REDUCTION OF HETEROCYCLIC AMINE FORMATION IN BEEF BY SURFACE APPLICATION OF SPICES

by

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Abstract

Heterocyclic amines (HCAs) are cancer causing compounds formed during the cooking of meat. Previous studies show that incorporating antioxidant spices into meat as well as marinating meat with antioxidant spices reduces formation of HCAs. The purpose of this study was to determine if commercially available spices applied to the surface of meat could effectively reduce HCA formation. Two commercially available spice blends and one blend of spices with known quantities of antioxidant spices were sprinkled onto the surface of beef just prior to pan-frying. The quantities of spices used were based on the amounts customarily consumed in typical Western cooking. The results of direct application were then compared to marinating with the same types and amounts of spices. The antioxidant potential of the spices was analyzed using DPPH and total phenolics methods. Results indicated that the spices would be effective antioxidants. Low recovery rates and problems during the extraction process made results inconclusive, but suggest that further research may find that applying spices directly to the surface of meat in consumer acceptable quantities may be as effective as marinating at reducing the formation of HCAs.

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Chapter 1 - Literature Review

Cancer is a broad term which affects different people in different parts of the body at different stages in life. The unknowns of cancer are enormous, but the desire to overcome the risks of cancer causes scientists to continue researching to find a cure. Cancer is the second most common cause of death in the United States, accounting for 29% of non-communicable causes of death (National Vital Statistics System, National Center for Health Statistics, CDC 2007; World Cancer Research Fund / American Institute for Cancer Research 2007). The occurrence of cancer in humans causes concern for both scientists and the general public.

It has been estimated that one-third of all cancer is related to food (Sugimura 2002). Food being carcinogenic is not a new phenomenon. The first study to correlate cancer to food was conducted in 1939 by Widmark. In this study, cooked horse meat extracts were applied to the backs of mice resulting in cancer (Dashwood 2002; Murkovic 2007). Since this original link between food and cancer, researchers have identified these cancer-causing compounds in food as heterocyclic amines (HCAs), also known as heterocyclic aromatic amines. The ten most common 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). Further study of HCAs has included determining human exposure to HCAs, their carcinogenicity and mutagenicity, kinetics of formation, occurrence in food, analysis procedures, as well as methods to minimize consumption.

Human Exposure to Heterocyclic Amines

While HCAs usually occur in foods at the ng/g range, their risk is still relevant to consumers (Murkovic 2007; NTP, U.S. Department of Health and Human Services, Public Health Service 2011). According to a 1997 study of an elderly Swedish populations' exposure to HCAs, the mean daily intake of IQ, MeIQ, MeIQx, DiMeIQx, and PhIP was found to be less than 200 ng/ day (Augustsson and others 1997). While this quantity seems low, this is still a level of concern. HCAs are present in the diet but the amount required to cause cancer is difficult to pinpoint (Felton and others 2007).

Estimates of intake of HCAs are based on questionnaires, national consumption surveys, doneness preference, as well as factors such as body weight, age, gender, ethnicity, etc. It has been estimated that the average total daily intake over a lifetime for adults is 9 ng/ kg / day (Bogen and Keating 2001). This correlates to a 68 kg (150 lb) person consuming approximately 613 ng/ day. This number seems low, but Sugimura (2002) suggests that carcinogenic compounds can build up in the body to produce cancer. A single agent or single occurrence is not likely to be the sole cause of cancer, but rather a combination of carcinogens adding up and working together (Sugimura 2002).

According to a 1993 monograph from the International Agency for Research on Cancer (IARC), one HCA, IQ, was identified as *probably carcinogenic to humans* while an additional nine were identified as *possibly carcinogenic to humans.* The United States 12th Report on Carcinogens (RoC) reported that, MeIQ, MeIQx, IQ, and PhIP are *reasonably anticipated to be human carcinogens* (NTP, U.S. Department of Health and Human Services, Public Health Service 2011). Further information about these HCAs including polarity and carcinogenicity is listed in Table 1.

Table 1. 10 most common HCAs (IARC 1993)

Name	Abbrevia-tion	Polarity	Carcinogenicity
2-amino-3-methylimdazo[4,5-f]quinoline	IQ	Polar	Probably
2-amino-3,4-dimethylimdazo[4,5-f]quinoline	MeIQ	Polar	Possibly
2-amino-3,8-dimethylimdazo[4,5-f]quinoxaline	MeIQx	Polar	Possibly
2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine	PhIP	Polar	Possibly
2-amino-9H-dipyrido[2,3-b]indole	AαC	Apolar	Possibly
2-amino-3-methyl-9H-dipyrido[2,3-b]indole	MeAαC	Apolar	Possibly
2-amino-6-methyl-dipyrido[1,2-a:3',2'-d]imidazole	Glu-P-1	Apolar	Possibly
2-amino-dipyrido[1,2-a:3',2'-d]imidazole	Glu-P-2	Apolar	Possibly
3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole	Trp-P-1	Apolar	Possibly
3-amino-1-methyl-5H-pyrido[4,3-b]indole	Trp-P-2	Apolar	Possibly

In agreement with the IARC findings, Sugimura found that IQ, MeIQ, MeIQx, Trp-P-1, Trp-P-2, Glu-P-1, and Glu-P-2 produced cancer in rodents (Arvidsson and others 1997; Murkovic 2007). The specificity of HCAs was studied by Nagao (1999) finding that different HCAs cause different mutations. For example, PhIP caused deletion of G in the GGGA DNA sequence in colon cancer cells (Nagao 1999). Similarly, Metry and others (2009) found that PhIP targets the colon, while MeIQx targets the liver (Metry and others 2009).

Understanding the correlation between level of exposure to HCAs and risk is also an important area of research. A study by Wei and others (2011) found that a no observable effect level existed in F344 rats. Rats were fed varying levels of IQ for 16 weeks, finding that a dose

level of 0.08 mg/ kg body weight/ day (1 ppm) or less did not show a carcinogenic effect on the liver or colon, the organs of interest. In contrast, concentrations greater than 1 ppm, specifically 10 ppm (0.76 mg/ kg body weight/ day) and 100 ppm (7.83 mg/ kg body weight/ day), did have carcinogenic effects (Wei and others 2011). It is important to note that this study was for a short period of time and therefore may not represent human exposure over a lifetime.

Mutagenicity and Carcinogenicity

Numerous methods have been developed to assess HCA cancer risks to humans. Studies utilize bacterial, animal, and now even human populations to determine if HCAs are carcinogenic, areas of the body prone to problems associated with HCAs, and associations between specific genotypes and increased cancer risk due to HCAs.

The Ames test utilizes specific strains of *Salmonella typhimurium* to determine the mutagenic potential of substances (Ames and others 1973). The two strains of *S. typhimurium* often used to identify mutagenic potential of HCAs are TA98 and TA100. TA100 is susceptible to base substitutions as compared to TA98 which is susceptible to frame-shift type mutagens. As indicated in Table 2 by the higher values for TA98 revertants /μg, HCAs more often cause frame-shift type mutagenicity (Sugimura and others 2004).

Human studies often utilize questionnaires to obtain information on consumption habits as well as doneness preferences. One issue found with doneness preferences is the variability of consumer doneness perception. In a study looking at consumer in-home cooking habits and HCA intake, it was found that many consumers did not perceive doneness in the same way (Keating and others 2000). This is an important consideration when basing results on consumer questionnaires. Nonetheless, questionnaires are still a valuable method of determining the association between eating habits and cancer risk.

Table 2. Mutagenicity of HCAs in *S. typhimurium* TA98 and TA100 with S9 mix (Sugimura and others 2004)

HCA	Revertants/ μg	
	TA98	TA100
MeIQ	661,000	30,000
IQ	433,000	7000
DiMeIQx	183,000	8000
7,8-DiMeIQx	163,000	9900
MeIQx	145,000	14,000
Trp-P-2	104,200	1800
4-Ch_2OH-8-MeIQx	99,000	3000
IQx	75,400	1500
Glu-P-1	49,000	3200
Trp-P-1	39,000	1700
Glu-P-2	1900	1200
PhIP	1800	120
AαC	300	20
MeAαC	200	120

Specificity of understanding HCA risk has improved to better comprehend how it affects humans. Since cancer encompasses the entire body, many studies look at specific regions of the body and their response to total HCA consumption, individual HCA consumption, as well as specific types of food products. There are numerous studies which found associations between HCA consumption and human cancer risk; however, there are also studies which did not find significant associations.

To asses the available information, the book entitled, *Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective* from the World Cancer Research Fund/

American Institute for Cancer Research (WCRF/AICR) summarized published research on cancer causing and preventative measures. The book is categorized based on the amount of information suggesting an association, the type of food product, and the location of cancer. The WCRF/AICR concluded that there is convincing evidence that exposure to HCAs increases the risk of certain cancers. HCAs, through consumption of red meat and processed meat, are associated most convincingly with colorectum cancer. While colorectum cancer is the only cancer categorized in the 'convincing' as well as the 'probable' areas, limited association has been found with cancers at other locations. Table 3 summarizes the information from the WCRF/AICR in regards to HCAs (World Cancer Research Fund / American Institute for Cancer Research 2007). The following sections look at some of the research available regarding the relationship between HCAs and specific organs.

