THE EVALUATION OF HETEROCYCLIC AMINE FORMATION IN CHEMICAL MODEL SYSTEMS

by

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Abstract

Heterocyclic amines (HCAs) are potentially carcinogenic and highly mutagenic by-products of the Maillard browning reaction that form specifically in high temperature cooked meat products. Consumption of HCAs has been associated with various cancers including prostate, breast, colon, and pancreatic cancers and efforts have been made to understand formation and inhibition of these compounds. Chemical model systems are a preferred method to study the in vitro formation and inhibition of HCAs as the complex matrix effects found in meat are eliminated. Two black pepper extracts were evaluated for their efficacy on PhIP formation in model systems, but no significant results were observed. Secondly, four Maillard reaction variables were evaluated for their effect on formation of five HCAs (IQ, IQx, MeIQ, MeIQx, and 4,8-DiMeIQx) in chemical model systems with an effort to define an ideal model system. Precursor molar concentration (0.2/0.2, 0.4/0.4, 0.6/0.6, and 0.8/0.8 mmol), water percentage (0, 5, 10, and 15%), sugar type (fructose, galactose, glucose, and lactose), and sugar molar amount (quarter, half, equi, and double molar) were the four Maillard variables examined in the study. Additionally, four antioxidants (butylated hydroxyanisole (BHA), epigallocatechin gallate (EGCG), rosmarinic acid, and naringenin) were evaluated for their effect on HCA formation in chemical model systems. All four Maillard variables had a significant effect ($p < 0.05$) on the formation of HCAs in the model system, with an interaction effect occurring between water percentage and precursor concentration. The four antioxidants had no effect on the formation of HCAs in the model system. A model system containing 0.6/0.6/1.2 mmol of threonine, creatinine, and glucose, with 15% water was determined to be the best representative chemical model system for the formation of HCAs commonly formed in meats.

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Chapter 1 - Literature Review

Introduction

In 1912, French chemist, Louis-Camille Maillard first described non-enzymatic browning reactions, hence the name Maillard browning reactions. These browning reactions involve a mixture of amino acids, reducing sugars, and pH-dependent solutions in a high temperature environment. Maillard reactions produce desirable flavors and colors in baked goods, beers, and roasted coffees.

Unfortunately, undesirable side products occur in browning reactions. One negative by-product formed through Maillard reactions is heterocyclic amines (HCAs). These potentially carcinogenic and highly mutagenic compounds form during the heating of muscle-rich food products (Murkovic 2004). Research shows creatin(in)e, an amino acid, and a reducing sugar, as well as a high temperature environment, are critical in the formation of HCAs.

Case study research may indicate that humans consume approximately 250 - 300 ng of HCAs per day (Nowell and others 2002; Li and others 2007) and with consumption of there is an association with breast, colon, prostate, and pancreatic cancers. Therefore, recent efforts are currently focused on the reduction of HCA development in cooked meats. Research shows antioxidant spices, added to food prior to cooking, reduce the formation of HCAs (Tsen and others 2006; Puangsombat and Smith 2010).

Chemical model systems allow for study of HCAs in a laboratory setting. Model systems allow researchers to vary precursor compositions and concentrations, while observing the effect time and temperature have on the reaction (Bordas and others 2004). Conflicting research indicates antioxidants both inhibit (Moon and Shin 2013) and promote (Zöchling and Murkovic 2002; Cheng and others 2007) formation of HCAs in model systems.

The following literature review will describe the health implications, formation, and current methods to inhibit formation of HCAs. The final section discusses the formation of HCAs in chemical model systems.

Health Implications of Heterocyclic Amines

The occurrence of carcinogenic substances in cooked meats dates back to 1939, when, Swedish chemist, E.M.P Widmark demonstrated both male and female mice developed

malignant tumors when topically exposed to organic solvent extracts of roasted horse meat (Widmark 1939). Since 1939, further research has identified four types of cancers potentially induced by consumption of HCAs: breast (Sinha and others 2000), colon (De Stefani and others 1997; Nowell and others 2002), prostate (Tang and others 2007), and pancreatic cancers (Li and others 2007).

Heterocyclic amines require metabolic activation to function as mutagens. Therefore, dietary intake and extent at which HCAs are metabolized determine carcinogenic risk for humans (Cross and Sinha 2004). The most abundant HCAs formed in cooked meat are 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4-8-trimethyl-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine (PhIP), with PhIP being the most abundant (Zheng and Lee 2009). The HCAs 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), are also present in meat. Case studies approximate human consumption to be approximately 250 – 300 ng per day, with the highest levels being PhIP (~160 – 240 ng), MeIQx (~70 – 90 ng), and 4,8-DiMeIQx (~4 – 6 ng) (Nowell and others 2002; Li and others 2007).

Various case studies have identified an association between the consumption of cooked meat (beef, poultry, pork, and fish) and the development of certain types of cancers. In a 2-year case study in Sweden, Gerhardsson de Verdier and others (1991) found a direct association among those who frequently consumed well-done meats and colon, rectum, and colorectal cancers. A case study completed in Uruguay presented similar associations, suggesting a strong association with meat intake and colon, rectum, and colorectal cancers (De Stefani and others 1997).

The National Toxicology Program has classified MeIQ, IQ, MeIQx, and PhIP as *reasonably anticipated to be human carcinogens* (U.S Department of Health and Human Services 2011). The International Agency for Research on Cancer has presented similar classifications: IQ as *probably carcinogenic to humans* (Group 2A) and MeIQ, MeIQx, and PhIP as *possibly carcinogenic to humans* (Group 2B) (International Agency for Cancer Research 1993). This indicates a significant carcinogenic potential when frequently exposed to these genotoxic compounds (Cheng and others 2006). Fully understanding the mechanism of reaction for HCAs may better clarify how these compounds are mutagenic and potentially carcinogenic to humans.

Formation of Heterocyclic Amines

To date, 25 HCAs have been identified by the Ames/*Salmonella* Test (Sanz Alaejos and others 2008) with concentrations in the parts per billion ranges. HCAs form in fried, grilled, baked, or roasted muscle meats and fish (Murkovic 2004); fried, grilled, and barbequed techniques producing highest amounts (Cross and Sinha 2004). HCAs occur at higher intensities in the crust of meats or fish and at a lesser extent in the interior, as cooking temperatures are greatest at the surface (Skog and others 1995). In cooked meat products, HCA formation has been found to be 3.5 times greater in well-done meat, as compared to medium-rare meat (Puangsombat and others 2012).

HCAs are present in low concentrations following boiling, but significantly increase above 150 ^{o}C (Murkovic 2004). In addition to the surface of muscle-rich foods, pan residues contain HCAs. An explanation for this phenomenon is an escape of precursors prior to or during cooking (Skog and others 1995). The formation and concentration of HCAs is dependent on numerous physical and chemical properties, including meat type, pH, water activity, and precursor amounts, including amino acids, creatin(in)e, and carbohydrates (Oz and Kaya 2011). Temperature also effects HCA formation; the reaction is initiated by temperature, but excessive heat causes HCA degradation.

Mechanism of Reaction

All HCAs contain one aromatic group and a heterocyclic structure; thus the name heterocyclic aromatic amines (Cheng and others 2006). Two main groups of HCAs exist: polar and non-polar moieties. The terms polar and non-polar identify the order of elution in reversed-phase chromatography as well as fluorescence of the substances. Polar HCAs elute first in reversed-phase chromatography with non-polar HCAs eluting last. Non-polar HCAs fluoresce intensely in polar solvents. PhIP, a polar HCA, also possesses fluorescent properties (Murkovic 2007).

Polar HCAs belong to a general class of compounds called aminoimidazo-azaarenes (AIAs). Of the AIAs, there are three subgroups. Most polar HCAs fall in the categories quinoline and quinoxaline types, but there are some pyridine HCAs (Murkovic 2004). Quinoline HCAs commonly known as IQ-type include IQ and MeIQ. Quinoxaline HCAs, or IQx-type, include

IQx and MeIQx, among others. PhIP, DMIP, and TMIP are pyridine-type HCAs (Cheng and others 2006).

All polar HCAs possess a five-membered imidazole ring. There is an amino group in the second position and a methyl group in the third position of the imidazole ring. Additionally, one or more six-membered aromatic rings are bound to the imidazole structure (Felton and others 1986).

Figure 1-1 illustrates various structures of the polar HCAs, specifically IQ and IQx-moieties. Structure A is the base structure of IQ-type HCAs. IQ and MeIQ are two IQ-type HCAs shown in Figure 1-1. Structure B is the base structure of IQx-type HCAs. IQx-moiety HCAs listed below in Figure 1-1 are IQx, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, and 4,7,8-DiMeIQx. The "R" character represents where functional groups interchange, based on the HCA. A hydrogen atom and a methyl group are the two functional groups that change among each of the HCA molecules.

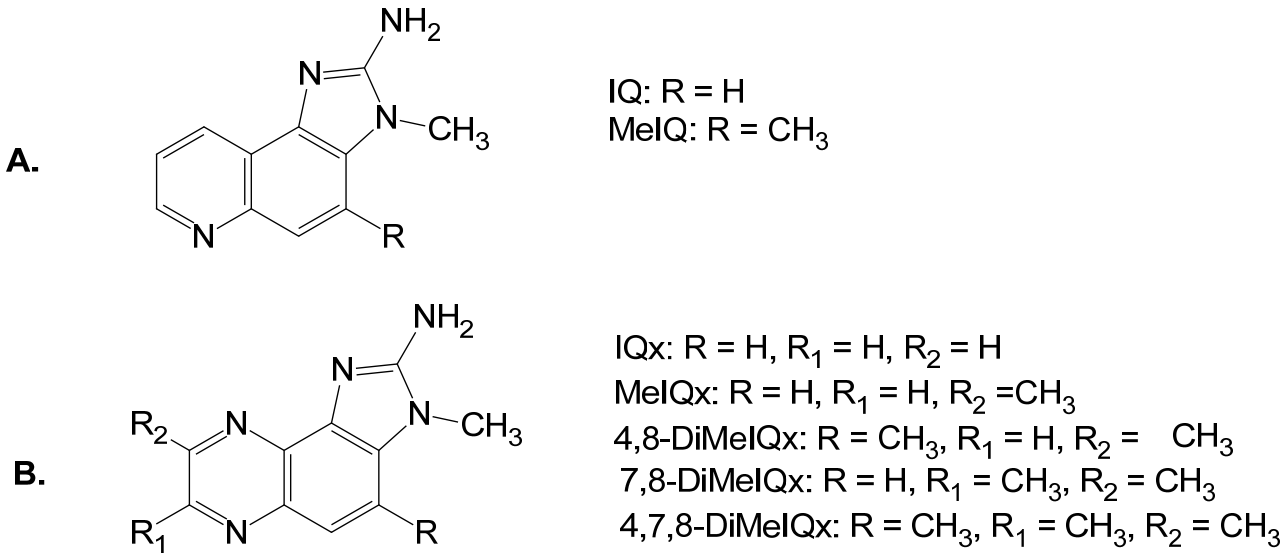


Figure 1-1: Chemical structures of polar HCAs. Structure A is representative of two IQ-type HCAs and structure B is representative of five IQx-type (Adapted from Murkovic, 2004)

Polar HCAs form as by-products of non-enzymatic browning reactions, known as Maillard reactions. Chemistry of these reactions is extremely complex, incorporating a network of various reactions. These molecules form in the temperature range of 150 to 200 ^{o}C via a reaction among essential precursors: an amino acid, a reducing sugar, and creatin(in)e (Murkovic

2004). Strecker aldehydes, products of Maillard reactions, and creatin(in)e are both necessary in formation of imidazo-type HCAs (Vitaglione and Fogliano 2004).

Free radical formation has two proposed pathways (Figure 1-2). The first pathway is formation of pyridine radicals through IQ-type HCA pathway. Glycol-aldehyde alkylimide oxidizes to glyoxal monoalkylimine, which then reduces to glyoxal. Glyoxal condenses, leading to pyridine free radicals. The second pathway is formation of dialkyl-pyrazine radicals, leading to formation of IQx-type HCAs. A biomolecular ring from the enaminol type of glycoaldehyde alkylimine forms. After an electron loss, formation of dialkyl-pyrazine radicals occurs (Pearson and others 1992; Jägerstad, M., Skog, K., Arvidsson, P. 1998).

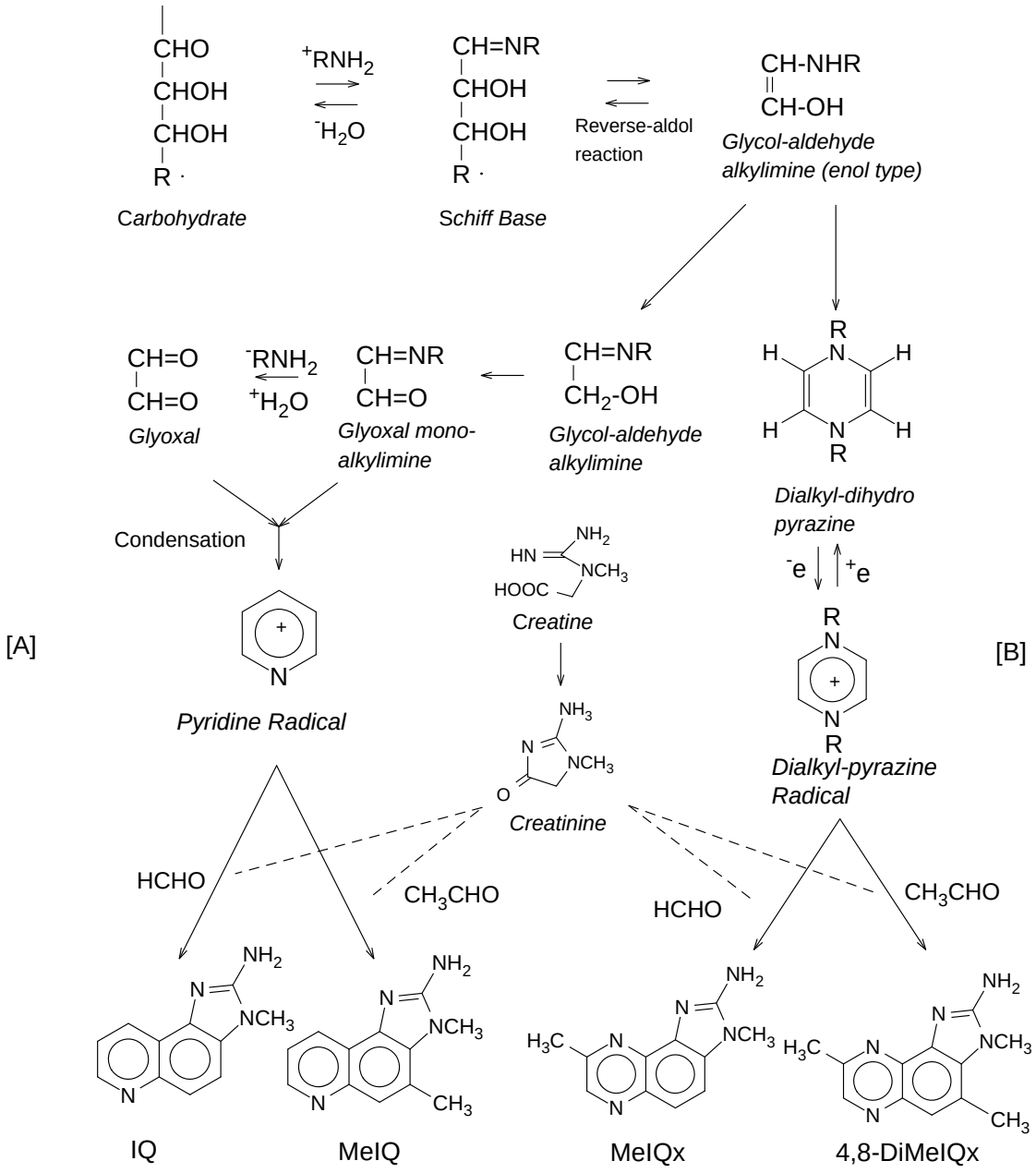


Figure 1-2: Suggested pathways of IQ [A] and IQx [B] compounds (Adapted from Vitaglione and Fogliano 2004)

Jägerstad and others (1983) proposed Maillard reaction intermediates, pyridines and pyrazines, react with aldehyde and creatin(in)e, producing IQ or IQx-containing compounds. The absence of creatin(in)e prevents formation of IQ and IQx-type HCAs. The glycoaldehyde pathway is a faster reaction. Therefore, the prominence of IQx moieties is greater in meat products than IQ-type compounds (Murkovic 2004).

Structures and abbreviations of 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), 3,4-cyclopentenopyrido[3,2-α]carbazole (Lys-P-1), 4-amino-6-methyl-1*H*-2,5,10,10*b*-tetraazafluoranthene (Orn-P-1), 1-methyl-9*H*-pyrido[3,4-*b*]indole (Harman), 9*H*-pyrido[3,4-*b*]indole (Norharman), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-aminodipyrido[1,2-α:3',2'-*d*]imidazole (Glu-P-2), 2-amino-6-methyldipyrido[1,2-α:3',2'-*d*]imidazole (Glu-P-1), 2-Amino-5-phenylpyridine (Phe-P-1), non-polar HCAs, can be found in Figure 1-3.

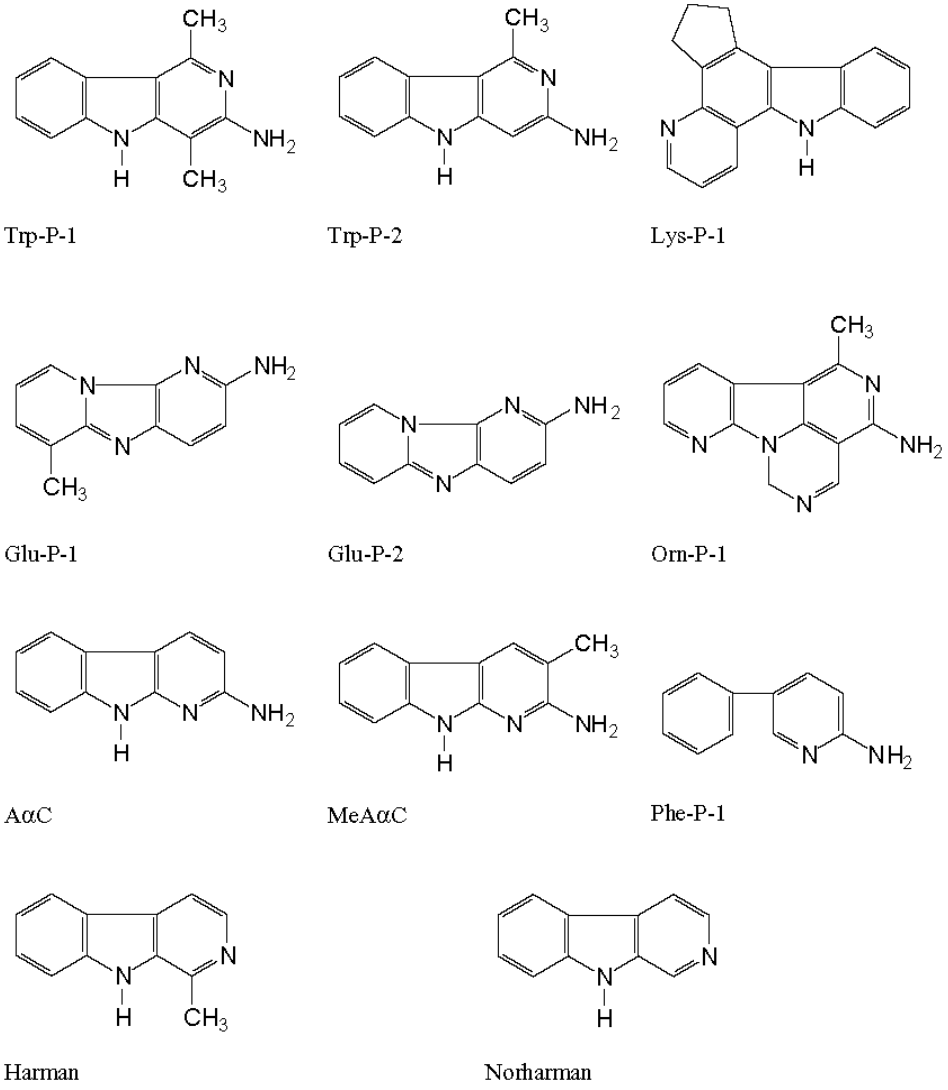


Figure 1-3: Chemical structures of non-polar heterocyclic amines (Murkovic 2004)

Non-polar HCAs have two classifications: 2-amino-pyridine or amino-carboline mutagens. Five subgroups further divide non-polar HCAs: pyridoindoles, pyridoimidazoles, phenylpyridines, tetraazafluoranthrenes, and benzimidazoles (Cheng and others 2006). Amino-carbolines possess two, six-membered aromatic rings with an imidazole ring in between. One or both of the six-membered rings can be a pyridine ring, where a nitrogen group replaces a methine group (CH). Unlike polar HCAs, if methyl groups are present in the structure, they attach to an aromatic ring (Cheng and others 2006).

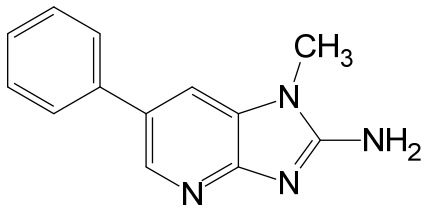
Formation of non-polar amines does not depend on creatin(in)e (Murkovic 2004; Cheng and others 2006). Often called protein pyrolysates (Cheng and others 2006), non-polar HCAs form as pyrolysis products of amino acids (Murkovic 2004; Murkovic 2007), favoring temperatures exceeding 300 ^{o}C (Cheng and others 2006).

Various hypotheses suggest mechanism of reactions for non-polar HCAs, including pathways involving free radicals and the subsequent fragments and products. Skog and others (2000) suggest amino-carbolines form through free radical reactions. These reactions produce reactive fragments that condense to form non-polar HCAs (Skog and others 2000). Because these reactions occur at such high temperatures, hypotheses prove difficult to research. Therefore, formation of non-polar HCAs has little evidentiary support (Cheng and others 2006).

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

PhIP is one of the most abundant HCAs formed in cooked meats under normal cooking conditions (Zöchling and Murkovic 2002). Research indicates this mutagenic compound induces tumors in laboratory animals, specifically prostate, liver, colon, and mammary carcinomas (Ito and others 1991; Sinha and others 2000; Tang and others 2007). In cooked foods, studies show PhIP levels average about 9.2 ± 1.20 ng/g in fried pork, 5.3 ± 0.81 ng/g in fried beef, and 6.1 ± 0.01 ng/g in fried chicken (skinless breasts) (Puangsombat and others 2012). Lynch (1992) has identified PhIP as one of the most absorbed HCAs by the body.

PhIP forms similar to other polar HCAs, via a pyrolysis reaction among creatin(in)e, amino acids, and reducing sugars (Skog and Jägerstad 1990). Like quinoline and quinoxaline compounds, PhIP contains an imidazole group (Shioya and others 1987) bound to a pyridine ring (Skog and Jägerstad 1990); therefore the classification as a pyridine-type HCA. Figure 1-4 illustrates the chemical structure of PhIP.



PhIP

Figure 1-4: Chemical structure of HCA, PhIP (Murkovic 2004)

Felton and others (1986) first isolated and identified PhIP from ground beef patties. Since identification, research from chemical model systems presents a description of a possible formation pathway of PhIP. Shioya and others (1987) identified phenylalanine, creatinine, and glucose as possible precursors of PhIP.

Zöchling and Murkovic (2002) describe the mechanism of PhIP formation beginning with thermal degradation of phenylalanine with glucose to form a Strecker aldehyde, phenylacetaldehyde. Strecker aldehydes form via *a*-amino acid degradation by reducing sugars, catalyzed by *a*-dicarbonyls (Hofmann and Schieberle 2000). Phenylacetaldehyde undergoes an adol condensation with creatinine (Figure 1-5 [A]) (Murkovic 2004) where carbon-5 of creatinine reacts with phenylacetaldehyde through a nucleophilic addition. This leads to dehydration and formation of an adol condensation intermediate (Figure 1-5, [B]).

Two theories exist as to how the PhIP forms from an adol intermediate. The first hypothesis of PhIP formation is that PhIP form as a Schiff's base. The reaction to form a Schiff's base occurs between creatinine portions of adol intermediates and phenylalanine. The second proposal is creatinine adol intermediates react with 2-phenylethylamine (Zöchling and Murkovic 2002). Murkovic (2004) suggests nitrogen of the pyridine ring of PhIP has two possible origins. The first explanation of the nitrogen forms from the amino group when creatinine reacts with oxo groups of adol intermediates. The second reasoning is nitrogen originates from the amino group of phenylalanine (Murkovic 2004). Figure 1-5 illustrates Murkovic's (2004) proposed formation of PhIP.

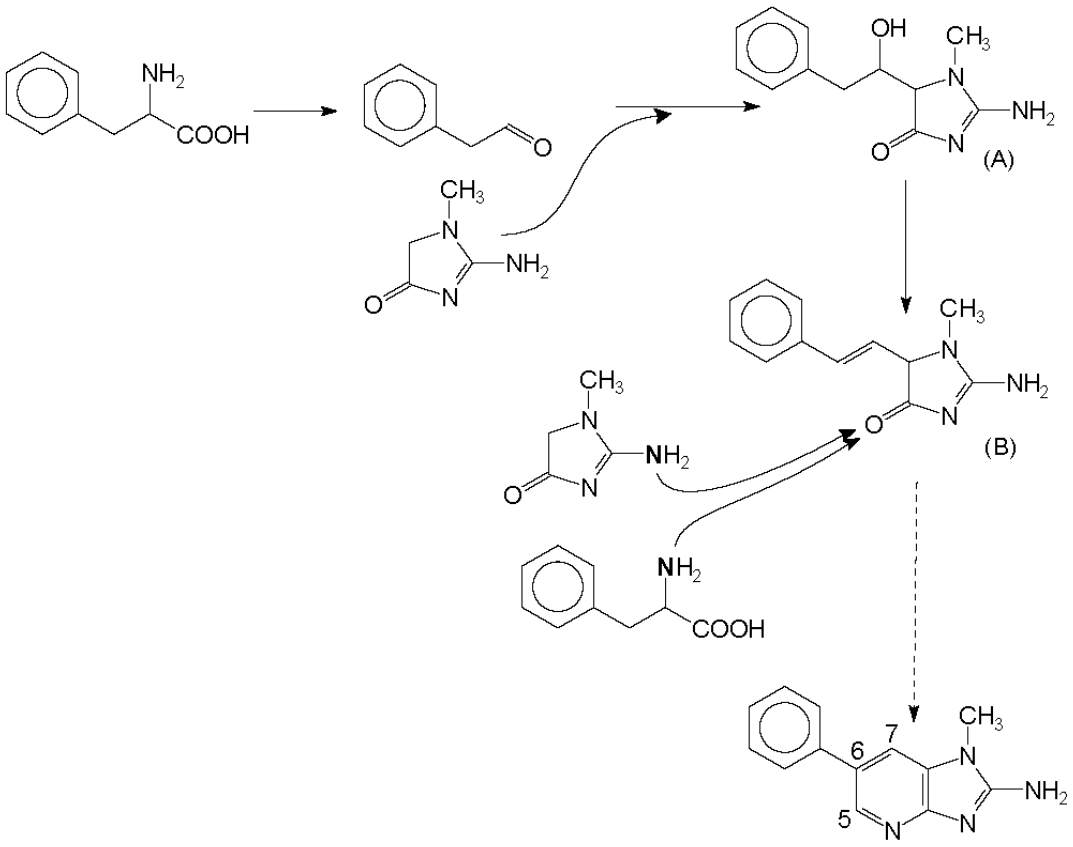


Figure 1-5: Proposed pathway of formation of PhIP. [A] is the addition product and [B] is the condensation product (Murkovic 2004)

Effect of Carbohydrates and Sugars

Sugar is an essential precursor of Maillard reactions, the origin of HCAs. By varying concentrations and types of mono- and disaccharides, HCA yields change greatly (Skog and Jägerstad 1990). When reducing sugars added to a meat system exceed the optimum level for HCA formation (2 to 4% in ground patties), alternate Maillard reactions are favored, further inhibiting the formation of HCAs (Skog and others 1992).

Formation of HCAs requires glucose, specifically, MeIQx and DiMeIQx (Skog and Jägerstad 1990). PhIP does form without glucose, but upon addition of glucose, PhIP formation increases three fold (Skog and Jägerstad 1991). Skog and Jägerstad (1990) suggest HCA formation is greatest when glucose is half-molar to other precursors. When equimolar or greater than creatin(in)e, remarkably lower yields of HCA formation occurs (Skog and Jägerstad 1990; Skog and Jägerstad 1991).

When compared to glucose, fructose produces more HCAs, though fructose has similar inhibitory effects. Lactose and sucrose effect formation of HCAs, but not as significantly as glucose (Skog and Jägerstad 1990). Though disaccharides exhibit similar results, monosaccharides have a more pronounced inhibitory effect (Skog and Jägerstad 1990). Starch, potato starch, and potato fiber inhibit HCAs in beef patties when added in excess amounts (Skog and others 1992).

Several hypotheses explain why inhibition transpires when sugars reach levels equimolar and greater than other precursors. Skog and Jägerstad (1990) suggest sugars, specifically glucose, added in excess, may react with creatin(in)e. Therefore, these reactions compete with the formation reaction of HCAs. Evidence supports this proposal, as glucose concentrations increase in model systems, unreacted creatin(in)e decreases. Persson and others (2004) also suggest carbohydrates participate in Maillard reactions, forming other products. Thus, these reactions hinder HCA formation.

An alternate explanation for HCA inhibition by reducing sugars is suppression of pyrazine cation radical Maillard intermediate formation. Kikugawa (1999) investigated this hypothesis with mixtures of 0-0.8 M glucose and 0.8 M glycine in a model system environment. Pyrazine cation radical intensities were highest at 0.4 M glucose levels. Additionally, mutagenicity formation was greatest at 0.4 M glucose concentration, thus supporting glucose levels directly affect pyrazine cation radicals and overall HCA formation (Kikugawa 1999).

Foods with high sugar contents, specifically honey, show effective inhibition of HCA formation and decreasing overall mutagenicity. Honey is rich in various compounds, including ascorbic acid, β-carotene, and α-tocopherol, which all act as antioxidants (Frankel and others 1998). In addition to the phenolic compounds, honey contains high amounts of sugar. When the antioxidant phenols and high sugar compounds combine, significant inhibition of HCAs occurs (Shin and others 2003). Buckwheat, clover, and sage unifloral honeys are dense in both fructose and glucose (34 – 40%) and contain lower amounts of maltose and sucrose.

In beef patties fried at 225 ºC for a total of 20 min, buckwheat honey inhibited the highest combined total of HCAs by 55%. Clover and sage varieties had similar effects, as clover inhibited by 52% and sage by 51%, when compared to the control patty (Shin and others 2003). Shin and Ustunol (2004) showed inhibition of PhIP, MeIQx, and 4,8-DiMeIQx in both fried beef patties and chicken breasts with honey-containing marinades. A 30% buckwheat marinade was

the most effective at reducing total HCA formation in beef patties by 41%. Inhibition of PhIP was greatest, with 43%, and both MeIQx, and 4,8-DiMeIQx were inhibited by 38% (Shin and Ustunol 2004).

