INHIBITION OF HETEROCYCLIC AMINES IN BEEF PATTIES BY SPICES

by

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Abstract

Heterocyclic amines (HCAs) formed during cooking of meats at high temperatures are suspected cancer causing compounds and efforts have been made to reduce their levels. Spices possessing high levels of antioxidants have been shown to inhibit these compounds when incorporated prior to cooking. Seven spices, black pepper, rosemary, turmeric, thyme, cinnamon, ginger and oregano were analyzed for their antioxidant capacity using three different assays. These spices were individually added at 0.25% to beef patties fried at 400 °F for 5 min per side to evaluate their effect on HCA inhibition. Black pepper was emphasized in this study by studying the effect of addition at different concentrations (0.25, 0.50, 1.00 %) on HCA reduction. It was found that patties treated with black pepper individually, as well in combination with other spices, greatly decreased the formation of HCAs. Black pepper at 0.25% level showed the highest inhibition of PhIP levels (85%). The spices were shown to have high levels of free radical scavenging activity as measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Overall, black pepper had significantly lower DPPH scavenging activity but still showed high HCA inhibition. All the spices individually inhibited HCA by 55-82%. Black pepper in combination with turmeric was the best combination, showing a 94.74% inhibition of HCA formation.

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Chapter 1 - Literature Review

Cancer is one of the most feared diseases which seems to affect people in some way or another in their life. Its cause and spread to different parts of body and it being the second most cause of death has a demand to study it and find the cause and cure. The general awareness of the public looks for more of prevention than cure.

The ten most common heterocyclic amines (HCAs) are 2-amino-3-methylimdazo[4,5-f]quinoline (IQ), 2-amino-3,4-dimethylimdazo[4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimdazo[4,5-f]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-9H-dipyrido[2,3-b]indole (AαC), 2-amino-3-methyl-9H-dipyrido[2,3-b]indole (MeAαC), 2-amino-6-methyl-dipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1), 2-amino-dipyrido[1,2-a:3',2'-d]imidazole (Glu-P-2), 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), and 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) (IARC 1993).

Consequently, finding strategies for decreasing HCA formation in foods is necessary. It has been reported that the level of HCAs in meat products can be reduced by cooking at lower temperature and time. The addition of compounds with antioxidant potential (Vitaglione and Foglino 2004) is another effective method for reducing HCAs in foods, because HCAs are formed through free radicals in the Maillard reaction (Vitagline and Fogliano 2004).

Human Exposure to Heterocyclic Amines

The epidemiological studies over the past 10 years have shown that high intake of well-done meat and high exposure to meat carcinogens, particularly HCAs, may increase the risk of human cancers (Zheng and Lee 2009). Four major cancers that have been reported to be associated with meat intake are colorectal, breast, prostate, and pancreatic cancers. The daily

consumption of meat was estimated to range from 80 to 160 g/day in United States, Sweden, New Zealand, and Japan (Augustsson and others 1997, Kobayashi and others 2002, Layton and others 1995).

The estimated HCA daily intake for the U.S. population have been reported at 6.3 ng/kg per day (Bryne and others 1998), 20.1 ng/kg per day (Layton and others 1995), and 2.3-6.6 ng/kg per day (Keating and Bogen 2004). Also, daily human exposure levels were estimated at the range of 0.3-3.9 g/g for MeIQx and 0.005-0.3 g/g for PhIP (Wakabayashi and others 1995). For a 54 kg individual, the reported average intake of PhIP was 899 ng/day, MeIQx was 141 ng/day, and DiMeIQx was 44 ng/day (Layton and others 1995).

PhIP is reported to be the most abundant HCA detected in foods as compared to the other HCAs. The PhIP levels in cooked muscle foods are normally higher than MeIQx and DiMeIQx. It has been detected in most types of meats and is commonly consumed in the United States. The average daily intake values of PhIP studied in various cohort studies ranged from 285.5 to 457 ng/day. The concentration of PhIP found in cooked beef is 0.56 to 48.5 ng/g (Report on Carcinogens, 2002). It has been shown to have the most positive association with cancer risk in a dose-response relationship. The large amounts of PhIP may occur because PhIP is formed by a different mechanism, which allows them to form more easily than other polar HCAs (Knize and Felton 1995). IQ and MeIQ are seldom detected in meat samples (Knize and Felton 1995).

It is difficult to measure the exact value or the mutagenicity of HCAs in an individual as intake is based on questionnaires, national consumption surveys, doneness preference, as well as factors such as body weight, age, gender, ethnicity, etc. (Sinha and others 2005). There are considerable uncertainties which are related to the estimation of HCA intake. Some challenges in estimating human exposure to HCAs are due to there being limited data on the HCA

concentration in meats cooked at home, in commercial meat products, and in, meats cooked at fast-food restaurants. Absence of complete information makes experimentally derived HCA concentrations to provide the best available data for estimated HCA intake in most studies (Skog 2002).

Sugimura (2002) suggests how these carcinogenic compounds being a part of our normal lifestyle can prove to be a cancer risk factor not just by themselves but even in combination with other carcinogens. It has been estimated that the average total daily intake over a lifetime for adults is 9 ng/ kg / day (Bogen and Keating 2001). The International Agency for Research on Cancer (1993) classifies HCAs into two groups based on carcinogenicity studies: MeIQ, MeIQx, PhIP, AαC, MeAαC, Trp-P-1, Trp-P-2, and Glu-P-2 as possible human carcinogens (group 2B), and IQ as a probable human carcinogen (group 2A).

Classification of HCAs

All the HCAs have at least one aromatic and one heterocylic structure (Cheng and others 2006). Murkovic (2007) classified HCAs either as polar or non-polar based on their structure. The classification as polar and non-polar expresses the order of elution in reversed-phase chromatography and also the fluorescence of the substances with the non-polar HCAs (Murkovic, 2007). Table 1-1 summarizes the chemical names and abbreviations of non-polar and polar heterocyclic amines.

Table 1-1 Non-Polar and Polar Heterocyclic amine chemical names (Sugimura and others 2004)

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

Polar HCAs

Polar HCAs are also known as amino-imidazo-azaarenes (AIA) and have been found to be responsible for most of the mutagenic activity in cooked foods, especially in Western diets (Felton and Knize, 1991). The polar HCAs are formed from amino acids and creatinine in the presence of carbohydrates. Polar HCAs can be identified by the 2-aminoimidazo group and a methyl group attached to one of the nitrogens in the imidazo ring (Jagerstad and others 1998). Polar HCAs are sub grouped depending on the groups that are attached to the 2-amino-imidazo. These subgroups are quinolines, quinoxalines, and pyridines. Quinolines are called IQ-type (e.g. IQ and MeIQ) while quinoxalines are called IQx type (e.g. IQx, MeIQx and DiMeIQx) (Pearson and others 1992). PhIP, DMIP, and TMIP are the important HCAs in the pyridine subgroup (Cheng and others 2006). The structures of polar HCAs are illustrated in Figure 1-1.

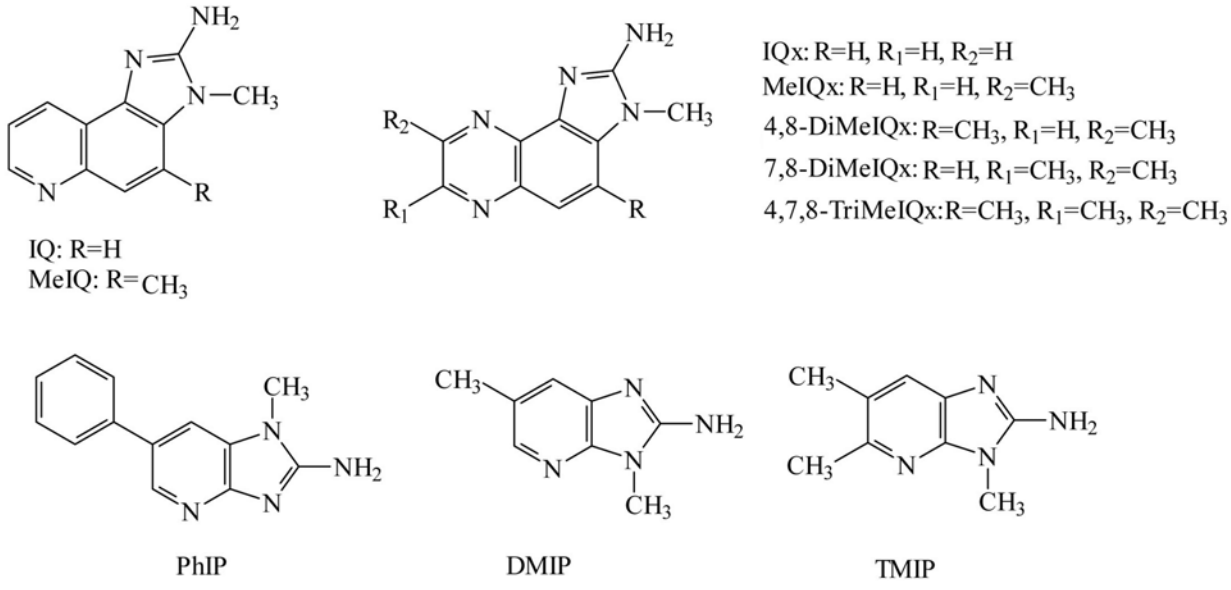

Figure 1-1 Chemical structure of polar heterocyclic amines (Cheng and others 2006).

Non-Polar HCAs

Non-polar HCAs, also known as amino-carbolines, are sub-divided into pyridoindole and dipyridoimidazole types (Murkovic 2007). They include five-membered heterocyclic aromatic rings sandwiched between two-six membered aromatic rings (Cheng and others 2006) as illustrated in Figure 1-2. They are formed at much higher temperatures (~300 °C and above) and thus have a lower occurrence in normally cooked foods. Due to the difficulty in manipulation of high temperatures required for experimental setup, non-polar HCA have been studied as extensively as polar HCAs (Cheng and others 2006).

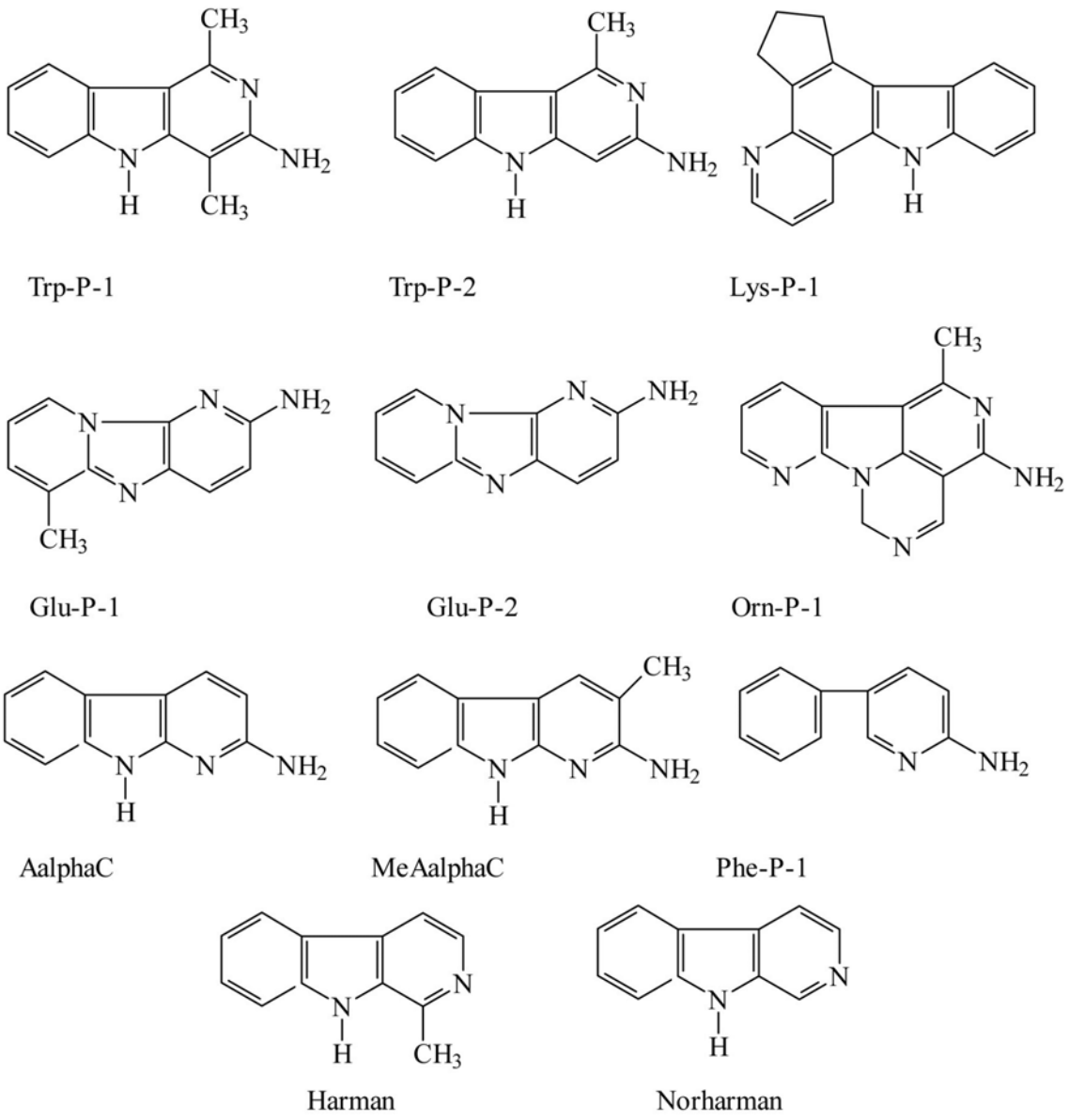

Figure 1-2 Chemical structure of non-polar heterocyclic amines (Murkovic 2004).

Formation of HCAs

The three main precursors leading to the formation of polar HCAs are creatine/creatinine, sugars, and amino acids originally found in muscle foods (Knize and Felton 2005). Maillard

browning is the reaction taking place during cooking where free radicals are formed which react with the creatinine producing HCAs (Pearson and others 1992; Kikugawa 1999).

The polar HCAs are formed from amino acids and creatinine where creatinine provides the imidazole ring for the condensed aromatic amines; in its absence IQ and IQx-type HCAs can not be formed (Murkovic 2007). The temperature needed for formation of polar HCAs is between 150 and 250 °C (Arvidsson and others 1997). These are generally the temperatures used during cooking of meat and so formation of polar HCAs is of importance in studies.

The IQ-type and IQx-type HCAs formed through the Maillard reaction as postulated by Pearson and others (1992), Jägerstad and others (1998), and Skog and others (1998) is shown in Figure 1-3. Herein, sugars and amino acids react producing intermediates that undergo reverse-aldol reaction to give enol type compounds which then further react with creatinine to give IQ and/or IQx type of HCAs. The imidazole ring is derived from creat(in)ine; therefore, formation of HCAs containing the imidazole ring (polar HCAs) does not occur without this precursor (Murkovic 2007). Formation of HCAs follows first-order kinetics (Arvidsson and others 1997; Kim and Lee 2010)

The mechanism of PhIP formation (Figure 1-4) is different from the mechanism that forms other polar HCAs. The PhIP mechanism starts with the reaction of two precursors, phenylalanine and creatinine, which forms phenylacetaldehyde. Then, the aldol condensation of phenylacetaldehyde with creatinine occurs and PhIP is produced as a final product. The nitrogen forming pyridine moiety in the PhIP molecule is originally derived from either an amino group of creatinine, or an amino group of phenylalanine or free ammonia (Pearson and others 1992).