Table 3. Association of specific cancer risk with certain types of foods (World Cancer Research Fund / American Institute for Cancer Research 2007)

Evidence indicating association	Type of Food	Location of Cancer
Convincing	Red Meat	Colorectum
	Processed Meat	Colorectum
Probable	N/A	N/A
Limited – suggestive	Red Meat	Oesophagus
		Lung
		Pancreas
		Endometrium
	Processed Meat	Oesophagus
		Lung
		Stomach
		Prostate
	Grilled (broiled) or barbecued (charbroiled) animal foods	Stomach
Limited – no conclusion	Meat	Mouth, pharynx, larynx
		Nasopharynx
		Breast (premenopause and postmenopause)
		Ovary
		Prostate
		Kidney
		Bladder

Colorectum

Colorectum cancer is the third most common type of cancer for both men and women in the U.S. (World Cancer Research Fund / American Institute for Cancer Research 2007). Cancer of the colon and/or rectum has also been associated with increased HCA intake; specifically with increased meat consumption (Shin and others 2007), increased red meat intake (World Cancer Research Fund / American Institute for Cancer Research 2007), and increased well-done meat intake (Cotterchio and others 2008). In a large prospective study, increased intake of MeIQx and

DiMeIQx showed an elevated risk of colon cancer but not rectal cancer (Cross and others 2010). A study of 10 humans exposed to a dietary-relevant dose of PhIP showed increased formation of DNA-adducts in the colon as well as urinary metabolites of PhIP (Malfatti and others 2006). While this is a very low sample size, this study is valuable in that it looked at human subjects rather than animal models.

In regards to colorectum cancer the WCRF/AICR concluded that there is convincing evidence that exposure to HCAs, specifically in red meat, increases the risk of colorectum cancer (World Cancer Research Fund / American Institute for Cancer Research 2007)

Prostate

One study specifically focused on PhIP and the prostate in F344 rats. It found that increasing exposure to PhIP increased the risk of prostate carcinomas (Shirai and others 1997). Studies by Sinha and others (2009) and John and others (2011) found an association between increased risk for all stages of prostate cancer and red meat consumption as well as an increased risk for advanced prostate cancer with higher consumption of processed meat. In contrast, no association for prostate cancer and white meat consumption was found. It should be noted however, that neither of these studies look specifically at HCAs but draw conclusions based on the concept that HCAs are present in cooked meat products. Other factors which are known to also be found in red meat, such as polycyclic aromatic hydrocarbons (PAH), may also contribute to the associations (Sinha and others 2009; John and others 2011).

A study by Tang (2007) looked at DNA adducts in human prostate tumor cells which are specific to PhIP. This study found a significant association between total meat consumption and total grilled red meat consumption with PhIP-DNA adducts. Furthermore, specific meats, including grilled hamburger, grilled steak, grilled pork chop, grilled hot dog, and grilled chicken

with skin were associated with the occurrence of PhIP-DNA adducts in prostate tumor cells (Tang and others 2007).

In contrast to these studies, a recent cohort study including 10 European countries did not find an association between meat intake or meat-doneness preference with increased risk of prostate cancer (Sander and others 2011). The WCRF/AICR has not seen enough evidence in support of an association between HCAs and prostate cancer to determine that a convincing association exists. Currently, the WCRF/AICR concludes that a limited amount of evidence suggests an association between processed meat consumption and risk of prostate cancer. Not enough information is available for the WCRF/AICR to draw conclusions regarding the association of meat consumption in general with prostate cancer (World Cancer Research Fund / American Institute for Cancer Research 2007).

Breast

Breast tissue is susceptible to PhIP-DNA adducts; therefore, increasing exposure to PhIP can increase the PhIP-DNA adducts and likely result in an increased risk of breast cancer (Zhu and others 2003). This study also used a questionnaire to find associations between meat consumption habits and potential of PhIP-DNA adducts. An association was found between well-done meat consumption and increased risk of PhIP-DNA adducts in the breast, but only in the presence of the rapid NAT2 genotype (Zhu and others 2003; Ambrosone and others 2007). In a study of German women, processed, fried, or stir-fried meat consumption also correlated to an increased risk of DNA adducts in the breast but this study did not specify the individual HCAs or the genotype of the women (Rohrmann and others 2009).

In a 2-generation study of F344 rats, PhIP showed carcinogenicity in the colon and mammary of first generation rats. In the second generation, mammary tumor development

significantly increased showing the transfer from one generation to the next of cancer-causing compounds (Ito and others 1997). Lauber and Gooderham (2001) took a different approach and analyzed not only the carcinogenic effect of PhIP but also the estrogenic activity of PhIP. Findings suggest that PhIP not only has the ability to propagate but also to promote cancer causing cells (Lauber and Gooderham 2011).

In contrast to these studies, research on U.S. women did not find an association between individual HCAs, total meat, red meat, or chicken consumption with breast cancer; this study did segregate by genotype for the rapid and slow acetylators (Mignone and others 2009). The WCRF/AICR had not seen enough evidence associating meat consumption, in general, with breast cancer risk, in either premenopausal or postmenopausal women (World Cancer Research Fund / American Institute for Cancer Research 2007).

Other Organs

Limited evidence is available linking HCAs to cancer in other organs. In the bladder, carcinogenesis has been found to positively correlate to red meat consumption, specifically PhIP intake (Ferrucci and others 2010). Similarly, CDF1 mice and F344 rats fed 50-80 ppm of HCAs developed tumors mainly in the liver but also in other organs (Nagao 1999). In contrast, consumption of meat has not been associated with increased occurrence of liver disease nor heptocellular carcinoma (Freedman and others 2010).

Genetic Variability

Some research indicates that using humans as a whole is too broad of group and that specific genotypes may increase or decrease the risks associated with HCA intake. A prospective study of a Danish population found that specific gene polymorphisms increased the risk of colorectal cancer when combined with increased intake of red and processed meats (Andersen

and others 2010). The difference between rapid acetylators and slow acetylators for N-acetyltransferase 1 (NAT1) and N-acetyltransferase 2 (NAT2) have shown increased risk for DNA-adducts in breast epithelial cells (Zhu and others 2003; Ambrosone and others 2007). Also, the presence of rapid NAT2 was found to increase the occurrence of MeIQx tumors (Metry and others 2009) and IQ and MeIQx DNA-adducts and mutagens (Metry and others 2010). In contrast, a multi-ethnic cohort study by Sharma and others (2010) did not find an increased risk for prostate cancer from consumption of well-done meat related to the slow or rapid NAT1 or NAT2 genotypes (Sharma and others 2010). Another study found similar results to this indicating that, while CYP1A1 likely activates PhIP, this is not dependent on gene polymorphisms in NAT2 (Bendaly and others 2009). The presence of CYP1A1 has also been found to increase the risk for colorectal cancer (Cotterchio and others 2008).

Classification of HCAs

Based on structure, HCAs are classified as either polar or non-polar (Murkovic 2007). The following sections describe the HCAs which fall into these categories as well as further classification.

Polar HCAs

Polar HCAs, also called amino-imidazo-azaarenes (AIA), are characterized by a 2-amino-imidazo group and a methyl group attached to nitrogen in an imidazole ring (Jagerstad and others 1998). This structure is then linked to one of three groups; imidazoquinolines (IQ and MeIQ), imidazoquinoxalines (MeIQx, 4,8-DiMeIQx, and 7,8-DiMeIQx), and imidazopyridines (PhIP) (Pearson and others 1992). The structures of polar HCAs are shown in Figure 1.