Effect of Temperature

As with sugars, HCA formation depends highly on cooking time and temperatures (Knize and others 1994). Polar HCAs predominantly form between normal cooking temperatures, 150 and 200 ^{o}C (Jackson and Hargraves 1995; Arvidsson and others 1997). Increased cooking time or temperatures have direct effects on HCA development (Knize and others 1994; Skog and others 1995). HCA formation does not begin immediately. There is a lag time after initial heating and initial observation of HCAs. Jackson and Hargraves (1995) suggest the reaction must meet a minimum temperature for precursors to initiate formation of HCA molecules.

Arvidsson and others (1997) studied kinetic formation of HCAs as illustrated in Figure 1-6. In this research, HCAs reached a maximum amount, 5 to 10 min after heating. Depending on the HCA, concentrations of mutagen levels reached a plateau or began to decrease. At 225 ^{o}C, MeIQx and 7,8-DiMeIQx undergo degradation, following a peak in concentration. At 225 ^{o}C, PhIP is no longer detectable. Jackson and others (1995) as well as Skog and others (1990) observed similar results.

At 200 ^{o}C and 10 min of heating, HCAs maximize and then plateau. Arvidsson and others (1997) suggest that the plateau indicates glucose is no longer available for use in the reaction. At higher processing temperatures (>200 ^{o}C), HCAs begin to decrease. Skog and Jägerstad (1990) postulate side-browning reactions occur during formation of HCAs. These competing reactions inhibit further formation of HCAs. Several explanations describe the occurrence of degradation. The first postulation is MeIQx and DiMeIQx react with other browning products to form melanoidins, or brown polymers, in the reaction (Skog and Jägerstad 1990; Jackson and Hargraves 1995). Side browning reactions may also occur, which compete with HCA formation (Skog and Jägerstad 1990). HCAs may endure thermal degradation and therefore, are not stable under excessive heating conditions (Jackson and Hargraves 1995; Arvidsson and others 1997).

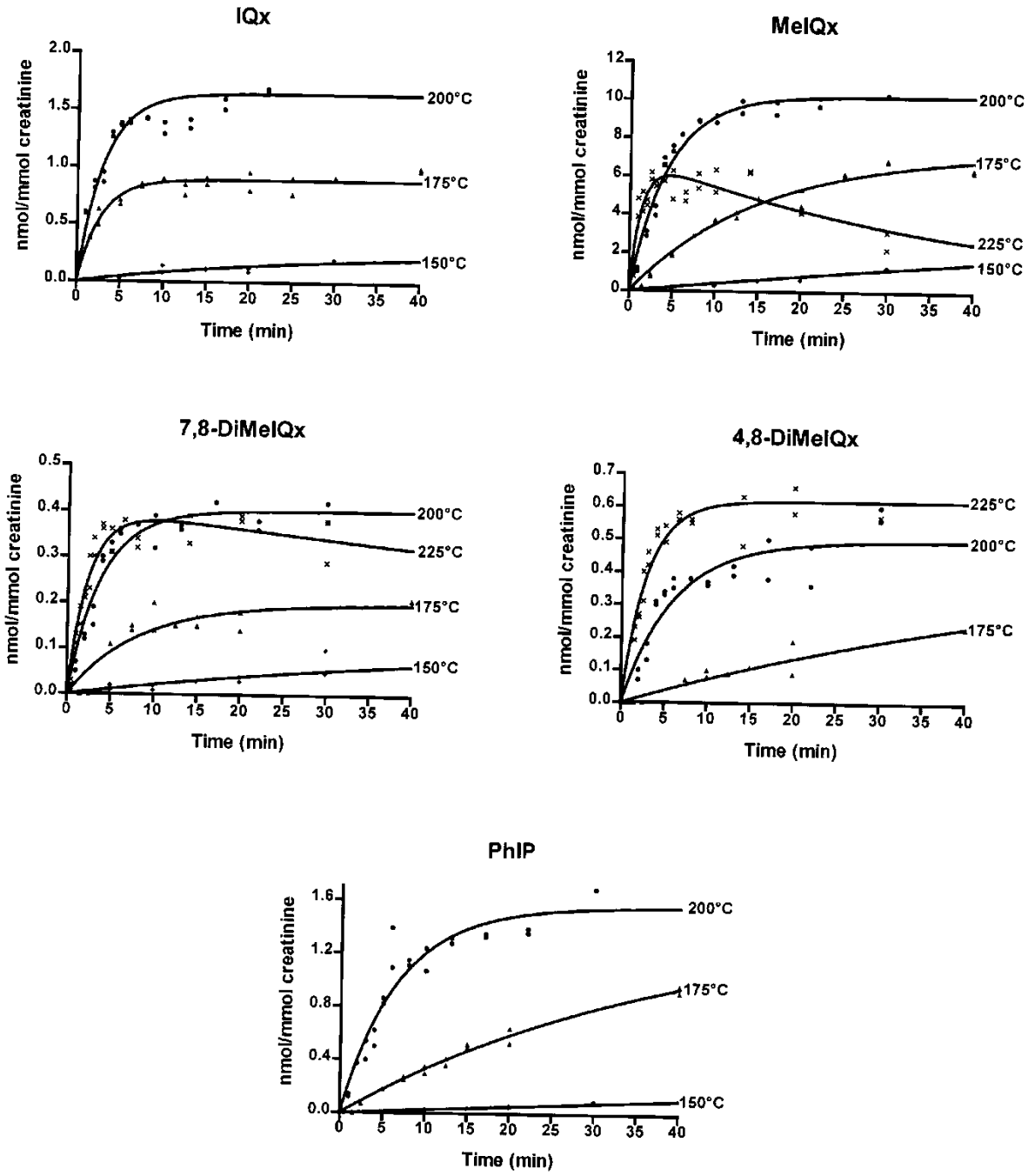


Figure 1-6: Formation of HCAs at 150, 175, 200, and 225 ^{o}C (Arvidsson and others 1997)

In addition to sugar and temperatures, other compounds effect formation of HCAs. Polyphenolic compounds, synthetic antioxidants, and antioxidant-containing spices reduce formation of HCAs. Unlike temperature and sugars, these compounds directly scavenge radicals formed during the reaction of HCAs.

Inhibiting Heterocyclic Amines

In years following the discovery of HCAs, research suggests unstable free radical Maillard reaction intermediates aid in formation of IQ and IQx-type HCAs (Pearson and others 1992). Variables, including pretreatment with marinades and microwave cooking, as well as addition of antioxidants have observable effects on HCA formation. Several different methods and treatments, including polyphenols, natural and synthetic antioxidants, and other inhibitory chemicals have great success in inhibiting HCAs in meat.

Research suggests antioxidants interfere at various point in HCA pathways, preventing formation of these mutagens. Antioxidants act as free radical scavengers at various steps in the HCA formation pathway, thus preventing the formation of HCAs (Figure 1-7). Vitaglione and Fogliano (2004) predict antioxidants scavenge pyridine and dialkyl-pyrazine free radicals in the IQ and IQx-type pathways; thus inhibiting IQ and IQx moieties.

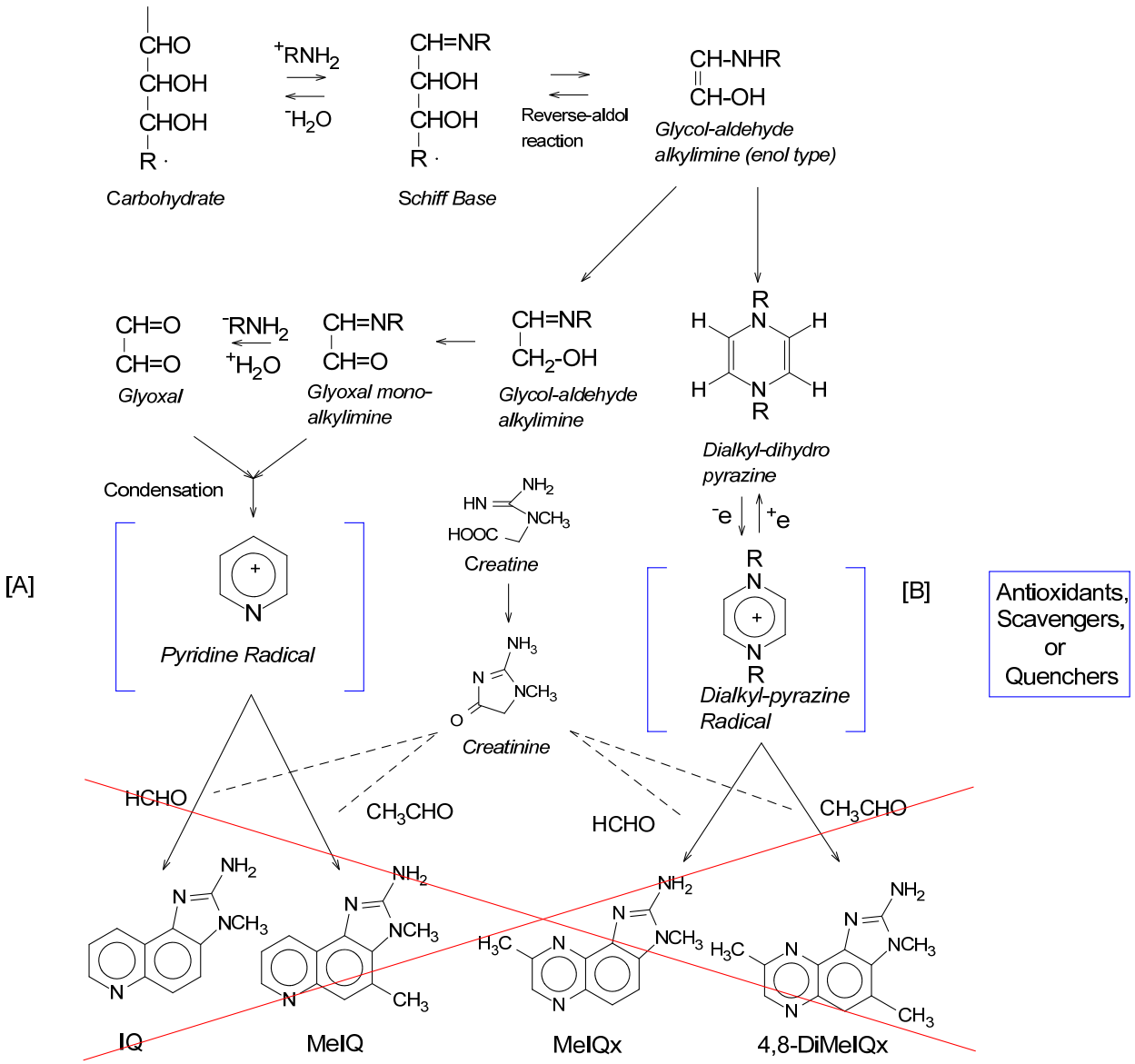


Figure 1-7: Pathway of HCA formation and suggested points where antioxidants impede the reaction (Adapted from Vitaglione and Fogliano 2004)

Synthetic antioxidants, tea polyphenols, and antioxidant rich spices contain high amounts of phenolic compounds. These molecules possess an aromatic ring with an attached hydroxyl group, which scavenge free radicals; thus reducing the formation of HCAs in meat. Research also suggests black pepper may have similar inhibitory effects on HCAs, as black pepper has antioxidant potential (Singletary 2010).

Antioxidants

Early studies on formation of HCAs in cooked meats suggest synthetic antioxidants reduce mutagenicity when added prior to frying. Wang and others (1982) reported a significant decrease of mutagen presence in fried beef with addition of butylated hydroxytoluene (BHT) prior to cooking as detected by the *Salmonella typhimurium* TA98 mutagenicity test. A 1% addition of ethylenediaminetetraacetic acid (EDTA) reduced mutagenicity by approximately 60% in fried beef (Barnes and Weisburger 1984).

Shin and others (2002) presented similar findings in beef patties treated with BHT. A total HCA inhibition of 60% was observed when compared with the control. The highest individually inhibited HCA was PhIP by 60%. MeIQx and 4,8-DiMeIQx also were inhibited by 52 and 50% (Shin and others 2002). In contrast, Lan and others (2004) noted BHT had only a minor effect on inhibition of HCA formation. Tert-butylhydroquinone (TBHQ) slightly inhibited PhIP, but only in chicken samples (Messner and Murkovic 2004).

Hathway (1966) found continuous consumption of synthetic antioxidants in laboratory animals caused teratogenic and carcinogenic effects. These effects may extend to humans, thus research has taken to investigating the effect of natural antioxidants on HCA reduction.

Tea Polyphenols

Tea polyphenolic compounds, known for free-radical scavenging abilities, inhibit HCA formation in meat. Black tea and green tea extracts have been observed to be effective inhibitors of HCAs. In a mutagenicity study using the Ames test, a purified black tea extract and a purified green tea extract, showed the ability to inhibit HCA formation (Weisburger and others 2002). Apostolides and others (1996) reported similar mutagen inhibition of PhIP with both purified black and green tea extracts using the Ames test.

Theaflavin 3,3'-digallate and epigallocatechin (EGC) reduce HCA formation when added prior to frying. Theaflavin 3,3'-digallate has a higher effect on HCA formation than EGC. In fried beef patties, theaflavin 3,3'-digallate reduced PhIP by 51%, MeIQx by 54%, and 4,8-DiMeIQx by 50%. Similarly, EGC reduced PhIP by 30%, MeIQx by 45%, and 4,8-DiMeIQx by 44% (Cheng and others 2007). Zhang and others (2013) observed similar inhibition of PhIP (34%), MeIQx (12%), and 4,8-DiMeIQx (23%) with tea polyphenols in fried pork samples.

Spices

Recent demands by consumers for "clean labeled" foods have caused a need to reduce use of synthetic antioxidants and increase the use of natural alternatives. Culinary herbs and spice extracts including rosemary, thyme, and sage, among many, show inhibitory effects on HCAs in meats (Murkovic and others 1998). In a comparison study of five Asian spices, turmeric (39%) and finger root (33%) exhibited the best ability to inhibit total HCA formation. Both of these two spices contained the highest phenolic content, thus having a higher scavenging activity. This indicates HCA inhibition with spices depends on phenolic content (Puangsombat and others 2011).

Lamiaceae Family

The *Lamiaceae* or *Libiatae* species, also known as the "mint" family is composed of numerous culinary herbs including rosemary, thyme, oregano, sage, marjoram, and basil, among many. Spices and their extracts have recently been studied for their antioxidant properties as natural antioxidants are in high demand. Capecka and others (2005) studied the antioxidant activity of three *Lamiaceae* species: oregano, peppermint, and lemon balm. Fresh and dried oregano had higher total antioxidant activity as compared to two other spices. When testing total phenolics, dried peppermint had the highest (2580 mg 100/g fresh matter), followed by fresh lemon balm (2253 mg 100/g fresh matter) and dried oregano (2221 mg 100/g fresh matter) (Capecka and others 2005). In a similar study, Hinneburg and others (2006) reported basil as having a total phenolic content of 147 (mg GA/g)

Wojdylo and others (2007) studied the total phenolic content (TPC) and total equivalent antioxidant capacity (TEAC), through 2, 2'azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$), 1,1-dipheenyl-2-picrylhydrazyl radical (DPPH), and ferric reducing/antioxidant power (FRAP) tests, of 32 herbs. Table 1.1 illustrates the TPC and TEAC of the selected *Lamiaceae* herbs in the study. Lemon balm and sage possessed the highest total phenolics, with oregano containing the least. Rosemary and thyme had relatively low levels of phenolics, but had the greatest TEAC, indicating both these spices have higher antioxidant activity than the other tested spices. Wojdylo and others (2007) also indicated that all of the *Lamiaceae* species had a positive association between total phenolic compounds (HPLC determined) and antioxidant activity.

Table 1-1: Differences averages ± standard deviations of total equivalent antioxidant capacity (TEAC) and total phenolic content (TPC) of selected *Lamiaceae* species (Adapted from Wojdylo and others 2007)

Common Name	Scientific Name	TPC (GAE/100 g of dry weight)	TEAC (μM trolox/100 g of dry weight)		
			$ABTS^{\cdot+}$	DPPH	FRAP
Sage	*Salvia officinalis*	8.25 ± 0.09	17.0 ± 0.23	41.2 ± 1.11	167 ± 1.01
Oregano	*Origanum vulgare*	0.15 ± 0.01	19.9 ± 1.00	79.6 ± 2.04	405 ± 2.22
Rosemary	*Rosmarinus officinalis*	1.71 ± 0.02	38.7 ± 0.11	513 ± 5.99	662 ± 4.66
White Horehound	*Marrubium vulgare*	3.86 ± 0.05	11.8 ± 0.43	22.5 ± 2.04	138 ± 3.01
Lemon Balm	*Melisa officinalis*	13.2 ± 0.13	10.6 ± 0.09	36.1 ± 1.03	61.8 ± 0.91
Thyme	*Thymus vulgaris*	0.58 ± 0.02	35.4 ± 0.12	295 ± 5.83	693 ± 5.87

[1]2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\cdot+}$), 1-dipheenyl-2-picrylhydrazyl radical (DPPH), and ferric reducing/antioxidant power (FRAP)

Research indicates that species in the *Lamiaceae* family possess the ability to inhibit the formation of HCAs in meat. Damasium and others (2011) assessed the effect that spice extracts had on HCA formation in meat. Thyme, rosemary, marjoram, oregano, and basil were spices in the *Lamiaceae* family. Each spice extract was studied at 0.2 and 0.5%. Greatest inhibition of PhIP formation was observed with both 0.2 and 0.5% oregano extract. Thyme inhibited PhIP formation, but not as significantly as oregano. Little effect on PhIP formation was observed with marjoram and rosemary. Basil at both percentages increased the levels of PhIP in the meat (Damasium and others 2011).

Puangsombat and Smith (2010) show phenolic content in rosemary extracts varied with amount of ethanol used in the extraction. Rosemary extracts of 10% and 20% ethanol contained three polyphenolic compounds: rosmarinic acid, carnosol, and carnosic acid. Rosemary extracts containing higher amounts of ethanol (40%) and containing water (100%) had only one polyphenolic compound (Puangsombat and Smith 2010).

In a study of the effect rosemary extracts have on HCA formation in Saudi Arabian food, a water extract at four levels was evaluated: 2, 5, 10, and 15%. Beef shawerma (beef grilled on a spit for long periods) and grilled chicken were assessed for HCA levels. Awney and Sindi (2010) observed rosemary extracts at 2, 5, and 10% inhibited the formation of PhIP, with 2% inhibiting

61% PhIP. An enhancement of formation was observed with the 15% rosemary extract. Inhibition of PhIP by 2 and 5% was also observed in grilled chicken. All extracts enhanced the formation of MeIQ and MeIQx in both the beef shawerma and grilled chicken (Awney and Sindi 2010).

Tsen and others (2006) investigated the effects of rosmarinic acid and rosemary powder on reduction of MeIQx and PhIP in beef patties. Rosemary powder had the greatest inhibition ability, significantly reducing MeIQx by 69% and PhIP by 66%. Rosmarinic acid had lower inhibitory effects, reducing MeIQx by 64% and PhIP by 48% (Tsen and others 2006). Cheng and others (2007) published similar results of the ability of inhibition of HCAs by rosmarinic acid.

Black Pepper

Piper nigrum L., a vine-like plant, produces black pepper and white pepper. Black pepper is the dried, unripe fruit and white pepper is the dehulled mature berry from *Piper nigrum*. Among one of the most widely used spices in the world, black pepper is indigenous to Southern India, islands of the Malay Archipelago, as well as Madagascar, and other African islands (Singletary 2010). Among the different producing countries, averages of 138,000 tons of black pepper are exported around the world each year (Zachariah and Parthasarathy 2008). Black pepper serves as a folk medicine in an assortment of cultures, cited as a treatment for gastrointestinal disorders, epilepsy, and most recently, chronic malaria (Singletary 2010).

Volatile oil and pungent compounds are two main components of white and black pepper. The hull contains fiber and some essential oils; therefore, black pepper has higher amounts of volatile oil than white pepper. The composition of black pepper is 2.0-2.6% volatile oils and 6-13% oleoresin (Zachariah and Parthasarathy 2008). Piperine is the major bioactive component in both black and white pepper (Singletary 2010). The pungency, or spice of black pepper, is attributed to piperine and volatile oil (Zachariah and Parthasarathy 2008).

Components of black pepper vary by cultivar and increase with age. Black pepper contains approximately 3.5% piperine, but levels increase with maturation and among cultivars (Zachariah and Parthasarathy 2008). Starch is also a component of black pepper; glucose accounts for 88% of the starch. Sugars also present are galactose, arabinose, galacturonic acid, and rhamnose, but in smaller quantities. Like piperine, starch increases with maturation and varies among cultivars (Zachariah and Parthasarathy 2008). Table 1-2 illustrates differences

found in chemical composition of black pepper cultivars during maturation. Non-volatile ether extracts appear to decrease with maturation, while volatiles are not affected by maturation.

Table 1-2: Differences in chemical compositions of two Indian black pepper cultivars during maturation (Adapted from Purseglove and others 1981)

Cultivar	Karimunda				Panniyur-1			
Stage of Maturation	Months after fruit setting				Months after fruit setting			
Volatile Oil (%)	6.8	10.4	4.4	3.6	6.4	7.6	2.8	2.0
Non-volatile Extract (%)	10.3	9.7	7.5	7.4	8.7	8.8	8.1	7.8
Piperine	1.9	2.4	3.1	3.1	1.9	2.6	3.1	3.5
Starch	2.6	4.9	15.3	15.3	2.5	3.7	10.2	16.8

Recent research suggests black pepper possesses antioxidant and free radical scavenging properties (Singletary 2010). Kapoor and others (2009) identified 54 components in essential oils of black pepper with β-caryophylline, limonene, and β-pinene as the major components. Piperine is the primary compound in both ethanol and ethyl acetate oleoresin extracts of black pepper (Kapoor and others 2009).

Table 1-3: Antioxidant and free radical scavenging properties of black pepper extracts compared to synthetic antioxidants (Adapted from Gulcin 2005)

	Total Antioxidant Activity (%)	DPPH Scavenging Activity (%)	Metal Chelating Activity (%)
Water extract	95.5	55 ± 4.2	84 ± 2.2
Ethanol Extract	93.3	48 ± 5.2	83 ± 4.4
Butylated hydroxyanisole	92.1	79 ± 3.2	69 ± 4.0
Butylated hydroxytolune	95.0	76 ± 4.4	66 ± 4.5

[1]1-dipheenyl-2-picrylhydrazyl radical (DPPH)

Table 1-3 illustrates the antioxidant and free radical scavenging properties of black pepper extracts. Essential oils and oleoresins extracted from black pepper using supercritical carbon dioxide also has high antioxidant and free radical scavenging abilities (Tipsrisukond and others 1998; Kapoor and others 2009). Gulcin (2005) identified both water and ethanol extracts

of black pepper having comparable total antioxidant activities to synthetic antioxidants, BHT, and butylated hydroxyanisole (BHA). Water and ethanol extracts have free radical scavenging properties, though at lower levels than BHT and BHA (Gulcin 2005).

Black pepper extract (50% acetone) has significant $ABTS^{\cdot+}$ scavenging capacity (39.9 ± 1.44 TE μmol/g botanicals) and strong Fe^{2+} chelating abilities (1.09 ± 0.16 EDTA Eq mg/g botanicals) (Su and others 2007). Strong hydrogen-donating and metal chelating abilities may attribute to the antioxidant abilities of black pepper (Kapoor and others 2009).

Vijayakumar and others (2004) showed black pepper and piperine provided significant protection against high fat induced oxidative stress in laboratory rats. Elevated levels of thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD) were evaluated in rats fed high fat diets. Elevated levels of TBARS and CD are indicative of peroxidative stress. Laboratory rats fed a black pepper and piperine supplemented diet had significantly lower TBARS and CD levels, similar to levels in control rats.

Glutathione peroxidase (GPx), glutathione-S-transferase (GST), and glutathione (GSH) levels increased in tissues of rats fed black pepper and piperine. GSH is a defense against peroxidative cell damage by detoxifying activated oxygen species and reducing lipid peroxides. GSH is also a substrate for GPx and GST. With induction of GPx, GST, and GSH, black pepper and piperine potentially have the ability to control radicals directly or indirectly in the human body (Vijayakumar and others 2004).

In addition to prevention of lipid oxidative stress, research suggests piperine is a potential treatment for obesity-related diseases. Park and others (2012) indicate piperine has the capability to inhibit fat cell differentiation. Piperine reduces transcriptional activity of peroxisome proliferator-activated receptors (PPARγ) (nuclear receptor proteins that regulate gene expression) during adipogenesis. PPARγ, necessary in adipogenesis, forms a heterodimer with the retinoid x receptor (RXR), while regulating expressions of specific gene subsets. Park and others (2012) suggest piperine prevents PPARγ binding to the coactivator cyclic adenosine monophosphate (cAMP)-response element-binding protein (CBP), therefore inhibiting PPARγ expression (Park and others 2012).

There is little research on the effects of black pepper on HCA formation in muscle-rich foods. One of the few documented studies by Oz and Kaya (2011) investigated the effect black pepper had on formation of HCAs in high-fat meatballs. At 200 °C, inhibition of IQ was 11% and

MeIQx was 25%. PhIP and 4,8-DiMeIQx were both undetectable. At 225 ºC, all four HCAs had 100% inhibition in black pepper treated meatballs. In control meatballs, individual HCAs were undetectable at all three temperatures (175, 200, and 225 ºC). At 175 ºC, IQ was the only detectable HCA and at 200 ºC, IQ and MeIQ were detectable. At 225ºC, MeIQ, 4,8-DiMeIQx and PhIP were all detectable, with IQ being the only HCA undetected. This data indicates black pepper may potentially reduce and inhibit HCA formation in meat (Oz and Kaya 2011) or HCA inhibition may be attributed to thermal degradation (Arvidsson and others 1997).

Chemical Model Systems

Chemical model systems are currently used to study the in vitro formation of HCAs. Some of the first identified HCAs were produced in model systems and later identified in meat. There is an elimination of complex side reactions and products, normally formed in protein-rich foods, when using model systems. Therefore, researchers find these models useful for studying HCAs (Murkovic 2004).

These models allow for variation of compositions and concentrations of precursors, while observing the effect of heating conditions (Bordas and others 2004). Adjustment of model system environments and conditions allow for better understanding of these complex reactions of HCAs (Messner and Murkovic 2004). Knowing the amount of creatin(in)e, carbohydrate, and the free amino acids in actual meat products allows for an accurate representation in model systems (Messner and Murkovic 2004).

Formation of Heterocyclic Amines in Model Systems

HCAs form via a reaction between amino acids and creatin(in)e, a reaction specific to muscle-dense foods. There are two types of HCAs: polar and non-polar. Polar HCAs form in a reaction among an amino acid, creatin(in)e, and a reducing sugar, in a temperature range of 150 to 200 ºC. Non-polar HCAs favor reaction temperatures exceeding 300 ºC and are not creatin(in)e dependent, like their polar counterparts (Cheng and others 2006).

Model system reactions occur as a dry or wet reaction, with diethylene glycol being a common medium used in wet reactions. Both dry and wet reactions produce similar quantitative results (Murkovic 2004). Diethylene glycol has a high boiling point and allows for an even and consistent heat transfer; therefore, a preferred method (Messner and Murkovic 2004). Additionally, incorporation of meat juices into model system allows study of all HCAs in meat

samples (Cheng and others 2006). In some studies, ethanolic environments have been used to imitate wines and/or liquors in cooking (Shen and Wu 2004; Wu and others 2011).

Polar HCAs form at normal cooking temperatures in protein-rich products, such as beef, chicken, pork, and fish; therefore, polar HCAs are readily studied. In model systems, the HCA(s) formed depends on the specific amino acid present in the reaction. For example: threonine forms IQ, IQx, MeIQx, and 4,8-DiMeIQx (Jackson and Hargraves 1995; Wu and others 2011); glycine forms MeIQx, IQx, and 7,8-DiMeIQx (Kato and others 1996; Murkovic 2004); and phenylalanine forms PhIP (Cheng and others 2007).

Inhibition of Heterocyclic Amines

Researchers study the inhibition of HCAs in model systems, as they do in meat systems. Phenolic antioxidants (BHA and sesamol), as well as natural phenol flavonoids, epigallocatechin gallate (EGCG), luteolin, and quercetin, have HCA reducing abilities in model systems, specifically PhIP, MeIQx, and IQ (Kato and others 1996; Oguri and others 1998). Kato and others (1996) isolated and identified pyrazine radicals generated in model systems. Phenolic antioxidants scavenge free radical pyrazine, thus inhibiting formation of both MeIQ and IQ (Kato and others 1996).

Moon and Shin (2013) studied the effect of six antioxidants on the formation of PhIP in model systems. BHA, α-tocopherol, diallyl disulfide, EGCG and naringenin (all at1000 ppm) and eugenol (500 ppm) caused the greatest PhIP inhibition in the model system (62.9 – 99.2% inhibition). EGCG (99.2%) and naringenin (97.5%) had similar effects on PhIP and had greatest inhibition at 1000 ppm (Moon and Shin 2013).

Olive oils, rich in phenolic compounds, also have HCA reducing abilities in model systems. Monti and others (2001) reported inhibition of IQx, MeIQx, and 7,8-DiMeIQx formation up to 50%, with addition of fresh olive oil. A year old olive oil also caused inhibition, but at significantly lower levels (Monti and others 2001).

Additionally, mono- and disaccharides prevent the formation of HCAs in chemical model systems. Glucose has an inhibitory effect on HCAs when added in equimolar or more to creatin(in)e and amino acids. Fructose, sucrose, and lactose all have similar effects in a model system (Skog and Jägerstad 1990; Skog and Jägerstad 1991). Bordas and others (2004) showed

inhibition of IQ in both dry and wet systems with an excess addition of glucose, as compared to other precursors.

Promotion of Heterocyclic Amines

Conflicting data from model systems reveals both enhancement and inhibition of HCA formation upon addition of both natural and synthetic antioxidants. Research indicates synthetic antioxidants, including BHA and BHT, cause an increase of both MeIQx and 4,8-DiMeIQx in model systems (Pearson and others 1992; Johansson and Jägerstad 1996). Pearson and others 1992) suggest alkylation of BHT adds a supplemental precursor to the reaction, thus allowing for more production of HCAs.

Rosmarinic, carnosic, and chlorogenic acids, as well as thyme, rosemary, and marjoram extracts increase the formation of PhIP. The increase in PhIP formation associates with antioxidant concentration in model systems. As antioxidant concentration increases, PhIP formation increases (Zöchling and Murkovic 2002; Cheng and others 2007). Zöchling and Murkovic (2002) suggest other components or compounds in the crude spices (thyme, rosemary, and marjoram) overwhelm the antioxidant molecules in the spices.