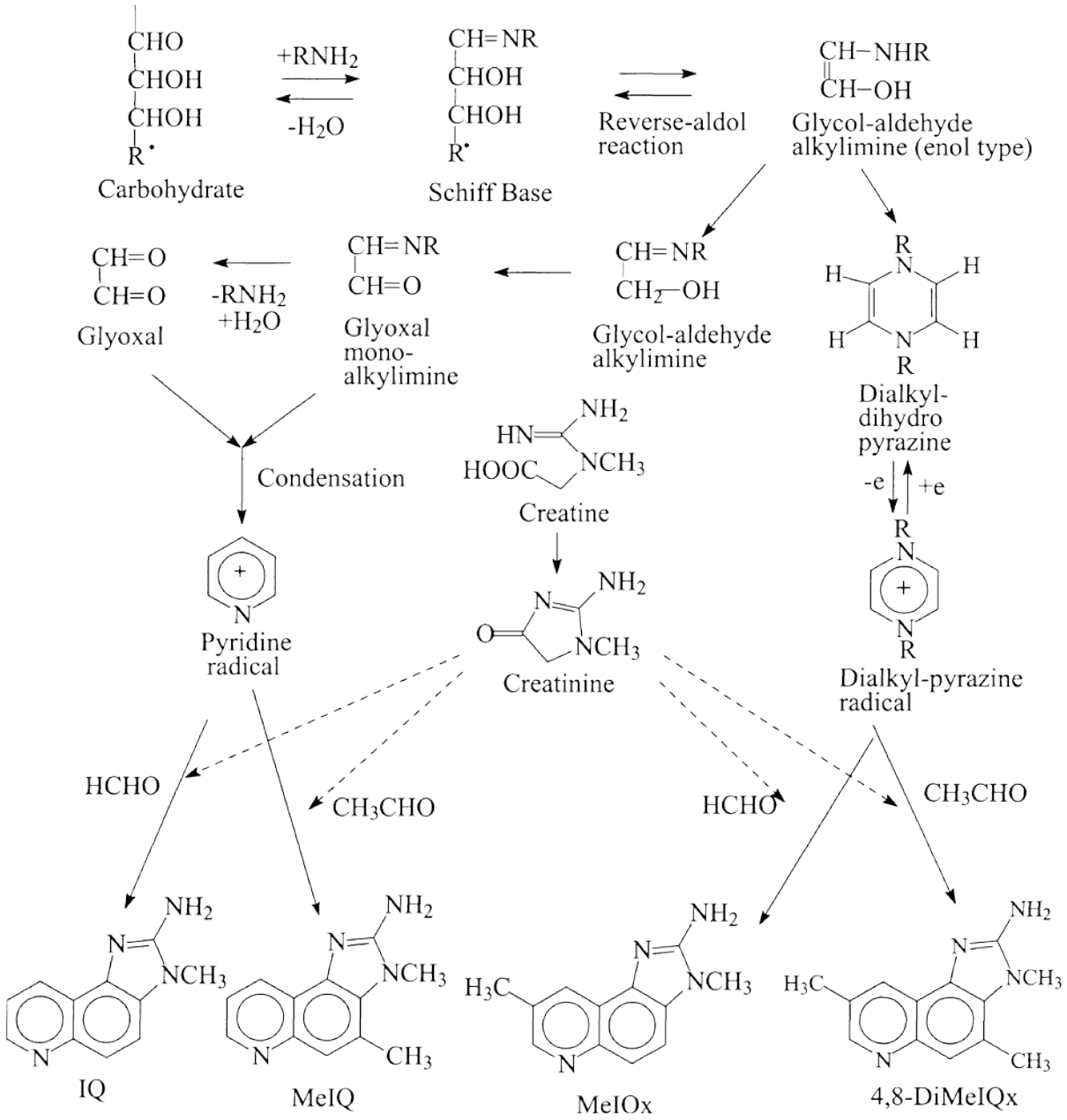

Figure 1-3 Suggested pathway for formation of IQ-like and IQx-like compounds (Pearson and others 1992)

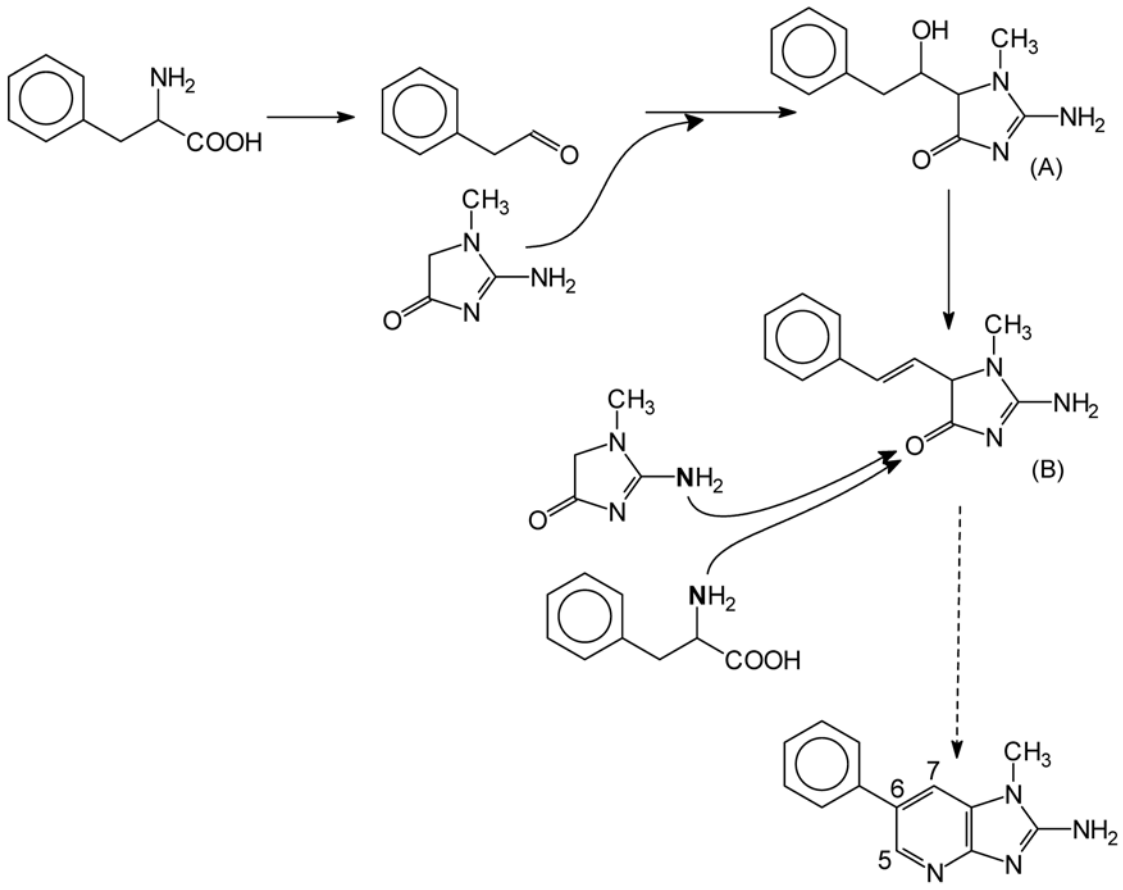

Figure 1-4 Formation of PhIP with identified intermediate reaction products (Murkovic 2004).

Analysis of HCAs

The analytical determination of HCAs is complicated, and several studies have been published to improve the analysis of these compounds. Various model systems and food matrices have been used to study HCAs and the extraction of the HCAs from these matrices makes the process complicated. Sample extraction is followed by cleanup, identification, and detection. Starting with extraction, the original method by Gross and Grüter (1992) became widely accepted and various modifications to this method were adopted. In this multi-step solid-phase extraction (SPE) method, the sample such as complex matrix as meat is saponified using sodium hydroxide. To avoid formation of a very stable emulsion during extraction of the saponified

liquid, diatomaceous earth is normally used to bind the liquid. Dicholoromethane or ethyl acetate is used to react and poured through the sludge. The soaked diatomaceous earth is placed in appropriate cartridges for subsequent extraction and two-step solid-phase extraction (SPE) is performed with a strong cation exchanger (e.g. propylsulfonyl sillica gel PRS) and octadecylsilane C18 material (Meester 1998, Toriobio and others 2000). Some studies also use replacement of the two-step procedure by a single step usage of novel Oasis MCX LP SPE cartridge (Murkovic 2007).

The original method utilizes high performance liquid chromatography (HPLC) with eluents of triethylamine (TEA) at pH 3.2 and 3.6 and acetonitrile; with a TSK gel ODS80 (Toyo Soda), 25 cm X 4.6 mm I.D. (5 μm particle size) column, and a Supelguard LC-8-DB (Supelco) guard column. The elution gradient can be seen as discussed in Table1- 2. Compounds were detected using ultraviolet diode array detection at 252 nm and fluorescence detection with excitation at 229 nm and emission at 437 nm (Gross and Grüter 1992; Puangsombat and Smith 2010).

Table 1-2 Gross and Gruter Elution Gradient (Gross and Gruter 1992)

Time, min	Eluent A (TEA pH 3.2), %	Eluent B (TEA pH 3.6), %	Eluent C (Acetonitrile), %
0	95	0	5
10	85	0	15
10.1	0	85	15
20	0	75	25
30	0	45	55
55	95	0	5

An optimization study by Gibis (2009) found that using mobile phases of TEA at pH 3.0 (Eluent A), TEA at pH 4.0 (Eluent B), and acetonitrile (Eluent C) with a TSK-gel Super ODS column, reversed-phase C-18, 10cm x 4.6mm I.D. (2 μm particle size) (Tosoh Bioscience, Stuttgart, Germany) improved separation and reduced run time as compared to the original method.

Less polar HCAs and PhIP are typically identified with fluorescence detection whose high sensitivity provides clean chromatograms (Ristic and others 2004). In contrast, the IQ- and IQx-type amines have no fluorescence characteristics; therefore they are measured with ultraviolet (UV) detection.

Despite the efforts of modifying the extraction, clean-up, and detection methods, the standard deviations in HCA studies are often high. In a study published by Santos and others (2004), the relative standard deviation between and within laboratories ranged from 8 to 24 and 3 to 38%, respectively. Other techniques such as time-of-flight mass spectrometry (LC-TOF-MS) (Barcelo and others 2004), enzyme-linked immunosorbent assay (ELISA) (Vanderlaan and others 1993) have shown to be used in identification of HCAs. Recent studies show UPLC method with ESI-MS/MS efficient to separate 16 HCAs in a complex food matrix in less than 2 min (Barcel and others 2006).

Inhibition of HCAs

Since the epidemiological studies have proven HCAs to have carcinogenic properties, the reduction of these compounds in foods is desirable. Since meat is an integral part of Western diets, methods to modify cooking can be an alternative to inhibit HCAs and their risks to produce cancers. Physical variables, such as temperature, time, and method of cooking significantly affect the mutagenic activity of cooked samples.

Cooking Time and Temperature

The formation of HCAs is highly dependent on time and temperature of cooking. Both the factors were seen to strongly impact the formation of HCAs (Knize and Felton 2005). Levels of HCAs are low or non-detectable in foods fried at 150°C, but a sharp increase is detected at higher cooking temperatures above 190°C; also, if the cooking temperature exceeds 200 °C, the total level of HCAs increases drastically ((Jackson and Hargraves 1994; Abdulkarim and Smith 1998).

When heat transfer is very efficient, the formation of HCAs begins immediately upon heating at around 200 °C, depending of the HCA, and within only 30 s surprisingly large amounts have been shown to be formed (Arvidsson and others 1998, 1999). In a study done by Bordas and others (2004), the effect of heating time was observed on the formation of HCAs by heating the lyophilized meat extract with water at temperature 175 °C. As the heating times were increased from 1 h to 2 h, the amounts of HCAs increased, especially for PhIP, which increased from 9.3 ng/g to 19.5 ng/g. The relation between different cooking temperature and levels of total HCAs (ng/g) in fried beef patties is illustrated in Figure 1-5. In this study the patties were heated till they reached the same internal temperature of 70 °C. Fried beef patties cooked at 140 °C had very low amounts of HCAs, and the levels of HCAs increased gradually when cooking temperature rose higher from 140 to 250 °C (Knize and Felton 2005).

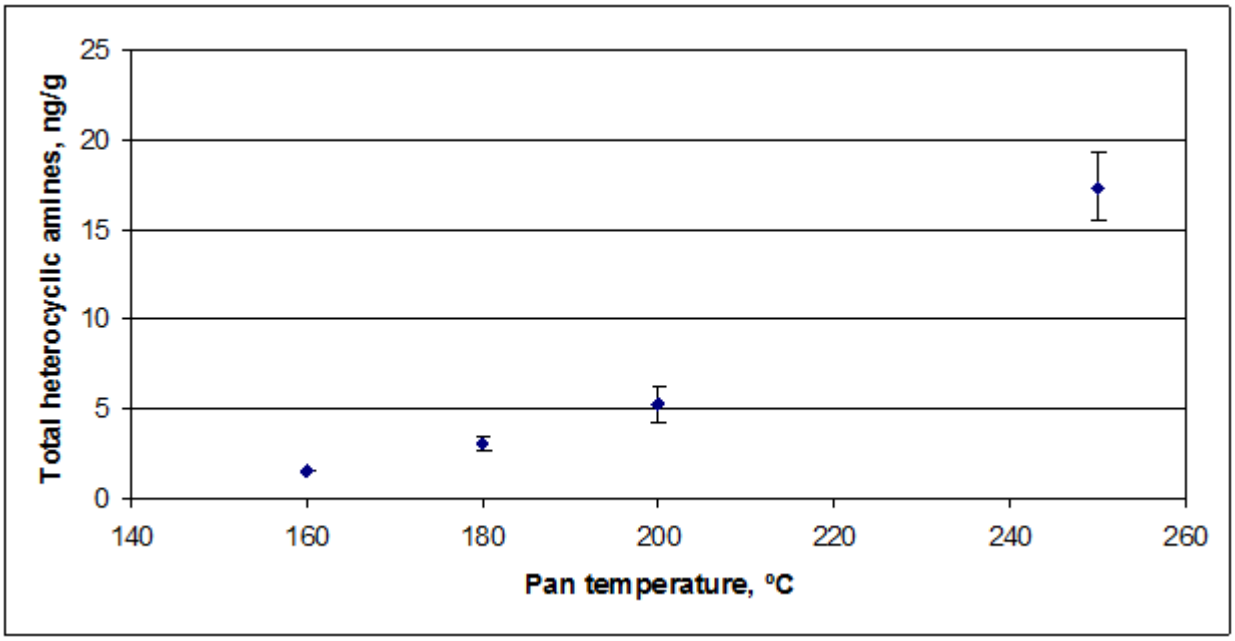

Figure 1-5 Formation of HCAs in beef patties after cooking to an internal temperature of 70ºC at different frying pan temperatures. Error bars are the standard error of four or five replicate cooking experiments (Knize and Felton 2005).

The HCAs are not very stable at 225 °C, and the rate of breakdown seems to vary slightly with pH. After 30 min heating at pH 6 or 8, about 25% to 50% of the HCAs are destroyed depending on the specific compound. PhIP is the less stable followed by 7,8-DiMeIQx, 8-MeIQx, 4,8-DiMeIQx, and IQx (Arvidsson and others 1997). However, during the first 25 min, while temperatures rise from 25 to 150 °C in fried beef, PhIP is formed predominantly, whereas MeIQ do not increase (Murkovic and Pfannhauser 2000).

These studies show that cooking meat longer or at higher temperatures produces a greater quantity of HCAs in both model systems and in various types of meat. Thus, consumer preference becomes complex when these values are related to the doneness of meat and the risk caused by HCAs.

Optimized Cooking Methods

The cooking method has a considerable influence on the formation of mutagenic activity (Wu and others 1997). The cooking method is believed to affect HCA formation and involves heat transfer and water loss (Skog and others 1995). Pan-frying, grilling, barbecuing, and broiling transfers high temperatures by radiation and conduction while oven-roasting, baking, and microwaving transfer medium amount of heats by indirect convention. In general, panfrying, grilling/barbecuing, and oven-broiling seem to cause the highest HCA concentrations, especially of PhIP (Zimmerli and others 2001; Keating and Bogen 2004) while oven-roasting, baking, and microwaving produced low or intermediate levels of HCAs (Skog and others 2003).

The parameters that appear to be important in forming HCAs are: direct contact of the meat with a hot flat surface such as in pan-frying or very high temperature such as in grilling/barbecuing (Sinha and others 1998). Another reason that has been pointed out is that when using these cooking methods, water is generally lost from the food item, resulting in decreasing water content from the inner to the outer parts, and in the formation of a relatively dry surface where the precursors tend to concentrate. These physical and chemical changes affect the mass and heat transport (Skog and others 2000).

Microwave pretreatment is one practical way to reduce the HCA level in meat products. Felton and others (1994) investigated the effect of microwave pretreatment on the reduction of HCAs in fried beef patties where, beef patties were microwaved for 0 to 3 min before being fried at 250 °C for 6 min on each side. Their results showed that fried beef patties that were microwaved for 3 min before being fried resulted in 3- to 9-fold decrease of MeIQx, IQ, DiMeIQx, and PhIP compared to non-microwaved beef patties, and also a decrease in mutagenicity of up to 95%. The elimination of the water soluble precursors of HCA (creatine,

sugar, and amino acids) by approximate 30% during microwaving might have caused reduction in the HCA formation (Felton and others 1994).

Studies have also shown some other cooking methods that help inhibit HCA formation, like the frequency of turning of the meat during cooking, a single turn or multiple turns, has been studied (Salmon and others 2000). Adding water-binding compounds, such as salts, starch, soy protein, and so on, can restrict the transport of precursors (Jagerstad and others 1998; Persson and others 2004). Stewing and boiling of poultry products did not lead to the formation of detectable amounts of HCAs, probably because cooking temperature does not exceed 100°C (Skog and Solyakov 2002). Also, foods that were steamed or poached, or smoke-dried, showed no detectable HCAs (Zimmerli and others 2001).

Marination

Lan and others (2004) described marination as a traditional Chinese cooking method, which is often conducted at about boiling temperature for an extensive period of time to enhance color, flavor, and texture attributes of foods in the presence of water and various ingredients such as soy sauce and sugar. This definition differs from what most people understand about marinating, which implies preincubation with a fluid of some sort to impart flavor prior to cooking. Xargayo and others (2001) mention marination as an important aspect to increase the yield of the raw meat, which can provide benefits to the producer and the consumers. The three methods for producing marinated products as described by them included immersion, injection, and vacuum tumbling. Since traditional times marinades were homemade which comprised of ingredients like spices, vinegars, lemon juice, wine, soy sauce, salt, and sugar. These marinades not only increased the tenderness of the meat but also flavor, and moistness of the cooked product.

In a study by Smith and others (2008) three different commercially available marinades, Caribbean, Southwest, and Herb were used to determine their effectiveness in reducing HCA formation. The various components of the marinades, including the liquid portion, non-spice portion, and the spice portion, were applied to beef steaks to determine which factor, if any, had the greatest ability to reduce HCA (MeIQx, PhIP) formation. Of the marinades studied the Caribbean mixture showed the highest decrease in the total HCA content (88%), followed by the Herb (72%) and Southwest (57%). Caribbean was the most effective at reducing the HCAs of interest (Smith and others 2008). In another study, the application of oil marinades with garlic, onion, and lemon juice achieved a reduction of MeIQx and PhIP formation in fried beef patties, and garlic was found to be the most active ingredient to have HCA inhibitory effect. The optimum amounts of onion, garlic, and lemon juice that achieved a maximum reduction of HCAs were calculated as 31.2%, 28.6%, and 14.6% in marinade. However, this study used a taste panel to evaluate the palatability of the garlic, onion, and lemon marinades and found that consumers did not like the garlic and onion marinades (Gibis 2007). Gibis and Weiss (2010) studied marinades containing different concentrations (0.2, 0.4, 0.6, 0.8 g/100 g) of hibiscus extracts on fried beef patties to observe significant reductions in MeIQx and PhIP with no negative results to the sensory attributes. In another study green tea extract marinades were used to marinate beef patties which were then pan fried at 200°C for 4 mins on each side. Significant reductions were observed in PhIP values and no significant differences in the marinade treated meat organoleptic characteristics (Quelhas and others 2010). Furthermore, meat marinating with alcoholic beverages, such as, beer and red wine can reduce significantly the formation of non-polar as well as polar HCAs (Melo and others, 2008).

Marinating meats with barbecue sauces has shown adverse effect on HCA concentration as shown in the study by Nerurkar and others (1999). Barbecued beef steaks marinated with commercial teriyaki sauce and turmeric-garlic sauce had a 40-60% reduction in PhIP and MeIQx levels; however, marinating with barbecue sauce caused up to a 5-fold increase in PhIP and MeIQx levels, which could be due to their use of high fructose corn syrup and honey in the sauce.