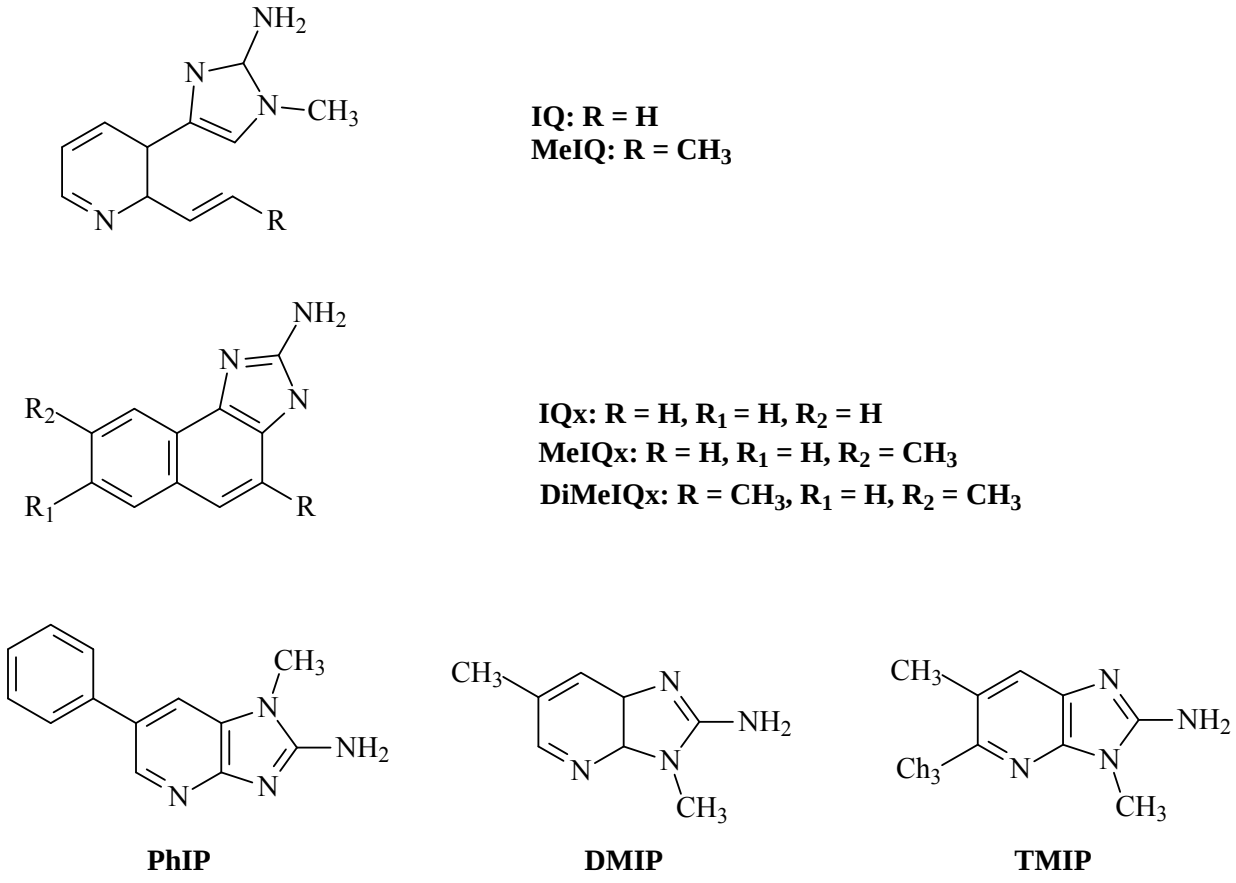
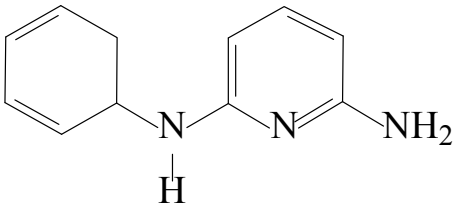


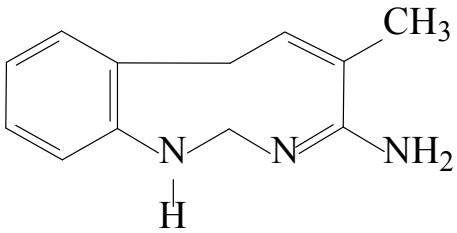
Figure 1. Chemical structures of polar HCAs (Cheng and others 2006)

Non-polar HCAs

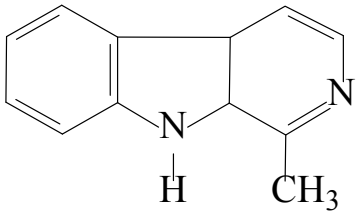
Non-polar HCAs, also known as amino-carbolines, are sub-divided into pyridoindole and dipyridoimidazole types (Murkovic 2007). The structures of these compounds are shown in Figure 2. While non-polar HCAs are also classified as possibly carcinogenic to humans (IARC 1993), formation occurs at temperatures higher than typical cooking conditions (Murkovic 2007).



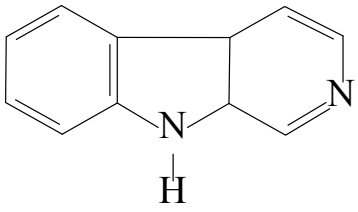
AaC



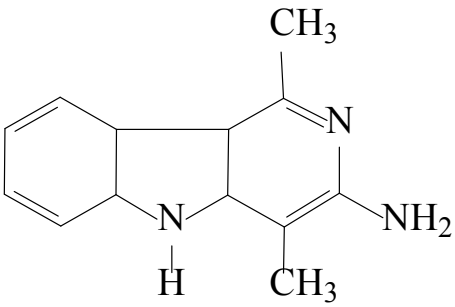
MeAaC



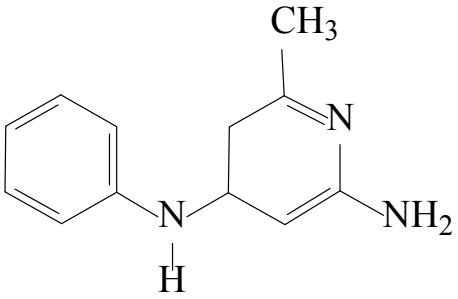
Harman



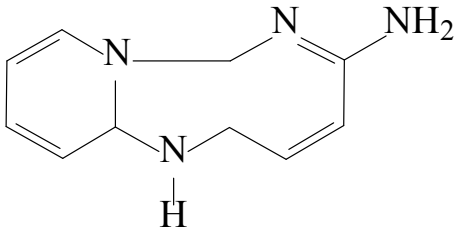
Norharman



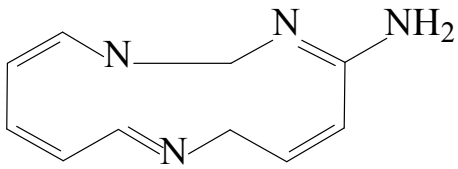
Trp-P-1



Trp-P-2



Glu-P-1



Glu-P-2

Figure 2. Chemical structures of non-polar HCAs (Cheng and others 2006)

Formation of HCAs

Cooking meat forms HCAs. The precursors of HCAs are amino acids, creat(in)ine, and carbohydrates (Murkovic 2004a). During cooking reactions, such as Maillard browning, free radicals are formed which can then react with other compounds, specifically creatinine, ultimately producing HCAs (Pearson and others 1992; Kikugawa 1999). Polar HCAs form at 150 - 200 °C (302 - 392 °F) while non-polar HCAs form at significantly higher temperatures (Murkovic 2007). Typical cooking temperatures therefore form polar HCAs with little formation of non-polar HCAs.

One suggested pathway of formation of IQ-type, polar HCAs is shown in Figure 3. The imidazole ring is derived from creat(in)ine; therefore, formation of HCAs containing the imidazole ring does not occur without this precursor (Murkovic 2007). Formation of HCAs follows first-order kinetics (Arvidsson and others 1997; Kim and Lee 2010). Formation of PhIP follows a slightly different pathway and requires the presence of the amino acid phenylalanine. The reaction steps and intermediates are shown in Figure 4.