As compared to other HCAs, PhIP has a different formation pathway, a potential explanation. Cheng and others (2007) postulate, that, unlike other HCAs, the rate-limiting step may not be the formation of free radicals. Therefore, free-radical scavenging compounds, such as antioxidants, will not have the ability to inhibit formation of PhIP in model systems (Cheng and others 2007). Phenolic antioxidants, ellagic, ferulic, and syringic acids, enhanced PhIP formation in model systems. Oguri and others (1998) propose the derivatized reactive groups from the acids aid in PhIP formation. Other explanations suggest the acids decrease the pH of the reaction or there are additional reactions between the amino group of phenylalanine and the carboxylic acid groups (Cheng and others 2007).

Conclusions

A stronger association between HCA consumption and human cancers still requires more research. Recent research indicates these mutagens are reducible in meat. Lowering cooking temperatures has the largest effect on HCA formation, but addition of antioxidants, tea polyphenols, spices, and high sugar marinades, prove a more effective method in reducing HCA levels in meat products.

Chemical model systems are useful in the laboratory study of HCAs. Precursors, environments, and temperatures, all controllable variables in model systems, allow for direct observation of HCAs. Unlike with meat systems, there are no side reactions in the system. Research indicates antioxidants decrease the formation of these mutagenic compounds in model systems, but also exhibits the ability to enhance formation.

Literature reveals conflicting data on HCA formation in model systems. Some research indicates HCA inhibition while other research suggests antioxidants promote formation. Black pepper appears to have desirable radical scavenging abilities in mice studies and HCA inhibition in meat. More study is needed in this area to explore the inhibition abilities of black pepper on HCA formation, specifically within a model system.

Though model systems are useful in the study of HCAs, the field lacks a well-defined HCA model system for HCA study. Precursor levels, water amounts, and a defined sugar are needed to develop and further refine a model system for HCA formation. Due to discrepancies in research literature of model systems, further studies defining an ideal model system is necessary to better understand the formation of HCAs in model systems. A more defined and affective model system will potentially lead to an improved study of antioxidant effects on HCA formation/inhibition/promotion in chemical model systems.

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Chapter 2 - The Effects of Black Pepper Oil and Extract on 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine (PhIP) Formation in Model Systems

Abstract

Heterocyclic amines (HCAs) are undesirable by-products formed during high temperature cooking of muscle-rich foods including beef, pork, chicken, and fish. Chemical model systems are currently used to study the formation of HCAs in laboratory environments. The mutagen, 2-amino-1-methyl-6-phenylimidazo [4,5-b]-pyridine (PhIP) was of interest as consumption of PhIP has been associated with breast, prostate, and colorectal cancers. Recently, antioxidant-containing spices have shown to inhibit the formation of HCAs in meat. Little research has been completed on black pepper (*Piper nigrum)*. Therefore, in this study, the effect that black pepper had on PhIP formation in a model system was observed. Model systems were dissolved in 80:20 diethylene glycol/water (v/v) solution and heated for 40 min at 175 ºC. Two types of black pepper solutions were studied: an 80% ethanol extract and a steam distilled black pepper oil. Both types of black pepper solutions were added in 36, 71, 142, 284, and 568 µL aliquots prior to heating of the model systems. The antioxidants, tert-butylhydroquinone (TBHQ) and butylated hydroxytoluene (BHT) (0.2 and 0.4 mmol) were added as positive controls. Data for both black pepper solutions were not significantly ($p < 0.05$) different from the control. The ethanol extract appeared to enhance PhIP formation to 71 µL. In model systems with more than 71 µL, PhIP appeared to decrease. With black pepper oil, PhIP increased and then decreased at 142 µL. In model systems with 142 µL and more of black pepper oil, PhIP appeared to increase. The positive controls, TBHQ and BHT, did not have a significant effect on the formation of PhIP. Though the data was not significant, there appeared to be a trend, indicating potential PhIP inhibiting abilities of black pepper in model systems.

Introduction

In 1939, Swedish chemist, E.M.P Widmark first described the occurrence of carcinogenic substances in cooked meat (Widmark 1939). Almost 80 years later, 25 heterocyclic amines (HCAs) have been isolated and identified in meat and model systems (Sanz Alaejos and others 2008). HCAs are highly mutagenic and potentially carcinogenic by-products of non-enzymatic browning reactions in muscle-rich foods. These compounds form in a complex network of reactions at temperatures exceeding 150 ^{o}C via a free amino acid, creatin(in)e, and a reducing sugar (Murkovic 2004).

The National Toxicology Program of the U.S. Department of Health and Human Services (2011) has classified four HCAs as *reasonably anticipated to be human carcinogens*: 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), and 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP). MeIQx, 4,8-DiMeIQx, and PhIP are the most abundant HCAs found in cooked meat (Zheng and Lee 2009). Case studies approximate human consumption to be approximately 250 – 300 ng per day, with the highest levels being PhIP (~160 – 240 ng), MeIQx (~70 – 90 ng), and 4,8-DiMeIQx (~4 – 6 ng) (Nowell and others 2002; Li and others 2007). Therefore, there is a necessity to study and understand the formation of these mutagens.

PhIP has been shown to induce greater mammalian cell damage than MeIQx and DiMeIQx (Lynch and others 1992). It is also one of the most abundant HCAs formed in cooked meats (Zöchling and Murkovic 2002; Zheng and Lee 2009). Development of breast (Sinha and others 2000), prostate (Tang and others 2007), liver, and colon (Ito and others 1991) cancers in laboratory rats is associated with the consumption of PhIP. Therefore, PhIP is of great concern to researchers in understanding the formation and possible inhibition of this compound.

Laboratory study of HCAs is currently conducted using chemical model systems. These model systems mimic the formation of HCAs, while eliminating complex side reactions that normally form in foods (Murkovic 2004). Model systems allow for composition and concentration precursor variation, while observing the effect of applied treatments (Bordas and others 2004). PhIP forms in model systems via the reaction among creatin(in)e, phenylalanine, and a reducing sugar (Murkovic 2004; Cheng and others 2007), in a temperature range of 150 to 200 ^{o}C (Cheng and others 2006).

During the formation of HCAs, free radical Maillard reaction intermediates form prior to the formation of IQ and IQx-type HCAs. Vitaglione and Fogliano (2004) suggest antioxidants scavenge these free radical intermediates, thus inhibiting the formation of HCAs. Antioxidants, butylated hydroxyanisole (BHA) and sesamol, as well as natural phenol flavonoids have been shown to reduce HCA formation in chemical model systems, specifically PhIP, MeIQx, and IQ (Kato and others 1996; Oguri and others 1998). Antioxidant containing spices, including rosemary, thyme, and sage, have inhibitory effects on HCA formation in meats (Murkovic and others 1998).

Black pepper (*Piper nigrum*), the dried, unripe fruit of the vine-like plant *Piper nigrum* L, is indigenous to Southern India, the islands of the Malay Archipelago, and Madagascar (Zachariah and Parthasarathy 2008). Approximately 138,000 tons of black pepper are annually exported around the world; making black pepper one of the most widely used spices in the world. Black pepper has been noted in folk medicine of an assortment of cultures, for treatment of gastrointestinal disorders, epilepsy, and chronic malaria (Singletary 2010).

There are two main components of black pepper: volatile oils and pungent compounds. The pungent compounds contain 1.9 – 3.5% piperine (Zachariah and Parthasarathy 2008), the major bioactive ingredient and attributor to spice of black pepper (Singletary 2010). Piperine varies in black pepper by maturation and cultivars. Generally, piperine content increases as the fruit ages (Purseglove and others 1981).

Water and ethanol extracts of black pepper have been identified to contain comparable total antioxidant activities to synthetic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytolune (BHT). Both extracts also possess free radical scavenging properties (Gulcin 2005). Oz and Kato (2011) investigated the efficacy of black pepper in reducing HCA formation in high-fat meatballs. PhIP inhibition was 100% at temperatures at and above 200 ºC, indicating black pepper potentially may inhibit HCA formation in meat (Oz and Kaya 2011). As little research on black pepper inhibition of HCAs have been completed, further studies are required in this area allowing a better understanding of black pepper and its inhibiting abilities. Therefore, the purpose of this research was to study the efficiency of two black pepper solutions, an ethanol extract, and black pepper oil, in inhibiting PhIP formation in a chemical model system.

Materials and Methods

Materials

McCormick Science Institute (Hunt Valley, Md., U.S.A.) provided a MSI standard sample of finely powdered, ground black pepper blend (total ORAC: 424 μmol TE / g; total phenolics: 8.2 mg GAE / g). Pure *Piper* nigrum oil (steam distilled black pepper oil) was purchased from Nükira Essential Oils (Moreno Valley, Calif., U.S.A.). The 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) standard was purchased from Toronto Research Chemicals (Ontario, Canada). D-(+)-Glucose, creatinine, L-phenylalanine, tert-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), triethylamine, and diethylene glycol (99%) were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). Methanol (*Optima*) was purchased from Fisher Scientific (Fairlawn, N.J., U.S.A.). Ethanol (200 proof) was purchased from Decon Labs, Inc (King of Prussia, Pa., U.S.A.). Deionized water was processed using a Sybron/Barnstead PCS unit (Barnstead/Thermolyne, Inc, Dubuque, Iowa, U.S.A.). Polyvinylidene fluoride (PVDF) membrane 17 mm 0.4 μm syringe filters were purchased from Agilent Technologies (Santa Clara, Calif., U.S.A.).

Preparation of Model Systems

A black pepper extract was prepared by shaking 1.0 g of black pepper in 100 mL 80:20 ethanol/water (v/v) solution. The black pepper solution was shaken for two hr 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) and a 7.5 mL aliquot of the filtrate was dried under nitrogen gas and reconstituted with diethylene glycol. The concentration of the final black pepper extract was 1.0 g/100 mL.

The model systems were prepared, as described by Cheng and Others (2007) with some modifications. The systems were prepared in 3 mL conical reaction vials (Sigma Aldrich, St. Louis, Mo., U.S.A.) and sealed with polytetrafluoroethylene (PTFE) lined, 20-400 thread, caps (Fisher Scientific, Fairlawn, N.J., U.S.A.). The precursor molar concentrations were 0.44/0.44/0.22 mmol. Phenylalanine (0.0740 g), creatinine (0.0500 g), and glucose (0.0400 g) were dissolved in 80:20 diethylene glycol/water (v/v) mixture, with a total volume of 2500 μL. High density, “threadmaster” PTFE thread seal tape (Mil-T-27730A) (Merco Hackensack, Inc., Hillburn, N.Y., U.S.A.) was used to seal the threading of the vial to the cap, maintaining an

enclosed system and reducing water loss (Figure 2-1). The mixture was vortexed until blended to ensure all solid-matter was well incorporated into the liquid matrix. The vials were then heated at 175 ^{o}C for 40 min in a heating mantel fitted for 3 mL vials (Figure 2-2).

Figure 2-1: Picture indicating how the PTFE thread tape was applied to the conical vial.

Figure 2-2: Picture of the heating block/mechanism used to heat the chemical model systems.

After 40 min, the model systems were cooled to room temperature before further examination. The effects of black pepper extract and black pepper oil were studied with the addition of each solution (36, 71, 142, 284, and 568 µL) to the model systems containing the

precursors mentioned above. TBHQ and BHT were added in 0.2 and 0.4 mmol amounts. The model systems were brought to a total volume of 2500 µL with 80:20 diethylene glycol/water (v/v) mixture. All model systems were syringe filtered and diluted 1:70 in methanol.

Analysis of Black Pepper Extract

Black pepper extract was analyzed using a HP 1090A Series II HPLC (Agilent Technologies, Santa Clara, Calif., U.S.A.) equipped with an HP 1046A programmable fluorescence detector. Separation of the extract was performed using reversed-phase chromatography using a TSKgel ODS-80T$_M$ (4.6 mm x 25 cm x 5 µm) column and a TSK guardgel ODS-80T$_M$ (3.2 mm x 1.5 cm) guard column (TOSOH Biosciences, Tokyo, Japan) with a mobile phase of 1% acetic acid (A) and acetonitrile (B). Separation of the extract was achieved using a linear gradient beginning with 70% A and 30% B, changing to 40% A and 60% B over 80 min at 1.0 mL/min and column temperature of 40 ^oC. After 80 min, the mobile phase returned to the initial ratio (70% A, 30% B) for column equilibration. For detection of the compounds present in the black pepper extract, the spectrophotometer wavelength was set at 343 nm. Solid-phase microextraction (SPME) headspace analysis coupled with GC-MS was performed on the black pepper oil (Appendix G).

Analysis of PhIP

PhIP was analyzed using a HP 1090A Series II HPLC (Agilent Technologies, Santa Clara, Calif., U.S.A.) equipped with an HP 1046A programmable fluorescence detector. Separation of the model system was performed by reversed-phase chromatography using a TSKgel ODS-80T$_M$ (4.6 mm x 25 cm x 5 µm) column and a TSK guardgel ODS-80T$_M$ (3.2 mm x 1.5 cm) guard column (TOSOH Biosciences, Tokyo, Japan) with a mobile phase of 0.01 M triethylamine pH 3.6 (A) and acetonitrile (B). Adjustments to mobile phase A were made with acetic acid.

A mobile system gradient was used, as described by Puangsombat and others (2012) with slight modifications. Separation of PhIP was achieved using a linear gradient beginning with 95% A and 5% B, changing to 75% A and 25% B in 30 min, keeping at this ratio until 35 min at 1.0 mL/min and column temperature of 40 ^oC. After 35 min, the mobile phase returned to the initial ratio (95% A, 5% B) for a ten min column equilibration. For detection of PhIP, the fluorescence detector operated at emission/excitation wavelengths of 229 nm and 437 nm.

Quantification and Statistical Analysis

A 100 ppm PhIP standard solution was prepared by dissolving 1.00 mg in 10.0 mL of methanol. A standard curve was prepared by analyzing PhIP standards at concentrations of 31.25, 62.5, 125, 250, and 500 ppb (Appendix A). The coefficient of determination (R^2) was 0.9998. Limit of Detection (LOD) of PhIP was 0.15 ng and the Limit of Quantification (LOQ) was 0.16 ng (Appendix B) (Smith 2010). SAS (Version 9.3, 2011, SAS Inst. Inc., Cary, N.C., U.S.A.) was used to analyze the six replications of data. The experiment was a completely randomized design. With SAS, an analysis of variance was completed to determine significant differences between the two treatments at $p < 0.05$.

Results

Black Pepper Extract and Black Pepper Oil

The ethanol black pepper extract was first separated on the HPLC to identify piperine, the chemical compound and major bioactive component. Piperine eluted at 42.3 min. A UV spectral match was made using HP 9000 series 300 Chemstation (Appendix E) to confirm the identity of piperine. Figure 2-1 is the HPLC chromatogram of black pepper extract, identifying the piperine peak. The UV spectral match to the piperine standard was 99%. Quantification of piperine was completed using a standard curve (Appendix A) created with purified piperine (Acros Organics, Fisher Scientific, Fairlawn, N.J., U.S.A.). The LOD of piperine was 95.2 ng and the LOQ was 96.0 ng (Appendix B). Piperine content was approximately 3.8% of the total black pepper extract.

An ethanol black pepper extract and a steam distilled black pepper oil were evaluated on their abilities to inhibit the formation of PhIP in a chemical model system. Black pepper was chosen as this spice has shown to possess high antioxidant, free radical scavenging, and Fe2+ chelating abilities (Tipsrisukond and others 1998; Gulcin 2005; Su and others 2007). Black pepper also inhibited PhIP and other HCAs when applied to high-fat meatballs prior to cooking (Oz and Kaya 2011), indicating black pepper may inhibit the formation of HCAs in both meat and model systems.

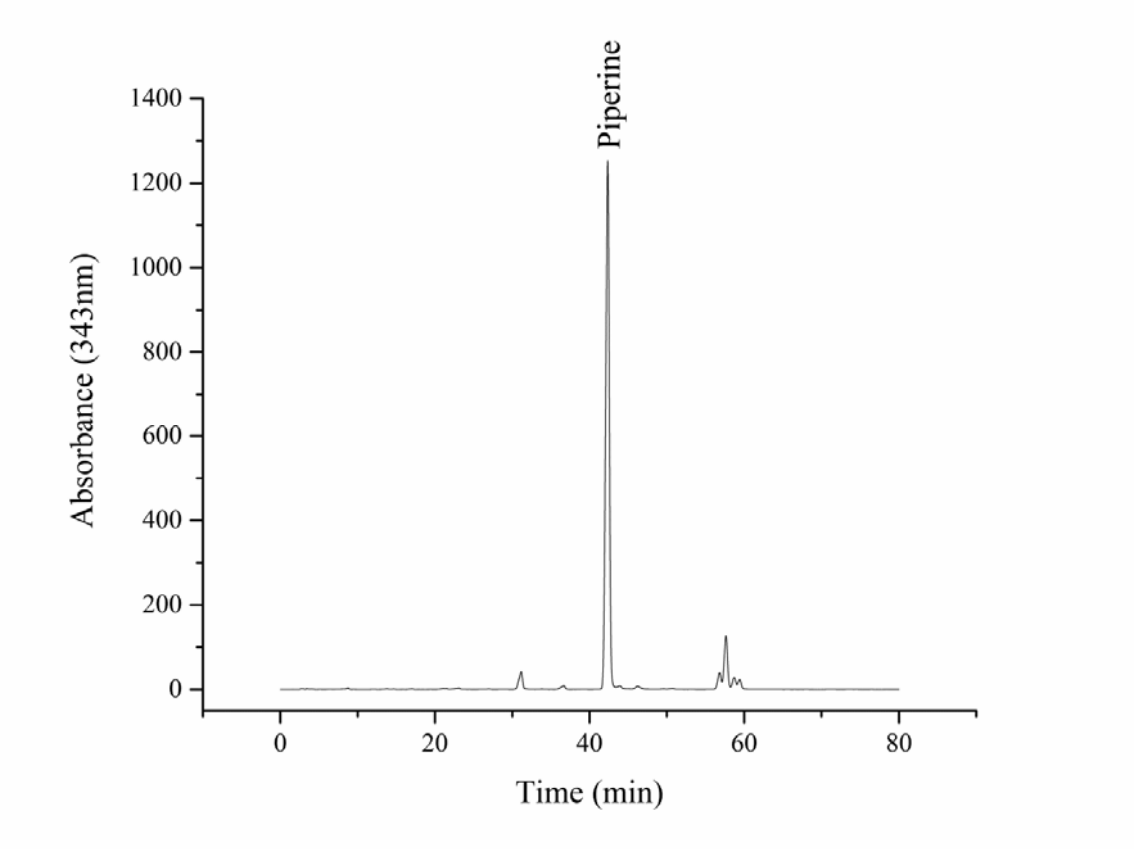

Figure 2-3: HPLC chromatogram of the injected black pepper extract

The ethanol extract did not have a significant effect on the formation of PhIP in the model system. Figure 2-2 illustrates the relationship between the ethanol black pepper extract and PhIP formation. Though the black pepper did not significantly inhibit the PhIP, a trend was observed in the data. At concentrations 36 and 71 μL, PhIP formation increases and then at 142 and 284 μL, PhIP formation decreases.

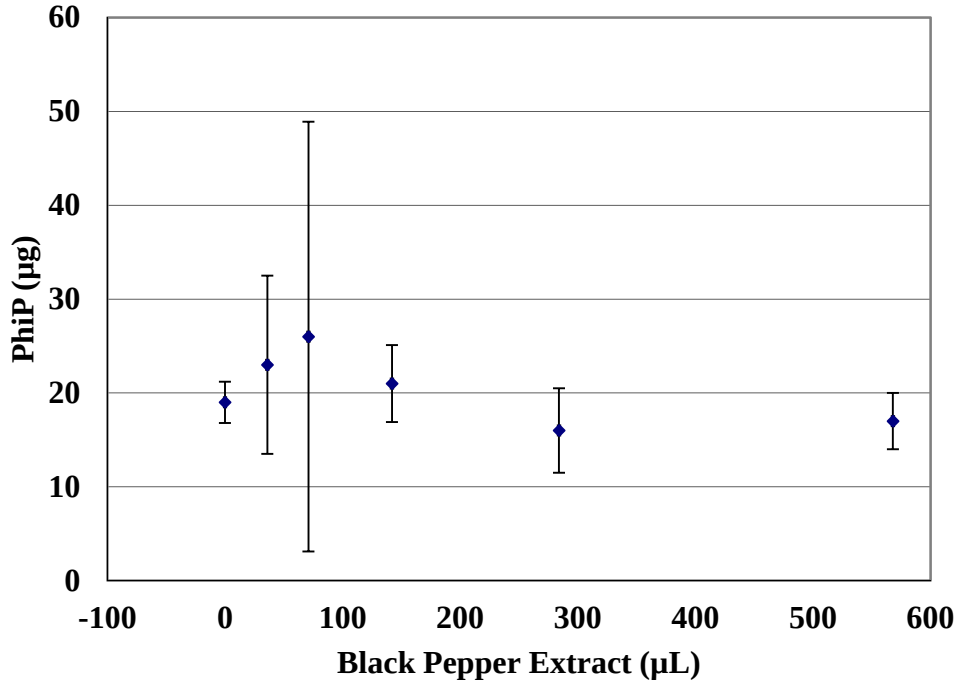

Figure 2-4: Relationship between black pepper extract and PhIP formation with means and error bars indicating standard deviations calculated in Excel

The steam distilled black pepper oil also did not significantly affect the formation of PhIP in the chemical model system. Figure 2-3 shows the relationship between the black pepper oil concentration and PhIP formation. There was a slight trend between PhIP and the concentration of oil. At 36 µL, PhIP, then decreased at 71 and 142 µL. Formation then increased from the control at 284 and 568 µL.

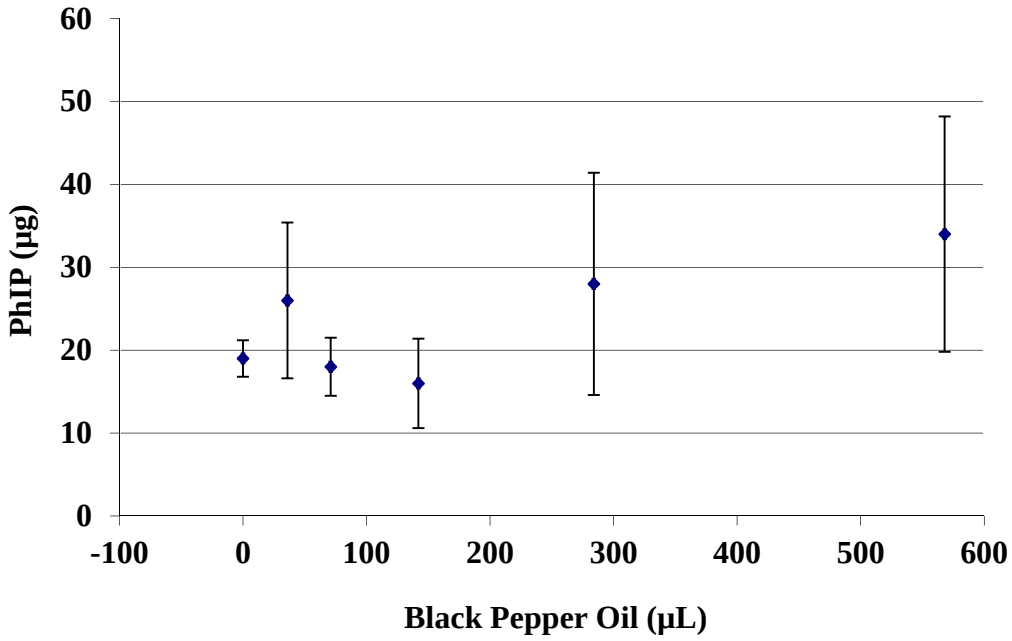

Figure 2-5: Relationship between black pepper oil and PhIP formation with means and error bars indicating standard deviations calculated in Excel.

The standard deviations of black pepper extract and black pepper oil concentrations were large, indicating high experimental error. Great variability was observed in the individual runs among the black pepper concentrations. The heating block is believed to be the source of the large error and variability amongst the PhIP amounts. To reduce this error from the heating block, a completely randomized block design would have been a more sound statistical design. Variability among the replications would have been less with the block design.

TBHQ and BHT

Research suggests that the antioxidants TBHQ and BHT inhibit the formation of HCAs in meat systems, specifically PhIP (Shin and others 2002; Messner and Murkovic 2004). Therefore, these two antioxidants were chosen as the positive controls for this study. Figure 2.3 shows the relationship of the two antioxidants on PhIP formation. BHT appears to have a dose-dependent relationship with PhIP formation; as BHT increases, formation of the mutagen also increases, though neither antioxidant had an overall effect on the formation of PhIP.

Table 2-1: Differences between antioxidants, TBHQ and BHT, and PhIP formation with means ± standard deviations calculated with Excel.

Treatment	Amount of PhIP (µg)
Control	19.4 ± 22
0.2 mmol TBHQ	21.4 ± 25
0.4 mmol TBHQ	17.5 ± 24
0.2 mmol BHT	26.2 ± 11
0.4 mmol BHT	28.8 ± 19

The standard deviations and experimental errors were high for both TBHQ and BHT. The large variation in the data is assumed to be attributed to the heating block. As before, a completely randomized block design would help decrease the variability amongst the replications of both antioxidants.

Discussion

Piperine content in the black pepper extract was approximately 3.8%. Zachariah and Parthasarathy (2008) indicate that black pepper contains approximately 3.5% piperine, varying with maturation and cultivar type. Therefore, the ethanol/water ratio used for extraction was optimal for an ideal piperine yield.

This present study indicates black pepper does not significantly affect PhIP formation in chemical model systems. Additionally, both positive controls, BHT and TBHQ, did not reduce the formation of PhIP. In some instances, PhIP formation appeared to be enhanced by the addition of black pepper extract (36 and 71 µL), black pepper oil (36, 284, and 568 µL), TBHQ (0.2 mmol) and BHT (0.2 and 0.4 mmol). Pearson and others (1992) observed similar promotion of HCAs with the addition of BHT. The increase of HCAs may be caused by alkylation of BHT, which adds a supplemental precursor to the reaction, enabling HCA formation (Pearson and others 1992).

Johansson and Jägerstad (1995) previously reported similar results with an addition of 100 ppm TBHQ in a glycine model system. MeIQx formation was enhanced by 220%, as compared to the control. A higher amount of TBHQ (1000 ppm) had no effect on MeIQx formation (Johansson and Jägerstad 1996).

PhIP has a different formation pathway than the other HCAs, which may cause the formation promotion with the addition of antioxidants. Cheng and others (2007) suggest the rate-limiting step of the PhIP pathway may not be the formation of free-radicals, like the other polar

HCAs (IQ and IQx-type HCAs). Therefore, free-radical scavenging compounds, such as antioxidants, will not inhibit the formation of PhIP in model systems.

Polar HCAs predominantly form between 150 and 200 ºC (Jackson and Hargraves 1995; Arvidsson and others 1997). In kinetic studies of PhIP, the mutagen was undetectable at 225 ºC. Thermal degradation of HCAs occurs at high processing temperatures (>200 ºC), causing a decrease in HCA formation (Arvidsson and others 1997). Oz and Kato (2011) indicated PhIP was undetectable at 200 and 225 ºC, attributing inhibition to the applied black pepper. The inhibition of PhIP may not have been caused by black pepper, but rather, PhIP underwent thermal degradation and was therefore, undetectable.

Conclusions

In this study, the mutagenic compound, PhIP was not significantly inhibited in a chemical model system by black pepper. Though the data was not statistically significant, a trend indicated that black pepper may inhibit PhIP formation. Further studies on the effect black pepper has on HCA formation in model systems would better clarify the inhibiting abilities of black pepper. Additionally, a defined chemical model system for HCA formation may eliminate the observed experimental error, thus allowing for a better understanding on how antioxidants effect HCA formation in model systems.

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Chapter 3 - Evaluation of Maillard Reaction Variables and Their Effect on Heterocyclic Amine Formation in Chemical Model Systems[1]

Abstract

Heterocyclic amines (HCAs), highly mutagenic and potentially carcinogenic by-products, form during Maillard browning reactions, specifically muscle-rich foods. Chemical model systems allow examination of in vitro formation of HCAs while eliminating complex matrices of meat. Limited research has evaluated the effects of Maillard reaction parameters on HCA formation. Therefore, four essential Maillard variables (precursors molar concentrations, water amount, sugar type, and sugar amounts) were evaluated to optimize a model system for the study of five HCAs: IQ, MeIQ, IQx, MeIQx, and 4,8-DiMeIQx. Model systems were dissolved in diethylene glycol, heated at 175 ºC for 40 min, and separated using reversed-phase liquid chromatography (TSKgel ODS-80T, 4.6 mm x 25 cm x 5 μm column). To define the model system, precursor amounts (threonine and creatinine) were adjusted in molar increments (0.2/0.2, 0.4/0.4, 0.6/0.6, and 0.8/0.8 mmol) and water amounts by percentage (0, 5, 10, and 15%). Sugars (lactose, glucose, galactose, and fructose) were evaluated in several molar amounts proportional to threonine and creatinine (quarter, half, equi, and double). Examined precursor levels and amounts of sugar were significantly different ($p < 0.05$) for total HCA formation, with 0.6/0.6/1.2 mmol producing higher levels. Water concentration and sugar type also had a significant effect ($p < 0.05$), with 5% water and lactose producing higher total HCA amounts. A model system containing threonine (0.6 mmol), creatinine (0.6 mmol), and glucose (1.2 mmol), with 15% water was determined to be the optimal model system with glucose and 15% water being a better representation of meat systems.

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Introduction

French chemist, Louis-Camille Maillard, first described non-enzymatic browning reactions in 1912, a reaction between amino acids and reducing sugars in a pH-dependent and high temperature environment. Browning reactions, or Maillard reactions, produce desirable flavors in roasted coffees, baked goods, and beers. In contrast, Maillard reactions also produce undesirable side products, one being heterocyclic amines (HCAs).

To date, 25 HCAs have been isolated and identified in both meat and chemical model systems (Sanz Alaejos and others 2008). Highly mutagenic and potentially carcinogenic non-enzymatic browning products, HCAs form specifically in grilled, baked, fried, or roasted muscle-rich foods. The formation reaction of HCAs occurs between amino acids, reducing sugars, and creatine/creatinine in a temperature range of 150 – 200 ºC (Murkovic 2004).

The most abundant HCAs found in meat are 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4-8-trimethyl-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), and 2-amino-1-methyl-6-phenylimidzo[4,5-b]-pyridine (PhIP) (Zheng and Lee 2009). HCAs 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) and 2-amino-3-methlimidazo-[4,5-*f*]quinoline (IQ) are also present in cooked meat.

Case studies approximate human consumption to be approximately 250 – 300 ng per day, with the highest levels being PhIP (~160 – 240 ng), MeIQx (~70 – 90 ng), and 4,8-DiMeIQx (~4 – 6 ng) (Nowell and others 2002; Li and others 2007) through the consumption of beef, chicken, pork, and fish products containing these compounds (Murkovic 2004). Swedish chemist, E.M.P. Widmark first described the carcinogenic substances when male and female mice developed tumors upon the application of roasted horse meat extracts (Widmark 1939).