Addition of Antioxidants

Antioxidants have been considered as free radical scavengers as per the hypothetical free radical pathway (pyrazines and pyridines radicals) forming HCAs as postulated by Vitaglione and Fogliano (2004).

Some of the synthetic known antioxidants such as butylated hydroxyanisole (BHA), propyl gallate (PG), and tert-butylhydroquinone (TBHQ) have been used for studying inhibition in HCA formation. The introduction of phenolic antioxidants, thiol compounds, BHA, PG, and reductones into a heated model system composed of glucose-glycine-creatinine have been shown to be effective to scavenge the intermediary pyrazine cation radical (Kato and others 1996; Kikugawa and others 2000). The amount of MeIQx in a fried beef patty was reported to decrease by 56% when BHA was added, 71% when PG was added, and 76% when TBHQ was added (Johansson and Jägerstad 1996). In contrast, a slight decrease of PhIP was found in chicken meat in the presence of TBHQ, but no influence on the formation of HCAs was seen in other kinds of meat at any TBHQ concentration added (Messner and Murkovic 2004).

Karre and others (2013) in a review described the recent claims of synthetic antioxidants to have potential to cause toxicological effects and consumers' increased interest in purchasing natural products, and the need of natural antioxidants by the meat and poultry industry.

Pomegranate, pine bark extract, cinnamon, and cloves have exhibited stronger antioxidant properties than some synthetic options (Karre and others 2013).

Phenolic compounds from medicinal herbs and dietary plants possess a range of bioactivities and play an important role in prevention of cancer. Most phenolic acids have antioxidant capacity, and the radical scavenging ability of phenolic acids depends on the number and position of hydroxyl groups and methoxy substituents in the molecules (Cai and others 2006). HCA inhibition by natural antioxidants has been successfully observed in previous studies when added as a whole food, or a specific compound of that food or in the form of extracts.

Inhibitory effects of antioxidants found in cherry tissue have been effective (Britt and others 1998). Cherry tissue and its methanolic extract inhibited the formation of 8-MeIQx, 4,8-DiMeIQx, and PhIP in pork patties fried at 225°C for 10 min/side (Shin 2005).

In a comparative study by Sanz and Afonso (2011), twelve tea phenolic compounds were evaluated in relation to their radical scavenging capacities and their inhibition on the formation of HCAs. Out of them four: epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate, and theaflavin- 3,3-digallate, which are tea polyphenols comprised highest antioxidant properties followed by carnosic and rosmarinic (found principally in rosemary) clorogenic acid, quercetin, quercetin- 3-glucoside, and rutin (found in several fruits and vegetables). Last ranked naringenin and hesperidin, flavonoids found in citrus fruits, were the less active free-radical scavengers. In ground beef patties, except for carnosic acid, all the compounds tested showed significant inhibition of the formation of PhIP, 8-MeIQx, and 4,8-DiMeIQx. But naringenin showed more than 50% of inhibition. However, in the model systems investigated (Cheng and others 2007), the poor correlation demonstrated between the radical scavenging capacity and their inhibitory activities in the formation of PhIP suggests that

antioxidation more specifically radical scavenging activity, may not be the principal mechanism of intervention of these phytochemicals. Naringenin's mechanism of action is illustrated in Figure 1-6 and its capability of simultaneously suppressing the formation of PhIP suggests a potential for practical application in daily cuisine (Cheng and others 2007). Also, the predicted pathways can be studied for compounds exhibiting low antioxidant property but high HCA inhibition.

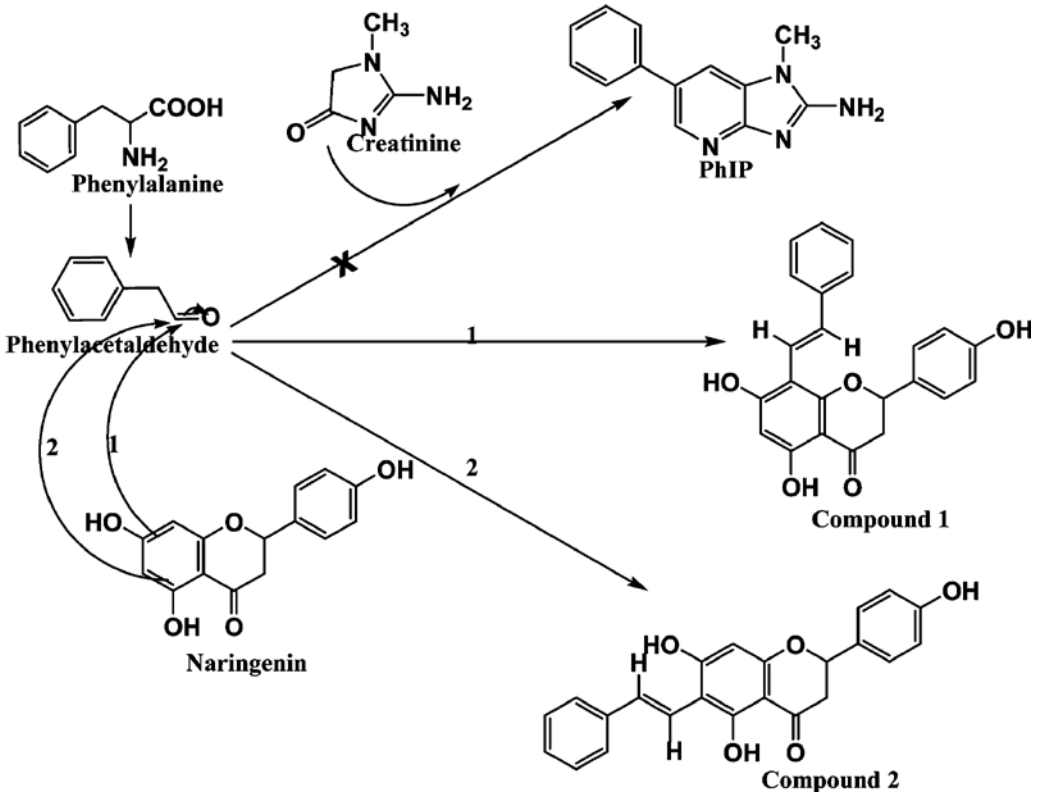

Figure 1-6 Hypothesized pathways for naringenin's inhibitory activity on PhIP formation (Cheng and others 2008).

Application of Spices

Spices contain antioxidants that can inactivate free radicals (pyrazinium and pyridinium) generated as intermediates during the Maillard reaction (Milic and Milic 1998). Therefore, spices can reduce the formation of HCAs during heating. All the investigated phenolic compounds

isolated from rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*), thyme (*Thymus* spp), and garlic (*Allium sativum*) show strong inhibitory effects on pyrazine cation free radical formation, precursors in the formation of 4,8-DiMeIQx (Murkovic and others 1998). Also, antimutagenic and anticarcinogenic effects were detected (Milic and Milic 1998).

Likewise, rosmarinic acid decreased the formation of MeIQx and PhIP in beef patties fried at 375 to 400 °F. The effects of the rosemary extracts were more dramatic when cooking temperature and time were increased, as rosmarinic acid was able to reduce MeIQx formation up to 70% and PhIP up to 64% and rosemary powder significantly reduced MeIQx up to 57% and PhIP up to 77% (Tsen and others 2006). Nerurkar and others (1999) studied beef steaks marinated with spice containing sauces consisting turmeric and garlic where the PhIP content was decreased by 49.5%.

One of the recent studies on application of dry Asian spices in beef patties and inhibition of HCAs, where turmeric (39.2% inhibition), fingerroot (33.5% inhibition), and galangal (18.4% inhibition) showed significant decreased HCAs compared with the control as illustrated in Table 1- 3 (Puangsombat and others 2011). The ability of these spices to inhibit HCAs is related to the significant amount of polyphenolic antioxidant compounds present in them.

Black pepper

Black pepper (*Piper nigrum*) is known as the “king of spices” belongs to the family Piperaceae. It originated in the western Ghats of India and is also grown in Malaysia, Singapore, Indonesia, Cambodia, Vietnam, Sri Lanka, Brazil and West Africa (Reineccuis 1994). Zachariah and Parthasarathy (2008) describe the quality attributes such as pungency and aroma with the

color aspect of black pepper. The phenols are known to contribute to the blackening of the fresh green peppers which is caused due to enzymatic oxidation of (3,4-dihydroxy phenyl) ethanol glycoside by an *o*-diphenol oxidase (PPO) present in the fresh fruit. This enzymatic oxidation also caused a 75% decrease in total phenolic content in the fruit. Black pepper initially gained prominence because of its pharmacological properties for digestion and improving appetite, as well as curing colds, coughs, and diseases of the throat, and intermittent fevers and malaria (Singletary 2010).

Zaria and others (2013) studied the levels of total phenolic content in black pepper and found 45.08 mg of gallic acid equivalent/g in the ethanol extract of P. *nigrum.* The result indicated clearly that the ethanol extract also which contained the highest amount of total phenolics, showed a high antioxidant activity of 65.59% at a concentration of 50 mg/ml. Also, they found piperine and piperic acid, to possess antioxidant and antibacterial activity (Zaria and others 2013). Unlike antioxidant studies on black pepper, there are limited studies carried out on black pepper and its property of HCA inhibition. Oz and Kaya (2011) studied black pepper for its HCA inhibiting capacity in high-fat meatballs and reported a 100% reduction in the PhIP concentration with black pepper treated meatballs compared to the control. The mechanism of black pepper and its compounds to inhibit HCAs is not well understood yet.

Table 1-3 Effect of spices on total HCA concentrations (ng/g) and percentage inhibition in beef patties cooked at 204 °C (400 °F) for 5 min on each side. (Puangsombat and others, 2011).

	HCAs (ng/g)			% inhibition
	MeIQx	PhIP	Total	
Control	7.00 ± 0.62 [a]	6.53 ± 0.19 [a]	13.53 ± 0.59 [a]	
Cumin	6.94 ± 0.33 [a]	6.14 ± 0.23 [a]	13.08 ± 0.10 [a]	3.33 %
Coriander seeds	6.78 ± 0.55 [ab]	6.15 ± 0.23 [a]	12.93 ± 0.32 [a]	4.46 %
Galangal	5.73 ± 0.48 [bc]	5.31 ± 0.32 [b]	11.04 ± 0.62 [b]	18.43 %
Fingerroot	4.86 ± 0.44 [cd]	4.13 ± 0.13 [c]	9.00 ± 0.31 [c]	33.51 %
Rosemary	3.50 ± 0.30 [e]	4.15 ± 0.16 [c]	7.65 ± 0.41 [d]	43.46 %
Turmeric	4.14 ± 0.06 [de]	4.08 ± 0.02 [c]	8.22 ± 0.05 [cd]	39.22 %

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

On the contrary, industrial flavor extracts of some spices like thyme, marjoram, rosemary, and Monascus red caused an increase of the concentration of PhIP in a model system (Zöchling and others 2002). A similar effect was seen when 1% concentration of natural extracts of pine bark (Pycnogenol®), oleoresin rosemary (Herbalox®), and grape seed (Acti VinTM) were added to ground beef and an increase in the formation of polar and nonpolar HCAs was noted. Thus Zöchling and others (2002) reported that the content of PhIP was increased

independent of pro- or antioxidative properties, concluding that radical involving reactions are not dominating the formation of PhIP.

Conclusion

The probability of presence of these HCAs in our consumed foods and the relation of HCAs to cancers requires more studies on these compounds and their chemistry. The different mechanisms by which HCAs are formed are of interest so as to understand the ways to inhibit these compounds.

Different factors affecting the formation of these compounds are still under study with researchers determining means to alternate these factors so as to have less human consumption of HCAs. Pre-cooking, optimizing cooking methods, time and temperatures, usage of antioxidants, marination, have shown to reduce HCAs but many questions remain unanswered. Mechanism of action of all these techniques is an area to explore. For these studies consumer studies on trends, surveys, and information from disease centers will help understand and support researchers their further studies.

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Chapter 2 - Inhibitory Activity of Black Pepper and Other Spices on Heterocyclic Amines in Cooked Beef Patties [1]

Abstract

Heterocyclic Amines (HCAs) are a risk factor for human cancers that form in meats cooked at high temperatures. Spices and their extracts can help inhibit HCAs. Seven spices were evaluated in this study: black pepper (*Piper nigrum*), turmeric (*Curcuma longa*), cinnamon (*Cinnamomum burmannii* with some *Cinnamomum verum*), rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*), ginger (*Zingiber officinale*), and oregano (*Origanum vulgare*). Total phenolics and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay were performed to evaluate the spices and their antioxidant potential. The spices' ability to inhibit HCAs was evaluated in beef patties cooked at 400°C (204°F) for 5 min on each side. Black pepper was studied at 0.25, 0.50, and 1% concentrations while the other spices were evaluated at the 0.25% concentration. Combinations of 0.25% black pepper and 0.25% of each spice were also evaluated. Treatments were evaluated by the percentage inhibition of PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine). Black pepper had significantly less ($P<0.05$) antioxidant capacity compared to the rest of the spices. Control patties had a concentration of 2.56 ng/g PhIP content ($P<0.05$) while in the 0.25% black pepper treated patties, an 81.77% decrease in PhIP was observed. All the spices individually inhibited HCA by 55-82%. Black pepper in combination with turmeric was the best combination, showing a 94.74% inhibition of HCA. This data suggests that black pepper can inhibit HCAs, and is more effective with other spices.

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Introduction

Cancer is the second most common cause of death in the United States (National Vital Statistics System, National Center for Health Statistics, CDC 2007); and in 1977, it was first reported that cooking meats at high temperatures generates mutagens/carcinogens (Sugimura 1997). Heterocyclic amines (HCAs), also known as heterocyclic aromatic amines, are cancer causing compounds, formed on the surface of meat during cooking. Since their discovery 30 years ago, more than 25 HCAs have been isolated and identified in cooked foods (Murkovic 2007). Formation of thermic HCAs is the result of complex reactions that involve creatine/creatinine, free amino acids, and sugars through the Maillard reaction, at temperatures between 150˚C and 250˚C (Jagerstad and others 1998; Nagao 1999), making them commonly found in food.

The most common HCAs found in foods are 2-amino-3-methyl-imidazo [4,5-f]quinoline (IQ), 2-amino-3-methylimidazo [4,5-f]quinoxaline (IQx), 2-amino-3,4-dimethylimidazo [4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo [4,5-f]quinoxaline (MeIQx), and 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) (Knize and others 1994). Some of these HCAs (IQ, MeIQ, MeIQx and PhIP) are listed as “reasonably anticipated to be a human carcinogen” by the U.S. Department of Health and Human Services’ 12th Report of Carcinogens (2011) where PhIP is reported to be the most abundant HCA detected in foods as compared to the other HCAs. It has been detected in most types of meats and is commonly consumed in the United States. The average daily intake values of PhIP studied in various cohort studies ranged from 285.5 to 457 ng/day. The concentrations of PhIP found in cooked beef is 0.56 to 48.5 ng/g as per Report of Carcinogens (2002).

To inhibit the formation of these carcinogens in cooked meats many strategies have been used such as reducing cooking time and temperature, and changing cooking styles have been tried. One effective and healthy ways of inhibiting HCA formation in meat is using antioxidant containing spices to help scavenge the pyridine and pyrazine intermediates, which form during the Maillard reaction (Balogh and others 2000; Tsen and others 2006; Cheng and others 2007). Marinades or extract of spices, such as rosemary, thyme, sage, garlic, and oregano, can be used to prevent formation of HCAs in cooked meats (Tikkanen and others 1996; Murkovic and others 1999; Murkovic 2007; Smith and others 2008; Damasius and others 2011). Puangsombat and others (2011) demonstrated that natural dried forms of rosemary, turmeric, and fingerroot also inhibited HCAs in beef patties.

Rosemary (*Rosmarinus officinalis*) is a well-known spice studied for HCA inhibition, and it contains antioxidants such as carnosol, rosmanol, and rosmarinic acid (Madsen and Bertelsen 1995). Ethanolic extracts of rosemary have been shown to decrease PhIP concentrations to 85.3% (Puangsombat and Smith 2010). Dried rosemary at concentrations of 0.2% has also shown inhibition of PhIP in beef patties cooked at 400 °F (Puangsombat and others 2011).

Turmeric (*Curcuma longa*) is one of the extensively used spices in Indian cuisine. Turmeric shows the presence of the major phenolic compound curcumin; and studies have shown that turmeric has high antioxidant properties (Chattopadhyay and others 2004), supporting its potential to prevent mutagenic activity of heterocyclic amines in cooked food (Shishu and Kaur 2008). In the Asian spice studies conducted by Puangsombat and others (2011), turmeric showed an inhibition of 39.22% when added at 0.2% concentration levels in cooked beef patties.

Cinnamon *(Cinnamomum verum)* is another antioxidant rich spice, which has health benefits when consumed regularly (Lin and others 2003). It contains mainly cinnamaldehyde (49.9%) and coumarin (16.6%), which are radical scavengers in the cinnamon species (Singh and others 2007). Thyme (*Thymus vulgaris* L) is a perennial herb indigenous to central and southern Europe, Africa, and Asia, and it is rich in essential oils and antioxidative phenolic substances (WHO 1999). Thyme has been shown to contain four flavonoids including epirosmanol, isorosmanol, galdosol, and carnosic acid. Two of these flavonoids, carnosol, and rosmanol, exhibited remarkably strong antioxidant activity (El Nekeety 2011; Miura and others 2002). Damasius and others (2011) evaluated oregano (*Origanum vulgare*) for its antioxidant properties and ability to reduce the PhIP content almost by half. Another important spice is ginger (*Zingiber officinale*) which has been used traditionally to treat gastric ailments. It possesses phytochemicals such as zingerone and 6-gingerol which have shown free radical scavenging activity (Haniadaka and other 2013) along with its usage in marinades inhibiting HCA formation (Viegas and others 2012).