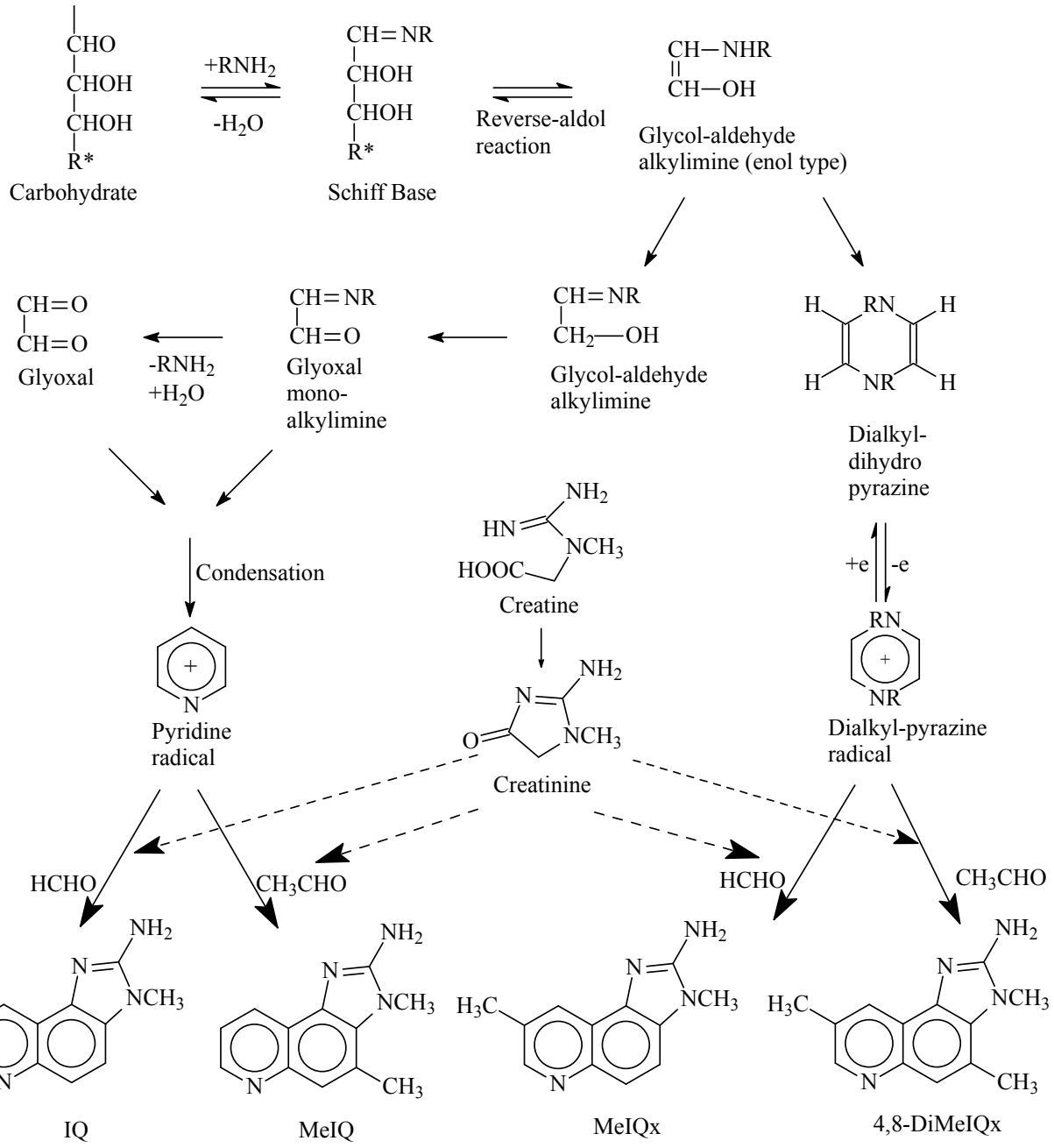


Figure 3. Suggested pathway for formation of IQ-like and IQx-like compounds (Pearson and others 1992)

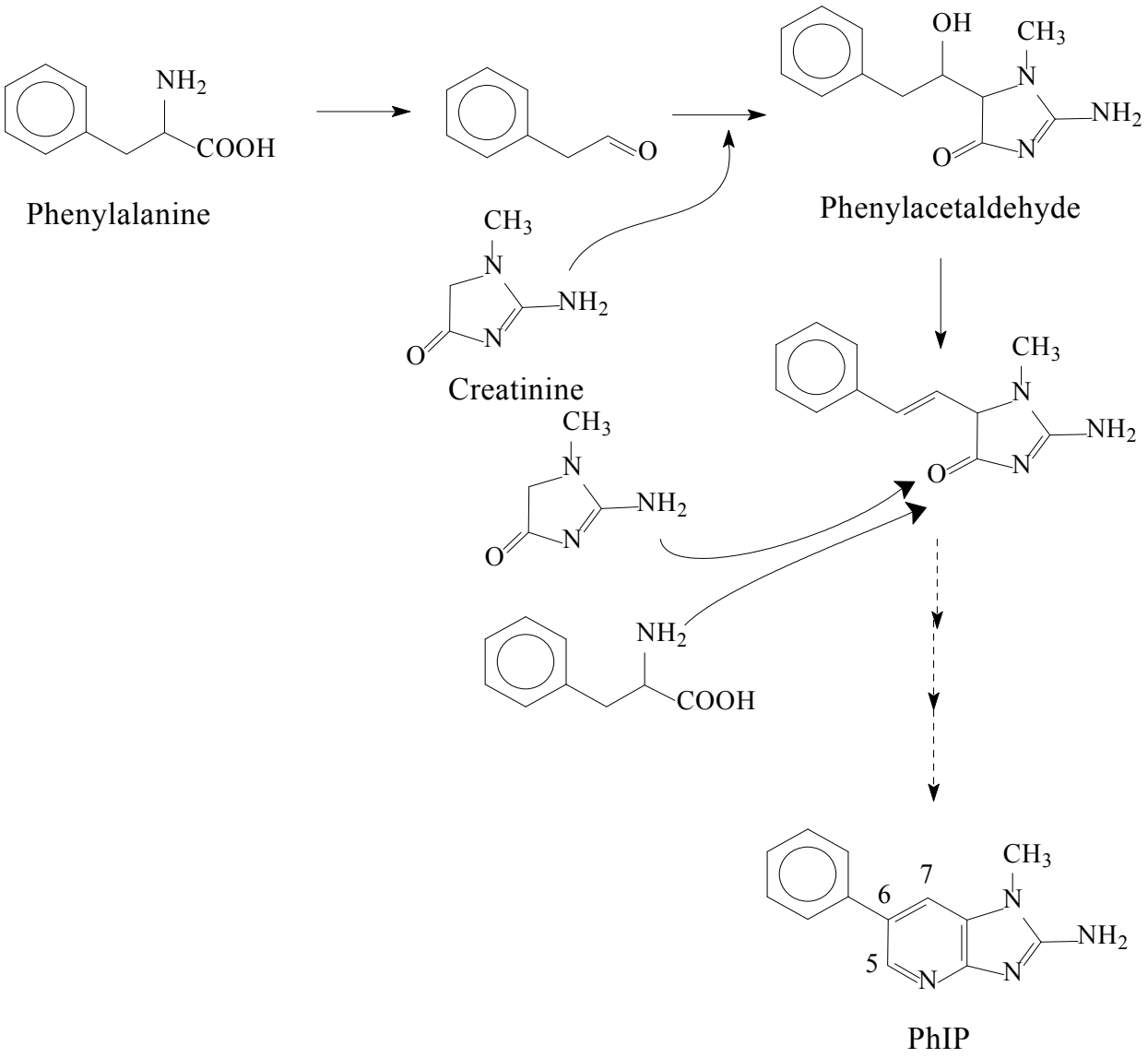


Figure 4. Formation of PhIP with identified intermediate reaction products (Murkovic 2004b)

Influence of Cooking Time and Temperature

The factors involved in cooking meat greatly influence the quantity and type of HCAs formed. Arvidsson and others (1997) used a model system containing creatinine, glucose, amino acids, and carnosine to determine the amount of HCAs formed at various temperatures from 0.5-120 minutes. As the temperature increased in 25 ºC increments from 150 ºC (302 ºF) to 200 ºC

(392 ºF) the quantity of the HCAs IQx, MeIQx, 7,8-DiMeIQx, 4,8-DiMeIQx, and PhIP, also increased (Arvidsson and others 1997). The graphs of each HCA can be seen in Figure 5.

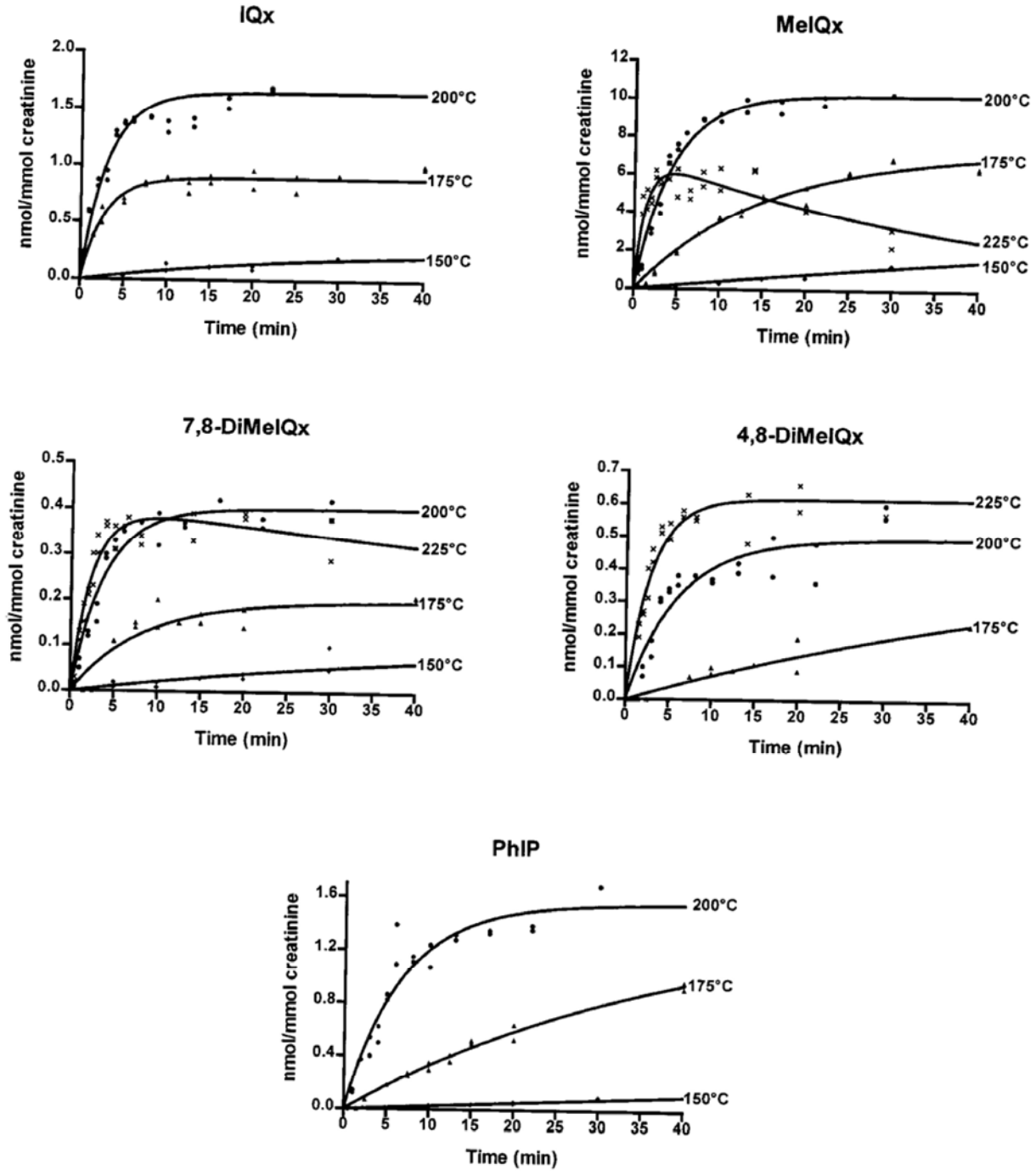


Figure 5. Formation of HCAs at 150, 175, 200, and 225 ºC (Arvidsson and others 1997)