Research indicates breast (Sinha and others 2000), colon (De Stefani and others 1997), prostate (Tang and others 2007), and pancreatic cancers (Li and others 2007) are associated with the consumption of HCAs. Consumption of IQ, MeIQ, and MeIQx HCAs has been associated with liver cancer (Ohgaki and others 1984; Ohgaki and others 1987; Kato and others 1988). Thus, the National Toxicology Program of the U.S. Department of Health and Human Services (2011) has classified MeIQ, IQ, MeIQx, and PhIP as *reasonably anticipated to be human carcinogens*. Therefore, understanding the formation of HCA compounds is necessary to inhibit formation.

Model systems are currently used to study the in vitro formation of HCAs. These systems were developed to understand the formation of HCAs and to identify the foods and cooking conditions responsible for the formation of these mutagens (Knize and Felton 2005). Model systems are beneficial for HCA research, as complex side reactions in meat matrices are eliminated so that chemical and physical parameters and reaction mechanisms of HCA formation are sufficiently studied (Jackson and Hargraves 1995).

Model systems allow for modification of precursor types, concentrations, and compositions, while adjusting the time and temperature of the reaction (Bordas and others 2004), unlike in meat systems. Factors including type of amino acids, precursor concentration, sugar type, sugar amount, and water all effect formation of HCAs. The type of amino acid present in the reaction greatly affects the type of HCAs formed in model systems. Research indicates threonine forms IQ, IQx, MeIQx, and 4,8-DiMeIQx (Jackson and Hargraves 1995; Wu and others 2011).

Type of sugars also effect formation of HCAs, specifically reducing sugars are required for HCA formation. Research indicates, specifically MeIQx and DiMeIQx, do not form without the presence of glucose in the reaction. Fructose has been shown to promote more HCA formation than glucose (Skog and Jägerstad 1990). Skog and Jägerstad (1990) suggest HCA formation is highest when sugars are in half-molar amounts to the amino acid and creatin(in)e. When sugars are equimolar and/or higher, an inhibitory effect on HCA formation occurs (Skog and Jägerstad 1990; Skog and Jägerstad 1991).

Therefore, the purpose of this research was to better understand how Maillard reaction variables affect the formation of IQ, IQx, MeIQ, MeIQx, and 4,8-DiMeIQx HCAs in chemical model systems and optimize a model system for further HCA study.

Materials and Methods

Materials

Threonine, creatinine, glucose, galactose, lactose, and fructose were purchased from Sigma-Aldrich Chemicals (St. Louis, Mo., U.S.A.). HCA standards, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx), 2-amino-3-methlimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), and 2-amino-3,4-8-trimethyl-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) were all

purchased from Toronto Research Chemicals (Ontario, Canada). Diethylene glycol and triethylamine were purchased from Sigma-Aldrich Chemicals. Acetic acid (HPLC grade) and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Fairlawn, N.J., U.S.A.). Polyvinylidene fluoride (PVDF) membrane 25mm 0.45 μm syringe filters were purchased from Derian (Shanghai, China). Deionized water was processed using a Sybron/Barnstead PCS filtration unit (Barnstead/Thermolyne, Inc, Dubuque, Iowa, U.S.A.).

Preparation of Model Systems

Model systems were prepared in two parts, with 16 model system combinations in each. The first part evaluated the adjustment of precursor amounts and water percentage, each with four treatment levels. In part 1, the precursors evaluated were threonine, creatinine, and glucose and reacted at: 0.4/0.4/0.2 mmol (control), 0.2/0.2/0.1 mmol, 0.6/0.6/0.3 mmol, and 0.8/0.8/0.4 mmol. Threonine was used as the amino acid, as it had been previously described for the formation of IQ, IQx, MeIQx, and 4,8-DiMeIQx (Jackson and Hargraves 1995; Wu and others 2011). Glucose was half molar of the other precursors as described by Skog and Jägerstad (1990). The mole amount of 0.4 was used based on previous studies (Kikugawa and others 2000; Cheng and others 2007). Water was evaluated at 4 levels: 0 (control), 5, 10, and 15% of the total volume.

Part 2 evaluated the adjustment of sugar amount and sugar type, each with four treatment levels. Glucose (control), lactose, fructose, and galactose were evaluated at molar amounts quarter, half (control), equi, and double of the threonine and creatinine.

All model systems were prepared in a 3 mL conical reaction vial and diluted to a total volume of 2500 μL with diethylene glycol as suggested by Messner and Murkovic (2004). To ensure no moisture loss during heating, vials were sealed with a high density, Threadmaster® polytetrafluoroethylene (PTFE) thread seal tape (Mil-T-27730A) (Merco Hackensack, Inc., Hillburn, N.Y., U.S.A.) and capped with PTFE lined, 20-400 thread, caps (Fisher Scientific, Fairlawn, N.J., U.S.A.) (see Chapter 2, page 37, Figure 2-1). The mixtures were vortexed until blended to ensure all solid-matter was well incorporated into the liquid matrix. The heating module was a sand bath containing a heating block fitted for 3 mL vials (see Chapter 2, page 37, Figure 2-2). The heating block was preheated for 2 hrs prior to heating the model system. The vials were then heated at 175 ^{o}C for 40 min. Temperature was monitored using a USB-TC

thermocouple and TracerDAQPro software (Measurement Computing™, Waltham, Mass., U.S.A.). After 40 min, the vials were removed from the heating block and cooled to room temperature prior to any further analysis. The reaction solution was removed from the vials, syringe filtered, and diluted 1:5 in mobile phase A.

Water Activity of Model Systems

The water activity was taken of the model systems in part one to see how water activity, compared to water, effected total HCA formation. The reactions for the 16 model systems were made for part one and analyzed using an AquaLab 4TE water activity meter with 47 mm diameter sample cups (Decagon Corp, Pullman Wash., U.S.A.). The instrument was calibrated using 0.500 and 0.760 a_w standards obtained from AquaLab. Because diethylene glycol has a slight vapor pressure (5.7X10-3 mm Hg at 25 °C) the meter mirror and thermophile had to be cleaned with 70% isopropanol every two test.

Heterocyclic Amine Analysis

The five HCAs were analyzed using a HP 1090A Series II HPLC (Agilent Technologies, Santa Clara, Calif., U.S.A.). Separation of the model system was achieved using reversed phase chromatography, with a TSKgel ODS-80T_M (4.6 mm x 25 cm x 5 µm) column and TSKgel guard column (Guardgel ODS80T_M) (3.2 mm x 1.5 cm) (TOSOH Biosciences, Tokyo, Japan). A mobile phase of 0.01 M triethylamine in water, pH 3.6 (A) and acetonitrile (B) was utilized. Acetic acid was used to adjust the pH of mobile phase A. Separation of the HCAs was best achieved when diluted in mobile phase A.

A mobile system gradient, as described by Puangsombat and others (2012) with modifications, was used for HPLC analysis. HCA separation was performed using a 30 min gradient program, beginning with 95% A and 5% B, changing to 87.5% A and 12.5% B at 11.5 min. From 11.5 min to 30 min, 87.5% A and 12.5% B was changed to 45% A and 55% B. The flow rate was 1 mL/min and column temperature was set to 40 °C. The equilibration time was 10 min. The spectrophotometer wavelength was set to 263 nm to detect IQx, MeIQx, and 4,8-DiMeIQx. Detection of IQ and MeIQ was at a wavelength of 258 nm (Jackson and Hargraves 1995; Wu and others 2011). The browning of the reaction was monitored at 420 nm (Ajandouz

and others 2001) using a Genesys 10vis spectrophotometer (Thermo Electron Corporation, Waltham, Mass., U.S.A.).

Quantification and Statistical Analysis

HCAs were quantified using stand curves prepared using pure HCA standards. Standard curves were prepared for each of the HCAs using pure standards at concentrations of 62.5, 125, 250, 500, 1000, 5,000, and 10,000 ppb. The coefficient of determination (R^2) for the standard curves were 0.9997 (IQx), 0.9994 (IQ), 0.9991 (MeIQx), and 0.9994 (4,8-DiMeIQx). A mixed standard of MeIQ and MeIQx was unable to be separated. Therefore, the assumption that MeIQ eluted at the same time as MeIQx in the model system was made. MeIQ was reported with MeIQx using the MeIQx standard curve. Table 3-1 illustrates the Limit of Detection (LOD) and Limit of Quantification (LOQ) for each of the HCAs (Smith 2010).

Table 3-1: Limit of Detection (LOD) and Limit of Quantification (LOQ) for the four HCAs

HCA	LOD (ng)	LOQ (ng)
IQx	1.3	1.4
IQ	0.8	0.8
MeIQx	1.3	1.4
4,8-DiMeIQx	2.0	2.0

To find the effect of the four Maillard variables on HCA formation, a randomized complete block design with days (n = 5) serving as blocks. The treatments were divided into two parts. Part 1 was precursor concentration and water amount and part 2 was sugar type and sugar amount. A four by four treatment structure was used (i.e. precursor concentration by water amount). The likelihood ratio test with a *p*-value cutoff of 0.01 (as advised in Millikan and Johnson 1993) was used to determine constant variance across days. The model was fit using PROC MIXED with SAS version 9.3 (2011, SAS Inst. Inc., Cary, N.C., U.S.A.) using either constant or nonconstant variance across days. Tukey-Kramer adjustments for multiple tests were used to determine difference among treatment levels, with $p < 0.05$ as the selected level decision for significant differences.

Results and Discussion

HCAs were identified in the model system by spiking the model system and confirming the identities of the compounds by retention times. Spectral data could not be captured from the standards and therefore, matches with the UV spectrum could not be made in the model systems. The HCAs eluted at approximately 11.8 min for IQx, 12.9 min for IQ, 16.4 min for MeIQx, and 19.8 min for 4,8-DiMeIQx. The MeIQ standard coeluted with MeIQx. In the model system samples, MeIQ was quantified with MeIQx. Figure 3-1 shows the HPLC chromatogram of the control system in part 1, including identification of the four HCAs in the present study. In previous HCA studies, research indicates IQ elutes prior to IQx (Wu and others 2011). The column and HPLC parameters in the study caused IQx to elute prior to IQ, as observed in Figure 3-1. IQ and IQx standards confirmed the change in elution of these two compounds. Murkovic (2004) also suggested that the glycoaldehyde pathway is a faster reaction, causing IQx-type HCAs to be present in greater amounts. When observing the data, as well as in Figure 3-1, IQ had the greatest overall formation, as compare to the other HCAs studied.

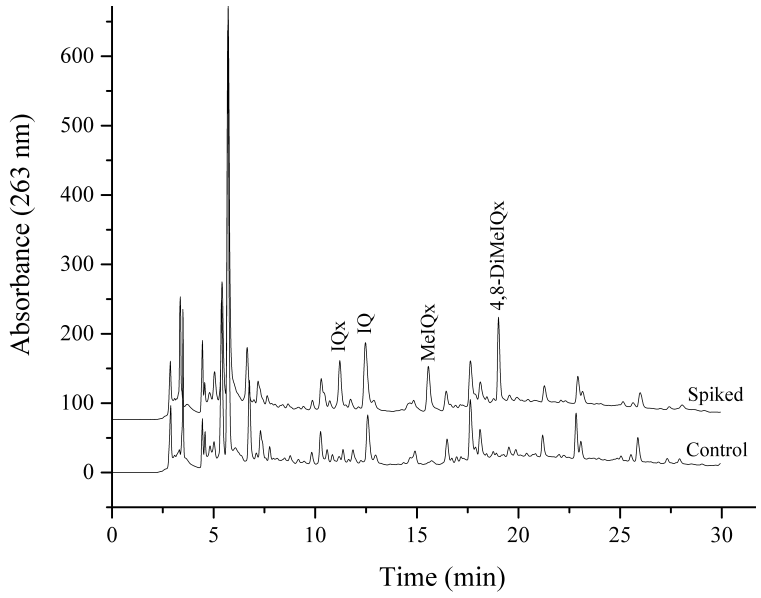

Figure 3-1: HPLC chromatogram of the control system (part 1) with an overlay of a spiked sample indicating the identity of the four HCAs. Both reactions were separated on a TSKgel ODS-80T$_M$ column as described in Section 2.3. The UV detector was set at 263 nm for IQx, MeIQx, and 4,8-DiMeIQx and 258 nm for IQ. The full loop injection volume was 20 µL.

Precursor Concentration and Water Amount

Table 3-2 summarizes the results of the effect of threonine and creatinine concentration on the formation of HCAs in chemical model systems. Precursor concentration had an overall effect on total HCA formation in the model system ($p < 0.05$). A positive association was observed between total HCAs formed and precursor concentration; as precursor concentration increased, total HCAs formed in the model system increased. A 65.6% increase was observed from the control (0.4 mmol) to 0.8 mmol, which produced the highest total HCAs with 80.8 µg. The highest increase of total HCAs are observed from 0.2 mmol to 0.8 mmol, with a 145% increase. There was only a 19.5% increase in total HCA formation from 0.6 to 0.8 mmol model systems. It appears from this data that the percentage change in HCA formation slows from 0.6 to 0.8 mmol systems. It is hypothesized that precursor amounts above 0.8 mmol will exhibit a similar slowing of HCA formation. This phenomenon could be attributed to an over saturation of precursors in the reaction.

Table 3-2: Effect of threonine and creatinine concentrations on HCA formation in chemical model systems heated at 175 °C for 40 min. Sugar was added in half-molar amounts to the threonine and creatinine.*

Threonine and Creatinine Concentration (mmol)	IQx (µg)	IQ (µg)	MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCAs (µg)
0.2	7.4 ± 1.5^{a}	18.6 ± 5.5^{a}	1.0 ± 1.3^{a}	2.3 ± 0.4^{a}	33.0 ± 3.7^{a}
0.4	13.9 ± 1.5^{b}	33.3 ± 5.5^{ab}	1.7 ± 1.3^{ab}	3.2 ± 0.4^{ab}	48.8 ± 3.7^{b}
0.6	23.6 ± 1.5^{c}	44.1 ± 5.5^{b}	2.6 ± 1.3^{b}	3.9 ± 0.4^{b}	67.6 ± 3.7^{c}
0.8	25.8 ± 1.5^{c}	47.4 ± 5.5^{b}	2.6 ± 1.3^{b}	3.4 ± 0.4^{b}	80.8 ± 3.7^{d}

Means with different superscript letters within the same column are significantly different at $p < 0.05$ with Tukey-Kramer adjustments.

*Values are means ± standard error of the class (column).

Borgen and others (2001) observed similar results; as precursor amounts increased, HCA formation increased. Chicken samples had almost twice the amount of free amino acids than

pork chop and roast beef samples. After heating, chicken breast samples had higher total HCA amounts than the other meat samples, indicating that the more free amino acids and precursors present, then the higher amounts of HCAs formed (Borgen and others 2001). This suggests that as the available precursors increase, the greater potential for HCA formation, therefore an increase in total HCAs in the chemical model systems.

For individual HCA formation, a positive association was observed between precursor amount and total formation for IQx, IQ, and MeIQx HCAs. MeIQx formation appeared to reach plateau in both 0.6 and 0.8 mmol model systems, indicating that 0.6 mmol is an optimal amount for MeIQx formation. A positive relationship was not observed in 4,8-DiMeIQx formation, as 0.8 mmol produced less than 0.6 mmol, though there was no significant difference between 0.8 and 0.6 mmol model system 4,8-DiMeIQx amounts. When comparing the individual HCAs, IQ had the highest formation, followed by IQx. In ethanolic model systems, highest formation are observed with IQx rather than IQ (Wu and others 2011). In the present study, the quinoline pathway was preferred over the quinoxaline. It is hypothesized that the dialkyl-pyrazine radical may react with other browning products in the reaction, thus inhibiting the formation of the IQx-type HCAs. In the present study, highest formation increase was 248% was observed in IQx from 0.2 to 0.8 mmol. MeIQx had an increase of 248% from 0.2 to 0.6 mmol and IQ increased 160% from 0.2 to 0.8 mmol model systems. HCA 4,8-DiMeIQx experienced the smallest increase, with only 79.6% from 0.2 to 0.6 mmol.

Water was the second Maillard reaction variable studied for the effect on HCA formation. Water is important in the transfer of water-soluble precursors in meat and therefore important for HCA formation in model systems (Jägerstad and others 1998). The effect of water was evaluated at four percentages: 0, 5, 10, and 15%. Table 3-3 summarizes the effect of water percentage on HCA formation results. Water significantly affected the formation of HCAs in the model system, though there was no positive association between water amount and HCA formation. It appeared that HCA formation increased, plateaued, and then decreased as water percentage increased. The 5 and 10% water-containing model systems both induced the highest HCA formation, though not significantly different from each other. A 42.7% increase was observed in total HCA formation from 0 to 5% water amounts. No significant difference was observed between 0 and 15% water content for total HCA formation. Water activity associated with the percent water and therefore, HCA formation was not attributed to water activity. It is hypothesized that the high presence of

diethylene glycol in the model systems did not allow the precursors to have an effect on water activity.

Table 3-3: Effect of water amount on HCA formation in chemical model systems heated at 175 °C for 40 min. Sugar was added in half-molar amounts to the threonine and creatinine.[1]

Water (%)	Water Activity[2]	IQx (µg)	IQ (µg)	MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCAs (µg)
0	ND	13.1 ± 1.5^{a}	34.6 ± 5.5^{a}	1.8 ± 1.3^{a}	2.8 ± 0.4^{a}	47.3 ± 3.7^{a}
5	0.363 ± 0.037	19.9 ± 1.5^{b}	39.9 ± 5.5^{a}	1.8 ± 1.3^{a}	3.1 ± 0.4^{a}	67.5 ± 3.7^{b}
10	0.464 ± 0.015	17.2 ± 1.5^{ab}	39.5 ± 5.5^{a}	1.7 ± 1.3^{a}	3.4 ± 0.4^{a}	62.0 ± 3.7^{b}
15	0.532 ± 0.006	20.5 ± 1.5^{b}	29.4 ± 5.5^{a}	2.8 ± 1.3^{a}	3.6 ± 0.4^{a}	53.4 ± 3.7^{a}

Means with different superscript letters within the same column are significantly different at $p < 0.05$ with Tukey-Kramer adjustments.

[1]Values are means ± standard error of the class (column).

[2]ND = not detected, values are ± standard deviation. Raw data is in Appendix I.

When investigating the individual HCAs, there appeared to be a positive association between water amount and HCA formation for IQx. The water percentage had an effect on IQx formation, as there was a 56.5% increase from 0 to 15% water. The water amount had no effect on IQ, MeIQx, and 4,8-DiMeIQx formation in the model systems. As observed with precursor amount, IQ was the highest HCA formed in water treated model systems.

Kato and others (1996) observed a decrease in HCA formation with an increase in water amount in model systems. Kikugawa (2004) had similar results in that mutagenicity in the model systems decreased as water content increased. It is proposed that the pyrazine radical in the formation pathway of HCAs is suppressed when water content increases (Kikugawa 2004). Water may react with the pyrazine and pyridine free-radical intermediates to form other browning products in the reaction and therefore, HCA formation is inhibited.

Interaction Between Precursor Concentration and Water Amount

There was an interaction effect ($p < 0.05$) observed between threonine and creatinine concentration and water percentage for overall total HCA formation. Table 3-4 summarizes the interaction effect between the two variables on total formation of HCAs. As predicted, total HCA

formation increased as precursor concentration increased with each water percentage present in the model systems. At 10% water, 0.6 mmol obtained the highest HCA formation, but was not significantly different from the 0.8 mmol model system. This was the same effect observed with the main effect of precursor concentration on HCA formation. At 5% water, overall HCA formation appeared to be the highest as compared to the other water treatments, with 0% producing the least. The 0.8 mmol model system had the highest HCA formation with 5% water, though it was not significant from 10 and 15% water.

Table 3-4: Interaction effect between threonine and creatinine concentration and water amount on HCA formation in chemical model systems heated at 175 ^{o}C for 40 min. Sugar was added in half-molar amounts to the threonine and creatinine.*

		Water Amount (%)			
		0	**5**	**10**	**15**
Threonine and Creatinine Concentration (mmol)	**0.2**	21.9 ± 4.9^{aw}	43.6 ± 4.9^{bw}	31.3 ± 4.9^{abw}	34.1 ± 4.9^{abw}
	0.4	41.9 ± 4.9^{ax}	62.5 ± 4.9^{bx}	49.1 ± 4.9^{abw}	40.9 ± 4.9^{awx}
	0.6	56.0 ± 4.9^{axy}	71.8 ± 4.9^{abx}	85.3 ± 4.9^{bx}	56.1 ± 4.9^{ax}
	0.8	68.7 ± 4.9^{ay}	91.6 ± 4.9^{by}	79.6 ± 4.9^{abx}	80.2 ± 4.9^{aby}

Means with different superscript letters within the same column row (a-d) and (w-z) are significantly different at $p < 0.05$ using Tukey-Kramer adjustments.

*Values are means ± standard error of the class (column).

The precursor concentration of 0.6 mmol was chosen for our optimized model system. Though the 0.8 mmol model system produced the highest HCAs, little research has studied levels at 0.6 mmol. Previous research on model systems have studied precursor concentrations at lower levels (0.2 and 0.4 mmol) (Jackson and Hargraves 1995; Arvidsson and others 1997; Wu and others 2011) and others have studied higher concentrations (0.9 mmol) (Johansson and Jägerstad 1996; Moon and Shin 2013). Therefore, 0.6/0.6 mmol was the selected concentration for further study in part 2.

For the individual HCAs, 15% water appeared to aid in HCA formation though this level did not produce the highest amount of total HCAs in the model systems. Insolubility of

precursors was also an issue in both part 1 and 2 of the experiment. More water present in the model systems was thought to help achieve better solubility of precursors. Previous studies also indicated that more water present in the model systems aided in HCA formation. High water percentages are found in raw meat. Water content of various raw meats are suggested to be 64% for ground beef (85% lean), 71% for whole beef brisket, and 66% for whole fryer chicken (USDA 2011). These values were too high to maintain in a model system due to pressure and water loss during heating. Therefore, 15% water was determined to be a better representation of water present in meat systems and the chosen amount in the optimized chemical model system.

Additionally, when looking at the interaction effect of the precursor amount and water percentage, 0.6 mmol and 15% water did not produce the highest amount of HCAs. These parameters were chosen to determine if insolubility of the reactants would be decreased with less precursors and more water.

Sugar Type and Sugar Amount

Sugars are an essential precursor in the formation of HCAs, specifically reducing sugars (Cheng and others 2006). Research indicates that HCA formation is dependent on the type of sugar, as glucose has been cited as necessary in the formation of MeIQx and DiMeIQx (Skog and Jägerstad 1990). Table 3-5 summarizes the effects of sugar type on HCA formation in the model systems.

Table 3-5: Effect of sugar type on HCA formation in chemical model systems heated at 175 °C for 40 min. The threonine and creatinine were 0.6 mmol and contained 15% water.*

Sugar Type	IQx (µg)	IQ (µg)	MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCAs (µg)
Fructose	24.0 ± 2.6^{a}	59.6 ± 4.7^{ab}	2.3 ± 1.5^{a}	2.7 ± 0.5^{a}	89.0 ± 5.8^{a}
Galactose	16.6 ± 2.6^{a}	48.3 ± 4.7^{a}	3.6 ± 1.5^{a}	6.4 ± 0.5^{c}	74.6 ± 5.8^{a}
Glucose	24.3 ± 2.6^{a}	59.5 ± 4.7^{ab}	4.5 ± 1.5^{a}	4.3 ± 0.5^{b}	92.3 ± 5.8^{a}
Lactose	22.0 ± 2.6^{a}	69.6 ± 4.7^{b}	15.2 ± 1.5^{b}	7.3 ± 0.5^{d}	115.4 ± 5.8^{b}

Means with different superscript letters within the same column row (a-d) and (w-z) are significantly different at $p < 0.05$ using Tukey-Kramer adjustments.

*Values are means ± standard error of the class (column).

The type of sugar significantly ($p < 0.05$) affected total HCA formation in the model system. Lactose produced the highest amount of total HCAs in the model system, followed by glucose. Though glucose, galactose, and fructose were not significantly different, galactose appeared to have produced the least amount of total HCAs in the chemical model systems. It is hypothesized that the carbonyl groups in galactose are sterically hindered in the α-position and therefore cannot react with the amino acid group to form more HCAs. Lactose was significantly different from the other three sugars, with glucose, galactose, and fructose not having significant differences. Lactose induced 54.7% higher HCA formation as compared to galactose initiated 25.0% more HCA formation than the control (glucose).

In a similar study observing the effects of various sugar types on PhIP formation, sucrose caused the highest PhIP formation, followed by glucose, and then lactose (Moon and Shin 2013). Skog and Jägerstad (1990) also observed greatest formation with sucrose. Skog and Jägerstad (1990) also indicated that fructose produces more HCAs as compared to glucose. Skog and others (1992) noted lactose as having inhibitory effects on mutagen formation in fried beef patties. As lactose percentage increased, mutagen formation decreased. Due to the discrepancies between the previous and the present study, it is hypothesized that lactose has more reactive groups available for reaction with the amino acid (i.e. the reducing end of glucose, the glycosidic bond, and free hydroxyl groups, etc.).

When comparing the individual HCAs, IQ had the highest formation out of the other three HCAs, which was observed in the precursor and water amounts. In addition to total HCA formation, lactose caused the highest IQ formation over the other three sugars. When compared to galactose, which had the lowest IQ formation in the model systems, lactose initiated 44.1% more IQ formation, though sugar type had no significant effect ($p < 0.05$) on HCA formation. MeIQx and 4,8-DiMeIQx formation was also highest with lactose. For IQ and IQx formation, glucose and lactose were not significantly different from each other. Though lactose produced a higher total HCA amount, glucose is a better representation of the sugars present in meat and therefore chosen as the sugar for the optimized model system.

Sugar concentration was the final Maillard variable examined in the study. Sugar amount greatly impacts the formation of HCAs. It has been stated that in model systems, sugar is optimal at half-molar to other precursors. Studies indicate that sugar added in excess of equimolar to

other precursors cause inhibition in HCA formation (Skog and Jägerstad 1990; Skog and Jägerstad 1991).

In the present study, four sugar amounts in comparison to threonine and creatinine were examined: 0.15, 0.2, 0.6, and 1.2 mmol (quarter, half, equi, and double molar, respectively). Table 3-6 summarizes the effects of the four sugar amounts on HCA formation in the model system. Sugar significantly affected ($p < 0.05$) the formation of HCAs in the model system, with 1.2 mmol producing the highest total HCAs. As observed in Table 3-6, there was a positive association between sugar amount and HCA formation; as sugars increased, HCA formation increased. When comparing the control (0.3 mmol) to 1.2 mmol sugar amounts, 1.2 mmol produced 63.8% more total HCAs. The 0.15 mmol sugar model system had the lowest HCA formation; the 1.2 mmol model system had 140% more HCA formation than the 0.15 mmol system.

Table 3-6: Effect of sugar amount on HCA formation in chemical model systems heated at 175 ^{o}C for 40 min. The threonine and creatinine were 0.6 mmol and 15% water was added to the model systems.*

Sugar Amount (mmol)	IQx (µg)	IQ (µg)	MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCAs (µg)
0.15	11.9 ± 2.6^{a}	34.7 ± 4.7^{a}	3.3 ± 1.5^{a}	4.8 ± 0.5^{a}	54.6 ± 5.8^{a}
0.3	18.1 ± 2.6^{ab}	52.4 ± 4.7^{b}	3.6 ± 1.5^{a}	4.8 ± 0.5^{a}	79.9 ± 5.8^{b}
0.6	25.5 ± 2.6^{bc}	68.0 ± 4.7^{bc}	7.9 ± 1.5^{ab}	4.8 ± 0.5^{a}	105.9 ± 5.8^{c}
1.2	31.4 ± 2.6^{c}	81.9 ± 4.7^{c}	10.7 ± 1.5^{b}	6.1 ± 0.5^{b}	130.9 ± 5.8^{d}

Means with different superscript letters within the same column row (a-d) and (w-z) are significantly different at $p < 0.05$ using Tukey-Kramer adjustments (column).

*Values are means ± standard error of the class (column).

A positive association between the increase in sugar content and HCA formation was also observed in the four individual HCAs. The 1.2 mmol model system also produced the highest individual HCAs as it did with total HCA formation. As previously observed, IQ had the highest overall formation when compared with the other three HCAs with 81.9 µg in the 1.2 mmol model system. MeIQx had the highest promotion of HCAs, with the 1.2 mmol model system

having an increase of 224% from the 0.15 mmol system. IQx had the second highest increase with 164% from 0.15 to 1.2 mmol model system. There was no association observed in the formation of 4,8-DiMeIQx but a significant increase was observed at 1.2 mmol.

Skog and Jägerstad (1990) evaluated the effect sugar type and sugar amount had on mutagenicity. Glucose, fructose, and lactose were evaluated at increasing concentrations. In all three sugar types, a trend was observed in relation to mutagenicity. As sugar concentration increased, mutagenicity increased, until the sugars reached equimolar to the amino acid and creatine. At levels above equimolar, mutagenicity decreased significantly. Fructose yielded a higher mutagenicity total at equimolar than glucose and lactose.

Moon and Shin (2013) studied the effect of sugar type and sugar amount on the formation of PhIP in model systems. Glucose, fructose, sucrose, and lactose were the four sugars evaluated. PhIP formation was highest when no sugar was present in the model systems. Unlike our study, Moon and Shin (2013) observed an inhibition in PhIP formation as sugar amounts increased, for all four sugars. When compared to previous studies, PhIP formation was greatest when no sugar was present, unlike the hypothesis that formation is greatest when sugars are half-molar to the other precursors.

In the present study, enhancement of HCA formation was observed. It is hypothesized that the excess sugar fully reacts with the threonine, as it has been suggested that the excess sugar reacts with creatinine. Another reason for the enhancement of HCAs is that the sugars undergo caramelization and the caramelization products are further reacting with the threonine, further promoting the formation of the HCAs in the model system.

From this study, it was determined that sugar in double molar to the other precursors promoted the highest total HCA formation in the model system. This same effect was observed for individual HCA formation. Therefore, 1.2 mmol of sugar was determined to be an ideal parameter for an optimized model system.

Absorbance

Absorbance of each model system was measured at 420 nm (Ajandouz and others 2001) to monitor the browning that occurred in each reaction. Figure 3-3 illustrates the relationship between sugar amounts and absorbance for the four sugars evaluated in the study. A positive association between sugar amounts and absorbance of each of the four sugars was observed. As

sugar amounts increased, absorbance increased, indicating that the browning of the reaction increased. Glucose, fructose, and galactose had similar absorbance values at each of the sugar concentrations. Lactose had the highest absorbance at each of the four sugar concentrations. Caramelization may attribute to the increase in browning within the model systems due to the increase in each of the four sugar amounts.