Black pepper (*Piper nigrum*) is the oldest and most widely used spice in the world, making it the king of spices (Nisha and others 2009). Black pepper initially gained prominence because of its pharmacological properties for digestion and improving appetite, as well as curing colds, coughs, and diseases of the throat, and intermittent fevers and malaria (Singletary 2010). Black pepper belongs to the Piperaceae family, and is native to India and grown in the tropical evergreen forest of the Malabar region of southern India and is also found in Vietnam, Lampong, and Brazil (Singletary 2010). It is appreciated for both its aroma and its pungency, one of the oldest spices in both the culinary and medicinal fields (Kapoor and others 2009). Black pepper

along with rosehip, nutmeg, cinnamon, and oregano may serve as potential dietary sources of natural antioxidants that could improve human nutrition and health (Su and others 2007).

There are two main components of black pepper, the volatile oil and the pungent compounds. The pungent compound in black pepper is primarily composed of 1.9 – 3.5% piperine (Zachariah and Parthasarthy 2008). Piperine content is directly related to black pepper's pungency (Reineccius 2004). Piperine is an alkaloid with the molecular formula of $C_{17}H_{19}O_3N$, which on hydrolysis with alkali gives piperic acid and piperidine (Majeed and Prakash, 2000). It is one of the active compounds in black pepper that has analgesic, antipyretic, and anti-inflammatory properties (Nisha and others 2009). Other major chemical components present in black pepper are sabinene (19.4%), limonene (17.5%), and caryophyllene (14.7%) (Lawrence 1981).

Black pepper has also shown favorable antimutagenic and anticarcinogenic properties, based on the Ames test (Deans 2001). Gülcin (2005) used water and ethanol extracts of black pepper to identify its antioxidant and radical scavenging activities. Oz and Kaya (2011) studied black pepper for its HCA inhibiting capacity in high-fat meatballs and reported a 100% reduction in the PhIP concentration with black pepper treated meatballs compared to the control. Limited studies have been performed on black pepper and its effects on HCA inhibition and published studies are not conclusive about which active component gives black pepper its HCA inhibiting property.

Therefore, the objective of this study was to determine the efficacy of black pepper, and several other spices (individually and in combination with black pepper) to inhibit PhIP formation in beef patties when cooked at 400°F.

Material and Methods

Materials

Seven spices (black pepper, turmeric, rosemary, thyme, ginger, cinnamon, and oregano), finely powdered, were provided by McCormick Science Institute (Baltimore, MD, U.S.A). The HCAs standards MeIQx (2-amino-3, 8-dimethylimidazo [4,5-*f*]quinoxaline), DiMeIQx (2-amino-3, 4, 8-trimethyl-3H-imidazo[4,5-*f*]quinoxaline), TriMeIQx (2-amino-3, 4, 7, 8-tetramethyl-3H-imidazo [4,5-*f*]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine) were obtained from Toronto Research Chemicals (Ontario, Canada).

Ethyl acetate, hydrochloric acid, hexane, acetone, acetic acid, methanol (HPLC grade), ammonium hydroxide, sodium carbonate, and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). Ammonium acetate and triethylamine were purchased from Aldrich Chemicals (Milwaukee, WI, U.S.A.). Phosphoric acid was obtained from Sigma-Aldrich Chemicals (St. Louis, MO, U.S.A.). Deionized water was processed by a Sybron/Barnstead PCS unit (Barnstead/Thermolyne, Inc.; Dubuque, IA, U.S.A.). The solid-phase extraction Extrelut NT 20 columns and diatomaceous earth refill material were purchased from VWR International (Bristol, CT., U.S.A.). Bond Elut propyl-sulfonic acid (PRS) cartridges, C-18 cartridges, and the coupling adaptors were purchased from Varian Sample Preparation (Harbor City, CA., U.S.A.). In addition, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu's reagent, sodium carbonate, and gallic acid were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A).

Preparation of Ground Beef Patty Samples

Fresh ground beef (90% lean) was obtained from a local supermarket in Manhattan, KS, (U.S.A). Fat and moisture was determined for the raw ground beef by rapid microwave drying

and nuclear magnetic resonance using the CEM Smart Trac system (CEM Corporation, Matthews, NC, U.S.A). Crude protein was determined with a LECO FP-2000 protein analyzer (Leco Corp, St. Joseph, MI, U.S.A). Sixteen treatments were designated, including a control. Turmeric, rosemary, thyme, ginger, cinnamon, and oregano were mixed directly with 100 g ground beef at a level of 0.25% and mixed using spatula. Black pepper was added at the following levels: 0.25, 0.5 and 1%. Finally, a combination of 0.25% black pepper and 0.25% of each spice was added to 100 g ground beef for an additional six combinations. The control patty samples were prepared with no spice added. Patties were uniformly prepared using a patty maker and a petri dish (10 cm × 1 cm). The prepared patties were wrapped in aluminum foil and refrigerated overnight at 4 °C. A non-stick electric griddle (Oster, Sunbean Corp., Boca Raton, FL, U.S.A.) was heated to a surface temperature of 400 °F and monitored using a direct-contact thermometer (Branant Company, Barrington, IL, U.S.A.). Spear-point thermocouple temperature probes were inserted horizontally to the midpoint of samples, and the internal temperature was monitored with a data logger (USB-TC model, Measurement Computing, Norton, MA, USA). Patties were fried individually on the griddle at 400 °F for 5 min on each side. Cooked patties were cooled to room temperature, homogenized with a food processor (KitchenAid, model KFP 750), and refrigerated at 4 °C. The extraction step was performed within 24 h of cooking.

Determination of Antioxidant Property of Spices

To study the antioxidant property of the spices, the total phenolic content and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was determined. The total phenolic assay was based on the method described by Chang and others (2006), using Folin-Ciocalteau's reagent. The powdered spice (1.00 g) was mixed with 10 mL of 95% ethanol and shaken on a wrist action shaker (Burrell Shaker model 75, Burrell Corp., Pittsburgh, PA, U.S.A.)

for 2 h at room temperature. Each sample was then filtered using Whatman #4 filter paper. Using 95% ethanol, a 1:5 (v/v) dilution of the extract was prepared. For each dilution, a 0.1 mL sample was mixed with 2 mL deionized water. Then, 0.2 mL of Folin-Ciocalteu reagent was added to the mixture and vortexed. The samples were held at room temperature for 6 min before adding 1 mL of 7.5% sodium carbonate solution. The samples were stored in the dark for 2 h at room temperature. The absorbance for each sample was measured at 765 nm with a Genesys 10 Vis spectrophotometer (Madison, WI, U.S.A). A standard curve was evaluated for a range of 25 – 250 μg/mL and reported as mg gallic acid equivalents per 1.0 g dried spice.

The DPPH radical scavenging activity of the spices was determined based on the method described by Singh and others (2002). Each spice (0.20 g) was mixed with 100 mL of 95% ethanol and shaken on a wrist action shaker for 2 h at room temperature. The sample was filtered through Whatman #4 filter paper, and then the extract was diluted to 1, 5, 20, 50, and 100 mg/mL with ethanol. An aliquot of 0.1 mL of each dilution was mixed with 2.9 mL of freshly prepared 0.1 mM DPPH methanolic solution, vortexed, and then stored in the dark for 30 min. Controls were prepared using 0.1 mL of 95% ethanol instead of spice extract and 2.9 mL of DPPH solution and subjected to same treatment. Ethanol (95%) was used as the blank. The absorbance of samples and controls was measured at 517 nm with a Genesys 10 Vis spectrophotometer (Madison, WI, U.S.A). The percentage radical scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})/\text{Abs}_{\text{control}}] \times 100$$

The Oxygen Radical Absorbance Capacity (ORAC) of the powdered spices was studied by extracting them using the method described by Wu and others (2004) with slight modifications. The extracted samples were then analyzed for ORAC assay using a Synergy™

HT system by BioTek (Winooski, VT, U.S.A). The Lipophilic- ORAC (L-ORAC) as well as the Hydrophilic-ORAC (H-ORAC) was performed using 0.5 g of the powdered sample which was first extracted with 10 ml of hexane, vortexed for 2 min. The extract was then centrifuged at 4000 rpm for 10 min. The hexane extract was dried under nitrogen flow in a 30 °C water bath, and the residue was reconstituted with 10 mL of acetone. After centrifugation this supernatant was used for L-ORAC analysis.

A similar hexane extract was prepared for H-ORAC with further extraction steps of adding 10 ml of acetone:water:acetic acid (70:29.5:0.5 v/v) in a 15 ml screw capped tube. The tube was vortexed for 30 sec and then sonicated at 37 °C for 5 min, shaking twice in the middle of sonication. The tube was kept at room temperature for 10 min. The tubes were vortexed for 30 s after 5 min. Ten minutes later, the tube was centrifuged at 4000 rpm for 10 min and the supernatant was removed. This extraction was performed twice, and the supernatants were combined. The combined supernatant was transferred to a 25 mL volumetric flask, and acetone:water:acetic acid was added to make the final volume 25 mL. This solution upon dilution was used for performing the H-ORAC assay.

Extraction and Analysis of HCAs

The HCAs were extracted and purified from meat by using the method described by Gross and others (1992) with slight modifications. The homogenized cooked beef patty (3.0 g) was mixed with 9 mL of 1 M NaOH in a commercial Waring blender (ThermoFisher Scientific Co., Pittsburgh, PA, U.S.A.) for 3 min and the homogenate was mixed with 18 g of Extrelut packing material (Merck, Darmstadt, Germany). The blender bowl was washed thoroughly with 3 mL of 1 M NaOH and added to the homogenate. This Extrelut packing material was filled in

an empty Extrelut 20 column. The HCAs were eluted from the Extrelut columns with 60 mL ethyl acetate (Borgen and others 2001) into a PRS cartridge pre-conditioned with 5 ml ethyl acetate with pressure, followed by 2 ml ethyl acetate without pressure. The PRS cartridge was then dried under vacuum for 4 min at 15 mm Hg. The PRS column was rinsed with 6 mL of 0.1 M HCl, 15 mL of methanol/0.1 M HCl (45:55 v/v), and 2 mL of distilled water to remove the nonpolar HCAs. The HCAs were eluted from the PRS cartridge with 20 mL of 0.5 M ammonium acetate pH 8 into 100-mg C-18 cartridges preconditioned with 5 mL of methanol and 5 mL of water. The C-18 cartridge was then rinsed with 2 ml distilled water followed by drying with N_2 pressure at 10 mm Hg for 10 min. The final step of elution was washing the dried C-18 cartridges with 1 mL of methanol/ammonium hydroxide (9:1, v/v) into a vial at 15 mm Hg pressure. The HCA extract was concentrated until dry under a stream of nitrogen and dissolved in 25 µL of methanol containing 250 ppb of TriMeIQx as an internal standard before being injected into the HPLC.

The HCAs were analyzed on a HP1090A Series II HPLC (Agilent Technologies, Palo Alto, CA, U.S.A) coupled to a photodiode array UV-visible detector (HP 1040) and a HP 1046A fluorescence detector. The column used was a TSK-gel Super ODS column, reversed-phase C-18, 10 cm x 4.6 mm I.D. (2 µm particle size) (Tosoh Bioscience, Stuttgart, Germany). The mobile phase was 0.01 M triethylamine, pH 3.0, 0.01 M triethylamine, pH 4.0, and acetonitrile as the gradient (Table 2-1; Gibis 2009). Adjustments to pH were made with phosphoric acid. The UV detector was set at 252 nm for MeIQx, DiMeIQx, and the fluorescence detector was run at excitation/emission wavelengths of 229 and 437 for PhIP. Spectral matching was performed using the HP 9000 series 300 Chemstation to confirm the identity of the compounds.

Table 2-1 HPLC mobile phase gradient using three phases in bottles A, B and C.

Time, min	Eluent A (TEA pH 3.0), %	Eluent B (TEA pH 4.0), %	Eluent C (Acetonitrile), %
0	46.5	50	3.5
5.5	35	40	25
6	25	40	35
6.5	15	50	35
11	10	55	35
12	0	25	75
16	46.5	50	3.5
20	46.5	50	3.5

Quantitation, Recovery, and Spectral Matching

HCA concentrations were measured as suggested by Lindsay (1992) using an internal standard method to compensate for variations in injection volume and also for any detector sensitivity changes. A known amount of TriMeIQx (used as internal standard) was added to samples before they were injected into the HPLC. The standard curve was plotted by analyzing different concentrations (25, 50, 100, 250, 500, and 1000) of the prepared pure PhIP standard with a coefficient of determination (R^2) of 0.9991.

Ultra Violet (UV) spectral matching to a spectral library was made from pure standard with match factors typically observed at 95% or higher. MeIQx, DiMeIQx were not found in detectable amounts. The average recovery for PhIP was around 30%. The recovery of PhIP is in agreement with previous studies (Oz and Kaya 2011; Gibis and Weiss 2012) where no detectable

levels were found. Puangsombat and others 2011 could not detect DiMeIQx levels, similar to the results obtained in this study.

Statistical Analysis

SAS (version 9.1, SAS Inst. Inc., Cary, NC, U.S.A.) was used to analyze the triplicate sets of data. The experiment was a randomized complete block design. An analysis of variance (ANOVA) was performed to determine significant differences among treatments at $P < 0.05$. Correlation and regression analysis was used to determine the relationship between the antioxidant property (total phenolic content and DPPH radical scavenging activity) and inhibition of HCA formation.

Results and Discussion

DPPH, Total Phenolics and ORAC

The seven spices, black pepper, turmeric, cinnamon, ginger, thyme, oregano, and rosemary were studied for their antioxidant properties and their effect on HCA formation. Figure 2-1 illustrates the concentration-dependent DPPH scavenging activity of the seven spices. Rosemary, turmeric, thyme, cinnamon, and oregano initially increased in DPPH activity, but after a certain concentration the scavenging activity became concentration independent. Black pepper and ginger showed increase in DPPH activity with increasing concentration from 0-100 mg/ml. The DPPH scavenging activity of the six spices at 50 mg/mL is summarized in Table 2-2. Rosemary had the highest DPPH scavenging activity (93.6%), followed by oregano and cinnamon having similar scavenging activity (around 90%). Thyme (88.5%), turmeric (87.7%), and ginger (84.0%) had similar high DPPH scavenging activity; whereas, black pepper showed significantly the lowest DPPH scavenging activity with 28.3% ($p<0.05$).

Similar DPPH scavenging activity has been seen in rosemary (95.7%), turmeric

(92.5%), cinnamon (86%), ginger (85%), oregano (89%), thyme (92%) and black pepper as (48%) in previous studies (Puangsombat and others 2011, Asimi and others 2013, Muchuweti and others 2007, Wang and others 2008, Gulcin 2005).

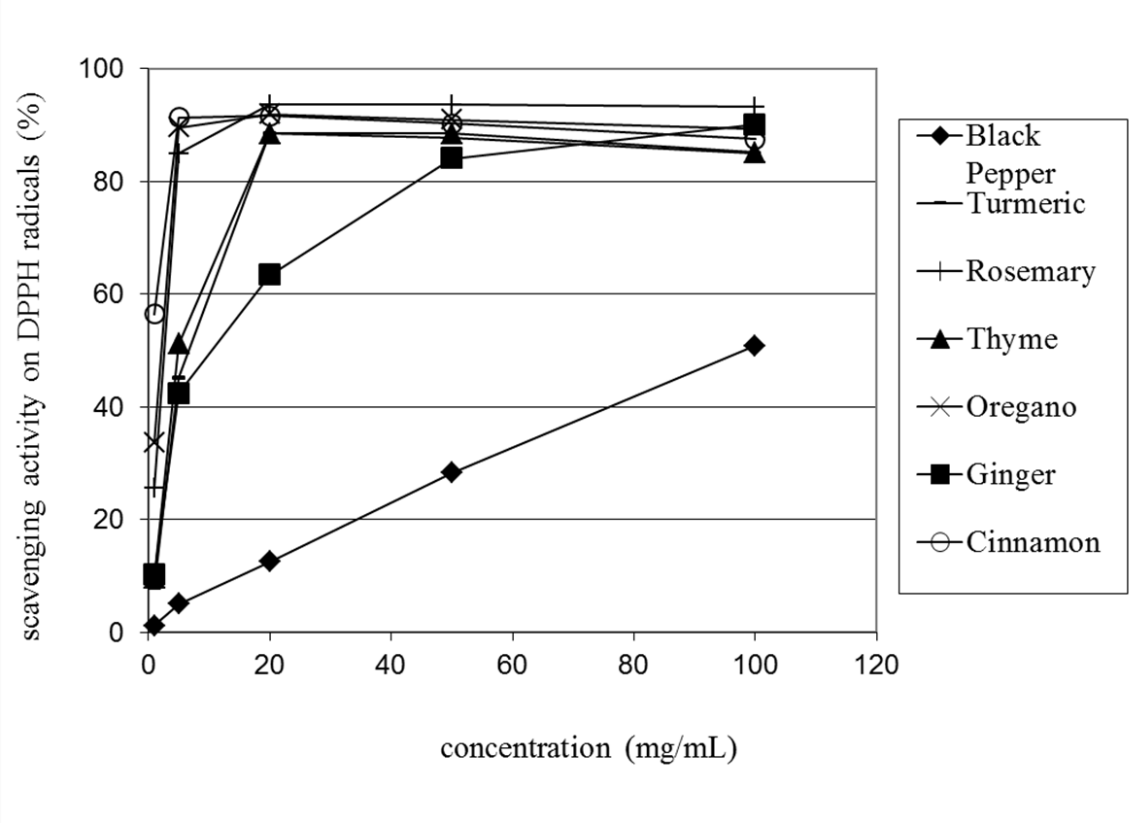

Figure 2-1 DPPH scavenging activity of the seven spices at concentrations from 0 to 100 mg/ml.

Table 2-2 Total phenolic content and scavenging effect on DPPH radicals present in spices.

Spice	Total Phenolic content (mg gallic acid equivalents/g dry weight)	Scavenging effect on DPPH radicals (%)[A]
Black Pepper	13.78 ± 0.46 [c]	28.31 ± 2.49 [e]
Turmeric	52.78 ± 3.41[ab]	87.68 ± 1.79 [cd]
Rosemary	55.42 ± 1.62 [a]	93.57 ± 0.54 [a]
Thyme	19.62 ± 0.51[c]	88.49 ± 0.33 [c]
Oregano	54.26 ± 0.72 [a]	90.90 ± 0.50 [b]
Ginger	20.84 ± 1.86 [c]	84.04 ± 0.52 [d]
Cinnamon	46.22 ± 1.24 [b]	90.28 ± 0.43 [b]

Means with different superscript letters within the same column are significantly different ($p<0.05$). n=3

[A] Scavenging effect on DPPH radicals of spices at concentration of 50 mg/mL.