While model systems are an effective method for seeing relationships between certain components and heating, actually cooking meat gives results which are more applicable to consumers. The method of heating influences the time and temperature necessary to cook a product; therefore, studies have compared various cooking methods to determine their effect on HCA formation. One study cooked meat samples for the same length of time, regardless of the final internal temperature. Among oven broiled, pan-fried, and boiled meat; pan-fried formed the most HCAs (Murkovic and others 1998; Shin 2005). Another study by Liao and others (2010) found that among pan-frying, deep-frying, charcoal grilling, and roasting of duck and chicken breasts, charcoal grilling had the highest concentration of HCAs followed by pan-frying (Liao and others 2010)

Balogh and others (2000) looked at a single cooking method, pan-frying, using three frying pan temperatures and two cooking times. This studied aimed to determine the conditions which form the greatest amount of HCAs in ground beef patties. The cooking temperatures were 175 ºC (347 ºF), 200 ºC (392 ºF), and 225 ºC (437 ºF) with cooking times of 6 or 10 minutes per side. The five HCAs studied were IQ, MeIQ, MeIQx, DiMeIQx, and PhIP Figure 6 shows that the total concentration of HCAs positively correlates to higher temperature and longer time. Note that, regardless of the temperature of the frying pan, increasing the time from 6 minutes to 10 minutes per side increased the total amount of HCAs. Also, regardless of the time per side, the HCA concentration increased with the temperature.

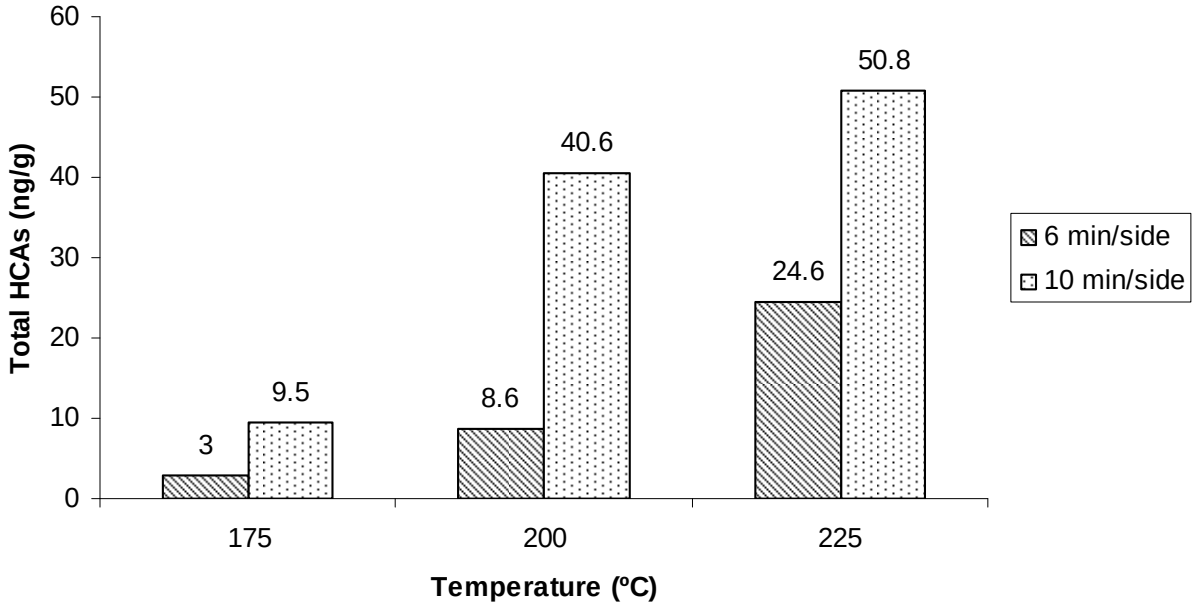


Figure 6. HCA concentrations (ng/ g) in fried ground beef patties using different time/temperature combinations of frying (Balogh and others 2000)

In general, the temperature at which HCAs can begin to be quantifiable is 150 -250ºF (66-121ºC) (Murkovic 2007). This temperature range includes the recommended minimum temperatures for most meat products. Table 4 summarizes the United States Department of Agriculture's (U.S.D.A.) safe minimum internal temperature (FSIS, United States Department of Agriculture 2006). These are the recommended internal temperatures, meaning that the surface temperature would be even higher. The U.S.D.A. considers these the safe temperatures to which consumers should cook meat. These recommendations only reflect destroying microorganisms, not chemical hazards.

Table 4. U.S.D.A. recommended safe minimum internal temperatures (FSIS, United States Department of Agriculture 2006; Bernard 2011)

Type of Meat	Product	Temperature (ºF)	Temperature (ºC)
Beef, Veal, and Lamb	Steaks and Roasts	145	63
Fish		145	63
Pork		145	71
Beef, Veal, and Lamb	Ground	160	71
Egg Dishes		160	71
Turkey, Chicken and Duck	Whole, Pieces, and Ground	165	74

Knize and Felton (2005) took a different approach by controlling the internal temperature of the beef patties and adjusting the temperature of the frying pan. Beef patties were cooked until the internal temperature registered 70 ºC (158 ºF). In Figure 7 we see that, even if the final internal temperature is constant, the total amount of HCAs increases with the pan temperature. The pan temperature, and more specifically the surface temperature of the meat, is heavily related to the amount of HCAs.

While the recommendations for safe cooking are based on temperature, consumers tend to base doneness on color (Louis and others 2007). According to a study by Liao and others (1998), as browning color increased, the amount of HCAs also increased (Liao and others 2009). In a study of pork products, Sinha and others (1998) associated doneness, as determined by color, to quantity of HCAs. Of the products studied, bacon was the only one which showed a positive correlation between color-doneness and HCA content (Sinha and others 1998). Another study by Louis and others (2007) showed that level of doneness correlated to the amount of PhIP but not to the concentration of Harman, a non-polar HCA (Louis and others 2007). Neither of the

studies which looked at doneness, as determined by color, took the initial temperature of the cooking surface or the final internal temperature of the product into consideration.

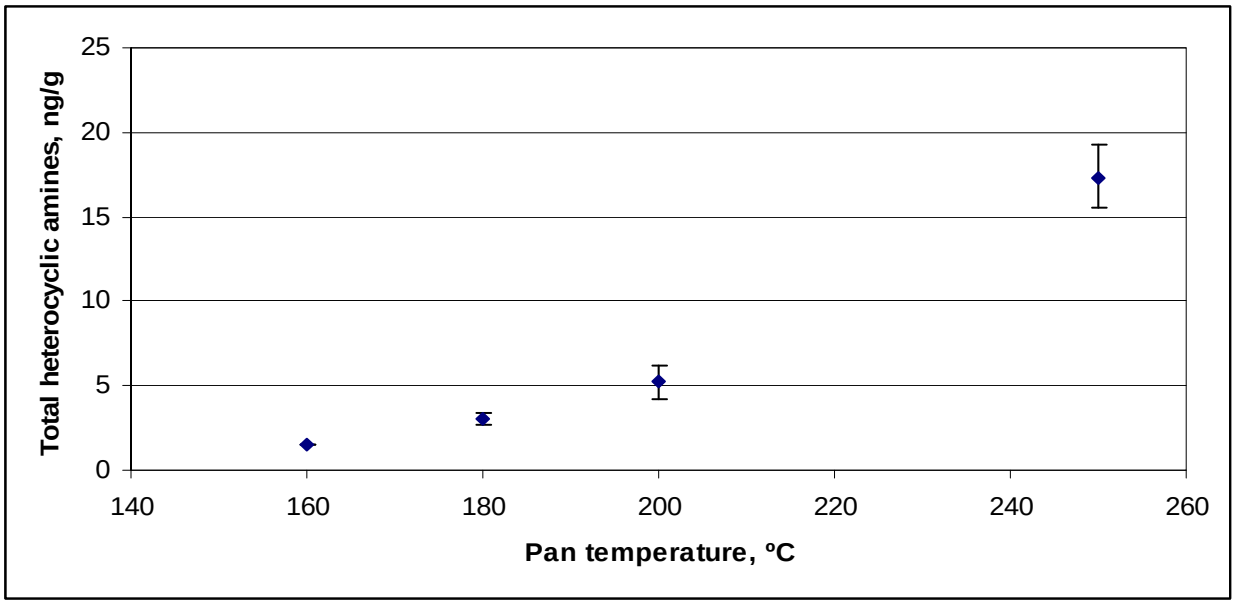


Figure 7. Formation of HCAs in beef patties after cooking to an internal temperature of 70ºC at different frying pan temperatures. Error bars are the standard error of four or five replicate cooking experiments (Knize and Felton 2005)

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. The method of cooking also influences HCA formation. This correlation seems direct, but when considering consumer cooking techniques, such as U.S.D.A. recommended safe internal temperatures and consumers monitoring doneness by color, the consumption of HCAs becomes much more complex.