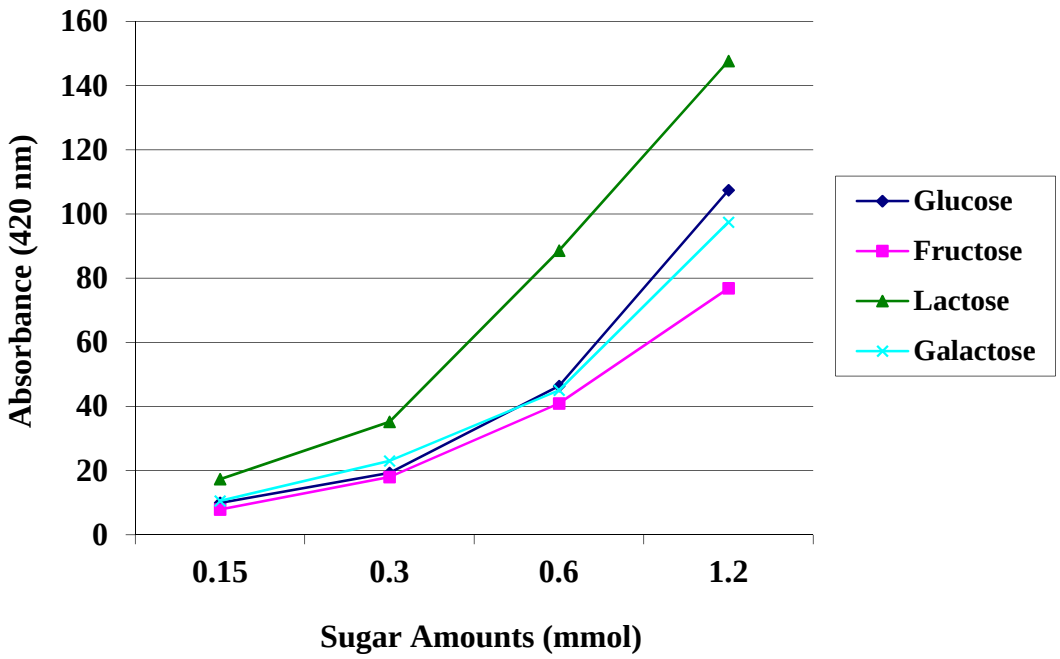

Figure 3-2: Relationship between sugar amounts and absorbance for four sugars

Insolubility and Temperature of Model Systems During Heating

Following heating of the model systems, it was observed that precursors did not fully dissolve during the heating process. Insolubility of the precursors is a large source of variation and error in the experiment. Insolubility and the result of remaining precursors indicate that the reaction among the threonine, creatinine, and sugar would not have fully come to completion. The inability of the reaction to fully react would cause model systems to produce different amounts of HCAs, initiating variation and possibly covariance inequality among the experimental replications.

Model systems may also be too concentrated, meaning there is an excess amount of precursors to the volume of liquid. An excessive amount of solids to a little volume of liquid may not allow precursors to fully react. According to the Merck Index (13th edition), creatinine is

soluble in 12 parts water and galactose is soluble in 0.5 parts water. One gram of glucose is soluble in 1 mL of water and 1 g of lactose is soluble in 2.2 mL of water. Fructose and threonine are both freely soluble in water (Merck Index, 13th edition). In part one, all model systems exhibited insolubility, which could be attributed to the variation of water present in the model systems in part 1.

In part 2, almost all of the lactose-containing model systems (except with 0.15 mmol lactose) had complete solubility of all three precursors. Greatest total HCA formation was observed in these model systems, as well as greatest browning, according to absorbance. Even with solubility of the precursors, lactose-containing model systems had the greatest standard deviation, as compared to other sugar model systems. All other sugar-containing model systems experienced insolubility of the three precursors.

Through temperature monitoring with a USB-TC thermocouple, it was observed that a significant decrease in temperature occurred within the first five min of heating the model systems (Figure 3-4). After five min, temperature of the heating module gradually increased, but never returned to the initial heating temperature. Arvidsson and others (1997) indicated the importance of temperature on HCA formation. Temperatures at and below 150 ºC are not ideal for HCA formation. At temperatures exceeding 225 ºC, degradation of HCAs occurs (Arvidsson and others 1997).

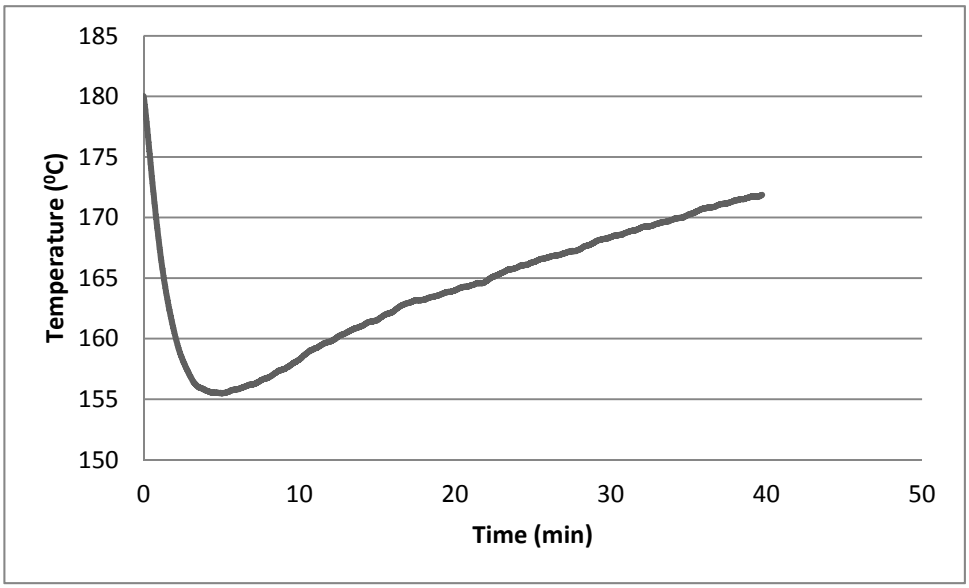

Figure 3-3: Graph representing the change in temperature during the 40 min heating of the model systems

The instability and dramatic decrease at the beginning of each heating period was attributed to the loss of heat when opening the heating module and inserting the reaction vials. The first five min is critical for HCA formation. According to Jackson and Hargraves (1995), there is an initial lag time of HCA formation in reaction mixtures. It has been postulated, that this increment of time is when precursors are brought to temperature (approximately 150 ºC), initiating a reaction among the amino acid, creatin(in)e, and sugar (Jackson and Hargraves 1995). Therefore, dramatic heat loss in the heating mantel may have delayed the reaction mixture from reaching 150 ºC, which is critical in the overall formation of HCAs in the model systems. This may attribute to variation observed in the total HCAs formed in each of the two experimental parts.

Additionally, the initial heat loss caused overall heating temperatures in the mantel to be lower than the desired 175 ºC. On average, overall heating mantel temperatures were 170 ºC. Though only 5º lower than the preferred temperature, the lower heating temperature in conjunction with the initial rapid heat loss of the mantel may have attributed to the insolubility of the precursors. If the heating mantel never reached or kept at the desired 175 ºC, the precursors never had the full opportunity to react during the set heating time. The variation in the temperature caused variation in the total HCA formation in each of the two parts of the entire experiment.

Conclusions

In this study, four Maillard reaction variables were evaluated for their effect on HCA formation in chemical model systems. Precursor concentration, water percentage, sugar type, and sugar amount were the four selected variables studied. Overall, all four variables significantly affected the total HCA formation in the chemical model systems. An interaction effect was also observed between precursor concentration and water percentage.

Precursor amount is important for HCA formation; as precursor concentrations increase in the reaction, HCA formation increases. Lower water percentages in the reaction also enhance formation of HCAs. Previous studies indicate lactose inhibits HCA formation, but our study indicates lactose does the opposite. Extensive studies illustrate sugars above equimolar to the amino acid and creatin(in)e inhibit HCA formation. In our study, a positive association was observed; as sugar amounts increased, HCA formation increased.

It was concluded from the analyzed data that the four variables were essential to HCA formation. A model system containing threonine (0.6 mmol), creatinine (0.6 mmol), and glucose (1.2 mmol), respectively, with 15% water was an optimal ratio for studying the formation of HCAs typically formed in meat.

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Chapter 4 - Evaluation of Four Antioxidants and Their Effect on the Formation of Heterocyclic Amines in Chemical Model Systems

Abstract

Potentially carcinogenic and highly mutagenic compounds, heterocyclic amines (HCAs), form in meat cooked at high temperatures via the Maillard browning reaction. Chemical model systems are a current method of in vitro study of HCAs, allowing variables to be controlled and adjusted. Human consumption of HCAs has been associated with breast, prostate, colon, and pancreatic cancers. Recent efforts have been made to develop methods in which HCA formation can be inhibited. Studies indicate that antioxidant and antioxidant-containing compounds inhibit the formation of HCAs in meat. Model system data is conflicting, as some studies state inhibition and others reported promotion of HCAs upon addition of antioxidants. Therefore, the current study evaluated four antioxidants (butylated hydroxyanisole (BHA), naringenin, rosmarinic acid, and Epigallocatechin gallate (EGCG) on their effect on the formation of five HCAs (IQ, MeIQ, IQx, MeIQx, and 4,8-DiMeIQx) in chemical model systems. Model systems were dissolved in diethylene glycol containing 15% water, heated for 40 min at 175 ^{o}C, and separated using reversed-phase liquid chromatography. Antioxidants were studied individually at six levels: 0, 125, 250, 500, 1000, and 2000 ppm. Overall, all four antioxidants had no significant effect ($p < 0.05$) on the formation of HCAs in the model systems. This study could not determine whether antioxidants effect the formation of HCAs in chemical model systems.

Introduction

Heterocyclic amines (HCAs) are potentially carcinogenic and highly mutagenic bi-products of the Maillard browning reaction. A reaction via amino acids, creatin(in)e, and reducing sugars, these compounds form specifically in muscle-dense food products that endure high temperature heat treatment (150 to 200 ºC) (Murkovic 2004). To date, 25 HCAs have been identified in both meat and model systems by the Ames/*Salmonella* test (Sanz Alaejos and others 2008) and are present in the parts per billion range (Murkovic 2004).

Case studies approximate human consumption to be approximately 250 – 300 ng per day, with the highest levels being PhIP (~160 – 240 ng), MeIQx (~70 – 90 ng), and 4,8-DiMeIQx (~4 – 6 ng) (Nowell and others 2002; Li and others 2007) through the consumption of grilled, fried, and roasted meat products. HCAs are most commonly found on the surface of meat products, including grilled, fried, baked, and roasted beef, chicken, pork, and fish (Murkovic 2004). The most abundant HCAs found in meat are 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4-8-trimethyl-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Zheng and Lee 2009). Also present in meat are 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) and 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ) HCAs.

In 1939, E.M.P Widmark first described the presence of potentially carcinogenic substances when male and female mice developed tumors upon topical application of roasted meat extracts (Widmark 1939). Since Widmark's 1939 discovery, research has indicated pancreatic (Li and others 2007), breast (Sinha and others 2000), prostate (Tang and others 2007), and colon/colorectal cancers (De Stefani and others 1997) are associated with HCA consumption. The National Toxicology Program of the U.S. Department of Health and Human Services (2011) has since classified MeIQ, IQ, and MeIQx as *reasonably anticipated to be human carcinogens*.

Laboratory and in vitro studies of HCAs are currently conducted using chemical model systems. Model systems are often a preferred method of HCA study as complex side reactions, products, and matrices found in meat are eliminated (Murkovic 2004). Model systems allow researchers to modify precursor compositions and concentrations while adjusting time and temperature of the reactions. As with meat systems, formation and concentration of HCAs in model systems depends on numerous physical and chemical properties, including meat type,

water activity, and pH (Oz and Kaya 2011), but also whether inhibitory chemicals are present in the reaction, such as antioxidants and free radical scavengers.

Pyridine and dialkyl-prazine free radicals form prior to IQ and IQx-type HCAs. Research indicates antioxidants may interfere with HCA development at various points in the HCA formation pathway and are suggested to act as free radical scavengers. Antioxidants and polyphenols contain phenolic compounds, a molecule possessing a phenolic ring with an attached hydroxyl group. Phenolic compounds are scavenging components of antioxidants.

In the formation pathway, antioxidants are suggested to scavenge pyridine and dialkyl-prazine free radicals in the IQ and IQx-type HCA pathways, thus inhibiting IQ and IQx moiety formation (Vitaglione and Fogliano 2004). Several various methods using polyphenols and natural and synthetic antioxidants have exhibited abilities to inhibit HCA formation in both meat and model systems (Oguri and others 1998; Tsen and others 2006; Moon and Shin 2013). Research also suggests that antioxidants and phenolic-containing compounds promote the formation of HCAs in chemical model systems (Johansson and Jägerstad 1996; Zöchling and Murkovic 2002; Cheng and others 2007).

Research indicates conflicting data pertaining to antioxidants and HCA formation in chemical model systems. Therefore, the objective of this study was to observe the effect that four free radical scavenging compounds, at varying concentrations, had on the formation of five HCAs in chemical model systems.

Materials and Methods

Materials

Rosmarinic acid (≥98%, HPLC grade), butylated hydroxyanisole (≥98.5%), and naringenin (≥98%) were all purchased from Sigma-Aldrich Chemicals (St. Louis, Mo., U.S.A.). Epigallocatechin gallate (≥98%) was purchased from Enzo Life Sciences (Farmingdale, N.Y., U.S.A.). Threonine, creatinine, glucose, ascorbic acid (99% ACS reagent), and gallic acid were also purchased from Sigma-Aldrich Chemicals. Sodium carbonate (certified ACS powder) was purchased from Fisher Scientific (Fairlawn, N.J., U.S.A.). HCA standards, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx), 2-amino-3-methlimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline

(MeIQx), and 2-amino-3,4-8-trimethyl-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) were all purchased from Toronto Research Chemicals (Ontario, Canada).

Diethylene glycol, and triethylamine were all acquired from Sigma-Aldrich Chemicals. Acetonitrile (HPLC grade), methanol (HPLC grade), and acetic acid (HPLC grade) were purchased from Fisher Scientific (Fairlawn, N.J., U.S.A.). Polyvinylidene fluoride (PVDF) membrane 4 mm 0.45 µm syringe filters were purchased from Agilent Technologies (Santa Clara, Calif., U.S.A.). A Sybron/Barnstead PCS filtration unit (Barnstead/Thermolyne, Inc, Dubuque, Iowa, U.S.A.) was used to process deionized water.

Preparation of Antioxidants

Four antioxidants were evaluated for their effect on HCA formation in chemical model systems: rosmarinic acid, butylated hydroxyanisole (BHA), naringenin, and epigallocatechin gallate (EGCG). Antioxidants were prepared in 5000 ppm solution for use in model systems by separately dissolving each antioxidant in diethylene glycol. Solutions were vortexed to ensure antioxidants completely dissolved and incorporated into the liquid matrix.

Preparation of Model Systems

Antioxidants were assessed at four concentrations in the model systems, similarly described by Johansson and Jägerstad (1996): 2000, 1000, 500, 250, and 125 ppm. The precursors threonine (0.2 mmol), creatinine (0.2 mmol), and glucose (0.1 mmol) were prepared in 3 mL conical reaction vials and diluted with 375 µL water. A spin vane was used to completely dissolve the precursors prior to further treatment. Vials were brought to a final volume of 2500 µL with diethylene glycol. Antioxidant solutions were added accordingly.

To prevent loss of water during heating, vials were fitted with high density polytetrafluoroethylene (PTFE) thread tape (Merco Hackensack, Inc, Hillburn, N.Y., U.S.A.) and capped with PTFE lined 20-400 thread caps (Fisher Scientific, Fairlawn, N.J., U.S.A.). The model system mixtures were vortexed to ensure a well-incorporated liquid matrix. The heating module was a sand bath containing a heating block fitted for 3 mL reaction vials. The heating block was preheated for 2 hr prior to heating the model systems. Vials were heated at 175ºC for 40 min. Temperature was monitored using a USB-TC thermocouple (Measurement Computing™, Waltham, Mass., U.S.A.). After 40 min, the model systems were removed and

brought to room temperature. Model systems were transferred to vials, diluted 1:4 with mobile phase A, and syringe filtered.

Heterocyclic Amine Analysis

The five HCAs were analyzed using a HP 1050 series HPLC (Agilent Technologies, Santa Clara, Calif., U.S.A.), equipped with a 21 sample HP 1050 series auto sampler. Reversed phase high-performance liquid chromatography, using a TSKgel ODS-80T$_M$ (4.6 mm x 25 cm x 5 μm) and guard column, TSK guardgel ODS80T$_M$ (3.2 mm x 1.5 cm) (TOSOH Biosciences, Tokyo, Japan) was used to achieve separation of the model systems. A mobile phase of 0.01 M triethylamine, pH 3.6 (A) and acetonitrile (B) was utilized. The adjustment of mobile phase pH was completed with acetic acid. Best achieved HCA separation occurred when model systems were diluted 1:4 with mobile phase A.

A mobile system gradient was used with modifications by Puangsombat and others (2012). A 30 min, linear-like, mobile phase gradient was used to separate HCAs within the model system. The gradient began with 95% A and 5% B, changing to 87.5% A and 12.5% B at 11.5 min and from 11.5 min to 30 min, A changed from 87.5 to 45% and B from 12.5 to 55%. After 30 min, the mobile phase returned to the original ratio (95% A and 5% B) for 10 min of column equilibration. The flow rate was 1.0 mL/min and the column temperature was 40 ^oC. For detection of IQx, MeIQ/MeIQx, and 4,8-DiMeIQx, the spectrophotometer wavelength was set at 263 nm. IQ was detected at 258 nm (Jackson and Hargraves 1995; Wu and others 2011). Browning of the model system reaction was monitored at 420 nm (Ajandouz and others 2001) using a Genesys 10vis spectrophotometer (Thermo Electron Corporation, Waltham, Mass., U.S.A.).

Quantification and Statistical Analysis

To quantify each HCA, standard curves of pure standards were prepared for each HCA at concentrations of 125, 250, 500, 1000, 5,000, and 10,000 ppb. A mixed standard curve for MeIQ and MeIQx was prepared, as separation of the two HCAs could not be achieved. MeIQ and MeIQx were reported as a mixed quantity. Coefficient of determination (R^2) for all four standard curves was 0.999. Table 4.1 illustrates the Limit of Detection (LOD) and Limit of Quantification (LOQ) for the four standards (Smith 2010).

Table 4-1: Limit of Detection (LOD) and Limit of Quantification (LOQ) for the four HCAs

HCA	LOD (ng)	LOQ (ng)
IQx	1.31	1.33
IQ	1.05	1.06
MeIQ/MeIQx	1.16	1.18
4,8-DiMeIQx	0.57	0.58

To find the effect of the four antioxidants on HCA formation, a randomized complete block design with days (n = 5) serving as blocks. The likelihood ratio test with a *p*-value cutoff of 0.01 (as advised in Millikan and Johnson 1993) was used to determine constant variance across days. The model was fit using PROC MIXED with SAS version 9.3 (2011, SAS Inst. Inc., Cary, N.C., U.S.A.) using either constant or nonconstant variance across days. Tukey-Kramer adjustments for multiple tests were used to determine difference among treatment levels.

Results and Discussion

All HCAs were identified in the model systems by spiking with a mixed standard and confirming the identities of the compounds via retention times (Appendix C). In all of the prepared and heated evaluated model systems, HCAs were observed and quantified via HPLC. Figure 4.1 illustrates the HPLC chromatogram of the control system, including identifiers for all five HCAs. HCAs eluted at approximately 11.3 min for IQx, 12.5 min for IQ, 15.3 min for MeIQ/MeIQx, and 19.1 min for 4,8-DiMeIQx. Wu and others (2011) reported IQ eluting prior to IQx. The column and HPLC parameters used for HCA analysis caused a reversal in elution, as IQx eluted prior to IQ as observed in Figure 4.1. IQ and IQx standards confirmed the change in elution for these two compounds.

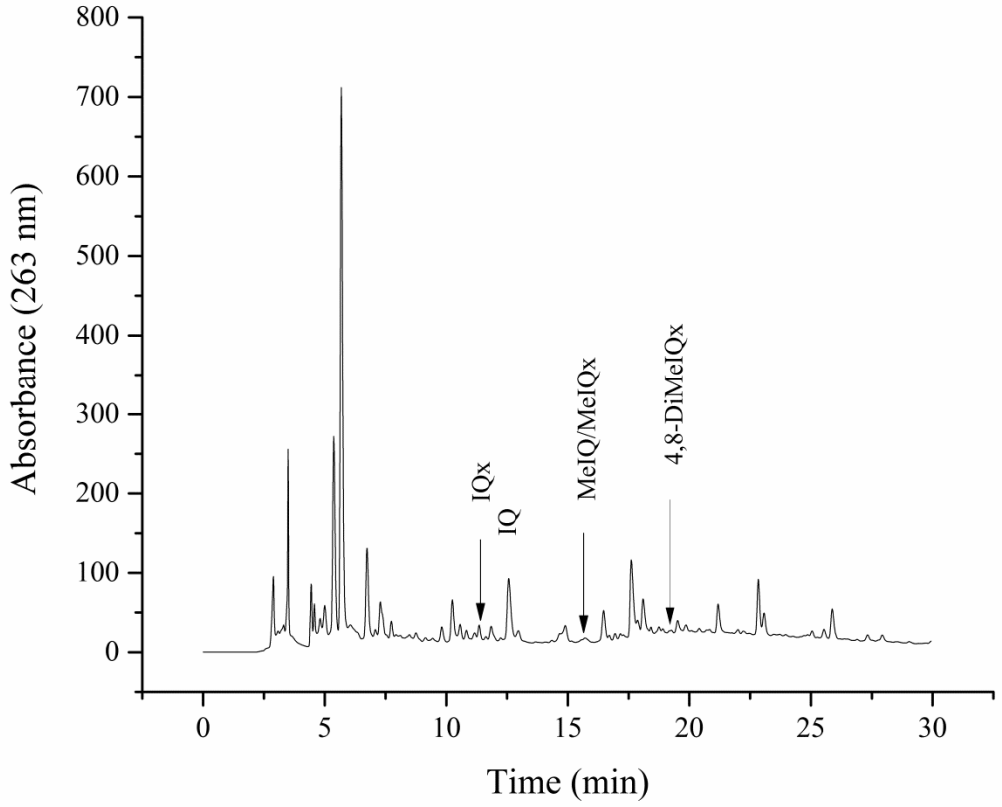

Figure 4-1: HPLC chromatogram of control model system

Antioxidants

BHA, a synthetic antioxidant, was the first evaluated antioxidant in the study. Table 4-2 summarizes the effects of BHA on total and individual HCA formation in the chemical model system. BHA at all five added levels had no significant effect ($p < 0.05$) on the formation of HCAs. The addition of BHA had neither an inhibiting effect nor an enhancing effect on HCA formation in the model systems. The addition of 2000 ppm appeared to inhibit total HCA formation as the total HCAs formed at 2000 ppm is lower, but no significant differences were found. BHA appeared to inhibit the formation of IQx, and MeIQ/MeIQx, but means were also not significantly different from each other. Possible enhancement of IQ formation was also observed.

Table 4-2: Effect of butylated hydroxyanisole (BHA) on HCA formation in chemical model systems heated at 175 °C for 40 min.*

BHA (ppm)	IQx (µg)	IQ (µg)	MeIQ/MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCAs (µg)
Control	6.8 ± 0.7	12.9 ± 1.1	4.4 ± 0.6	1.2 ± 0.2	25.8 ± 1.5
125	5.9 ± 0.7	14.1 ± 1.1	3.1 ± 0.6	1.2 ± 0.2	24.5 ± 1.5
250	5.6 ± 0.7	14.3 ± 1.1	3.0 ± 0.6	1.0 ± 0.2	24.1 ± 1.5
500	5.8 ± 0.7	15.2 ± 1.1	2.8 ± 0.6	1.3 ± 0.2	24.2 ± 1.5
1000	7.0 ± 0.7	12.9 ± 1.1	3.1 ± 0.6	1.1 ± 0.2	24.2 ± 1.5
2000	6.1 ± 0.7	14.7 ± 1.1	2.9 ± 0.6	1.4 ± 0.2	21.5 ± 1.5

No significant differences were observed at $p < 0.05$.

*Means ± standard error of the class (column)

Unlike the present study, BHA has been noted in the literature to inhibit the formation of HCAs in model systems and meat products. Moon and Shin (2013) reported a 65.3% inhibition of PhIP upon the addition of 1000 ppm BHA. Reduction in mutagenicity was observed upon the direct addition of BHA to beef extracts using the Ames test (Wang and others 1982). Pearson and others (1992) suggest BHA performs as a free radical scavenger due to the methoxy group becoming a quinone-like compound, blocking the reaction to form IQ and IQx-type HCAs. In contrast, Oguri and others (1998) saw no effect on MeIQx levels with the addition of BHA in model systems. Johansson and Jägerstad (1996) noted a significant promotion of MeIQx upon the addition of 10,000 ppm BHA in model systems.

Naringenin, a grapefruit flavonoid, was the second antioxidant evaluated in the study. Table 4-3 summarizes the effect of naringenin addition on HCA formation in the chemical model systems. There was no significant effect ($p < 0.05$) observed upon the addition of all five levels of naringenin. Based on the data, it is unsure whether naringenin had an effect on total HCA formation. The addition of 2000 ppm appeared to enhance the formation of IQx and 4,8-DiMeIQx, whereas 2000 ppm appeared to inhibition formation of IQ and MeIQ/MeIQx formation. No significant differences were observed among the individual HCAs as well.

Table 4-3: Effect of naringenin on HCA formation in chemical model systems heated at 175 ºC for 40 min.*

Naringenin (ppm)	IQx (µg)	IQ (µg)	MeIQ/MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCAs (µg)
Control	6.2 ± 0.7	14.2 ± 1.1	3.1 ± 0.6	1.2 ± 0.2	23.7 ± 1.5
125	5.9 ± 0.7	14.7 ± 1.1	2.5 ± 0.6	1.1 ± 0.2	24.3 ± 1.5
250	6.1 ± 0.7	15.1 ± 1.1	3.0 ± 0.6	1.2 ± 0.2	24.3 ± 1.5
500	5.5 ± 0.7	14.9 ± 1.1	2.9 ± 0.6	1.0 ± 0.2	23.6 ± 1.5
1000	5.9 ± 0.7	14.2 ± 1.1	3.3 ± 0.6	1.1 ± 0.2	24.1 ± 1.5
2000	7.5 ± 0.7	13.6 ± 1.1	2.8 ± 0.6	1.4 ± 0.2	23.2 ± 1.5

No significant differences were observed at $p < 0.05$.

*Means ± standard error of the class (column)

Limited research has studied the effect of naringenin on HCA formation in meat and model systems. The little research that has been conducted has suggested that naringenin does inhibit the formation of HCAs, specifically when added to model systems. Moon and Shin (2013) reported a 97.6% inhibition of PhIP from the control and a model system containing 1000 ppm naringenin. It was also noted that as naringenin concentration increased, PhIP formation decreased (Moon and Shin 2013). Cheng and others (2007) noted similar observations as naringenin significantly inhibited the formation of PhIP in model systems. In the study, naringenin had the greatest inhibition abilities as compared to the other evaluated antioxidants (Cheng and others 2007). Naringenin also inhibited 70% of total HCA formation when 0.1% was added to beef patties prior to frying. An inhibition of PhIP (74%), 4,8-DiMeIQx (63%), and MeIQx (67%) HCAs were observed (Cheng and others 2007).

Rosmarinic acid is a phenolic compound found in rosemary and the third antioxidant compound evaluated in the study. Rosemary and its extracts have been reported as having antioxidant properties (Wojdylo and others 2007). Table 4-4 summarizes the effect of rosmarinic acid on total and individual HCA formation in the chemical model system. Rosemarinic acid had no effect ($p < 0.05$) on HCA formation. The addition of 125 and 250 ppm appears to have inhibited total HCA formation, whereas 2000 ppm enhanced the formation. The addition of 500

and 2000 ppm rosmarinic acid acted as to also have enhanced the formation of IQx. Rosmarinic acid at 2000 ppm also appeared to have an enhancement effect on 4,8-DiMeIQx. In contrast, 500 ppm appeared to have inhibited the formation of MeIQ/MeIQx and possibly IQ.

Table 4-4: Effect of rosmarinic acid on HCA formation in chemical model systems heated at 175 ^{o}C for 40 min.*

Rosmarinic Acid (ppm)	IQx (µg)	IQ (µg)	MeIQ/MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCAs (µg)
Control	6.1 ± 0.7	14.8 ± 1.1	3.4 ± 0.6	1.2 ± 0.2	25.7 ± 1.5
125	5.7 ± 0.7	15.5 ± 1.1	2.7 ± 0.6	1.3 ± 0.2	25.0 ± 1.5
250	6.3 ± 0.7	14.1 ± 1.1	3.2 ± 0.6	1.1 ± 0.2	24.0 ± 1.5
500	7.6 ± 0.7	13.3 ± 1.1	3.5 ± 0.6	1.1 ± 0.2	24.5 ± 1.5
1000	6.9 ± 0.7	14.7 ± 1.1	2.8 ± 0.6	1.2 ± 0.2	25.1 ± 1.5
2000	8.2 ± 0.7	15.1 ± 1.1	2.6 ± 0.6	1.7 ± 0.2	26.5 ± 1.5

No significant differences were observed at $p < 0.05$.

*Means ± standard error of the class (column)

Research indicates rosmarinic acid possesses inhibitory qualities towards HCA formation in both meat and model systems. In beef patties, rosmarinic acid showed inhibitory effects on HCAs, reducing PhIP by 48% and MeIQx by 64% (Tsen and others 2006). Zöchling and Murkovic (2002) and Cheng and others (2007) identified a association between PhIP formation and the concentration of rosmarinic acid in model systems. As the concentration of rosmarinic acid increased, PhIP formation increased. When added to beef patties prior to frying (0.1%), rosmarinic acid caused 42% inhibition of total HCAs. Individual HCAs were inhibited by 33 (PhIP), 46 (4,8-DiMeIQx), and 48% (MeIQx) (Cheng and others 2007).

EGCG, a tea flavonoid, was the final antioxidant compound evaluated in the study. Table 4-5 summarizes the effect of EGCG on total and individual HCA formation in the model systems. EGCG had no significant effect ($p < 0.05$) on the formation of HCAs. It appeared as with total HCAs, as well as IQ and MeIQ/MeIQx, formation was inhibited upon the addition of 500 ppm EGCG. At this same level, IQx and 4,8-DiMeIQx HCAs appeared to be promoted in formation. At 2000 ppm, IQ and MeIQ/MeIQx formation appeared to also be inhibited, whereas

total HCAs, IQx, and 4,8-DiMeIQx levels increased. At 250 ppm, IQ appeared to be inhibited by EGCG. Again, no significant differences were observed at these levels.

Table 4-5: Effect of epigallocatechin gallate (EGCG) on HCA formation in chemical model systems heated at 175 °C for 40 min.*

EGCG (ppm)	IQx (µg)	IQ (µg)	MeIQ/MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCAs (µg)
Control	6.3 ± 0.7	14.0 ± 1.1	3.6 ± 0.6	1.1 ± 0.2	25.8 ± 1.5
125	5.9 ± 0.7	13.8 ± 1.1	3.3 ± 0.6	1.1 ± 0.2	24.5 ± 1.5
250	7.9 ± 0.7	12.0 ± 1.1	3.0 ± 0.6	1.2 ± 0.2	24.0 ± 1.5
500	6.3 ±0.7	14.3 ± 1.1	2.8 ± 0.6	1.3 ± 0.2	24.5 ± 1.5
1000	6.2 ± 0.7	13.9 ± 1.1	3.0 ± 0.6	1.9 ± 0.2	24.5 ± 1.5
2000	6.4 ± 0.7	12.8 ± 1.1	2.7 ± 0.6	2.0 ± 0.2	27.3 ± 1.5

No significant differences were observed at $p < 0.05$.