Total phenolic content of these spices was also determined because phenols are the major groups of compounds that act as primary free radical scavengers (Moreno and others 2006; Tabart and others 2009). The total phenolic content varied greatly among the seven spices, ranging from 13.8 to 55.4 mg GAE/g dw. Ginger (20.84 mg GAE/g dw), thyme (19.62 mg GAE/g dw), and black pepper (13.78 mg GAE/g dw) had significantly ($p<0.05$) lower total phenolic content than rosemary (55.42 mg GAE/g dw), oregano (54.26 mg GAE/g dw), turmeric

(52.42 mg GAE/g dw), and cinnamon (46.22 mg GAE/g dw) (Table 1-2). Overall, spices containing a high amount of total phenolic content had high scavenging activity except ginger. Black pepper had significantly the lowest value ($p<0.05$) in total phenolic content as well as DPPH scavenging activity indicating low antioxidant property of black pepper compared to the other spices.

The L-ORAC values observed are as shown in Table 2-3. Wu and others (2004) reported the L-ORAC values to be as, turmeric (1193.46 μmol TE/g), oregano (169.88 μmol TE/g), cinnamon (34.53 μmol TE/g), black pepper (88.13 μmol TE/g), and ginger (1.43 μmol TE/g). The H-ORAC spice extract concentrations were too high to be read by the micro plate analyzer.

Table 2-3 L-ORAC (μM TE/g) values of spices.

Treatment	L-ORAC (μmol TE/g)	L-ORAC (μmol TE/g) Literature Values
Black Pepper	5527	88 [a]
Rosemary	10981	19 [b]
Cinnamon	3314	34 [a]
Oregano	10865	170 [a]
Turmeric	2105	1193 [a]
Thyme	3508	19 [b]
Ginger	181	1 [a]

[a] Wu and others (2004), [b] Zheng and Wang (2001).

The spice blends and values obtained from McCormick Science Institute were also compared for total phenolic content which were very much similar to the observed values in this study except for cinnamon (Table 2-4). The L-ORAC values were not in agreement. The discrepancies in the observed values could be due to different assay methods and techniques. Black pepper was observed to possess the lowest total phenolic content in both studies.

Table 2-4 Total phenolic content and ORAC values of spices (McCormick Science Institute, 2009, Sample characterized library).

Spice	Total Phenolic (mg GAE/g)	L-ORAC (μmol TE/g)	H-ORAC (μmol TE/g)	Total ORAC (μmol TE/g)
Black Pepper	8.2	237	187	424
Rosemary	49.8	518	1186	1704
Cinnamon	105.3	25	1875	1900
Oregano	63.6	157	2500	2657
Turmeric	33.9	1097	735	1832
Thyme	44.7	327	1310	1637
Ginger	10.2	202	62	264

Individual Spice Treatment

The beef patties used in this study were prepared with locally available meat and consistent size, dimensions, and weight. The ground beef used to make the patties contained 69.45% moisture, 19.96% crude protein, and 9.33% fat. The cooking temperature used was 204 °C (400 °F), and the patty was cooked for 5 min on each side producing medium-well-done cooked patties. The internal temperature of the patties reached a temperature of around 155-160 °F. All the patties were detected for the presence of PhIP with the control having highest concentration of 2.64 ng/g. MeIQx and DiMeIQx were in non-detectable quantities in all samples. These results were similar to those reported by Knize and others 1994; Balogh and others 2000; Gibis 2009.

In the individual spice study 0.25% concentration of each spice was added to the patty, and was extracted for PhIP. Table 2-3 summarizes the PhIP (ng/g) content in each spice treatment and the percentage inhibition observed. The descending order of PhIP content was as follows: ginger (1.15 ng/g), turmeric (1.14 ng/g), thyme (0.97 ng/g), oregano (0.87 ng/g), cinnamon (0.58 ng/g), rosemary (0.54 ng/g) and black pepper (0.47 ng/g), with no significant difference ($p<0.05$) among each spice treatment. Black pepper showed the highest percentage inhibition (81.8%) compared to the other spice treatments.

Table 2-5 Effect of individual spices at 0.25% level concentration on PhIP levels (ng/g) in beef patties cooked at 204 ˚C (400 ˚F) for 5 min on each side (mean ± SE).

Treatment	PhIP (ng/g)	% inhibition
Control	2.56 ± 0.35 [a]	
Black Pepper	0.47 ± 0.02 [b]	81.8 %
Rosemary	0.54 ± 0.01 [b]	78.9 %
Cinnamon	0.58 ± 0.12 [b]	77.3 %
Oregano	0.87 ± 0.34 [b]	66.2 %
Turmeric	1.14 ± 0.32 [b]	55.5 %
Thyme	0.97 ± 0.80 [b]	62.0 %
Ginger	1.15 ± 0.42 [ab]	55.2 %

Means with different superscript letters within the same column are significantly different at ($p < 0.05$). n=3

Similar findings were seen by Murkovic and others (1998) where rosemary and thyme showed a 75% decrease in PhIP content in fried meat. Smith and others (2008) observed up to an 88% decrease in total HCA content when commercial marinades rich in spices such as thyme and rosemary were used on grilled beef steaks. Puangsombat and others (2011) found that 0.2% turmeric had a 40% inhibition in HCAs in ground beef patties cooked at 400 °F for 5 min on each side. Oregano at 0.2% levels showed PhIP concentrations of 0.38 ng/g in cooked beef

(Damasius and others 2011). Viegas and others (2012) studied ginger containing marinades and showed they inhibited PhIP formation in pan-fried beef by 30%.

Black Pepper in Combination with Other Spices

Combining spices (0.25% individually) with black pepper (0.25%) decreased PhIP content significantly ($p<0.05$) compared to individual spices (Table 2-1). PhIP content in the combinations as shown in Table 2-4 were found to be as: black pepper with oregano 0.77 ng/g (71.8% inhibition), black pepper with ginger 0.65 ng/g (76.0% inhibition), black pepper with thyme 0.51 ng/g (81.9% inhibition), black pepper with rosemary 0.43 ng/g (84.2% inhibition), black pepper with cinnamon 0.41 ng/g (84.9%), and black pepper with turmeric 0.14 ng/g (94.7% inhibition). All combinations showed high inhibition of PhIP, with the highest inhibition of 94.7% in the black pepper-turmeric combination.

Studies involving grilled beef steaks treated with spice-containing marinades rich in polyphenols, resulted in PhIP percentage inhibition (Smith and others 2008). Nerurkar and others (1999) also observed 45% inhibition in PhIP concentration when Asian marinades containing spices such as turmeric and garlic were used to marinate beef steaks.

Table 2-6 Effect of combination of spices (0.25% black pepper with 0.25% of the other spice) on PhIP content (ng/g) in beef patties cooked at 204 ˚C (400 ˚F) for 5 min on each side (mean ± SE).

Treatment	PhIP (ng/g)	% Inhibition
Control	2.71 ± 0.41 [a]	
Black Pepper +Rosemary	0.43 ± 0.15 [b]	84.2%
Black Pepper + Cinnamon	0.41 ± 0.25 [b]	85.0 %
Black Pepper + Oregano	0.77 ± 0.53 [b]	71.8 %
Black Pepper + Turmeric	0.14 ± 0.07 [b]	94.7 %
Black Pepper + Thyme	0.51 ± 0.30 [b]	81.1 %
Black Pepper + Ginger	0.65 ± 0.12 [b]	76.0 %

Means with different superscript letters within the same column are significantly different at ($p<0.05$). n=3

Black Pepper Treatment at Different Concentrations

The concentration effect of black pepper on HCA inhibition was studied by using three concentrations (0.25%, 0.50%, and 1%). The three concentrations of black pepper were seen significantly ($p<0.05$) different from the control (3.03 ng/g). The amount of PhIP found in 0.25% black pepper was 0.47 ng/g (84.6 % inhibition); whereas, 0.50% black pepper had similar PhIP levels of 0.45 ng/g (85.0 % inhibition), but black pepper at 1% level had significantly higher ($p<0.05$) PhIP concentration of 1.16 ng/g (61.8 % inhibition) compared to 0.25% and 0.50% black pepper treatments (Table 2-5). Lan and others (2004) explained a similar concentration

effect, where a decrease in percentage inhibition of HCA was observed after a certain increase in spice concentration.

Table 2-7 Effect of black pepper levels on the formation of PhIP in beef patties cooked at 204 ˚C (400 ˚F) for 5 min on each side (mean ± SE).

Treatment	PHIP (ng/g)	% Inhibition
Control	3.03 ± 0.36 [a]	
0.25 % Black Pepper	0.47 ± 0.02 [b]	84.6 %
0.50 % Black Pepper	0.45 ± 0.07 [b]	85.0 %
1% Black Pepper	1.16 ± 0.08 [c]	61.8 %

Means with different superscript letters within the same column are significantly different at ($p < 0.05$). n=3

In the limited literature on black pepper and its effects on HCA formation, Oz and Kaya (2011) studied high fat meatballs treated with 1% dry black pepper for HCA formation. The control (meatball with no added spice) sample was observed to have PhIP levels of 31.80 ng/g. At a cooking temperature of 225 °C PhIP was not detected in the meatballs. The effects of black pepper were studied on high-fat meatballs but the effect varies depending on the meat cooking temperature and the type of HCA (Oz and Kaya 2011).

Correlation Analysis between Antioxidant Property and HCA inhibition

A correlation and regression analysis was performed to determine the relationship between HCA inhibition in cooked beef patties to which spices were added and the scavenging activity of spices, as well as the relationship between HCA inhibition and total phenolic contents

and DPPH scavenging activity of spices. No correlation was observed between percentage HCA inhibition and total phenolic content or DPPH scavenging activity (Figure 2-2).

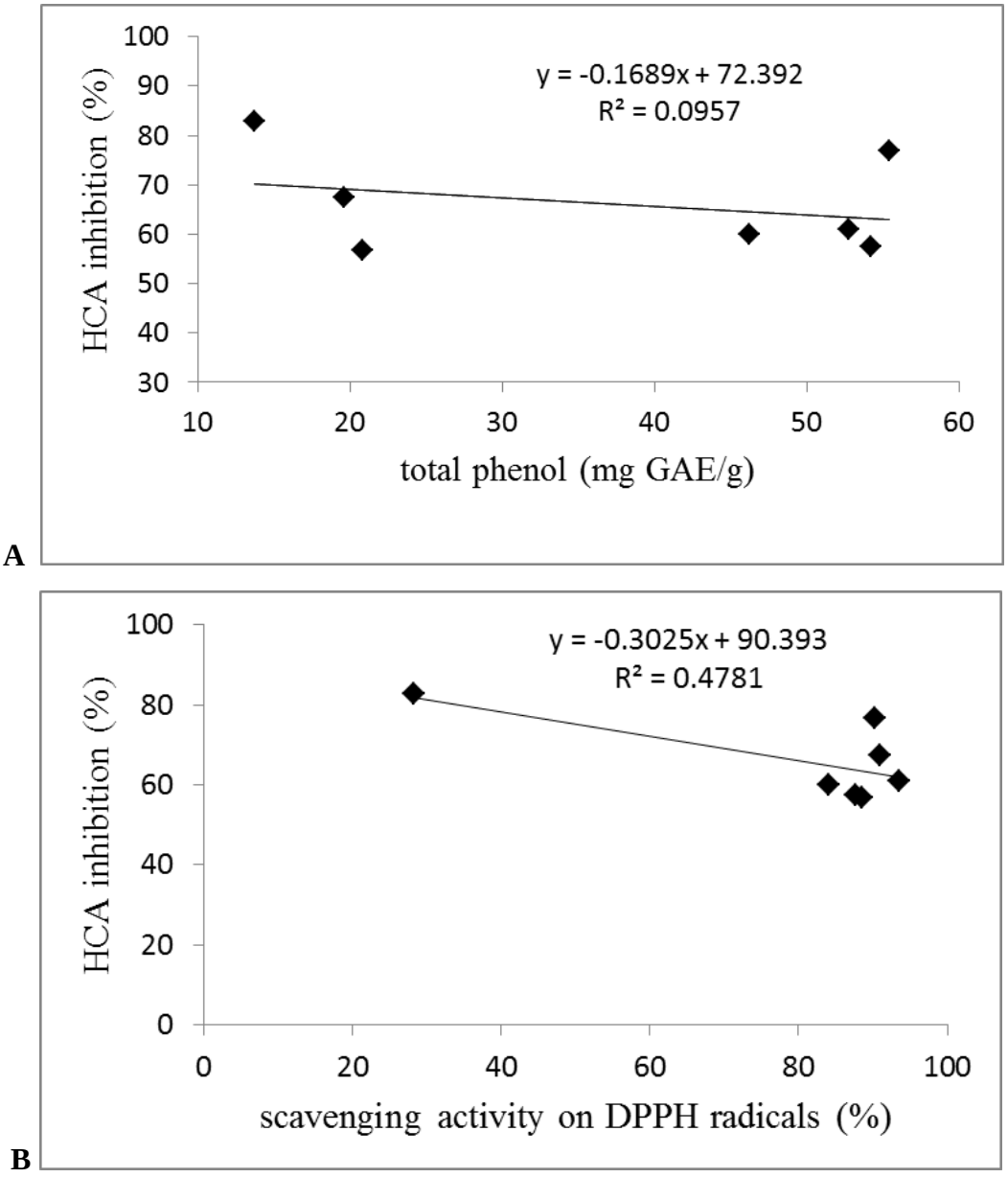

Figure 2-2 Correlation analysis between the percentage HCA inhibition in cooked patties with spice treatments and (A) total phenolic activity, (B) scavenging activity on DPPH radicals of spices.

Similar results with no correlation between HCA inhibition and antioxidant capacity were seen by previous researchers (Viegas and others 2012, Damasius and others 2011). Cheng and others (2008) hypothesized naringenin a flavonoid found in citrus fruits having low radical scavenging activity as compared to many polyphenols but 50% higher HCA inhibiting capability. Naringenin was hypothesized to be forming adducts with phenylacetaldehyde inhibiting the formation of PhIP. A similar chemical reaction may occur between black pepper components, inhibiting the formation of PhIP in a similar fashion.

Conclusion

Black pepper showed the highest percentage inhibition of PhIP, even when it has far less antioxidant properties than rosemary, turmeric, oregano, and cinnamon. Further studies on the composition and constituents of black pepper would help explain how black pepper inhibits HCAs formation. Because pepper is readily available and commonly used around the world, cooking with black pepper to inhibit HCAs would benefit consumers and the meat industry.

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Chapter 3 - Model Systems and Heterocyclic Amines

Abstract

This chapter focuses on the importance to study model systems and heterocyclic amines (HCAs). As per literature review, model systems are an easier approach compared to meat systems to study the formation of HCAs, factors affecting their production, and the means to inhibit their formation. In this research, a model system was tried to evaluate HCAs, using precursors such as glucose, glycine, creatinine, and phenylalanine. The precursors were used at specific concentrations to mimic the meat system. The reaction was carried out in a "MS" micro reactor made of stainless steel at a temperature of 180°C for 30 min. The samples from the reaction were cleaned up and extracted before analyzing them using high performance liquid chromatography. The major HCAs studied were MeIQx, DiMeIQx and PhIP. The identification and quantitation of these HCAs using the model system was unsuccessful due to several possible reasons. The model system equipment was not very functional and leaked during the experiments resulting in silicon oil contamination of the samples. The ensuing sample contamination led to incomplete separation of the HCAs formed. A better reaction vessel, heating conditions and HPLC analysis parameters might help in improving the model systems. If a functional model system was obtained, various factors in the HCA formation and mechanism would be easier to study.

Introduction

The formation of HCAs depends heavily on the amounts of precursors, enhancers, inhibitors, time, temperature, water activity, and pH, since it forms as a result of a chemical reaction. Cooking conditions, specifically heating times and temperatures, influence the yield of heterocyclic amines in model systems and foods (Knize and others 1994; Arvidsson and others 1997). To study the factors affecting HCA formation, a model system may be used. A model system was recently developed and applied to kinetic studies investigating and confirming the mechanism for HCA formation (Knize and Felton 2005). Mixtures of glucose, creatinine and amino acids, such as phenylalanine, glycine, alanine, lysine, methionine, and threonine heated at different temperatures in diethylene glycol and water, and also in some cases in dry conditions, allow the formation of MeIQ, IQ, IQx, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx and PhIP (Chen and others 1999; Skog and others 1992; Johansson and others 1995). Johansson and others (1995) studied different model systems in aqueous solutions of creatinine, glucose, and arginine one of the amino acids frequently found in meat proteins, which were heated at 180 °C for 10 min and showed the presence of the IQx, MeIQx and 7,8-DiMeIQx. In order to investigate and confirm the mechanism of PhIP formation, Murkovic used a model system containing phenylalanine and creatinine in diethylene glycol with the mixture heated at temperatures ranging from 120 to 200 °C (Murkovic and others 1999). The objective of this study was to prepare a model system for analyzing HCAs and to observe the effect of the precursors and inhibitors on HCA formation.

Cooking Methods

The cooking method is believed to affect HCA formation and involves heat transfer and water loss (Skog and others 1995). High temperature means of cooking observed in pan-frying, grilling, and broiling have shown to produce high concentrations of HCAs (Chen and Chiu 1998) compared to oven-roasting and baking (Skog and others 2003) which produce intermediate amounts. Boiling and steaming are normally achieved at temperatures below 100 °C (212 °F) and usually produce low or undetectable level of HCAs (Chen and Chiu 1998).

Temperature and Time of Cooking

Arvidsson and others (1997) examined the effect of heating time and temperature on HCA formations by heating a mixture of creatine, glucose, and amino acids at 150 to 225 °C for 0.5-120 min. The results showed that HCA formation was a time and temperature function as illustrated in Figure 3-1. Both time and temperature were found to have a strong impact on the formation of HCAs (Knize and Felton 2005). Each HCA in a complex meat system is formed differently at certain temperature and time points. Temperature and time are directly proportional to the production of HCAs. They are produced at a temperature of 150 °C and above and are seen to increase as temperature rises. Time at this temperature from when they start formation is around 20 minutes.