Analysis of HCAs

Analysis of HCAs is a multi-step process. Initially, either a food sample or a model system is heated using various methods. Once sufficient heat is applied, extraction of any HCAs

from the complex food matrix or the model system is necessary. In accordance with the original HCA extraction method by Gross and Gruter (1992), a sample of meat is solubilized in sodium hydroxide then the HCAs are extracted with dichloromethane through multiple steps of solid phase extraction (SPE). The sample preparation and extraction methods have been adjusted slightly to improve the original method (Gross and Gruter 1992; Borgen and others 2001; Tsen and others 2006; Smith and others 2008; Gibis 2009).

The original method utilizes high performance liquid chromatography (HPLC) with eluents of triethylamine (TEA) at pH 3.2 and 3.6 and acetonitrile. The column used in the original method was a TSK gel ODS80 (Toyo Soda), 25 cm X 4.6 mm I.D. (5 μm particle size), with a Supelguard LC-8-DB (Supelco) guard column. The elution gradient can be seen in

Table 5. 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 Gruter 1992; Puangsombat and Smith 2010).

Table 5. Gross and Gruter HPLC 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

Variations on the original method alter the pH of the mobile phase as well as the type of column. 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. (2µm particle size) (Tosoh Bioscience, Stuttgart, Germany) improved separation and reduced run time as compared to the original method. The adjusted mobile phase gradient is shown in Table 6.

Table 6. Gibis HPLC elution gradient[a] (Gibis 2009)

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
13.5	10	55	35
15	0	25	75
17	46.5	50	3.5
21	46.5	50	3.5

[a] Flow rate = 1.1mL/min, temperature = 25°C

Inhibition of HCAs

The significant amount of research showing the risk associated with consuming HCAs, as well as the presence of HCAs in meat, indicate that reducing HCA formation is desirable. What then, is the best approach to minimize exposure to these cancer causing compounds? Murkovic (2004) states that the first step in reducing HCA intake is limiting consumption of certain foods, specifically meats.

While this sounds simple, meat is a significant part of the Western diet. It is possible, however, to encourage consumers to avoid consuming burnt meat. The relationship between increased temperature and increased HCA concentration suggests that the exterior portions of meat would have increased amounts of HCAs. The external portions of meat are exposed to the most direct cooking and therefore, reach the highest temperature. These high temperatures correlate to higher amounts of HCAs, especially in portions that achieve such high temperature they are considered 'burnt'. Murkovic (2004a) states that one way to decrease HCA consumption is to limit consumption of burnt meat. Dashwood (2002) agrees that limiting intake is the simplest way to reduce exposure to HCAs, but also suggests two other methods to reduce intake. The first is suppressing formation, which means limiting the ability of HCAs to form. The second is blocking formation, such as by adding an ingredient which would prevent formation.

Another approach to minimizing the potentially harmful effects of HCAs is to limit the gentoxicity of these substances after they are in the body. A recent study by Platt and others (2010) found that consumption of certain fruits and vegetables limits the genotoxicity of IQ and PhIP in Chinese hamster lung fibroblasts, with analysis of specific gene polymorphisms. The theory is that, even though HCAs may be present in the food, by decreasing or preventing activation by enzymes in the body the genotoxicity will also be decreased or prevented. It was found that certain fruit and vegetable juices were effective in decreasing the genotoxicity of IQ and PhIP by decreasing the activation of specific enzymes. Various points in the genotoxic activation of HCAs were studied with efficacy depending on the activation step as well as the type of fruit or vegetable juice. Sweet cherries, plums, blueberries, kiwi fruit, spinach, and onions were all found to be effective at limiting HCA genotoxicty, although at varying points in

activation (Platt and others 2010). While this is an area which merits further study, other works have emphasized the ability to limit the formation of HCAs.

Optimized Cooking Conditions

Optimizing the cooking conditions of meat is proposed to be an effective method for reducing HCA formation. As previously noted, the cooking conditions, specifically temperature and time significantly influence formation of HCAs (Balogh and others 2000; Knize and Felton 2005). The surface of the meat and the internal temperature of the product are important safety checkpoints for consumers. A study by Salmon and others (2000) showed that the way in which the meat reaches the desired internal temperature, in this case 70 °C (158 °F), is also important. Turning the meat every minute rather than just once midway through cooking, reduced the combined total of MeIQx, DiMeIQx, and PhIP. As Figure 8 shows, at every pan temperature, turning the patty multiple times resulted in a lower concentration of HCAs (Salmon and others 2000).

Another method for reducing HCAs through cooking techniques is through microwave pre-treatment. According to Felton and others (1994) microwave pretreatment suppresses HCA formation. This study looked at the effect of 1.0, 1.5, 2.0, and 3.0 minutes of microwave pre-treatment followed by pan-frying at 200 °C (392 °F) and 250 °C (482 °F) on beef patties. The liquid portion derived from microwaving was found to contain increasing concentrations of HCA precursors, namely creatine, glucose, and amino acids as well as fat, with increasing microwave treatment. The HCAs found in the pan-fried meat samples were IQ, MeIQx, DiMeIQx, and PhIP. The overall trend showed a reduction in precursors and a reduction in HCAs with increased microwave pre-treatment; however, PhIP did not follow this trend exactly. Conclusions can be

drawn stating that, by microwaving for 2 minutes, the amount of HCA precursors can be reduced by up to 30% and the total amount of HCAs can be reduced up to 90% (Felton and others 1994).