*Means ± standard error of the class (column)

In contrast to the current study's data, literature states that EGCG is a strong inhibitor of HCAs in model systems. Oguri and others (1998) reported 35% inhibition of MeIQx and 46% decrease in mutagenicity upon the addition of EGCG in chemical model systems. Cheng and others (2007) noted similar results as EGCG significantly inhibited ($p < 0.05$) PhIP as compared to the control. In another study, a 99.2% inhibition of PhIP was observed upon the addition of 1000 ppm EGCG. A association was observed that as EGCG concentration increased, PhIP formation decreased (Moon and Shin 2013).

There are several explanations as to why all four antioxidant compounds did not inhibit or even enhance the formation of HCAs in the model system. In several of the studies where inhibition of HCAs occurred in model systems, precursor concentrations were higher (0.4 and 0.9 mmol) (Cheng and others 2007; Moon and Shin 2013). When comparing our model system to these, our precursor concentration was 0.2 mmol. It may be that the systems were not concentrated enough to form an adequate amount of HCAs and therefore, no change in HCA formation by the antioxidants was observed. The reason for having a lower concentrated model system was to combat the insolubility and high variability issues observed in prior studies. It was

rationalized that if insolubility was no longer an issue, variation would decrease, and the ability for inhibition of HCAs would occur. Variation did decrease (Appendix D), but the antioxidants had no effect on HCA formation.

Another reason may be that the concentration of the antioxidants is not ideal for this model system. Though these levels are similar to other studies (Moon and Shin 2013), change in formation may not be occurring due to too much or too little. Therefore, it has been determined that further studies should be conducted evaluating the precursor and antioxidant concentrations in the model systems in hopes to determine an effect on HCA formation.

Conclusions

This study evaluated the effect of four antioxidant compounds (BHA, naringenin, rosmarinic acid, and EGCG) on the formation of five HCAs (IQ, MeIQ, IQx, MeIQx, and 4,8-DiMeIQx) in chemical model systems. All four antioxidants had no significant effect ($p < 0.05$) on the formation of total and individual HCAs in the model systems. Further studies are required to further determine what ratios of precursors and antioxidants are ideal to inhibit the formation of HCAs in chemical model systems.

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Appendix A - Standard Curves

Chapter 2 Standard Curves

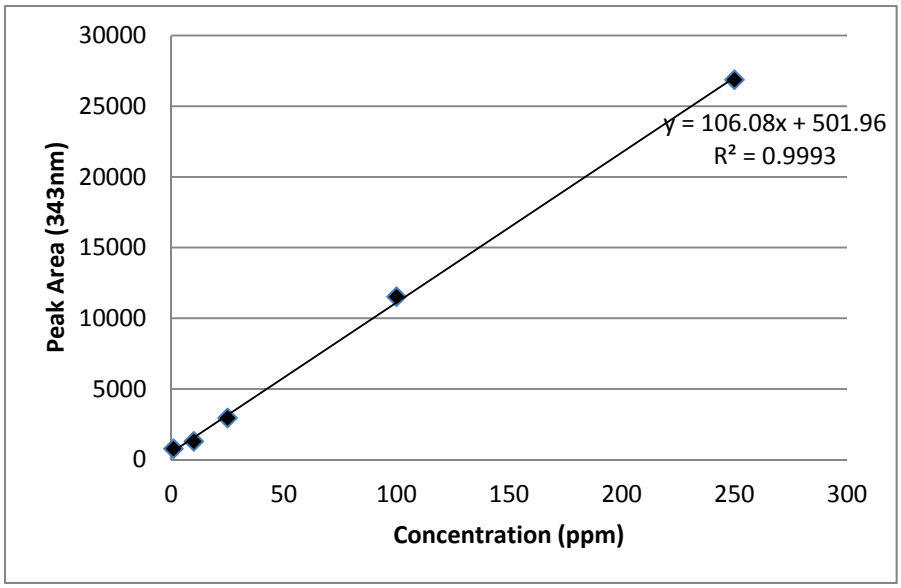

Figure A-1: Piperine standard curve obtained by HPLC UV at 343 nm

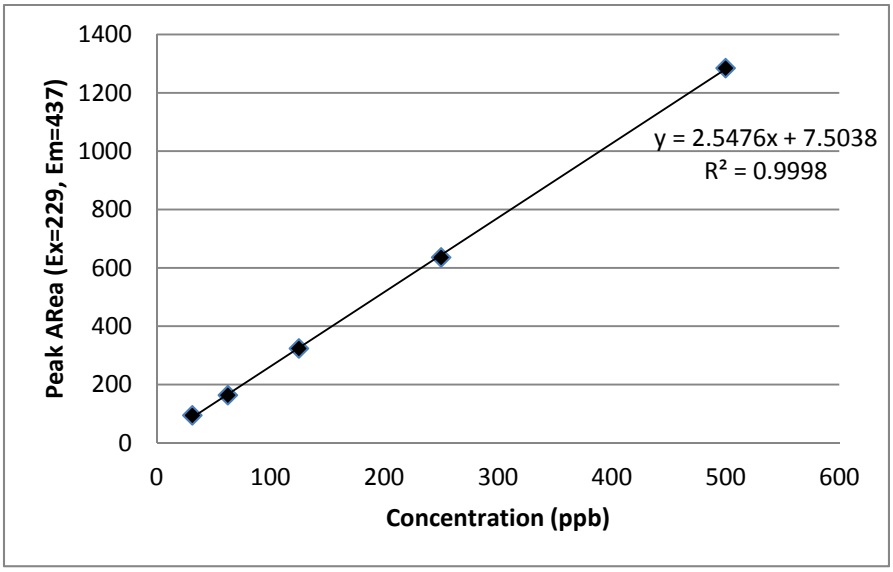

Figure A-2: PhIP standard curve obtained by HPLC fluorescence at Ex = 229, Em = 437

Chapter 3 Standard Curves

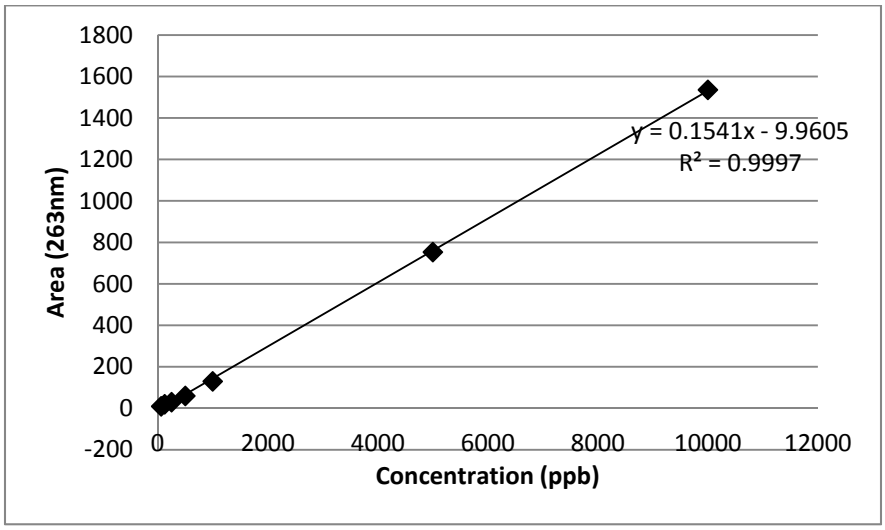

Figure A-3: IQx standard curve obtained by HPLC UV at 263 nm

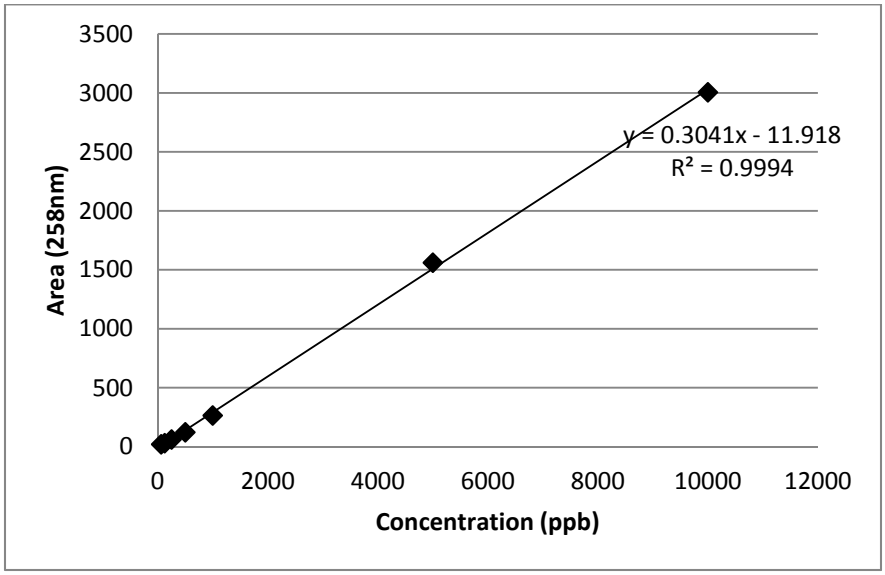

Figure A-4: IQ standard curve obtained by HPLC UV at 258 nm

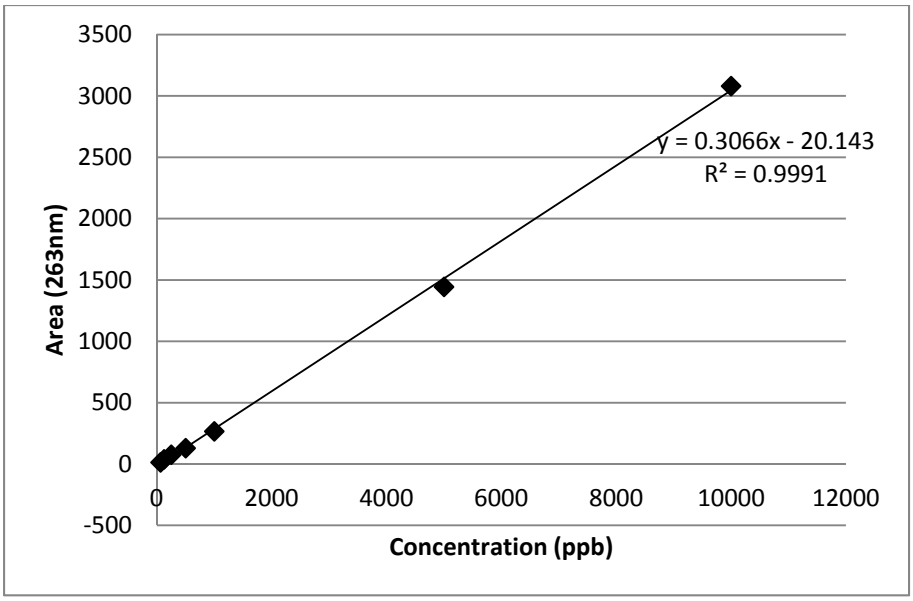

Figure A-5: MeIQx standard curve obtained by HPLC UV at 263 nm

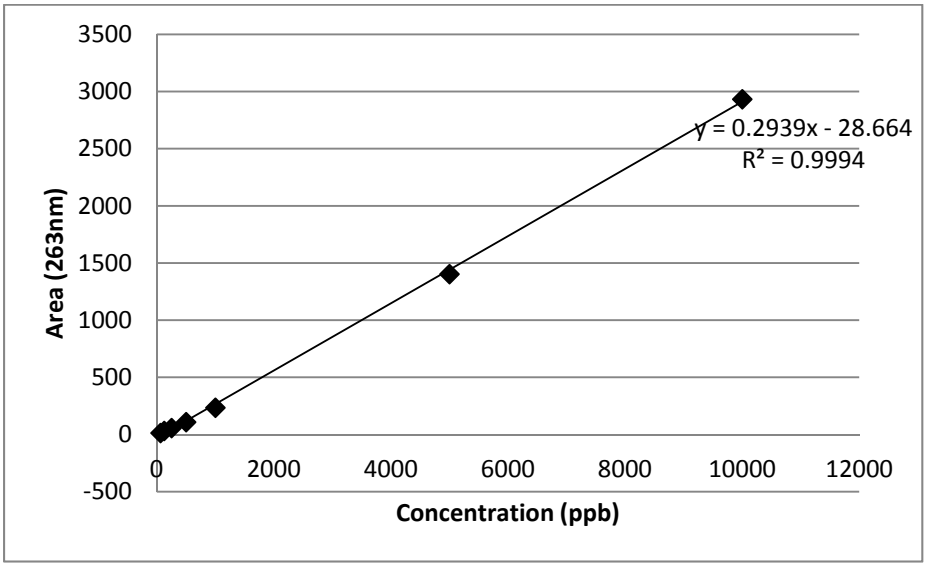

Figure A-6: 4,8-DiMeIQx standard curve obtained by HPLC UV at 263 nm

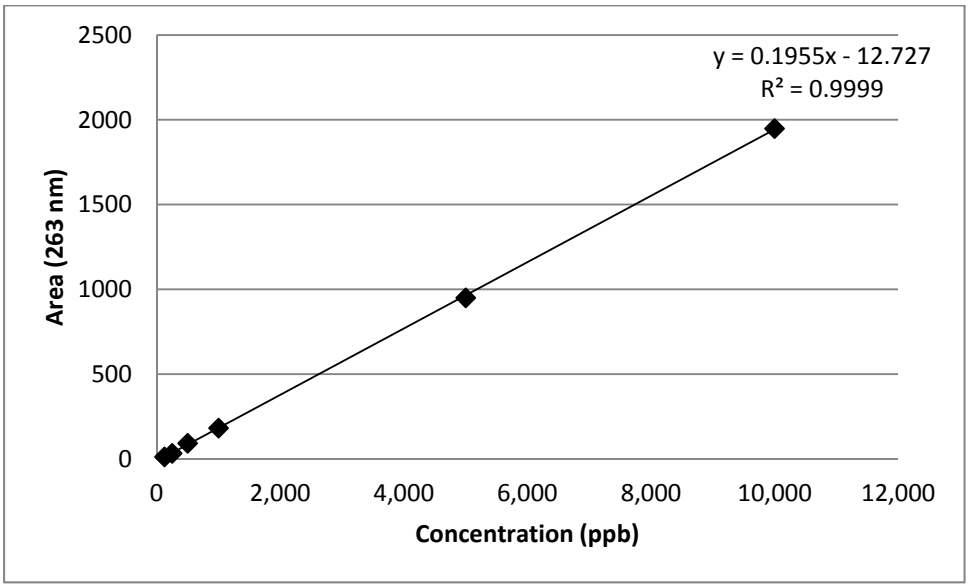

Figure 4-7: IQx standard curve obtained by HPLC UV at 263 nm

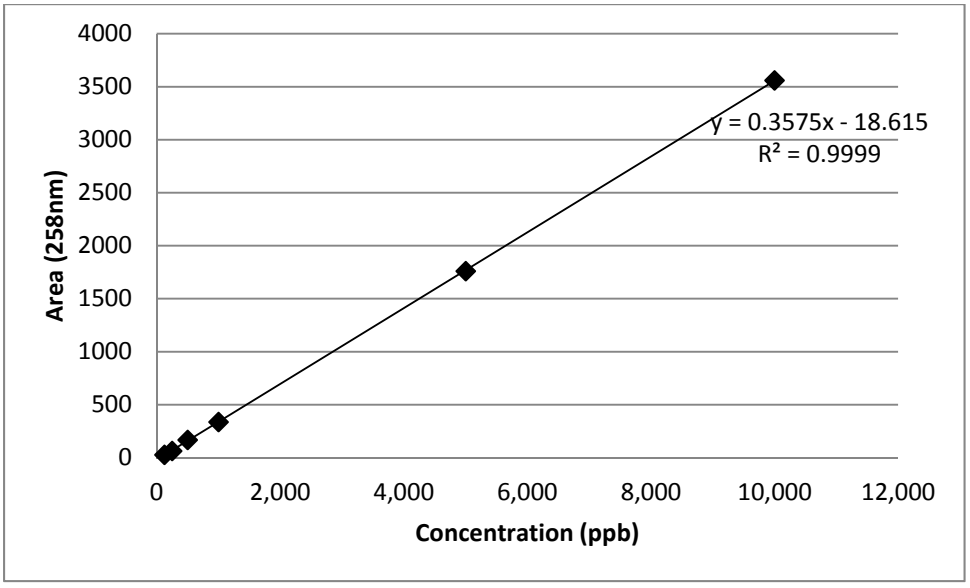

Figure 4-8: IQ standard curve obtained by HPLC UV at 258 nm

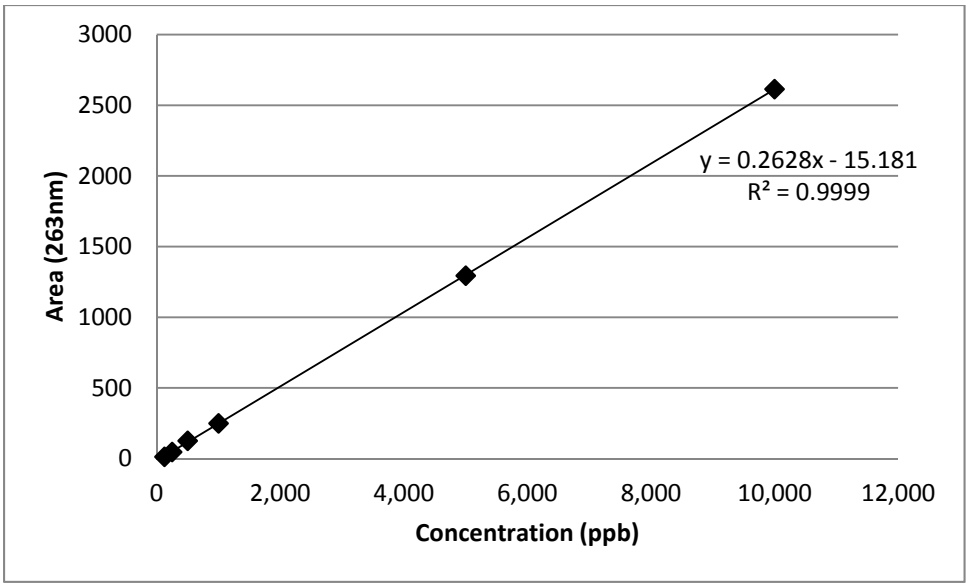

Figure A-9: MeIQx and MeIQx Mixed standard curve obtained by HPLC UV at 263 nm

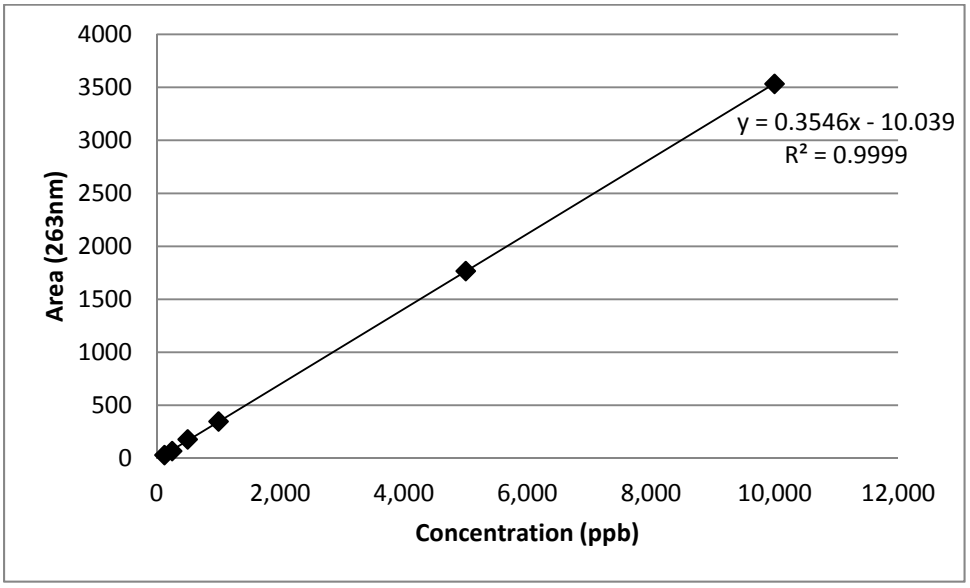

Figure A-10: 4,8-DiMeIQx standard curve obtained by HPLC UV at 263 nm

Appendix B - LOD and LOQ Calculations

$$X_{LOD} = X_{Blk} + 3\,x\,SD_{Blk}$$

$$X_{LOQ} = X_{Blk} + 10\,x\,SD_{Blk}$$

X_{Blk} = signal of blank
SD_{Blk} = standard deviation of the blank readings
X_{LOD} = minimum detectable concentration
X_{LOQ} = minimum quantifiable concentration

Table B-1: Table denoting the LOD and LOQ for piperine in Chapter 2

Peak Number	**Peak Area**
1	1.73
2	1.23
3	2.65
4	1.20
AVERAGE	*1.70*
STANDARD DEVIATION	*0.586*
LOD	**95.2 ng**
LOQ	**96.0 ng**

Table B-2: Table denoting the LOD and LOQ for PhIP using the HP 1090 HPLC fluorescence at Ex = 229, Em = 437 in Chapter 2

Peak Number	**Peak Area**
1	0.041
2	0.030
3	0.043
4	0.072
5	0.044
AVERAGE	*0.050*
STANDARD DEVIATION	*0.0147*
LOD	**0.15 ng**
LOQ	**0.16 ng**

Table B-3: Table denoting the LOD and LOQ for HCAs using HP 1090 HPLC UV at 263 nm in Chapter 3

Peak Number	**Peak Area**
1	0.238
2	0.103
3	0.165
4	0.117
5	0.080
AVERAGE	*0.140*
STANDARD DEVIATION	*0.056*
IQx LOD	**1.33 ng**
IQx LOQ	**1.38 ng**
IQ LOD	**0.80 ng**
IQ LOQ	**0.82 ng**
MeIQx LOD	**1.33 ng**
MeIQx LOQ	**1.36 ng**
4,8-DiMeIQx LOD	**1.97 ng**
4,8-DiMeIQx LOQ	**1.99 ng**

Table B-4: Table denoting the LOD and LOQ for HCAs using HP 1050 HPLC UV at 263 nm in Chapter 4

Peak Number	**Peak Area**
1	0.314
2	0.266
3	0.172
AVERAGE	0.251
STANDARD DEVIATION	0.0719
IQx LOD	**1.31 ng**
IQx LOQ	**1.33 ng**
IQ LOD	**1.05 ng**
IQ LOQ	**1.06 ng**
MeIQ/MeIQx LOD	**1.16 ng**
MeIQ/MeIQx LOQ	**1.18 ng**
4,8-DiMeIQx LOD	**0.57 ng**
4,8-DiMeIQx LOQ	**0.58 ng**

Appendix C - Standard Chromatograms

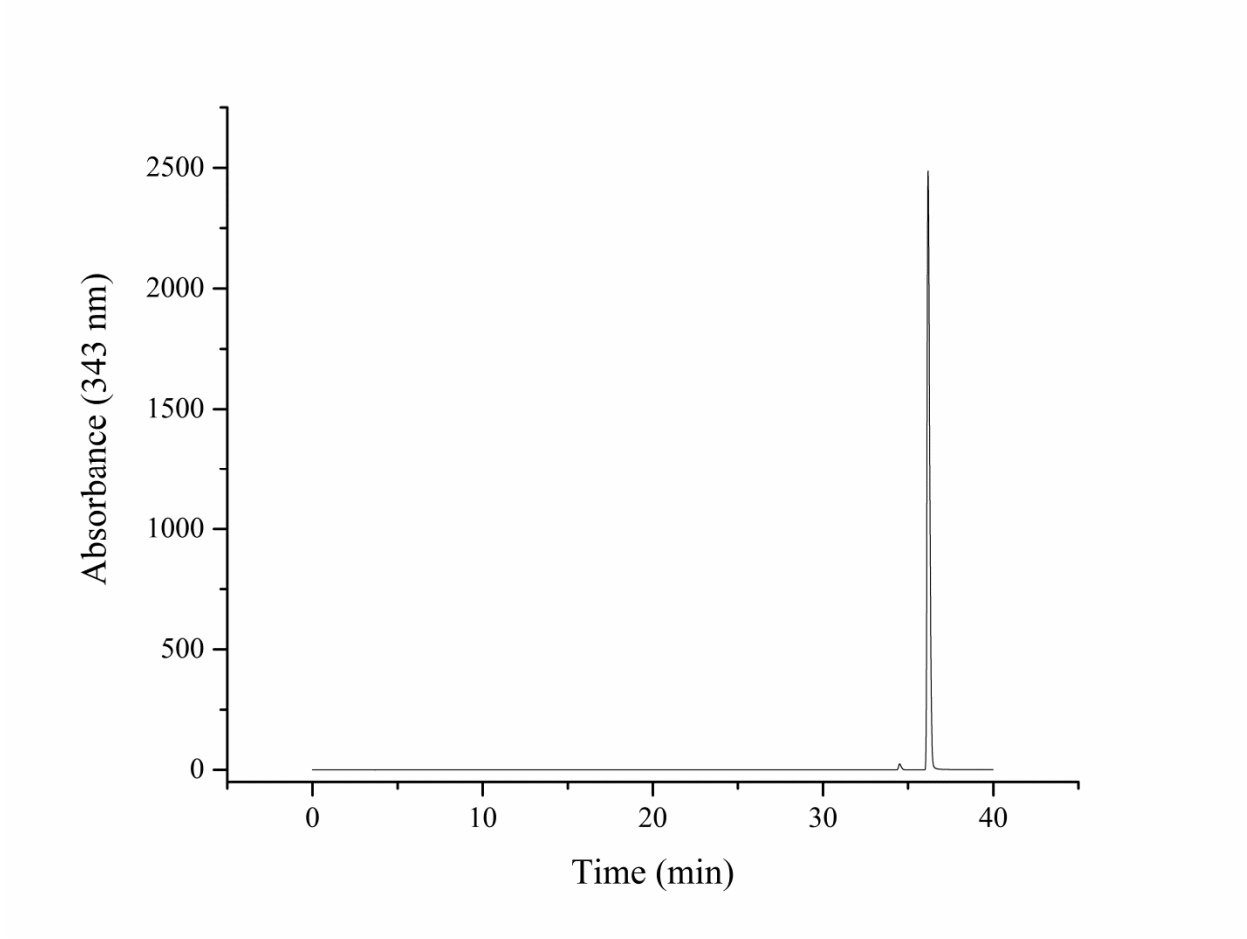

Figure C-1: HPLC chromatogram of 250 ppm piperine standard separated on a TSKgel ODS-80TM column described in Chapter 2 (page 36). The UV detector was set at 343 nm. The full loop injection volume of the sample was 20 μL.

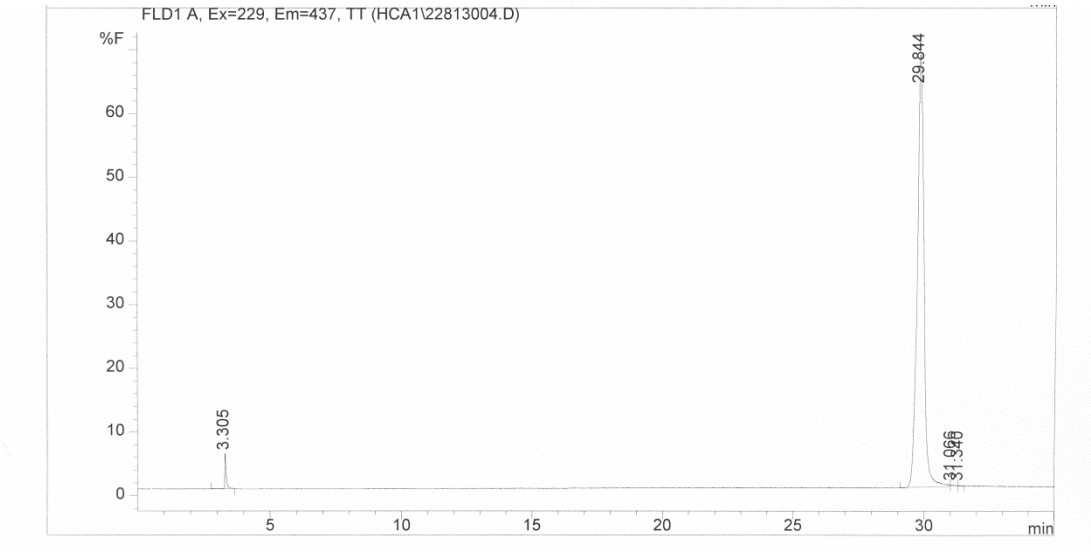

Figure C-2: HPLC chromatogram of 500 ppb PhIP standard separated on a TSKgel ODS-80TM column described in Chapter 2 (page 36). The FLD detector was set at Ex = 229 and Em = 437. The full loop injection volume was 20 µL.

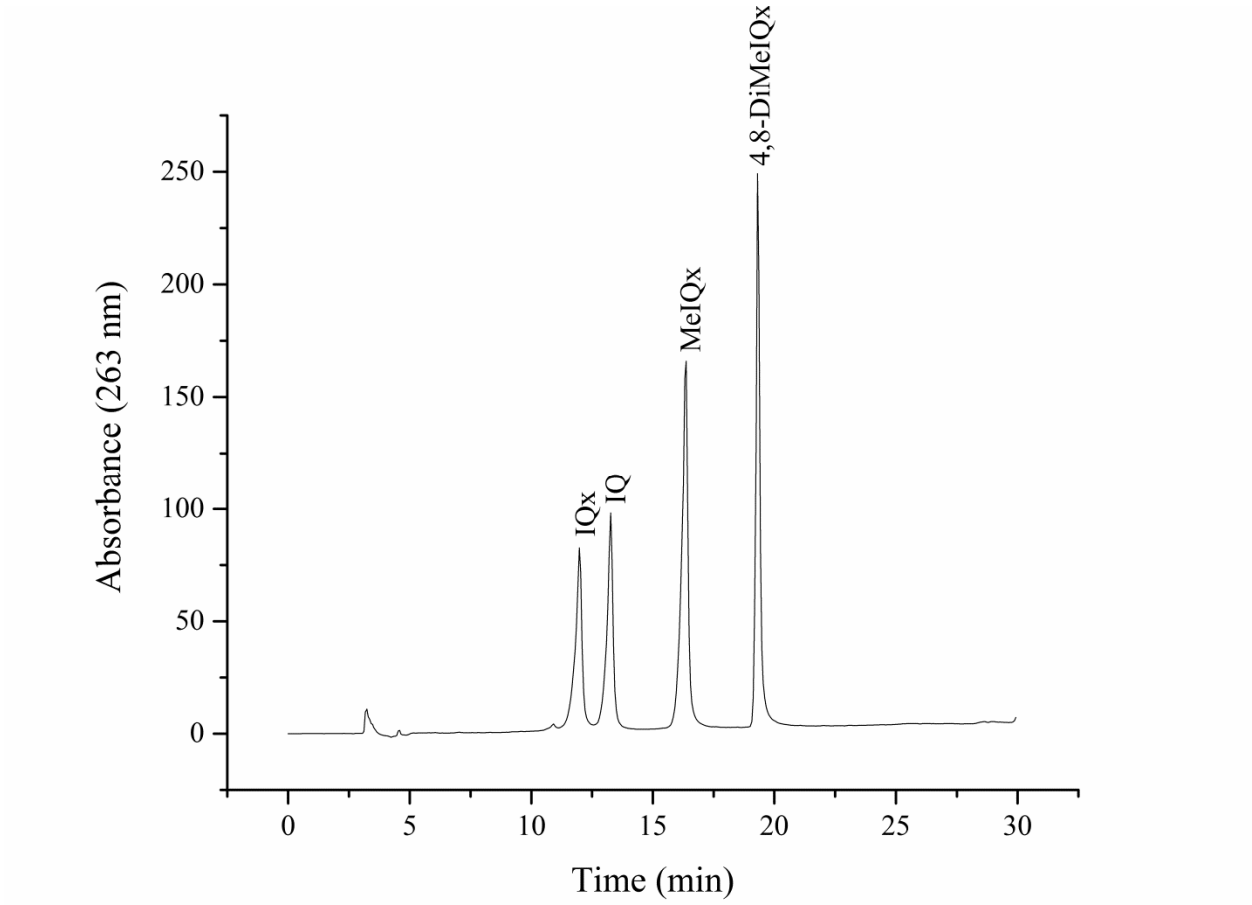

Figure C-3: HPLC chromatogram of a 1000 ppb HCA standard mix separated on a TSKgel ODS-80TM column described in Chapter 3 (page 50). The UV detector was set for 263 nm for IQx, MeIQx, and 4,8-DiMeIQx and 258 nm for IQ. The full loop injection volume was 20 µL.