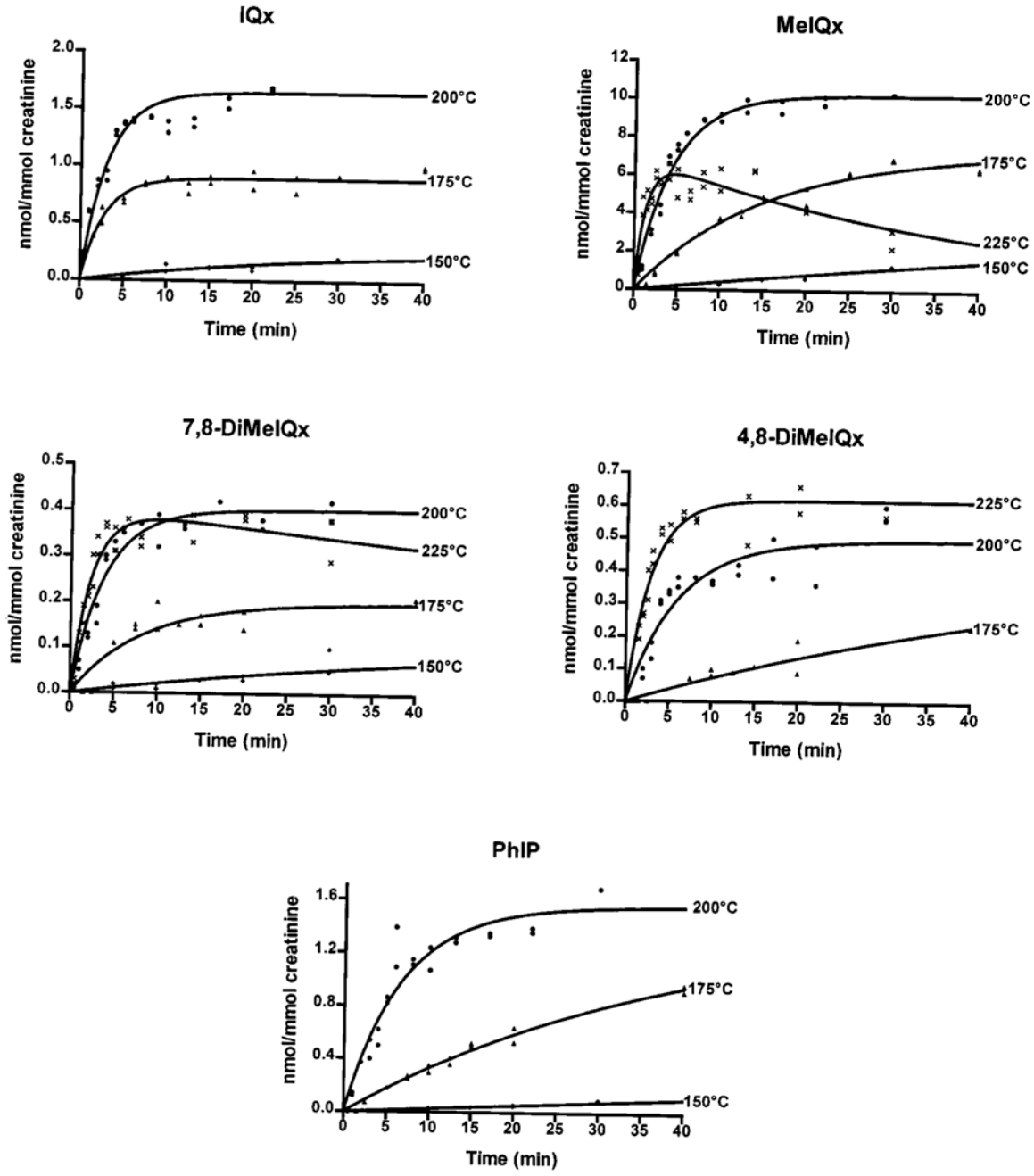

Figure 3-1 Formation of HCAs at 150, 175, 200, and 225 ºC (Arvidsson and others 1997).

Concentration of Precursors

HCAs are formed through complex chemical reactions that depend on the amounts of the main precursors, creatine or creatinine, sugar, and amino acids, as well as intermediates (e.g. Amadori compounds, Strecker dehydration, pyridine and pyrazine) that are present in a meat system.

An increase in the amount creatinine showed an increase in the concentration of PhIP but a decrease in the amounts of IQ and IQx type compounds (Bordas and others 2004). In general high concentration of glucose showed an inhibitory effect on formation of HCAs (Skog and Jägerstad 1990, Skog and others 1998, Skog and others 2000). The addition of glycine, alanine, and phenylalanine in 50-fold amounts to lyophilized meat extract, increased the formation of MeIQx and PhIP (Skog and others 1998). Phenylalanine, leucine, isoleucine, and tyrosine have been reported to be precursor amino acids for the formation of PhIP (Pais and others 1999).

Figure 3-2 shows the results for the six model systems containing precursors corresponding to the levels in the six meats. The cooking conditions were set at a heating time of 30 min and temperatures of 225 °C for the model systems. The error bars correspond to the standard deviations and are ~10%, indicating good reproducibility of the model system, the heating conditions, and the analysis method for the three replicate experiments. In all model systems IQ was detected except for turkey. Although, the concentrations of IQ in the beef and both chicken models were low (<1 ng/g). The highest amount was formed in the fish model. Chicken thigh, pork, and fish model systems, were also detected with MeIQ at very low concentrations (0.3-1 ng/g). Like IQ, the higher concentration was also in the fish model. These results were in accordance with those of Kasai and coworkers (Kasai and others 1980, 1981), who first isolated IQ and MeIQ from broiled fish. A model system of boiled pork juice heated under reflux formed IQ and MeIQ (Lee and others 1994).

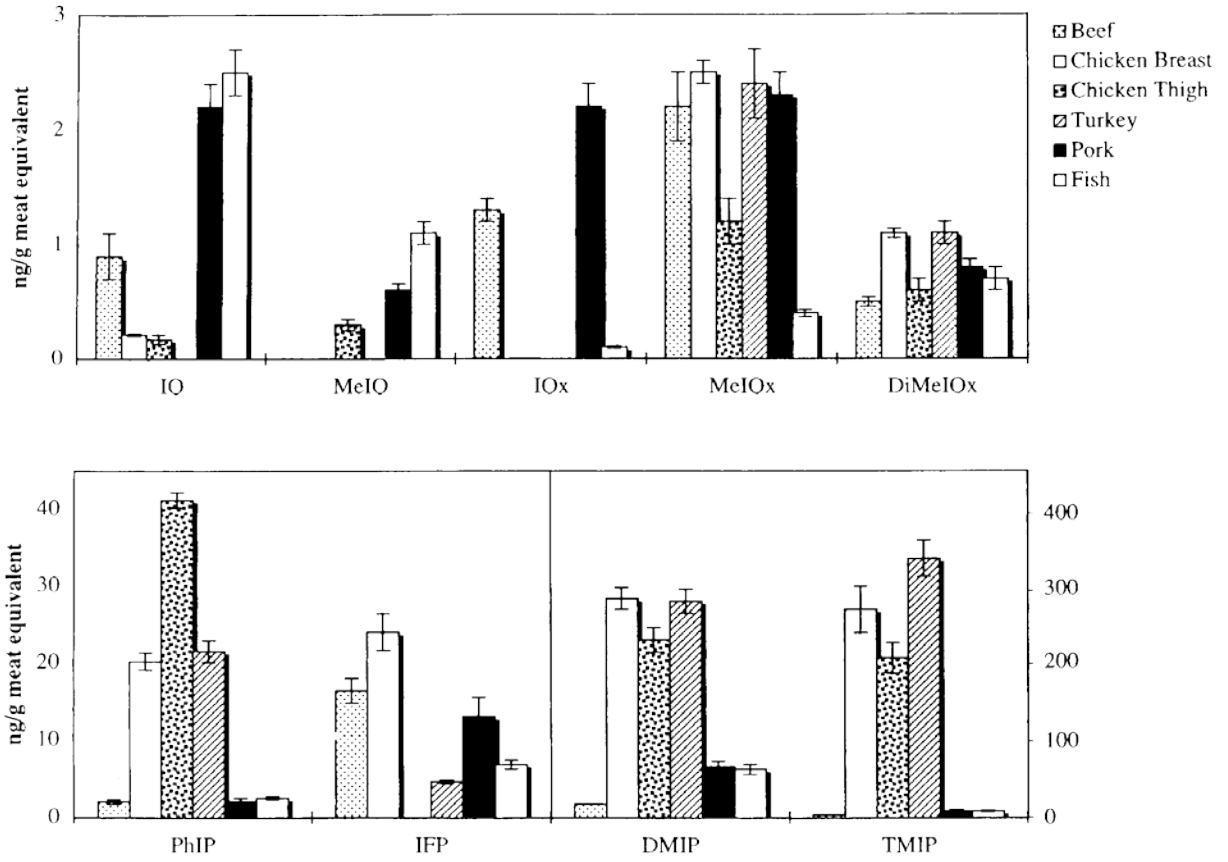

Figure 3-2 Concentrations of heterocyclic amines (ng/g of meat equivalent) found in the six model systems (Pais and others 1999).

A model system of dry-heated beef juice was also reported to form IQ (Taylor and others 1986). Pork and beef model systems due to higher content of arginine were observed to contain IQx, which is only reported to be formed from arginine in a water based model system (Johansson and others 1995). All the model systems showed the presence of 8-MeIQx and 4,8-DiMeIQx. There were no significant differences among the MeIQx concentrations in the model systems, except for the chicken thigh and fish. The unexpectedly low PhIP concentrations in beef and pork may be explained due to the sugar content in these meats. Sugar affects the formation of PhIP, and the concentration decreases with an increase in the amount of glucose (Taylor and others 1987). Aminodimethylimidazofuropyridine (IFP) was formed in all of the models except

the chicken thigh. The concentrations of IFP (4.6-24 ng/g) were comparable to the PhIP concentrations formed in the model. The 2-amino-1,6-dimethylimidazo-[4,5-*b*]pyridine (DMIP) and 2-Amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine (TMIP) concentrations of (4.6-24 ng/g) are in accordance with the earlier studies conducted on them. These concentrations give us an insight to the amount of heterocyclic amines in general in beef, chicken breast, chicken thigh, turkey, pork, and fish.

Moisture and Lipid Content

Increased moisture content showed a decrease in the PhIP content while moisture promoted IQ, IQx formation. Lipid content and its effects still need to be studied more as they show an increase as well as a decrease in the HCA formation.

These factors and parameters can only be efficiently studied using model systems. Figure 3-3 represents a chromatogram of the 10 major heterocyclic amine (HCA) standards after separation by HPLC chromatography. The chromatogram is a reference to compare and quantify the actual amount of HCAs produced when a meat product is cooked. The abundance versus time ratio is specific for each HCA standard as well as the m/z ratio, which helps to identify the HCA. From the chromatogram the order of elution is, DMIP, IQ, MeIQx, MeIQ, 4, 8-DiMeIQx, Tri-MeIQx, Trp-P-2, Trp-P-1, PhIP, AαC, and MeAαC, showing that DMIP, has the least affinity for the column, and carried better in the mobile phase, while AαC has the least affinity towards the mobile phase, and interacts more with the column. Tri-MeIQx is used as the internal standard. All the compounds are eluted between 0 to 30 minutes.

The addition of the HCA standard to the HPLC, of a known concentration (200ng/g in this case) also helps to know the recovery, accuracy and efficiency of the instrument. The Limit

of Detection is calculated using the sound to noise ratio from the standard chromatograms, and when compared to the limit of quantification of the sample, gives the recovery of the sample.

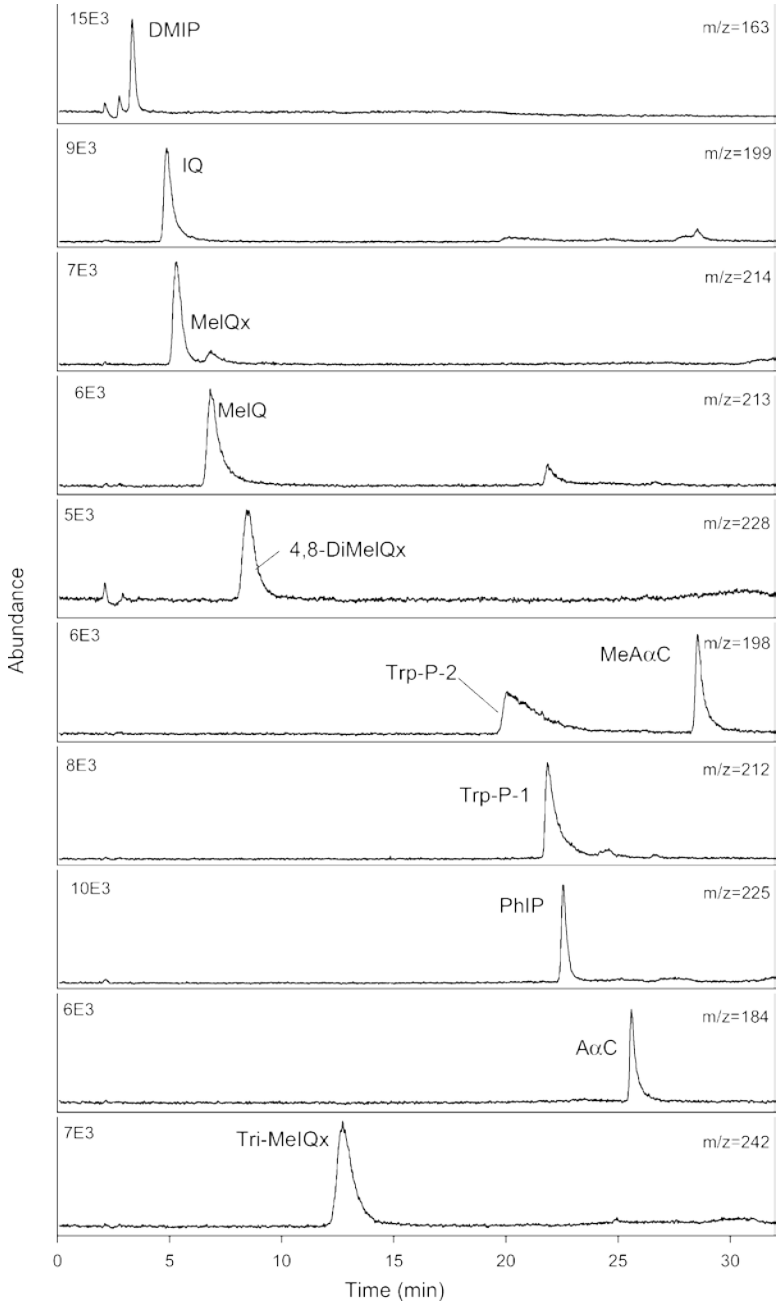

Figure 3-3 LC–MS chromatogram of a solution of 10 different HA standards in MeOH (each 200 ng/g) and TriMeIQx as internal standard (Murkovic and others 2004).

Materials and Methods

The model system was prepared in a stainless steel high pressure "MS" Micro Reactor by High Pressure Equipment Co. (Erie, PA, U.S.A) Figure 3-4. The Micro Reactor was designed for numerous applications including small volume testing of components and miniature scale reaction tests. Standard material of construction is Type 316 stainless steel. The length of the reactor was around 6.5" with an internal diameter of 5/16" and external diameter of 9/16". The capacity of the reactor was 5 ml and rated to withstand a pressure of 20,000 psi. The reactor had a high pressure coned and threaded tubing giving it a good sealing property.

Operation at temperatures up to 800°F is made possible by the metal to metal seal construction. Working pressures should be reduced by approximately 15% at the maximum 800°F temperature level. Glucose (BioXtra; ≥99.5% purity), glycine (≥99% purity), phenylalanine (≥98% purity) and creatinine were purchased from Sigma Chemicals (St. Louis, Missouri, U.S.A.). A High-Temp Bath 160A was used for providing heat from Fisher Scientific (Fairlawn, New Jersey, U.S.A.) with silicone oil type 200.50 from Fluke Hart Scientific (Everett, WA, U.S.A) as a source of heat distributor. The HCAs standards MeIQx (2-amino-3, 8-dimethylimidazo [4,5-*f*]quinoxaline), DiMeIQx (2-amino-3, 4, 8-trimethyl-3H-imidazo[4,5-*f*]quinoxaline), TriMeIQx (2-amino-3, 4, 7, 8-tetramethyl-3H-imidazo [4,5-*f*]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine) were obtained from Toronto Research Chemicals (Ontario, Canada).

Figure 3-4. “MS” Micro Reactor (High Pressure Equipment Co.).

Model System Preparation, Heating, and Extraction

The constitution of the chemical content in the model system was based similar to as suggested by Shin and others (2002). Powdered forms of glucose (0.3 mmol.), glycine (0.6 mmol), phenylalanine (0.6 mmol.) and creatinine (0.6 mmol.) were mixed together in deionized water. For the control, the mixture was prepared with no creatinine in it. Then 5 ml of the mixture solution was added into the MS micro Reactor through one end and then both the ends were tightly sealed using a torque wrench. The reactor was then immersed carefully into the silicone oil bath pre-heated to 180 °C. The temperature of the bath was monitored using a thermocouple and the reactor was immersed in a manner to absorb equivalent heat from all directions. After the given time the reactor was taken out of the oil bath and cooled with crushed ice. The contents from the reactor were then carefully transferred to a glass vial. The contents were then extracted by the method described by Gross and Grüter (1992) with slight modifications. Heated sample around 3 ml was mixed in 12.0 mL of 1 M sodium hydroxide and mixed with Extrelut refill material (approx. 20 g) and placed in a 20 mL Extrelut column. After filling, each Extrelut column is connected to PRS cartridges and the HCAs are eluted with ethyl

acetate. Then the PRS cartridges were rinsed with hydrochloric acid, and connected to C-18 (100 mg) cartridges to concentrate polar HCAs. The adsorbed HCAs were eluted into vials using a methanol ammonium hydroxide solution. The collected samples were dried under nitrogen for several hours and dissolved in 25 µL of methanol.