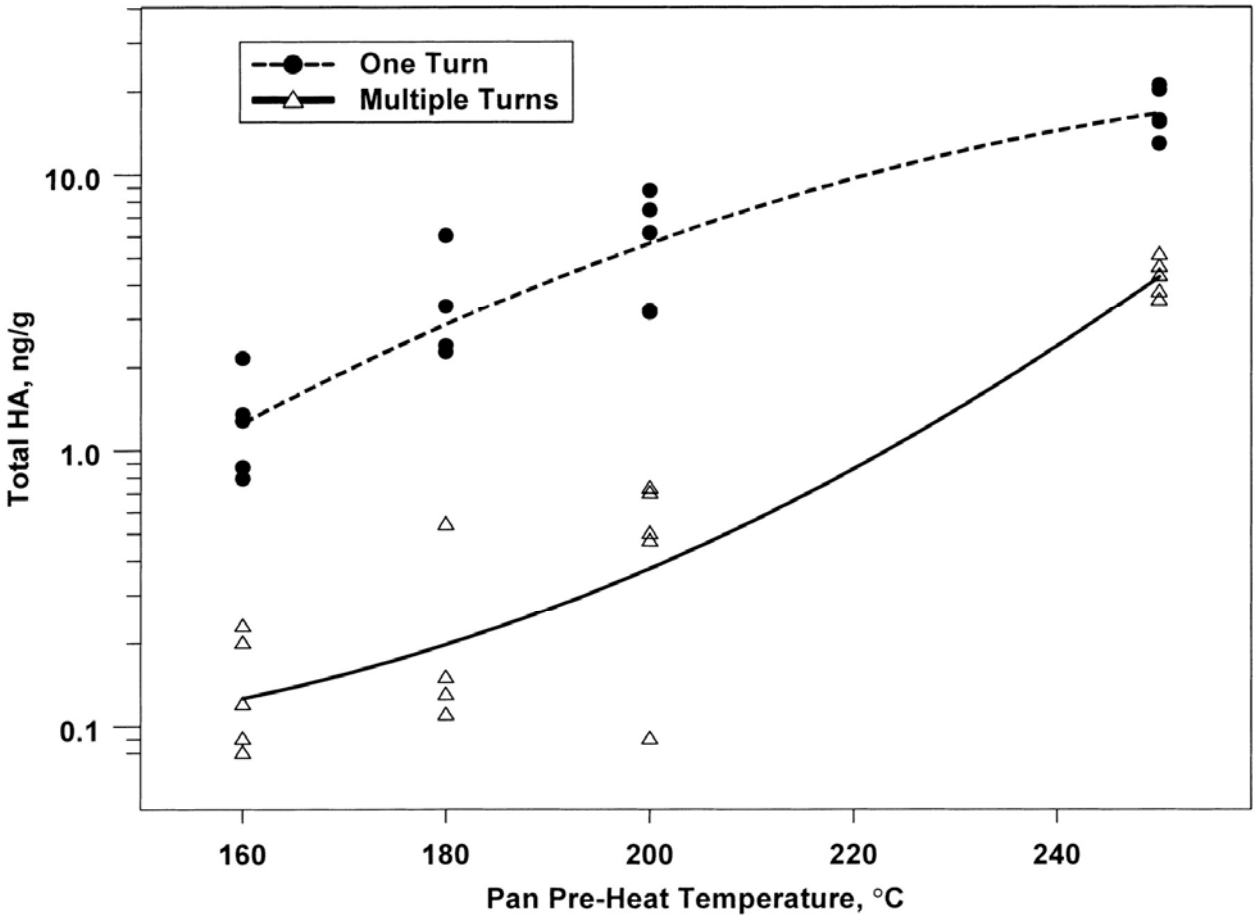


Figure 8. Total HCA (MeIQx, DiMeIQx, and PhIP) concentration (nanograms per gram of patty) in ground beef patties fried to a nominal internal temperature of 70°C as a function of pan temperature and whether the patty was turned once or multiple times. Curves show quadratic fits of the logarithm of total HCA concentration on pan temperature. Since the magnitude of the difference in log-total HCA depended on pan temperature, separate fits were performed for each turning method (Salmon and others 2000)

Blocking Formation

Adding inhibitory ingredients is another proposed method for blocking the formation of HCAs. Shin (2005) studied pork patties and found that certain inhibitory ingredients reduced HCA formation. These ingredients, listed in descending order of efficacy, were: cherry tissue extract, cherry tissue, sodium ascorbate, vitamin E, sodium nitrite, and sodium triphosphate. Prevention of free-radicals, as an intermediate in the Maillard reaction, would likely decrease the formation of HCAs (Murkovic and others 1998; Kikugawa 1999). Formation of free radicals occurs during the Maillard reaction and antioxidants scavenge these free radicals to prevent them from forming HCAs. Some natural sources of antioxidants include rosemary, thyme, and sage. Chemically derived antioxidants, including butylated hydroxyanisole (BHA), propyl gallate (PG), sesamol, exculetin, and epigallocatechin gallate (EGCG), have also proven to decrease HCA formation (Kikugawa 1999).

Following the same free radical theory, Murkovic and others (1998) looked at the antioxidants rosemary, sage, thyme and garlic as well as Fe^{2+} as a pro-oxidant in myoglobin applied to beef. The antioxidants had the anticipated effect of reducing HCA formation. Likewise, the addition of the pro-oxidant myoglobin caused an increase in IQ, MeIQx, 4,8-DiMeIQx, and PhIP formation. A decrease in MeIQx was seen with the addition of myoglobin. The overall trend shows that the addition of antioxidants is a probable method of blocking HCA formation.

However, studies by Cheng and others suggest that it is not the free-radical scavenging capabilities of these phytochemicals which reduces HCA formation (Cheng and others 2007; Cheng and others 2008; Cheng and others 2009). These studies analyzed the HCA inhibition of naringenin, a compound found in citrus fruit. Based on the Trolox equivalent antioxidant

capacity analysis, naringenin is not a strong antioxidant. Instead of naringenin following the free-radical-scavenging mechanism of HCA inhibition, an alternative pathway is suggested by Cheng in Figure 9. Compounds 1 and 2 show the result of naringenin preventing creatinine from reacting with phenylalanine and thereby producing PhIP (Cheng and others 2007; Cheng and others 2008).

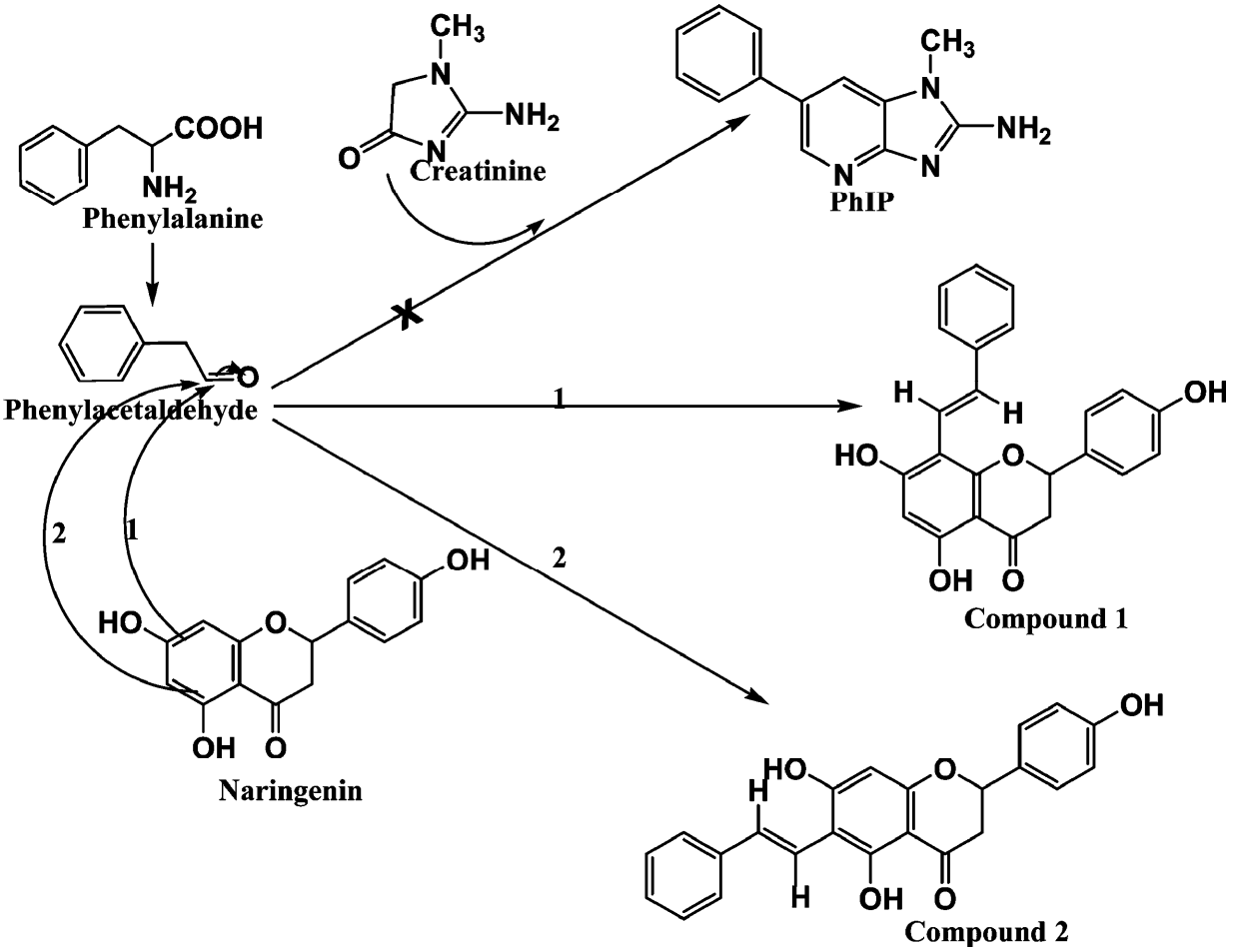


Figure 9. Postulated pathways for naringenin's inhibitory activity in PhIP formation (Cheng and others 2008)

In contrast, certain concentrations of antioxidants increase HCA content in specific applications. Tai and Chen (2001) found that the antioxidants vitamin C, α-tocopherol, and butylated hydroxy toluene did not reduce HCAs in fried fish fiber (Tai and others 2001). Liao and others (1998) found similar results that vitamin C at various concentrations did not reduce HCAs (Liao and others 2009). Another study by Zochling and others (2002) looked at industrially produced flavors in a model system to determine if they could reduce PhIP formation. The flavors studied were thyme, rosemary, and marjoram, as well as Monascus red. These flavors did not decrease the formation of PhIP (Zochling and others 2002). It should be noted that these were industrially produced flavors, which did not necessarily contain antioxidant potential.

While these instances show that antioxidants do not inhibit HCAs all the time, they do not disprove that antioxidants can be beneficial in some situations. Numerous studies show that antioxidants can aid in minimizing the risk from HCAs. The next three sections look at methods for applying antioxidants, including incorporating antioxidants into meat, marinating, and surface application.

Incorporation of Antioxidants

One application of blocking formation of HCAs is by incorporating antioxidants into the meat. In this method, antioxidants are mixed throughout the system, meaning that they are available to stop free radicals no matter where they form. The time allowed after the antioxidants are added to the meat but prior to cooking varies between 1 hour and 2 hours depending on the study (Balogh and others 2000; Tsen and others 2006; Cheng and others 2007; Cheng and others 2007). Scientists are studying the effectiveness of both synthetic and natural antioxidants.

