;
1063    1530
1134    1530
1074    1530
970.9   1530
978.0   1530
1626    1530
1425    1530
1654    1530
1208    1530
1091    1530
;
run;
  proc ttest;
    paired Cont2*Trt2;
  run;

  data piperine3;
 input Trt3 Cont3;
datalines;
1968    1854
1727    1854
1504    1854
1607    1854
1395    1854
1402    1854
1327    1854
1130    1854
1061    1854
1063    1854
;
run;
  proc ttest;
    paired Cont3*Trt3;
  run;

   data piperine4;
 input Trt4 Cont4;
datalines;
1658    1909
1538    1909
1260    1909
1186    1909
1122    1909
1509    1909
1416    1909
1476    1909
1102    1909
1331    1909
;
run;
  proc ttest;
    paired Cont4*Trt4;
  run;

data rosemary;
 input Trt1 Cont1;
datalines;
1226    1847
1117    1847
```

```
852.0    1847
1108     1847
921.3    1847
1709     1847
1427     1847
1694     1847
1360     1847
1943     1847
;
run;
 ods graphics on;
  proc ttest;
    paired Cont1*Trt1;
  run;

data rosemary2;
 input Trt2 Cont2;
datalines;
1005     1470
992.6    1470
962.1    1470
1032     1470
1062     1470
1412     1470
1465     1470
1155     1470
1163     1470
1197     1470
;
run;
  proc ttest;
    paired Cont2*Trt2;
  run;

  data rosemary3;
 input Trt3 Cont3;
datalines;
1066     1536
1430     1536
1379     1536
1165     1536
1366     1536
1240     1536
1122     1536
986.0    1536
944.3    1536
840.7    1536
;
run;
  proc ttest;
    paired Cont3*Trt3;
  run;

   data rosemary4;
 input Trt4 Cont4;
datalines;
718.8    1693
1084     1693
1066     1693
973.6    1693
861.6    1693
1129     1693
1008     1693
900.8    1693
853.3    1693
897.1    1693
;
run;
  proc ttest;
    paired Cont4*Trt4;
  run;

 data rosemary5;
 input Trt5 Cont5;
datalines;
1320     1495
1884     1495
784.2    1495
855.6    1495
1462     1495
777.6    1495
650.0    1495
893.2    1495
863.0    1495
801.4    1495
;
run;
  proc ttest;
    paired Cont5*Trt5;
  run;

data tbhq1;
 input Trt1 Cont1;
datalines;
1364     1682
1395     1682
1389     1682
1206     1682
1461     1682
;
run;
 ods graphics on;
  proc ttest;
    paired Cont1*Trt1;
  run;

data tbhq2;
 input Trt2 Cont2;
datalines;
1648     2086
1826     2086
1534     2086
```

```
1545    2086
1370    2086
;
run;
  proc ttest;
    paired Cont2*Trt2;
  run;

  data tbhq3;
 input Trt3 Cont3;
datalines;
1711    2009
1950    2009
1116    2009
1437    2009
1625    2009
;
run;
  proc ttest;
    paired Cont3*Trt3;
  run;

   data tbhq4;
 input Trt4 Cont4;
datalines;
891.5   871.8
927.8   871.8
1039    871.8
914.1   871.8
816.2   871.8
;
run;
  proc ttest;
    paired Cont4*Trt4;
  run;
```