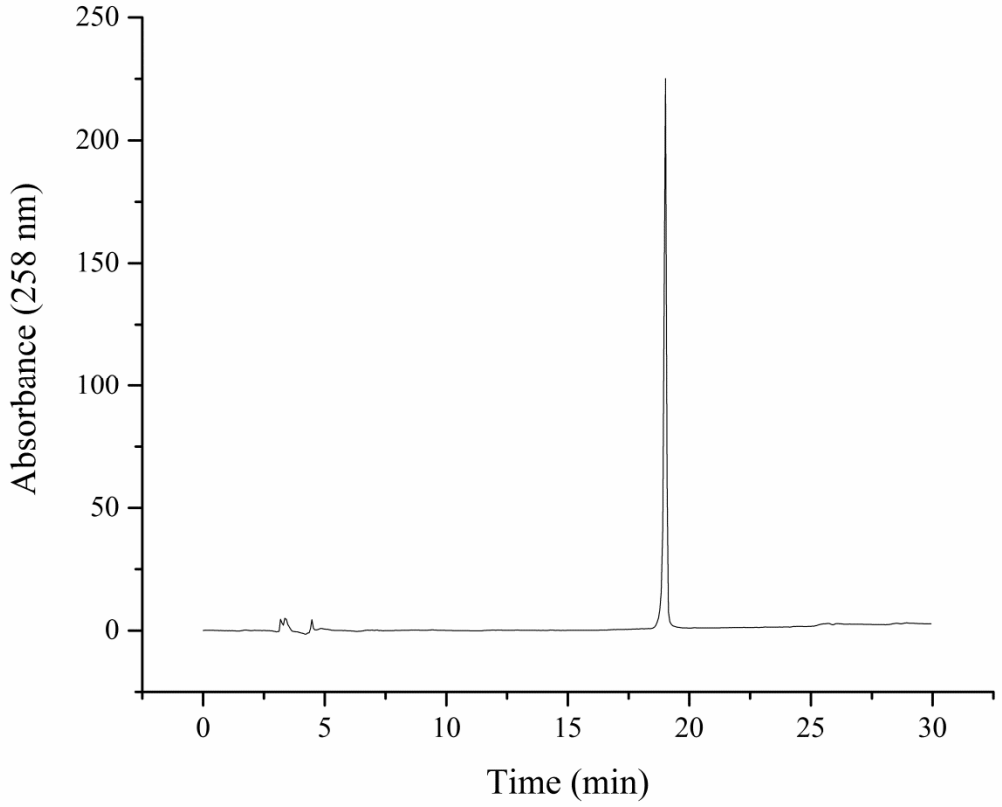

Figure C-4: HPLC chromatogram of a 1000 ppb MeIQ standard separated on a TSKgel ODS-80TM column as described in Chapter 3 (page 50). The UV detector was set for 258 nm and the full loop injection volume was 20 µL.

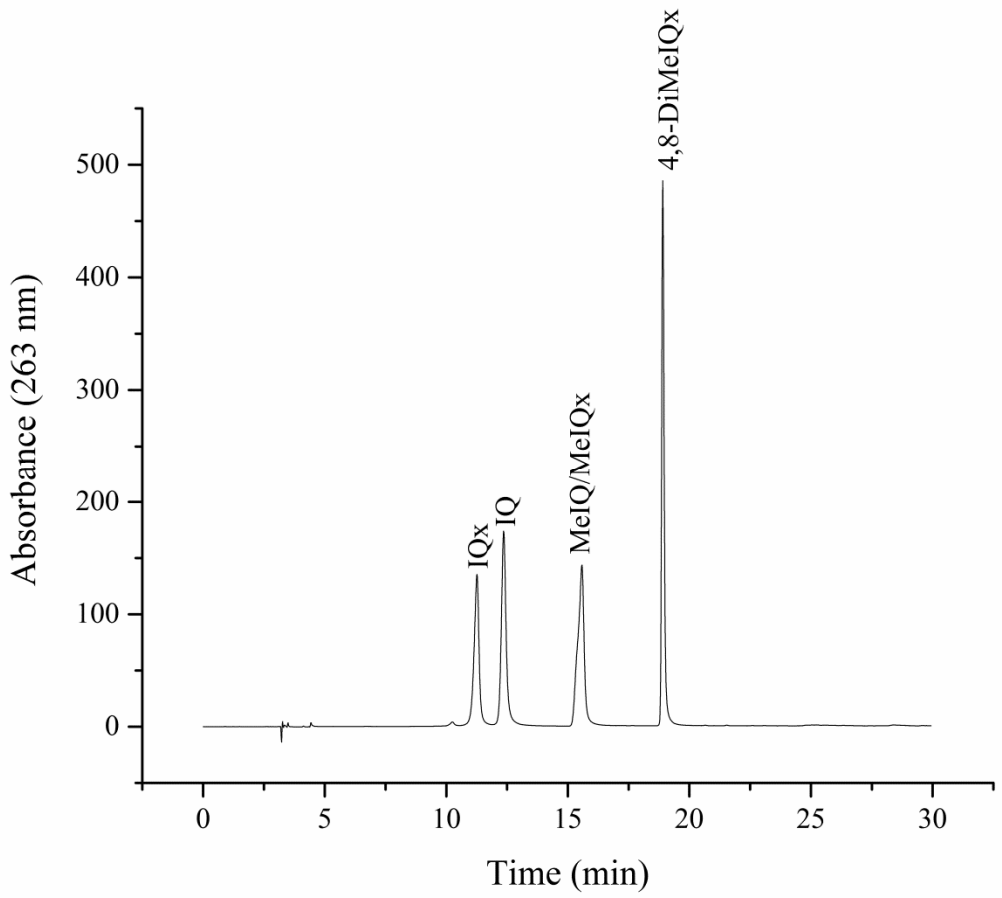

Figure C-5: HPLC chromatogram of a 1000 ppb mixed standard separated on a TSKgel ODS-80T¬M¬ column as described in Chapter 4 (page 71). The UV detector was set for 263 nm for IQx, MeIQ/MeIQx, and 4,8-DiMeIQx and 258 nm for IQ. The full loop injection volume was 20 µL.

Appendix D - HPLC Chromatograms

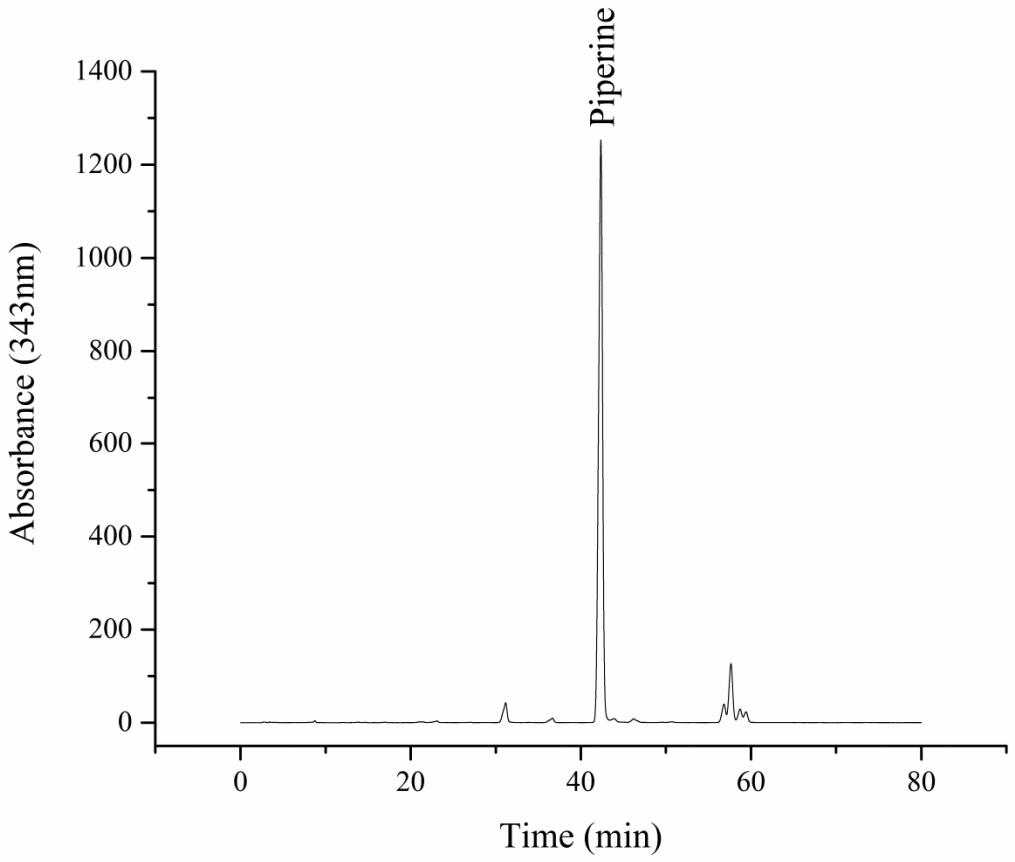

Figure D-1: HPLC chromatogram of black pepper extract separated on a TSKgel ODS-80TM column described in Chapter 2 (page 36). The UV detector was set at 343 nm for detection of piperine. The full loop injection volume of the sample was 20 μL.

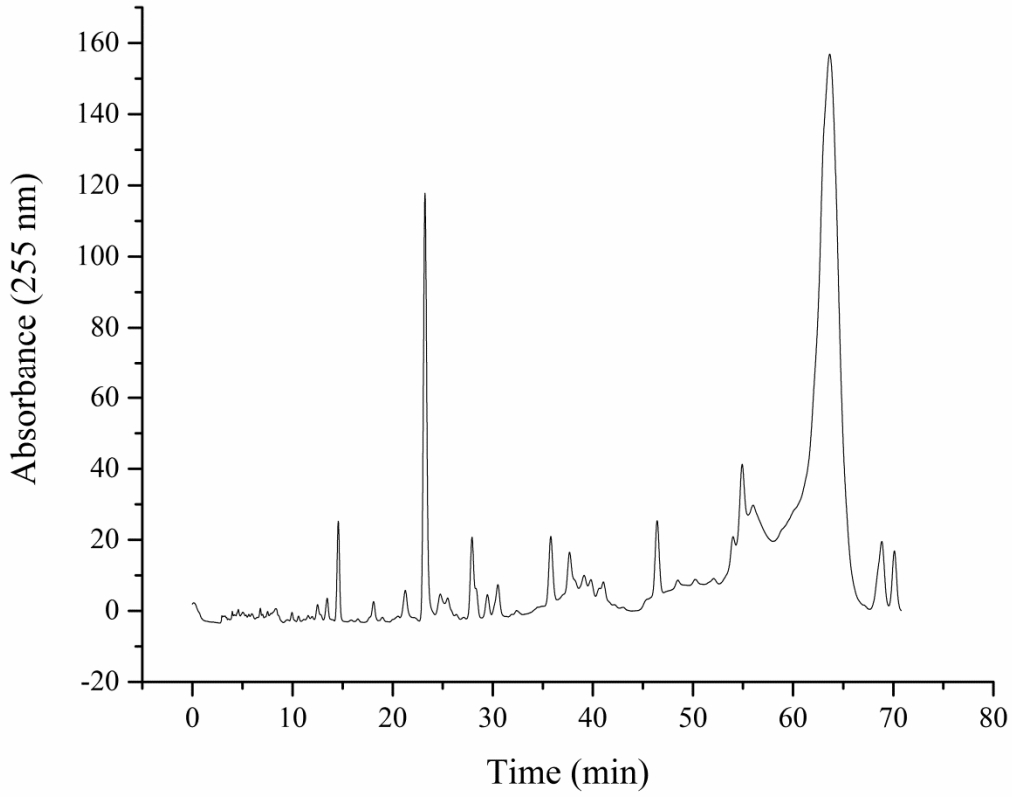

Figure D-2: HPLC chromatogram of 10% black pepper oil in acetonitrile separated on a TSKgel ODS-80TM column described in Chapter 2 (page 36). The UV detector was set at 255 for best absorbance (see 3D plot in Appendix E, Figure E-3). The full loop injection volume was 20 µL.

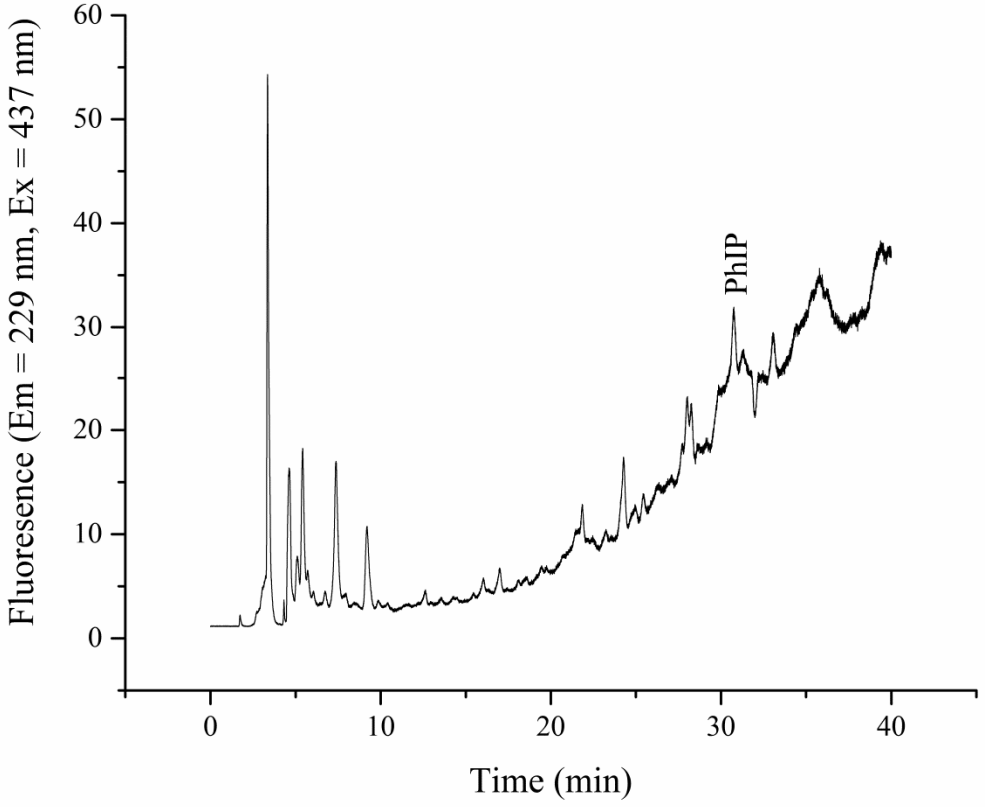

Figure D-3: HPLC chromatogram of the phenylalanine model system separated on a TSKgel ODS-80TM column as described in Chapter 2 (page 36 and 37). The fluorescence detector was programmed to emission/excitation wavelengths of 229 nm and 437 nm for PhIP. The full loop injection volume was 20 µL.

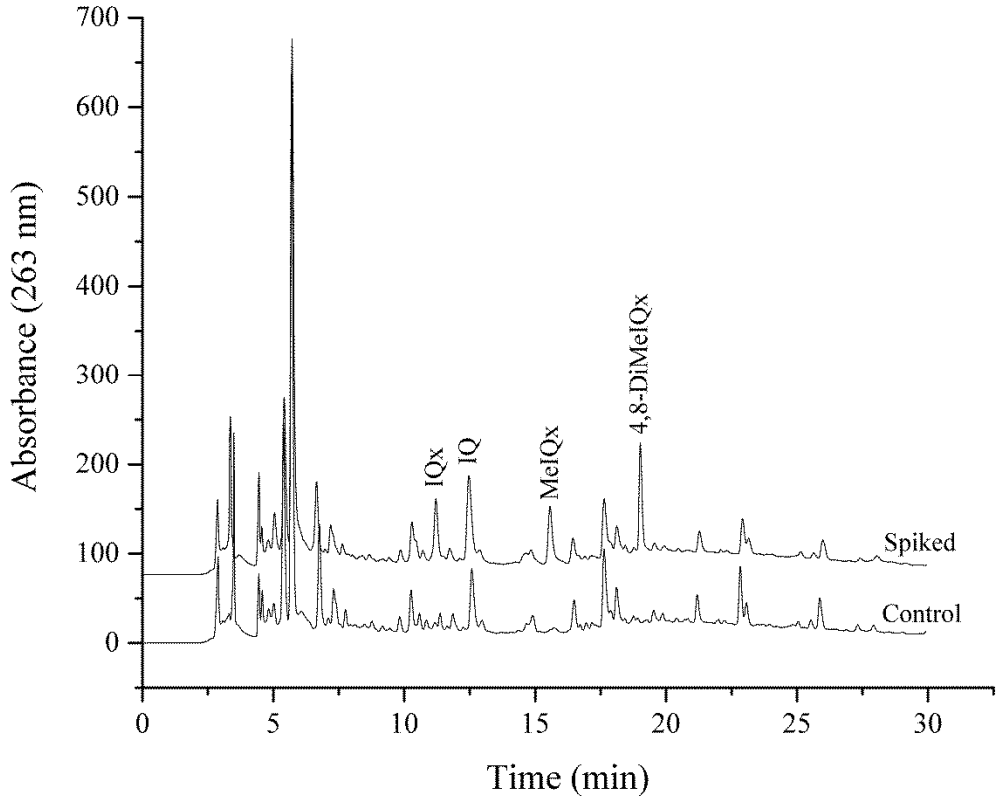

Figure D-4: HPLC chromatogram of the control system (part 1) with an overlay of a spiked sample indicating the identity of the four HCAs. Both reactions were separated on a TSKgel ODS-80T$_M$ column as described in Section 2.3. The UV detector was set at 263 nm for IQx, MeIQx, and 4,8-DiMeIQx and 258 nm for IQ. The full loop injection volume was 20 µL.

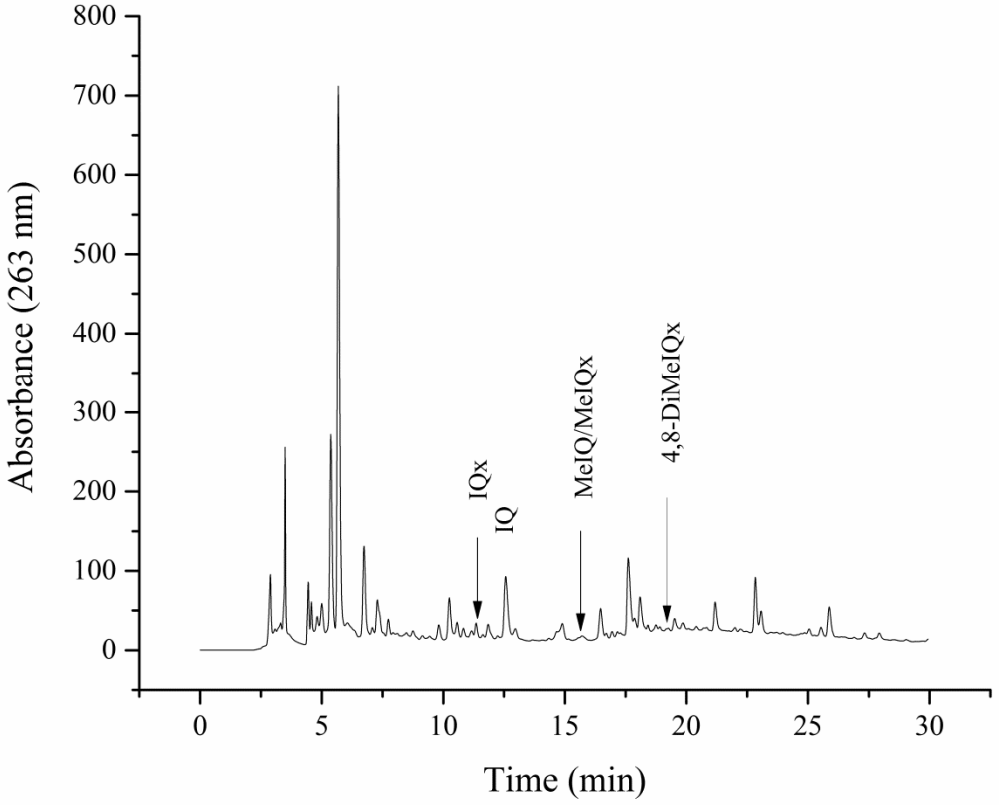

Figure D-5: HPLC chromatogram of the threonine model system separated on a TSKgel ODS-80TM column as described in Chapter 4 (page 71). The UV detector was set for 263 nm for IQx, MeIQ/MeIQx, and 4,8-DiMeIQx and 258 nm for IQ. The full loop injection volume was 20 µL

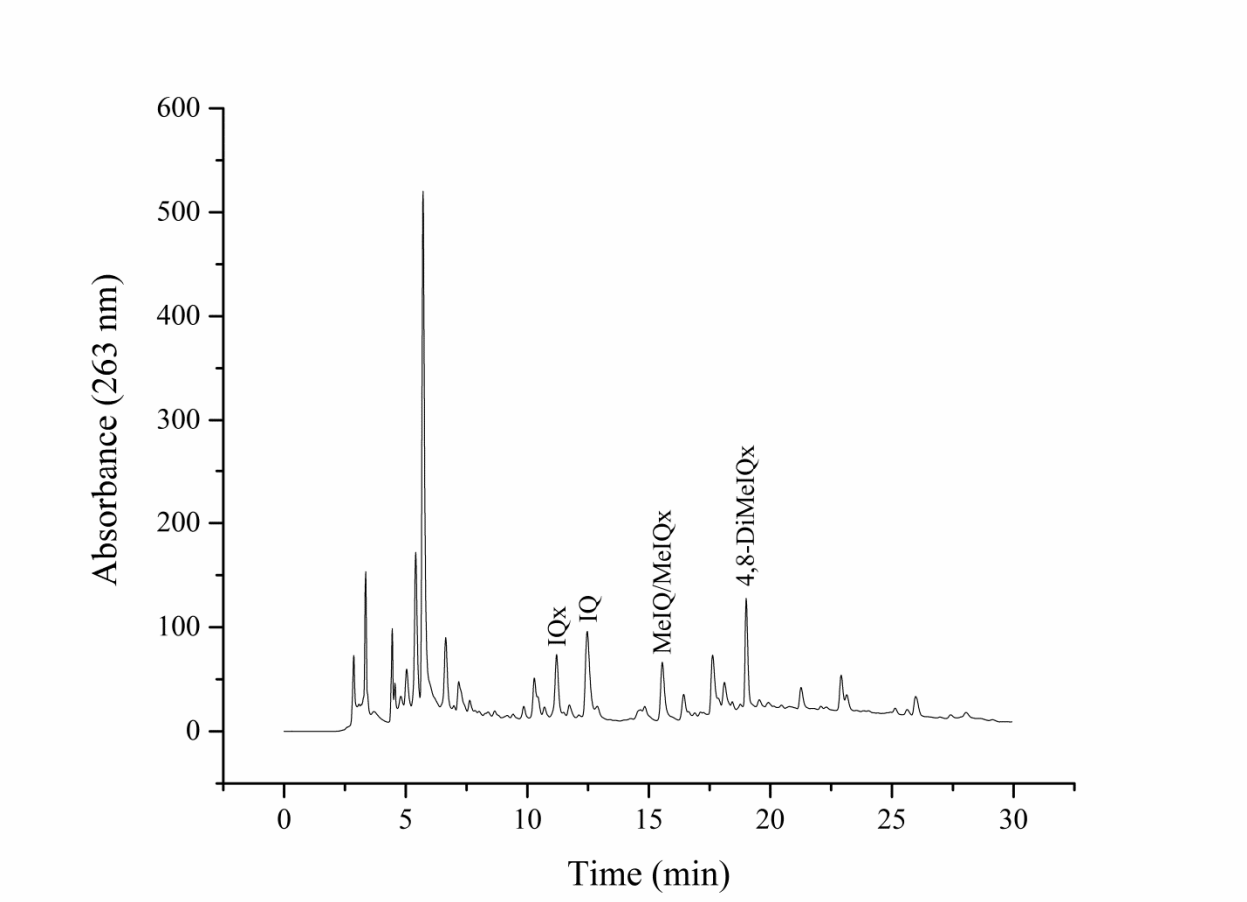

Figure D-6: HPLC chromatogram of the spiked threonine model system separated on a TSKgel ODS-80TM column as described in Chapter 4 (page 71). The UV detector was set for 263 nm for IQx, MeIQ/MeIQx, and 4,8-DiMeIQx and 258 nm for IQ. The full loop injection volume was 20 µL.

Appendix E - Spectral Data

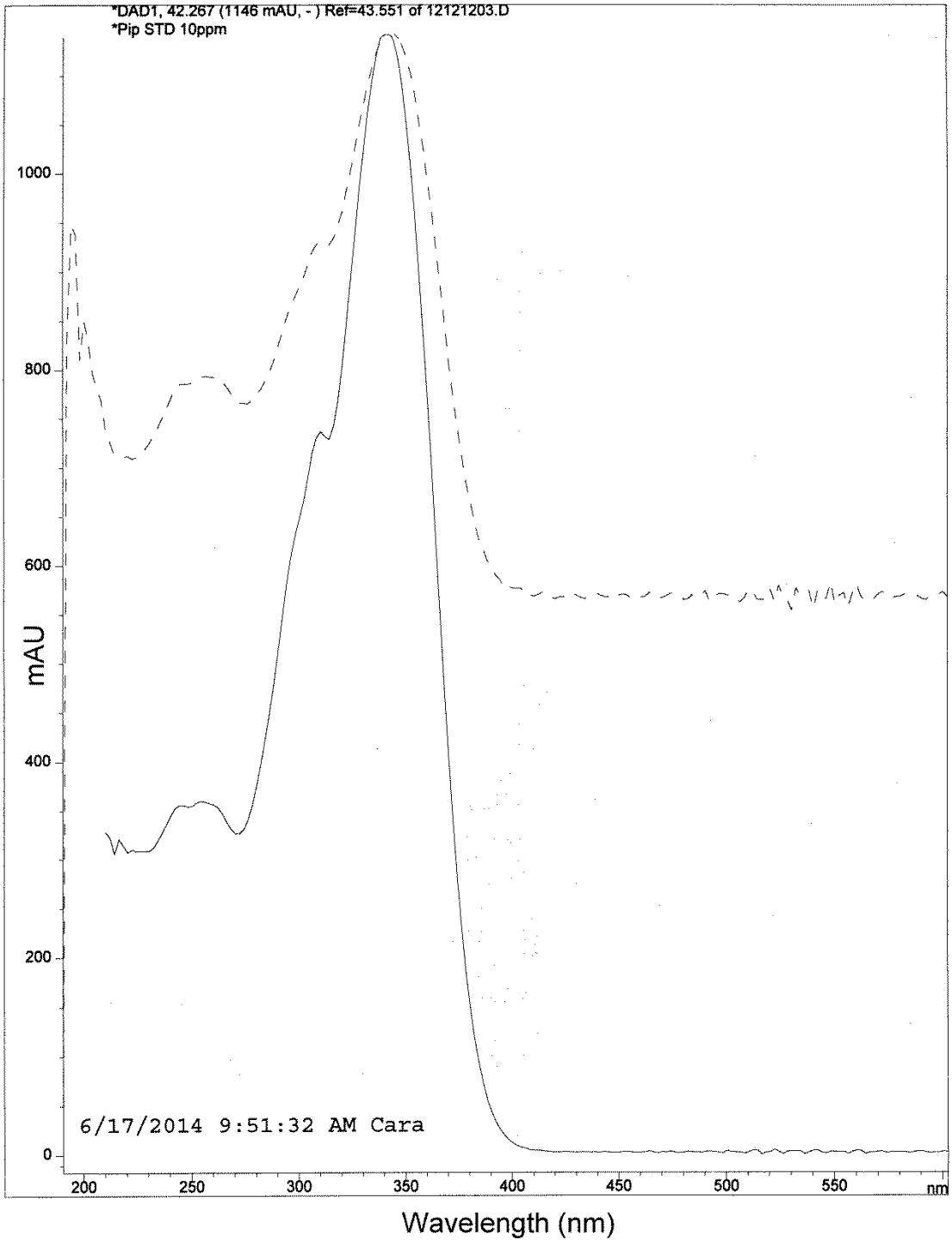

Figure E-1: Spectral match of piperine (dashed lined) with a component in black pepper (solid line) (peak at 43.132 min). The spectral match was 991/1000 (Figure E-2).

Library search results

#	Match	Entry	Time [min]	Name
1	991.6408	1	43.132	Pip STD 10ppm

Figure E-2: Matching results of peak at 43.132 min with known piperine standard.