Results and Discussion

The model system was evaluated for studying the different factors influencing HCA formation; time, temperature, and concentration of precursors. A standard run of 250 ppb of mixed HCA standards (MeiQx, DiMeIQx, TriMeiIQx and PhIP) was injected into the HPLC system and the chromatogram as observed in Figure 3-5 was obtained. MeIQx, DiMeIQx, TriMeiIQx were observed in the UV range at retention times of 3.838, 4.683, and 5.414 min, respectively. PhIP was observed in the fluorescence range at a retention time of 6.247 min. A lot of noise was observed in the chromatogram, which could be due to the HPLC instrumental analysis or contamination of the standard.

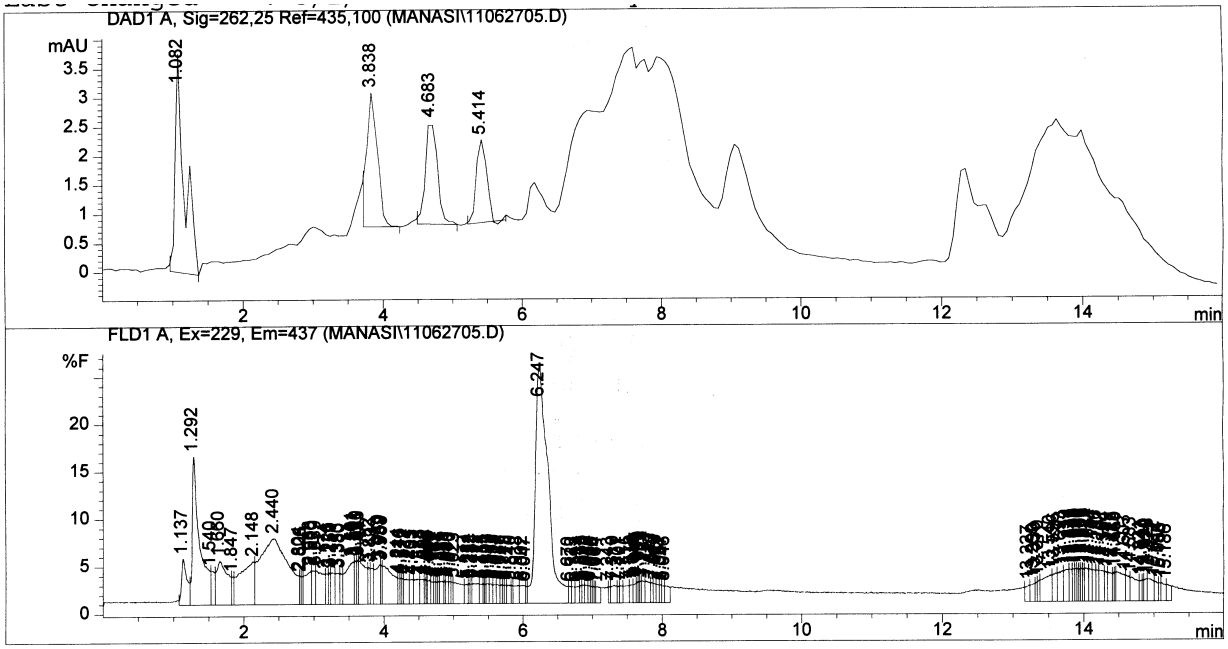

Figure 3-5. HPLC chromatogram of 250 ppb mixed standard containing MeIQx, DiMeIQx, TriMeiIQx and PhIP which was separated on a TSK-gel Super ODS column as described in Chapter 2 (page 44). The UV detector was set at 252 nm for MeIQx, DiMeIQx, and TriMeIQx detectios, and the fluorescence detector was programmed accordingly to the excitation/emission wavelengths of 229 and 437 nm for PhIP. The injection volume of the sample was 20 µl.

After the standards the model system samples were injected into the HPLC for identifying and quantitating the HCA content. Figure 3-6 illustrates one of the chromatograms observed upon injection of a model system sample heated at 180 °C for 30 min.

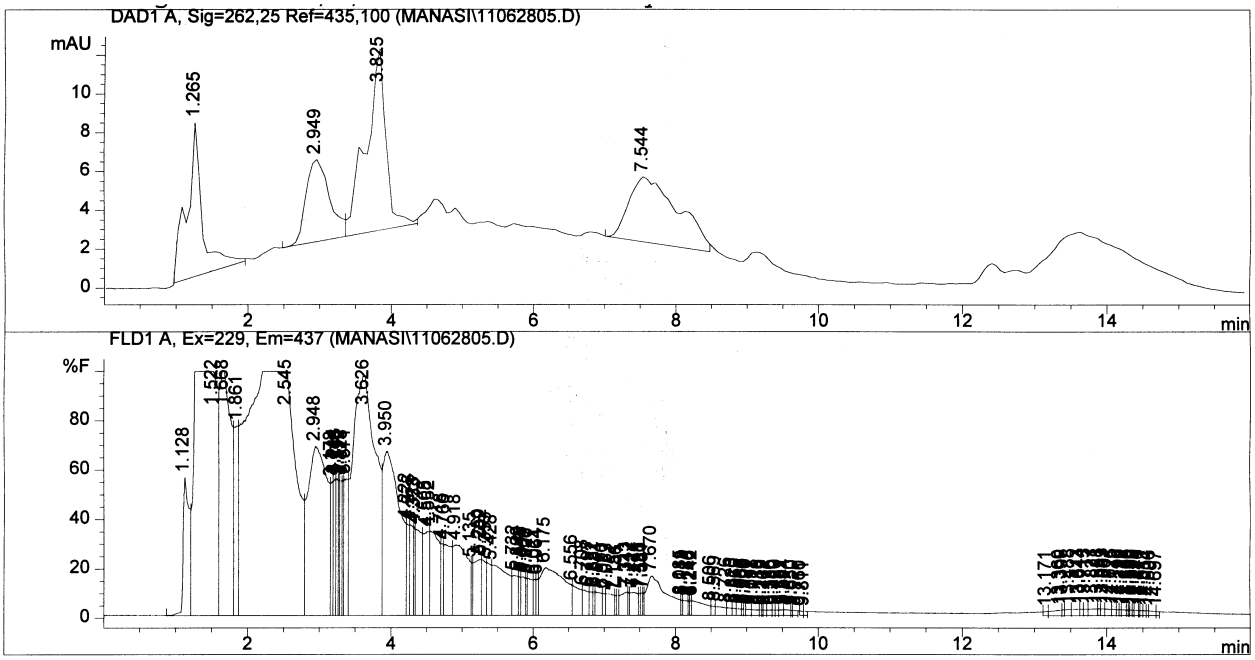

Figure 3-6. HPLC chromatogram of model system sample (heated at 180 °C for 30 mins) which was separated on a TSK-gel Super ODS column as described in Chapter 2 (page 44). The UV detector was set at 252 nm for MeIQx, DiMeIQx, and TriMeIQx detections, and the fluorescence detector was programmed accordingly to the excitation/emission wavelengths of 229 and 437 for PhIP. TriMeIQx (250 ppb) was used as internal standard. The injection volume of the sample was 20 µl.

A number of difficulties were approached in the process of functioning the model system. The reactors due to not being air tight led to the contamination of the contents inside them with silicone oil from the bath. Each sample was observed to be very dark (caramelized) in color with even a few of them having floating particles. This could have been due to the insolubility of the precursors before the reaction. Also, after extraction and clean up of the samples the spectra observed on the HPLC had a lot noise which made it not possible to detect the HCAs. Overall, the model system was not successfully functional during the research unlike previous researchers.

Conclusion

This research attempted to study heterocylic amines using model systems. The model system prepared was cumbersome to operate. The quantitation of the HCAs was not possible due to the difficulty in the clean up of the sample which led to unclear chromatograms and non-identification of the HCAs. Further studies on the model system would be helpful where an easy to operate model can be functioned. The extraction method for the sample clean up is also complex and a short method for the same can help obtain results faster.

The results obtained by a successful model system can be extrapolated to the meat and then applied in the meat industry or in general while cooking meat. These HCA and their related studies can be much easier, faster, and simpler to perform when conducted in a model system compared to the meat. Using meat involves cost, wastage of meat, and a much more complicated system to work with. The extraction and recovery of the HCAs produced can be better when using a model system.

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Appendix A - Standard Curves and Chromatograms

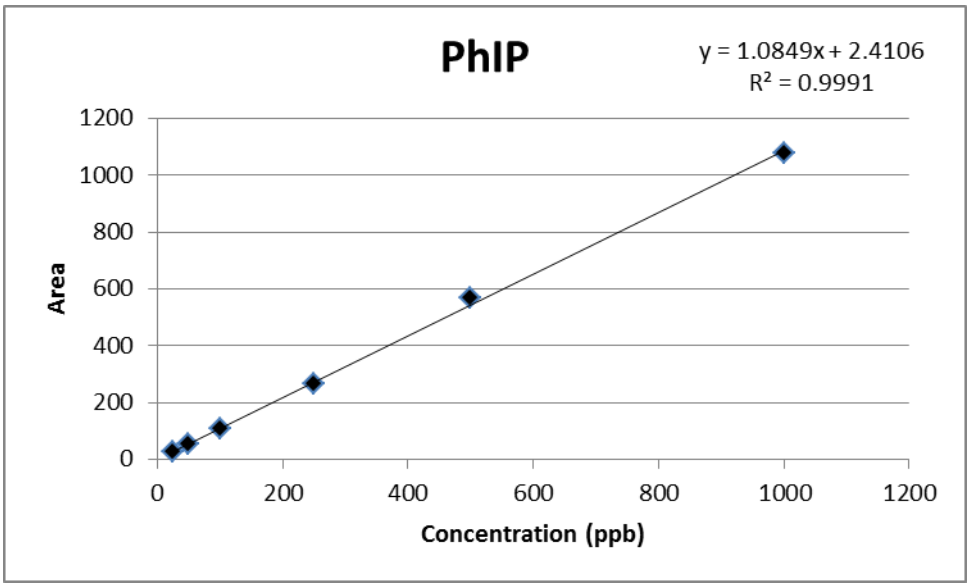


Figure A-1 Standard curve obtained by HPLC UV detection for PhIP at 252 nm.

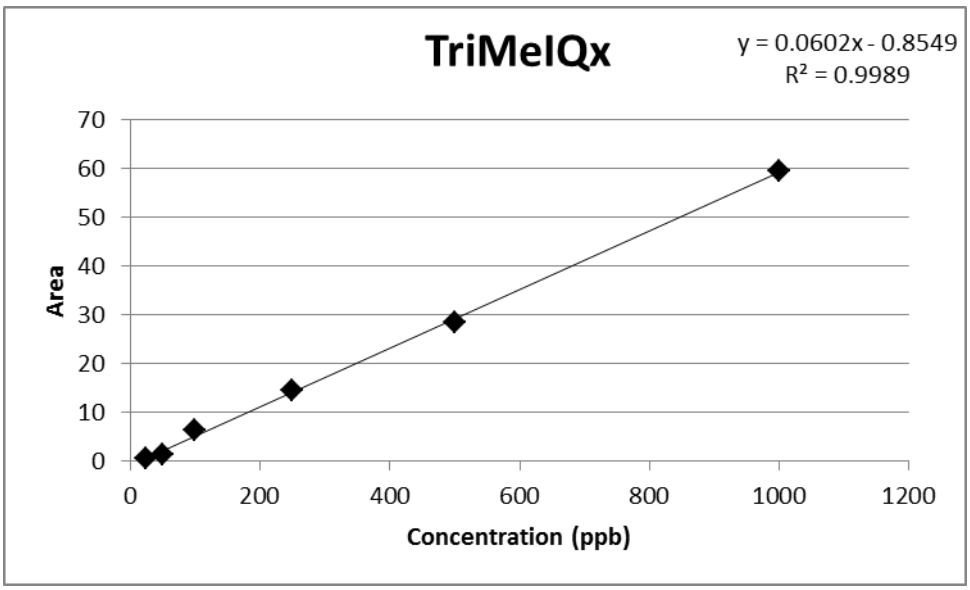


Figure A-2 Standard curve obtained by HPLC UV detection for TriMeIQx at 252 nm.

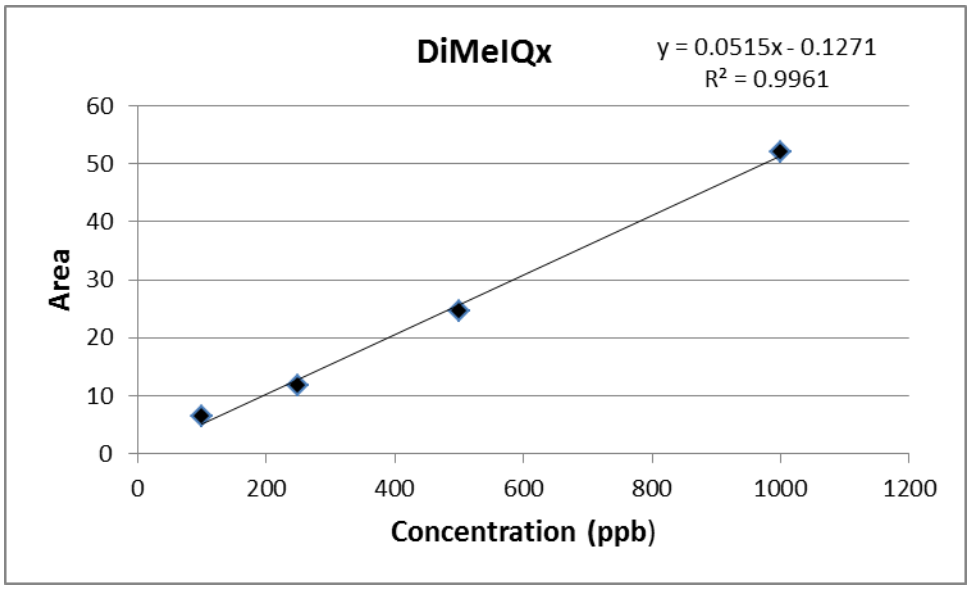


Figure A-3 Standard curve obtained by HPLC UV detection for DiMeIQx at 252 nm.

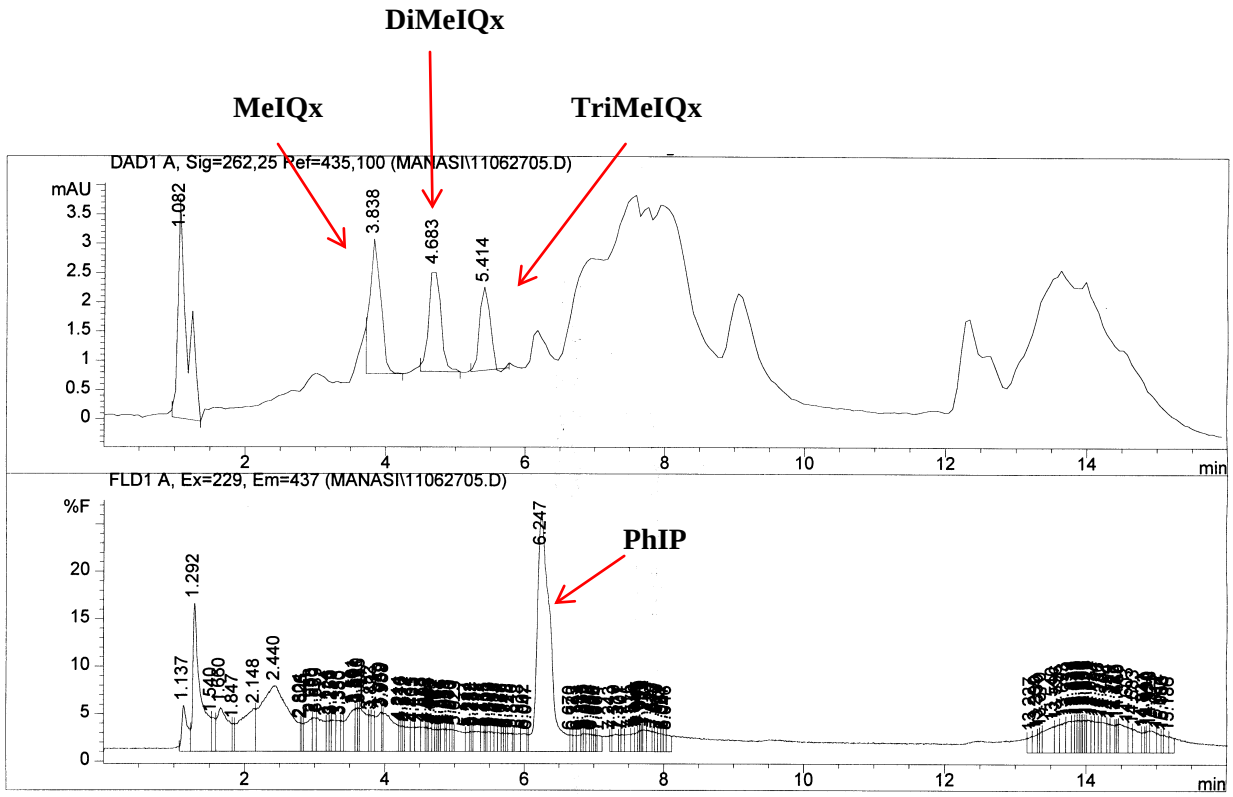


Figure A-4 HPLC chromatogram of a mixed standard (MeIQx, DiMeIQx, TriMeIQx, and PhIP) separated on a TSK-gel Super ODS column as described in Chapter 2 (page 44). The concentration of the mixed standard was 250 ppb (20 µl injection volume). The UV detector was set at 252 nm for MeIQx, DiMeIQx, and TriMeIQx detections, and the fluorescence detector was programmed accordingly to the excitation/emission wavelengths of 229 and 437 nm for PhIP. TriMeIQx (250 ppb) was used as internal standard.