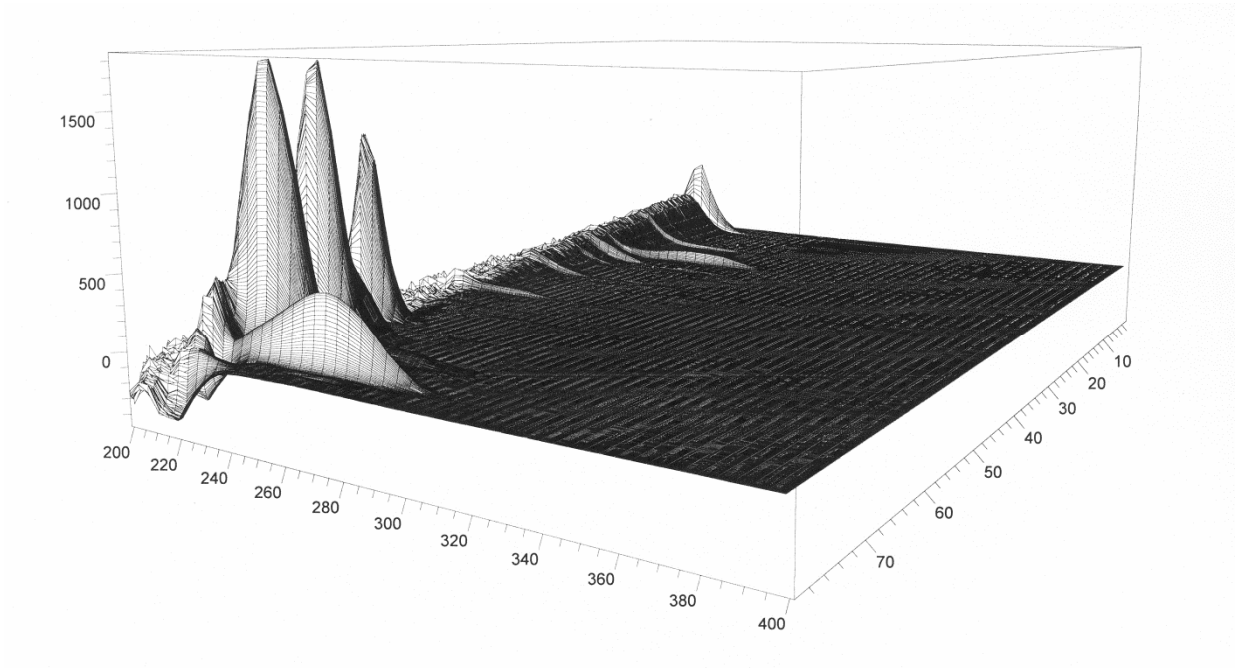

Figure E-3: 3D UV spectrum of 10% black pepper oil in acetonitrile separated on a TSKgel ODS-80TM column described in Chapter 2 (page 36). The full loop injection volume of the sample was 20 µL.

Appendix F - Excel Data

Table F-1: Effect of precursor concentration (threonine and creatinine) on total HCA formation in chemical model systems heated at 175 °C for 40 min. Values are means ± standard deviation calculated from excel.

Precursor Concentration (mmol)	IQx (µg)	IQ (µg)	MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCAs (µg)
0.2	7.4 ± 3.9	18.6 ± 16.5	0.8 ± 1.9	2.3 ± 1.2	29.1 ± 8.2
0.4	13.9 ± 5.6	33.3 ± 7.9	1.9 ± 1.0	3.5 ± 1.8	52.6 ± 14.8
0.6	23.6 ± 9.0	44.1 ± 26.4	3.1 ± 2.7	4.1 ± 2.2	75.0 ± 24.0
0.8	25.8 ± 9.1	47.4 ± 21.7	3.3 ± 3.2	3.4 ± 1.9	79.9 ± 18.1

Table F-2: Effect of water on HCA formation in chemical model systems heated at 175 °C for 40 min. Values are means ± standard deviation calculated from excel.

Water (%)	IQx (µg)	IQ (µg)	MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCA Formation (µg)
0	13.1 ± 6.2	34.6 ± 17.6	1.9 ± 1.8	2.6 ± 0.8	52.2 ± 24.3
5	20.0 ± 12.0	39.9 ± 18.8	2.0 ± 2.3	3.3 ± 1.4	65.2 ± 22.3
10	17.2 ± 11.2	39.5 ± 27.1	1.8 ± 1.8	3.3 ± 1.4	62.2 ± 31.0
15	20.5 ± 10.1	29.4 ± 23.8	3.3 ± 3.3	3.6 ± 3.1	57.1 ± 27.3

Table F-3: Interaction effect between precursor concentration and water amount on total HCA formation in chemical model systems heated at 175 ºC for 40 min. Values are means ± standard deviation calculated from excel.

		Water Amount (%)			
		0	**5**	**10**	**15**
Precursor Concentration (mmol)	**0.2**	22.5 ± 5.9	36.8 ± 8.10	30.4 ± 6.4	26.9 ± 6.1
	0.4	44.3 ± 10.3	65.0 ± 13.1	50.8 ± 12.6	50.4 ± 17.6
	0.6	65.7 ± 14.2	73.5 ±17.1	90.7 ± 33.5	58.3 ± 36.7
	0.8	76.3 ± 17.8	85.2 ± 15.1	77.1 ± 23.0	81.1 ± 20.5

Table F-4: Differences among sugar types on the amount of HCAs produced in chemical model systems heated at 175 ºC for 40 min. Values are means ± standard deviation calculated from excel.

Sugar Type	**IQx (µg)**	**IQ (µg)**	**MeIQx (µg)**	**4,8-DiMeIQx (µg)**	**Total HCAs (µg)**
Fructose	24.1 ± 15.6	59.6 ± 24.8	2.3 ± 2.6	3.2 ± 2.1	89.1 ± 35.0
Galactose	16.6 ± 9.5	48.3 ± 21.5	3.6 ± 4.0	6.1 ± 3.2	74.6 ± 27.6
Glucose	24.3 ± 13.7	59.5 ± 23.6	4.5 ± 4.5	4.0 ± 2.4	92.3 ± 35.5
Lactose	22.0 ± 11.8	64.7 ± 27.9	15.2 ± 14.1	8.5 ± 6.4	110.4 ± 44.8

Table F-5: Differences among sugar amounts on the HCA formation in chemical model systems heated at 175 ºC for 40 min. Values are means ± standard deviation calculated from excel.

Sugar Amount (mmol)	IQx (µg)	IQ (µg)	MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCAs (µg)
0.15	12.0 ± 5.2	33.5 ± 15.8	3.3 ± 4.5	4.7 ± 3.8	56.0 ± 19.7
0.3	18.1 ± 6.6	52.5 ± 18.3	3.6 ± 6.2	5.7 ± 3.5	79.9 ± 23.3
0.6	24.3 ± 11.9	67.7 ± 15.8	7.9 ± 13.8	4.5 ± 2.0	100.9 ± 31.6
1.2	31.4 ± 17.0	77.8 ± 25.7	10.7 ± 8.1	6.9 ± 6.7	125.9 ± 38.2

Table F-6: Effect of butylated hydroxyanisole (BHA) on HCA formation in chemical model systems heated at 175 ºC for 40 min. Values are means ± standard deviation calculated from excel.

BHA (ppm)	IQx (µg)	IQ (µg)	MeIQ/MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCAs (µg)
Control	6.8 ± 1.2	12.9 ± 1.6	4.4 ± 5.2	1.2 ± 0.3	25.3 ± 2.3
125	5.9 ± 1.0	14.1 ± 1.5	3.1 ± 1.6	1.2 ± 0.6	24.3 ± 3.4
250	5.6 ± 0.8	14.3 ± 1.9	3.0 ± 1.6	1.0 ± 0.3	23.8 ± 3.0
500	5.8 ±0.9	15.2 ± 1.9	2.8 ± 1.4	1.3 ± 0.4	25.0 ± 3.5
1000	7.0 ± 2.8	12.9 ± 3.8	3.1 ± 1.2	1.1 ± 0.5	24.0 ± 2.5
2000	6.1 ± 1.6	14.7 ± 1.0	2.9 ± 2.1	1.4 ± 0.4	25.2 ± 2.3

Table F-7: Effect of naringenin on HCA formation in chemical model systems heated at 175 ºC for 40 min. Values are means ± standard deviation calculated from excel.

Naringenin (ppm)	IQx (µg)	IQ (µg)	MeIQ/MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCAs (µg)
Control	6.2 ± 0.5	14.2 ± 1.2	3.1 ± 1.2	1.2 ± 1.2	24.8 ± 2.4
125	6.2 ± 0.7	14.4 ± 1.2	3.0 ± 1.4	1.4 ± 1.2	24.2 ± 3.6
250	5.9 ± 0.9	14.7 ± 1.5	2.5 ± 1.2	1.2 ± 1.1	25.2 ± 2.8
500	6.1 ± 0.8	15.1 ± 1.7	2.9 ± 1.6	1.6 ± 1.1	24.3 ± 3.0
1000	5.5 ± 1.0	14.9 ± 1.4	2.9 ± 1.0	1.0 ± 1.0	24.5 ± 1.9
2000	7.5 ± 1.3	13.6 ± 0.3	2.8 ± 2.1	1.4 ± 0.2	25.2 ± 1.2

Table F-8: Effect of rosmarinic acid on HCA formation in chemical model systems heated at 175 ºC for 40 min. Values are means ± standard deviation calculated from excel.

Rosmarinic Acid (ppm)	IQx (µg)	IQ (µg)	MeIQ/MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCAs (µg)
Control	6.1 ± 0.7	14.9 ± 1.7	3.4 ± 1.0	1.2 ± 0.4	25.5 ± 2.4
125	7.1 ± 0.7	14.6 ± 0.8	2.3 ± 0.6	1.2 ± 0.6	25.3 ± 2.0
250	6.1 ± 0.8	14.7 ± 1.8	3.0 ± 0.7	1.2 ± 0.4	24.6 ± 1.3
500	5.7 ± 0.5	15.6 ± 1.1	2.8 ± 1.1	1.2 ± 0.6	25.6 ± 2.2
1000	6.2 ± 1.0	14.0 ± 2.6	3.2 ± 1.1	1.1 ± 0.5	25.6 ± 1.4
2000	8.2 ± 0.4	15.1 ± 1.9	2.6 ± 1.9	1.7 ± 0.3	27.6 ± 3.0

Table F-9: Effect of epigallocatechin gallate (EGCG) on HCA formation in chemical model systems heated at 175 ºC for 40 min. Values are means ± standard deviation calculated from excel.

EGCG (ppm)	IQx (µg)	IQ (µg)	MeIQ/MeIQx (µg)	4,8-DiMeIQx (µg)	Total HCAs (µg)
Control	6.3 ± 0.9	14.1 ± 1.2	3.6 ± 1.4	1.4 ± 1.2	25.8 ± 2.8
125	6.1 ± 0.9	14.6 ± 3.6	2.7 ± 0.9	1.9 ± 0.6	24.3 ± 3.0
250	6.4 ± 1.1	14.1 ± 1.4	3.0 ± 1.5	1.1 ± 0.6	24.0 ± 2.9
500	5.9 ±0.9	13.9 ± 2.8	3.3 ± 1.5	1.1 ± 0.5	24.7 ± 3.1
1000	7.9 ± 4.1	12.0 ± 4.9	3.0 ± 1.0	1.2 ± 0.5	24.9 ± 3.0
2000	6.4 ± 2.8	12.8 ± 5.4	2.7 ± 2.0	2.0 ± 1.1	23.8 ± 10.5

Appendix G - SPME Chromatograph of Black Pepper Oil

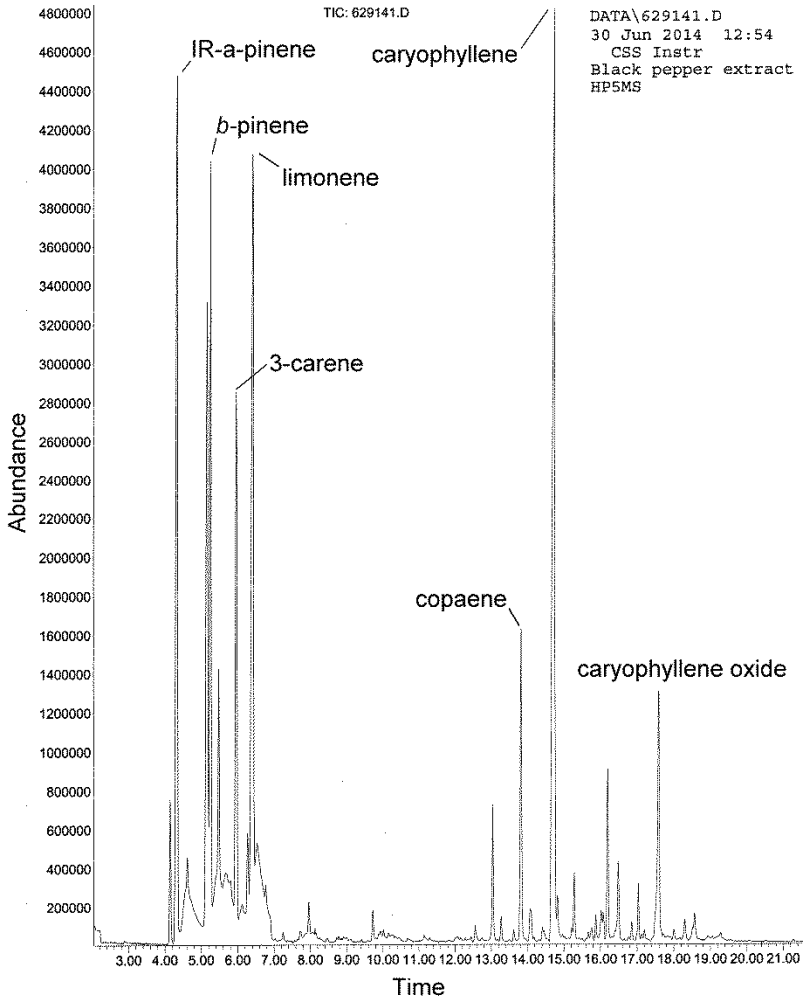

Figure G-1: SPME chromatography of black pepper oil was according to the methods described by Puangsombat and Smith (2010). In a 5 mL sealed vial, 0.1 g of oil was heated for 15 min at 60 °C with a CAR-PDMS fiber (Supelco, Bellefonte, PA) inserted in the headspace. After extraction, the fiber was removed and placed in the injection port of the GC-MS for 15 min. GC-MS was performed with an HP5890 GC fitted with a HP-5 MS column (cross-linked 5% Ph Me siloxane, 30 m x 0.22 mm x 0.25 µm film thickness) and an HP MSD 5070 detector. GC-MS conditions were: oven temperature program set with an initial oven temperature of 70 °C for 3 min, followed by a temperature increase at a rate of 7 °C/min to 180 °C and then held for 5 min. The injector was set at 260 °C and the detector at 300 °C. Helium was the carrier gas with a flow rate of 1 mL/min. Volatile compounds were identified by comparing spectra with known spectra in the NIST98/Wiley library.

Table G-1: Table denoting the chemical name and CAS numbers of the identified compounds in the black pepper oil using SPME (see Figure H-1).

Chemical Name	**CAS #**
1R-α-pinene	007785-70-8
β-pinene	000127-91-3
3-carene	013466-78-9
Limonene	005989-54-8
Copaene	003856-25-5
Caryophyllene	000087-44-5
Caryophyllene Oxide	001139-30-6

Appendix H - Solubility of Model Systems

		Water Concentration (%)			
		0	**5**	**10**	**15**
Threonine and Creatinine Concentration (mmol)	**0.2**	No	No	No	Yes
	0.4	No	No	No	No
	0.6	No	No	No	No
	0.8	No	No	No	No

Figure H-1: Table indicating whether the solid precursors were completely dissolved following heating in Part 1 of Chapter 3 (yes indicates full solubility, no indicates insolubility).

		Sugar Concentration (mmol)			
		0.15	**0.3**	**0.6**	**1.2**
Sugar Type	**Fructose**	No	No	No	Yes
	Galactose	No	No	No	Yes
	Glucose	No	No	No	Yes
	Lactose	No	Yes	Yes	Yes

Figure H-2: Table indicating whether the solid precursors were completely dissolved following heating in Part 2 of Chapter 3 (yes indicates full solubility, no indicates insolubility).

Appendix I - Water Activity Data

Table I-1: Raw data collected for the water activities of the model systems in Part 1. Water activity was determined using an AquaLab 4TE meter using 2.5 mL of sample in a 47 mm diameter sample cups. Water activity was unable to be determined for the 0% water samples (ND). The standards read; 0.500 std, 0.5011 and 0.760 std, 0.7603.

	0.2 mmol Model System			
Water %	**Run 1**	**Run 2**	**Run 3**	**Average**
0	ND	ND	ND	ND
5	0.3233	0.319	0.3101	0.317
10	0.4329	0.4473	0.4432	0.441
15	0.5313	0.5375	0.5331	0.534
	0.4 mmol Model System			
Water %	**Run 1**	**Run 2**	**Run 3**	**Average**
0	ND	ND	ND	ND
5	0.3573	0.368	0.3351	0.353
10	0.4919	0.4773	0.4465	0.472
15	0.5413	0.5482	0.5173	0.536
	0.6 mmol Model System			
Water %	**Run 1**	**Run 2**	**Run 3**	**Average**
0	ND	ND	ND	ND
5	0.4005	0.418	0.3977	0.405
10	0.4678	0.4937	0.4465	0.469
15	0.5238	0.5254	0.5173	0.522
	0.8 mmol Model System			
Water %	**Run 1**	**Run 2**	**Run 3**	**Average**
0	ND	ND	ND	ND
5	0.3605	0.3872	0.3829	0.377
10	0.4668	0.4986	0.4524	0.473
15	0.5349	0.543	0.5251	0.534

% Water	**Overall Average**	**SD**
0	ND	---
5	0.363	0.037
10	0.464	0.015
15	0.532	0.006

Appendix J -SAS Codes

The following code was written for SAS statistical analysis of Chapter 3 and 4.

```
/*----------------------Cara's Analysis----------------------*/
/* This macro takes the response variable (HCA), the data,  */
/* and the predictors, and gives outputs the model, pairwise*/
/* contrasts, and residual output for the correct model. If */
/* we have nonconstant variance, I have also written a piece*/
/* defining where the differences lie and trying to use less*/
/* than 5 variance components for day.                      */
/*----------------------------------------------------------*/
/*Data info:
        */
/*Response=Total HCAs
        */
/*Block=day                                          (5) levels        */
/*Treatments=c(water,precursor)                  (4,4) levels      */
/*----------------------------------------------------------*/
/*Model info:
        */
/*the study design is RCBD where days is our blocking term  */
/*----------------------------------------------------------*/


%macro CaraMacro(response,dataset,x1,x2,chisq);
/*Running model assuming constant variance*/
        ods listing close;
                data &dataset;
                        set &dataset;
                        mer=1;
                run;
                proc mixed data=&dataset covtest cl maxiter=1000;
                  class day &x1 &x2;
                  model &response= &x1|&x2;
                  random day;
                  ods output FitStatistics=FitStats1;
                run;
/*Running assuming non-constant variance assumption
        */
/*if there is a difference, we may want to look into how many       */
/*difference variance components we should be using
        */
                proc mixed data=&dataset covtest cl maxiter=1000;
                        class day &x1 &x2;
                        model &response = &x1|&x2;
```

```
                random day;
                repeated /group=day;
                ods output FitStatistics=FitStats2;
        run;
/*Checking nonconstant variance by creating a dataset of the difference */
/*between the two -2Res Log Likelihoods
        */
        data Fitstats1(drop=Value line);
                set Fitstats1;
                Nonconstant=Value;
                line=_n_;
                if line lt 2
                        then output;
        run;

        data Fitstats2(drop=Value line);
                set Fitstats2;
                Constant=Value;
                line=_n_;
                if line lt 2
                        then output;
        run;
        data OverallFit;
                merge Fitstats1 Fitstats2;
                Difference=Nonconstant-Constant;
                if Difference>=&chisq then Analysis=2; else Analysis=1;*This will give a
p-value .01;
                mer=1;
        run;

        data OverallFit2 (keep=Analysis);
                set OverallFit;
                call symput('indicator',Analysis);
        run;

    ods listing;
/*Checking normality of residuals assumption*/
        %if &indicator eq 2 /*#######################################*/
                %then %do;
                        proc mixed data=&dataset covtest cl maxiter=1000;
                          class day &x1 &x2;
                          model &response = &x1|&x2/outpred=resid;
                          random day;
                          repeated /group=day;
                          lsmeans &x1 &x2/pdiff adjust=tukey cl;
                          lsmeans &x1*&x2/pdiff adjust=tukey cl;
```

```
                                    title 'Assuming NONconstant variance by day';
                                run;
                        %end;
                %if &indicator eq 1
                        %then %do;
                                proc mixed data=&dataset covtest cl maxiter=1000;
                                  class day &x1 &x2;
                                  model &response = &x1|&x2/outpred=resid;
                                  random day;
                                  lsmeans &x1 &x2/pdiff adjust=tukey cl;
                                  lsmeans &x1*&x2/pdiff adjust=tukey cl;
                                  title 'Assuming Constant variance by day';
                                run;
                        %end;
                proc univariate normal plot data=resid;
                   var resid;
                   title4 'checking normality (all data)';
                run;
                data resid2;
                        set resid;
                run;
                proc sort data=resid2;
                        by day;
                run;
                proc univariate normal plot data=resid2;
                   var resid;
                        by day;
                   title4 'checking normality (by day)';
                run;
                proc datasets;
                        delete Fitstats1 Fitstats2 Overallfit2 Resid Resid2;
                run;
%mend CaraMacro;


/*##########################################################################
######*/
/*_____Read in
data_____*/
/*##########################################################################
######*/
proc import out= WORK.CaraPart1a datafile="E:\New folder\Liquid data.csv" dbms=csv
replace;
        getnames=yes;
run;
```

```
proc import out= WORK.CaraPart1b datafile="E:\New folder\Sugar data.csv" dbms=csv
replace;
        getnames=yes;
run;
proc import out= WORK.CaraPart2 datafile="E:\New folder\AntiOxidants.csv" dbms=csv
replace;
        getnames=yes;
run;
data CaraPart1a(drop=MeIQ Total_HCAs);
        set CaraPart1a;
        Total=Iqx+IQ+MEIQx+DiMeIQx;
run;
data CaraPart1b(drop=MeIQ Total_HCAs);
        set CaraPart1b;
        Total=Iqx+IQ+MEIQx+DiMeIQx;
run;



/*##########################################################################
######*/
/*_____________________________Run Macros on dataset
1a_____________________________*/
/*##########################################################################
######*/
%CaraMacro(Total,CaraPart1a,Precursor_amount,water,13.2767);
        /* -2(L0-L1)=17.61     */
        /* Our assumption of normality holds */
        /* outliers: 12,27,31*/
        /*Lets try 3 variance levels for day 1,3,5(medium) and day 2(large) and day 4(small)*/
        data Part1aTotal;
                set CaraPart1a;
                if Day eq 4
                        then DayGroup=1;
                if Day eq 1 or Day eq 3 or Day eq 5
                        then DayGroup=2;
                if Day le 2
                        then DayGroup=3;
        run;
        proc mixed data=Part1aTotal covtest cl maxiter=1000;
                class DayGroup day Precursor_amount water;
                model Total = Precursor_amount|water/outpred=resid;
                random DayGroup;
                repeated /group=DayGroup;
                lsmeans Precursor_amount water/pdiff adjust=tukey cl;
                lsmeans Precursor_amount*water/pdiff adjust=tukey cl;
```

```
        run;
                proc univariate normal plot data=resid;
                   var resid;
                   title4 'checking normality (all data)';
                run;
                data resid2;
                        set resid;
                run;
                proc sort data=resid2;
                        by day;
                run;
                proc univariate normal plot data=resid2;
                   var resid;
                        by day;
                   title4 'checking normality (by day)';
                run;

%CaraMacro(Iqx,CaraPart1a,Precursor_amount,water,13.2767);
        /* -2(L0-L1)=4.32                */
        /* Our assumption of normality holds for all but day 3*/
        /* outliers: NULL*/
%CaraMacro(IQ,CaraPart1a,Precursor_amount,water,13.2767);
        /* -2(L0-L1)=10        */
        /* Our assumption of normality holds */
        /* outliers: 27 31*/
%CaraMacro(MeIQx,CaraPart1a,Precursor_amount,water,13.2767);
        /* -2(L0-L1)=18.9 */
        /*        Our assumption of normality holds all but day 3       */
        /* outliers: 48 80 59*/
        /* Lets try 2 variance levels for day 3,4,5 and day 1,2*/
        data Part1aMeIQx;
                set CaraPart1a;
                if Day ge 3
                        then DayGroup=1;
                if Day le 2
                        then DayGroup=2;
        run;
        proc mixed data=Part1aMeIQx covtest cl maxiter=1000;
                class DayGroup day Precursor_amount water;
                model MeIQx = Precursor_amount|water/outpred=resid;
                random DayGroup;
                repeated /group=DayGroup;
                lsmeans Precursor_amount water/pdiff adjust=tukey;
                lsmeans Precursor_amount*water/pdiff adjust=tukey;
        run;
%CaraMacro(DiMeIQx,CaraPart1a,Precursor_amount,water,13.2767);
```

```
/* -2(L0-L1)=20                    */
/* Our assumption of normality holds all but day 1   */
/* outliers: 19 20 28 32 74 76 80*/
/* Lets try 2 variance levels for day 2,5 and day 1,3,4*/
data Part1aDiMeIQx;
        set CaraPart1a;
        if Day eq 2 or Day eq 5
                then DayGroup=1;
        if Day eq 1 or Day eq 3 or Day eq 4
                then DayGroup=2;
run;
proc mixed data=Part1aDiMeIQx covtest cl maxiter=1000;
        class DayGroup day Precursor_amount water;
        model DiMeIQx = Precursor_amount|water/outpred=resid;
        random DayGroup;
        repeated /group=DayGroup;
        lsmeans Precursor_amount water/pdiff adjust=tukey cl;
        lsmeans Precursor_amount*water/pdiff adjust=tukey cl;
run;
        proc univariate normal plot data=resid;
           var resid;
           title4 'checking normality (all data)';
        run;
        data resid2;
                set resid;
        run;
        proc sort data=resid2;
                by day;
        run;
        proc univariate normal plot data=resid2;
           var resid;
                by day;
           title4 'checking normality (by day)';
        run;


/*#########################################################################
######*/
/*_____Run Macros on dataset
1b_____*/
/*#########################################################################
######*/
%CaraMacro(Total,CaraPart1b,Sugar_Type,Sugar_Amount,13.2767);
        /* -2(L0-L1)=9.3 */
        /* Our assumption of normality holds all but day 1 and 4     */
        /* outliers: 12 60 76*/
```

```
%CaraMacro(Iqx,CaraPart1b,Sugar_Type,Sugar_Amount,13.2767);
        /* -2(L0-L1)=8.3 */
        /* Our assumption of normality holds for all but day 1 and 5 */
        /* outliers: 40 68 72*/
%CaraMacro(IQ,CaraPart1b,Sugar_Type,Sugar_Amount,13.2767);
        /* -2(L0-L1)=3.5 */
        /* Our assumption of normality is weak but we have*/
        /* outliers in both directions so a gamma dist will fail*/
%CaraMacro(MeIQx,CaraPart1b,Sugar_Type,Sugar_Amount,13.2767);
        /* Our assumption of normality fails so we will try a gamma dist*/
        /* outliers: 12 27*/
%CaraMacro(DiMeIQx,CaraPart1b,Sugar_Type,Sugar_Amount,13.2767);
        /* -2(L0-L1)= 29.7     */
        /* Our assumption of normality holds for all but day 1 */
        /* outliers: 12 49 61 64 65*/
        /* Lets try 2 variance levels for day 2,3,4,5 and day 1*/
        data Part1bDiMeIQx;
                set CaraPart1b;
                if Day eq 2 or Day eq 3 or Day eq 4 or Day eq 5
                        then DayGroup=1;
                if Day eq 1
                        then DayGroup=2;
        run;
        proc mixed data=Part1bDiMeIQx covtest cl maxiter=1000;
                class DayGroup day Sugar_Type Sugar_Amount;
                model DiMeIQx = Sugar_Type|Sugar_Amount/outpred=resid;
                random DayGroup;
                repeated /group=DayGroup;
                lsmeans Precursor_amount water/pdiff adjust=tukey cl;
                lsmeans Precursor_amount*water/pdiff adjust=tukey cl;
        run;
                proc univariate normal plot data=resid;
                   var resid;
                   title4 'checking normality (all data)';
                run;
                data resid2;
                        set resid;
                run;
                proc sort data=resid2;
                        by day;
                run;
                proc univariate normal plot data=resid2;
                   var resid;
                        by day;
                   title4 'checking normality (by day)';
                run;
```

```
/*##########################################################################
######*/
/*_____Run Macros on dataset 2
_____*/
/*##########################################################################
######*/
%CaraMacro(Iqx,CaraPart2,Antioxidants,ppm,13.2767);
/*If we remove day 4, the LR is defined (done below in the comments).*/
/*I believe that since the variance component for Day 4 goes negative, the LR is undefined.*/
/*This is fine since it seems that there is little or no difference across days*/
/*Our assumption of normality fails by p-values but visual inspection shows acceptable
normality*/
/* outliers: 11 26 34 104 105*/


/*
	data CaraPart2b;
		set CaraPart2;
		if day ne 4
			then output;
	run;

	proc mixed data=CaraPart2b covtest cl maxiter=1000;
		class  day Antioxidants ppm;
		model IQx = Antioxidants|ppm/outpred=resid;
		random Day;
		repeated /group=Day;
		lsmeans Antioxidants ppm/pdiff adjust=tukey cl;
		lsmeans Antioxidants*ppm/pdiff adjust=tukey cl;
	run;
*/


%CaraMacro(IQ,CaraPart2,Antioxidants,ppm,13.2767);
/*-2(L0-L1)=492-462=9.41*/
/*Our assumption of normality fails by p-values but visual inspection shows acceptable
normality*/    /* outliers: 11 26 105*/

%CaraMacro(MeIQx,CaraPart2,Antioxidants,ppm,13.2767);
/*-2(L0-L1)= 353-340=12.96*/
/* Our assumption of normality holds for all but day 2 and 4 */
/* outliers: 86*/

%CaraMacro(DiMeIQx,CaraPart2,Antioxidants,ppm,13.2767);
/*-2(L0-L1)= 128-126=1.66*/
```

```
/* Our assumption of normality holds for all but day 2 and 4 */
/* outliers: 14 20 26 38*/

%CaraMacro(Total,CaraPart2,Antioxidants,ppm,13.2767);
/*-2(L0-L1)=518-482=36.59 */
/* Our assumption of normality holds for all but day  */
/* outliers: 26*/
/*Lets try 3 variance levels for day 1,3,4(medium) and day 2(small) and day 5(big)*/
        data Part2Total(keep=total DayGroup Day ppm Antioxidants);
                set CaraPart2;
                if Day eq 2
                        then DayGroup=1;
                if Day eq 1 or Day eq 3 or Day eq 4
                        then DayGroup=2;
                if Day eq 5
                        then DayGroup=3;
        run;
        proc mixed data=Part2Total covtest cl maxiter=1000;
                class DayGroup day Antioxidants ppm;
                model Total = Antioxidants|ppm/outpred=resid;
                random DayGroup;
                repeated /group=DayGroup;
                lsmeans Antioxidants ppm/pdiff adjust=tukey cl;
                lsmeans Antioxidants*ppm/pdiff adjust=tukey cl;
        run;
                proc univariate normal plot data=resid;
                   var resid;
                   title4 'checking normality (all data)';
                run;
                data resid2;
                        set resid;
                run;    proc sort data=resid2;
                        by day;
                run;
                         proc univariate normal plot data=resid2;
                   var resid;
                        by day;
                   title4 'checking normality (by day)';
                run;
```