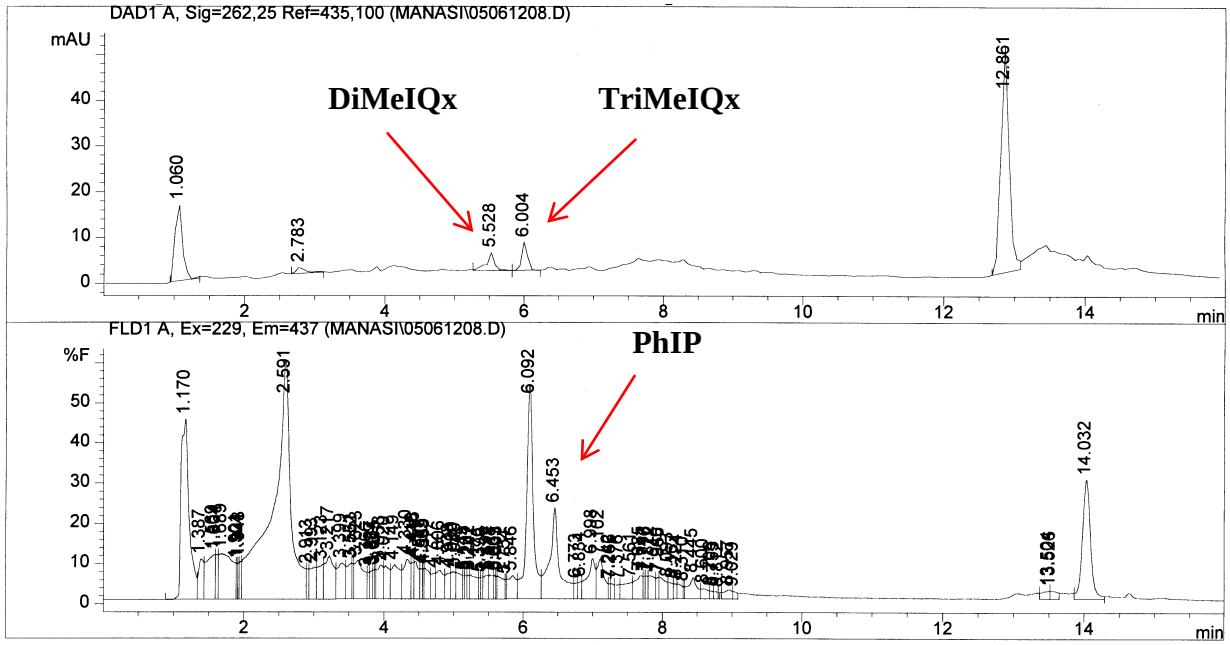
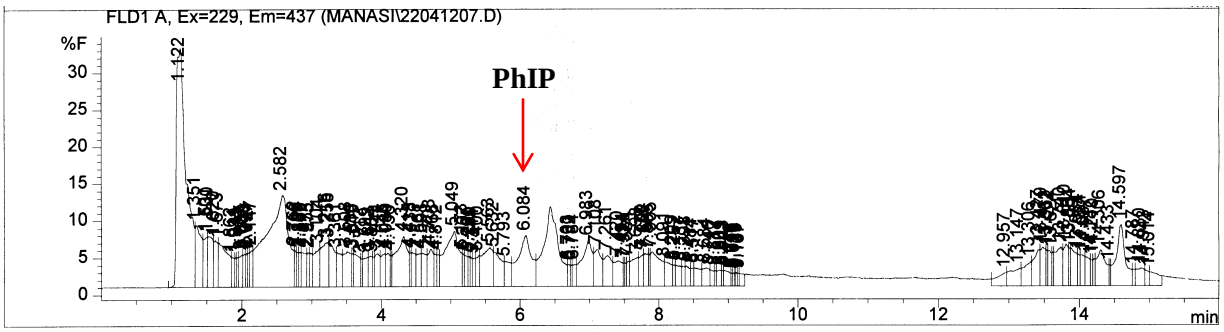


Figure A-5 HPLC chromatogram of control sample (patty with no spice) which was separated on a TSK-gel Super ODS column as described in Chapter 2 (page 44). The UV detector was set at 252 nm for MeIQx, DiMeIQx, and TriMeIQx detections, and the fluorescence detector was programmed accordingly to the excitation/emission wavelengths of 229 and 437 nm for PhIP. TriMeIQx (250 ppb) was used as internal standard. Injection volume of the sample was 20 µl.

(a).



(b).

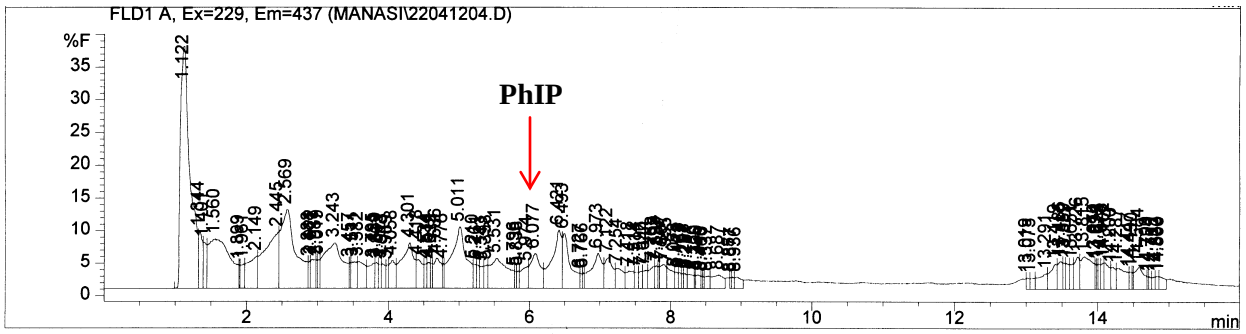


Figure A-6 HPLC chromatogram of 0.25% individual spice treated beef patty samples (a). 0.25% turmeric and (b). 0.25% rosemary which was separated on a TSK-gel Super ODS column as described in Chapter 2 (page 44). The fluorescence detector was programmed accordingly to the excitation/emission wavelengths of 229 and 437 nm for PhIP. The injection volume of the sample was 20 µl.

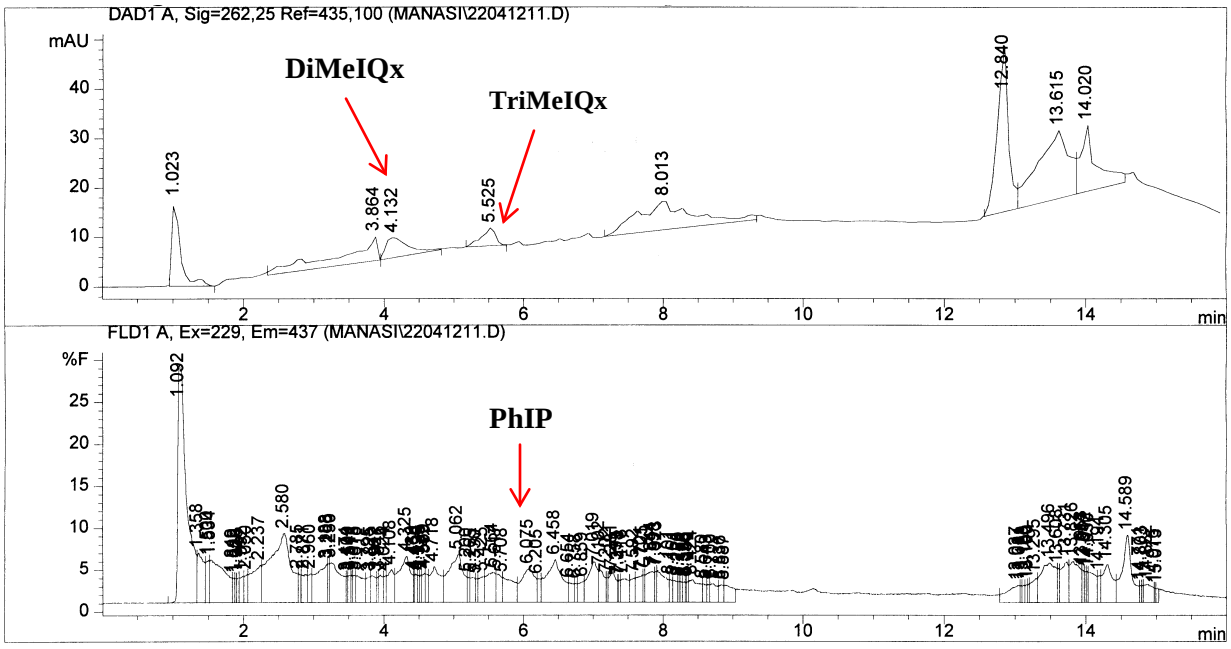


Figure A-7 HPLC chromatogram of 0.25% black pepper treated beef patty sample which was separated on a TSK-gel Super ODS column as described in Chapter 2 (page 44). The UV detector was set at 252 nm for MeIQx, DiMeIQx, and TriMeIQx detections, and the fluorescence detector was programmed accordingly to the excitation/emission wavelengths of 229 and 437 nm for PhIP. TriMeIQx (250 ppb) was used as internal standard. The injection volume of the sample was 20 µl.

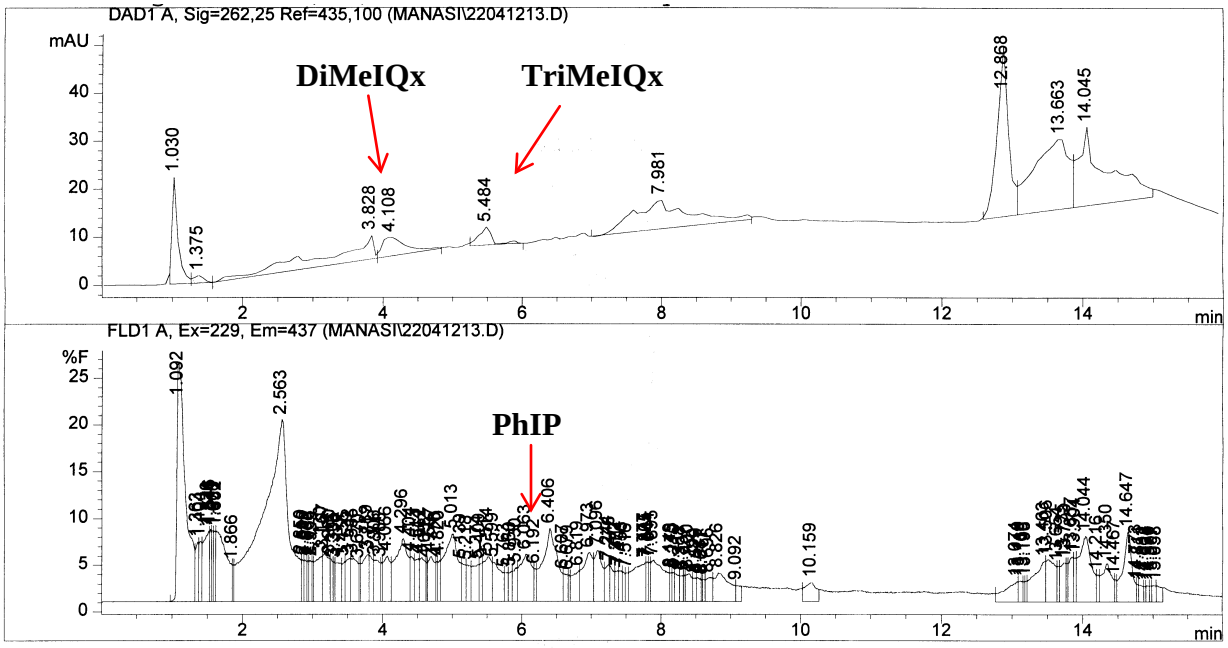


Figure A-8 HPLC chromatogram of 1.00% black pepper treated beef patty samples which was separated on a TSK-gel Super ODS column as described in Chapter 2 (page 44). The UV detector was set at 252 nm for MeIQx, DiMeIQx, and TriMeIQx detections, and the fluorescence detector was programmed accordingly to the excitation/emission wavelengths of 229 and 437 nm for PhIP. TriMeIQx (250 ppb) was used as internal standard. The injection volume of the sample was 20 µl.

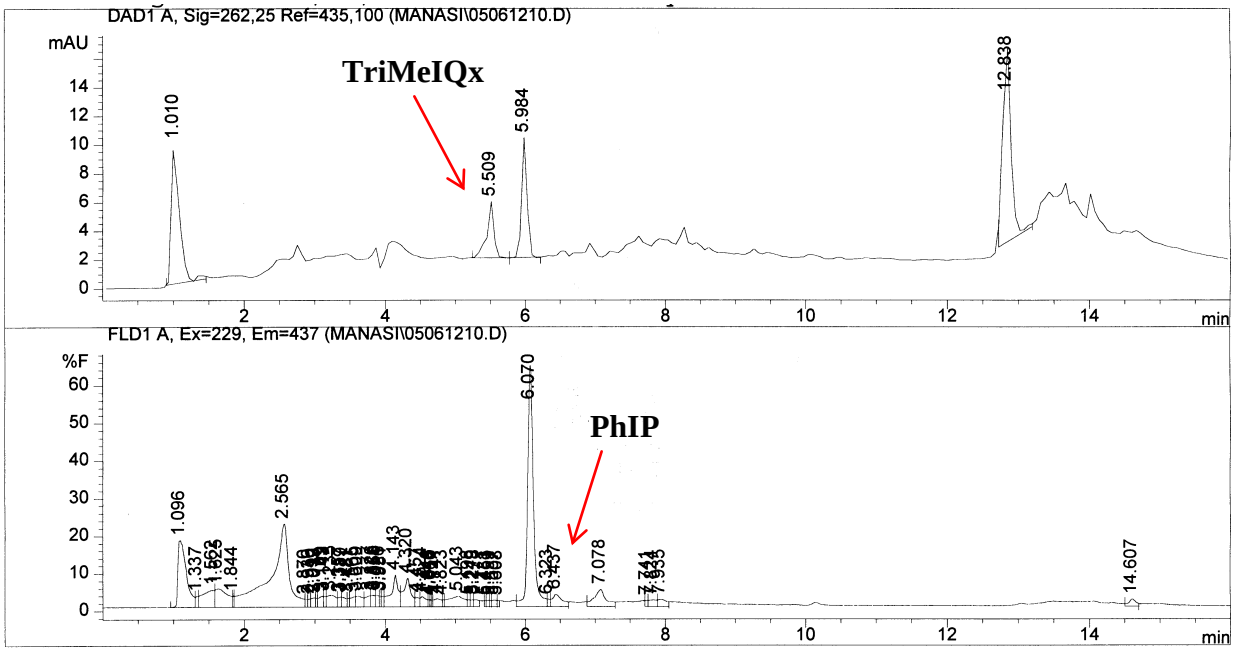


Figure A-9 HPLC chromatogram of beef patty sample treated with combination of 0.25% black pepper and 0.25% rosemary, which was separated on a TSK-gel Super ODS column as described in Chapter 2 (page 44). The UV detector was set at 252 nm for MeIQx, DiMeIQx, and TriMeIQx detections, and the fluorescence detector was programmed accordingly to the excitation/emission wavelengths of 229 and 437 nm for PhIP. TriMeIQx (250 ppb) was used as internal standard. The injection volume of the sample was 20 µl.

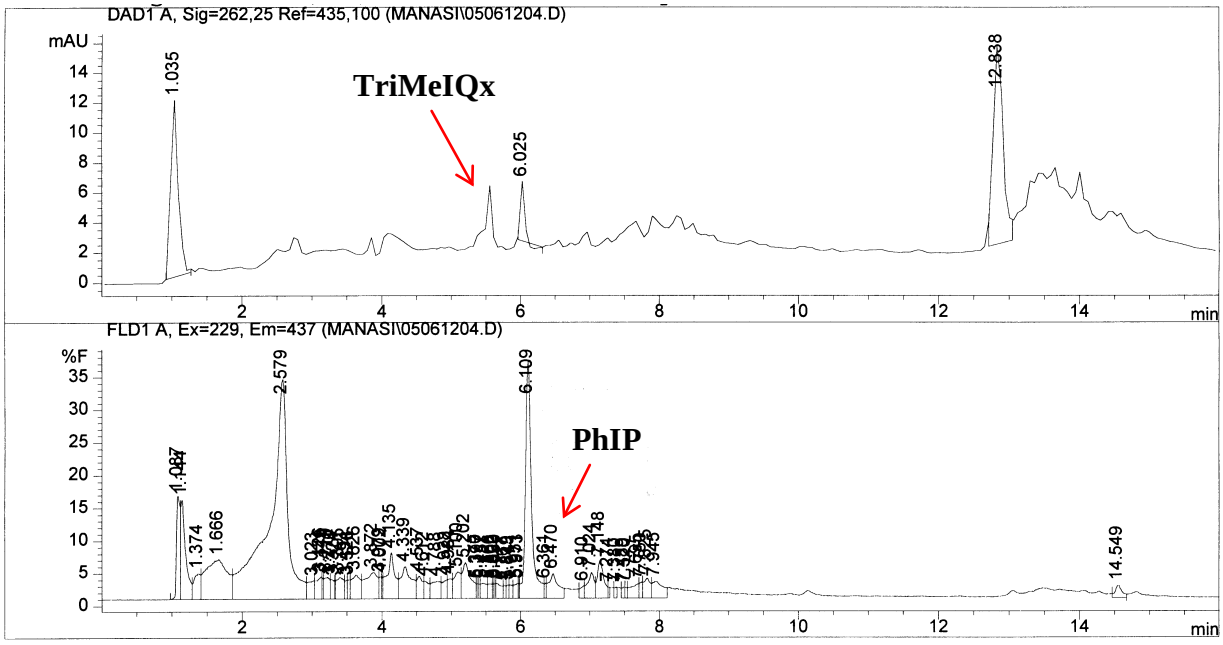


Figure A-10 HPLC chromatogram of beef patty sample treated with combination of 0.25% black pepper and 0.25% turmeric, which was separated on a TSK-gel Super ODS column as described in Chapter 2 (page 44). The UV detector was set at 252 nm for MeIQx, DiMeIQx, and TriMeIQx detections, and the fluorescence detector was programmed accordingly to the excitation/emission wavelengths of 229 and 437 nm for PhIP. TriMeIQx (250 ppb) was used as internal standard. The injection volume of the sample was 20 µl.