Incorporating synthetic antioxidants, specifically vitamin E, reduce HCA formation. In a study of pork floss, 0.1 % addition of vitamin E significantly reduced norharman, PhIP, AαC, and MeAαC (Liao and others 2009). Another study by Balogh and others (2000) compared antioxidant treatments in ground beef. Concentrations of 1 % and 10 % (fat based) vitamin E and oleoresin rosemary were incorporated into the meat two hours prior to pan-frying. The 1 % vitamin E treatment significantly reduced IQ by 85 %, MeIQ by 78.6 %, MeIQx by 48.0 %, DiMeIQx by 79.2 %, and PhIP by 69.0 %. However, the treatments of 10 % vitamin E, 1 % oleoresin rosemary, or 10 % oleoresin rosemary only significantly reduced IQ and PhIP (Balogh and others 2000).

Incorporating natural antioxidants is another option. Tsen and others (2006) found that formation of MeIQx and PhIP was significantly reduced by the addition of antioxidant powders derived from rosemary. In this study, varying amounts of rosmarinic acid and rosemary powder were mixed with ground beef 2 hours prior to grilling on a non-stick surface at a temperature of either 375 °F or 400 °F. The addition of 0.02 % rosmarinic acid significantly reduced ($p < 0.05$) MeIQx by 69.6 % and PhIP by 63.8 %. Also, the addition of 0.3 % rosemary powder significantly reduced MeIQx by 57 % and PhIP by 77.1 % (Tsen and others 2006).

A study by Cheng and others (2007a) looked at phenolic antioxidant compounds derived from various sources and their effectiveness in reducing PhIP, 4, 8-DiMeIQx, and MeIQx when mixed in to ground beef patties. The 12 phenolic compounds with their corresponding origin were chlorogenic acid, quercitin, quercitin-3-glucoside, and rutin from fruits or vegetables; naringenin and hesperidin from citrus fruit; rosmarinic acid and carnosic acid from rosemary; and epicatechingallate, epigallocatechin, and theaflavin-3,3'-digallate from tea. Findings showed that inhibition varied based on the type of phenolic compound as well as the source of the

phenolic compound. Of the compounds studied, naringenin was the most effective with a 70% reduction in total HCAs (Cheng and others 2007).

Another study by Cheng and others (2007b) was based on the idea that extracting a specific compound from a fruit and using it as a single antioxidant may not be as effective as using a whole fruit extract; the idea was that the combination of compounds in fruit could work in unison to decrease HCAs. The fruit extracts tested were apple, grape-seed, pineapple, and elderberry. Dry powders of the extracts were incorporated into ground beef patties 60 minutes prior to pan-frying. Apple and grape-seed were the most effective at reducing HCAs. The specific phytochemicals in apple extract, proanthocyanidins, phloridzin, and chlorgenic compounds, were further studied in a model system. Proanthocyanidins were found to be the most effective single phytochemical in the apple extract; effectively reducing both PhIP and MeIQx (Cheng and others 2007). More research is necessary to determine if a single antioxidant can stop all HCA formation or if multiple antioxidants are more effective.

Surface Application of Antioxidants

Similar to the antioxidants found to reduce HCAs when incorporated into meat, antioxidants applied to the surface of meat can also reduce HCA formation. Weisburger (2002) analyzed the inhibition of HCA genotoxicity through application of tea polyphenols to the surface of ground beef patties 60 minutes prior to pan frying. Polyphenon B, a polyphenon found in black tea, significantly reduced the genotoxicity of cooked meat as determined by the Ames test. Table 7 shows the results of the Ames tests as well as the quantities of powder added to the surface of the meat (Weisburger and others 2002).

Ground spice powders have also been found to be effective inhibitors of HCAs through direct surface application. Murkovic (1998) applied the ground powders of rosemary, sage,

thyme, and garlic, individually, to the surface of beef 24 hours prior to pan-frying. However, the quantity of spice powder applied to the surface of meat was not reported. Sage followed by thyme were the most effective of the spices studied at reducing the formation of IQ, MeIQ, MeIQx, 4,8-DiMeIQx, and PhIP (Murkovic and others 1998).

Table 7. Effect of application of powdered polyphenon B to surface of meat patties prior to frying (Weisburger and others 2002)

Treatment	Revertants per plate (mean)
Control	1065.0 ± 42
Polyphenon B 159 mg	215.5 ± 22
Polyphenon B 175 mg	244.0 ± 30
Polyphenon B 521 mg	49.5 ± 8.0
Polyphenon B 597 mg	39.5 ± 14

Similarly, the efficacy of Vitamin E, another source of antioxidant activity, was analyzed for limiting formation of IQ, MeIQ, MeIQx, DiMeIQx, and PhIP by Balogh and others (2000). Vitamin E at 1% of the total meat weight was applied to the surface of ground beef patties 30 minutes prior to pan-frying, As Table 8 shows, Vitamin E application resulted in a significant reduction ($p < 0.006$) in formation of all HCAs of interest (Balogh and others 2000).

Table 8. Formation and inhibition of heterocyclic aromatic amine in fried ground beef patties following surface application of vitamin $E_{a,b,c}$ (Balogh and others 2000)

Compounds	IQ	MeIQ	MeIQx	DiMeIQx	PhIP
Control	16.4 ± 3.8a	12.3 ± 4.8c	7.1 ± 0.4e	5.9 ± 3.5g	53.7 ± 20.9i
Vitamin E	6.4 ± 5.8b	3.0 ± 3.0d	3.9 ± 1.2f	1.8 ± 0.7h	14.1 ± 6.0k
Inhibition (%)	61.0	75.6	45.1	74.6	73.0

[a]Vitamin E was added at 1% level based on fat content (15%) of ground beef patties.

[b]Each value represents the mean ± SD for duplicate analyses of seven replications.

[c]Means in columns with different letters are significantly different at $p < 0.006$.

Marinating

Not only can consumers incorporate antioxidants into a meat mixture or apply them directly to the surface, but marinating has also been proven to effectively reduce HCAs. Marinades can be home-made or store-bought and vary greatly in ingredients, including spices, juice, and/or beverages.

One study by Gibis (2007) found that, with ground beef patties, marinades which contain garlic or onion reduce HCAs but marinades which contain lemon juice did not. Interestingly, 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). This shows that while an ingredient may be effective in reducing HCAs, it still must be palatable to consumers.

To overcome the flavor issues of marinating with garlic and onion, a study of meat marinated in hibiscus extracts found a reduction in PhIP and MeIQx without a negative impact on sensory characteristics (Gibis and Weiss 2010). Similarly, green tea marinades reduced HCAs while maintaining consumer acceptability (Quelhas and others 2010). These studies show that it

is possible to marinate meat in a way that is desirable to consumers while also reducing formation of HCAs.

Smith and others (2008) took a different approach by analyzing commercially available Caribbean, Southwest, and Herb marinades 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 formation. Of the marinades studied, Caribbean was the most effective at reducing the HCAs of interest. However, significant reductions ($p < 0.05$) were seen across all of the marinades studied for the portions which contained the spices (Smith and others 2008).

Similar to the other methods, more research is necessary to determine the type of marinade, method of application, or combination of factors which reduce HCAs the most while maintaining consumer acceptability.

Consumer Trends

According to an April 2009 *Food Technology Magazine* article about the top ten food trends, consumers are likely to be ready to accept new products aimed at reducing HCAs (Sloan 2009). The number one trend of consumers is "Cooking Again", meaning cooking at home. Consumers are looking for products which help them create restaurant style foods conveniently at home. The third trend is "Gourmetization", or making eating experiences gourmet. There is an increased demand for unique flavors, including extreme spices or ethnic seasonings. The eighth food trend also applies to HCA reduction, "Me M.D.". Consumers are paying attention to, and looking for, the health benefits of food. This means eating more functional foods, particularly naturally functional foods. The article states that naturally functional foods are likely to be a top

food trend for the next ten years. Each of these trends offers an opportunity to market ways to reduce HCAs.

Conclusions

The multitude of research relating HCAs to cancer, the presence of HCAs in commonly consumed foods, and the high cancer-related death rate results in the need to prevent the consumption of these compounds. Reducing consumption through optimizing cooking conditions and blocking formation through added ingredients all lend to minimizing human exposure to HCAs. Methods of reducing HCAs are available although more research is necessary to understand the mechanisms by which HCA formation can be prevented.

Pre-cooking, incorporating antioxidants, surface application of antioxidants, and marinating all can reduce HCAs, but many questions are still unanswered. Further information is needed to determine the cooking methods which can affectively reduce HCA formation, the compounds which are most affective at reducing HCA formation while maintaining palatability, and, overall, the best method to limit consumption of HCAs. These and many more questions arise when studying previous work on HCAs. Future research must keep the consumer in mind. Palatability and ease-of-home-preparation must be constant considerations for researchers.

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Chapter 2 - Effect of Direct Application of Spices to Beef on Heterocyclic Amine Formation

Abstract

Heterocyclic amines (HCAs) are cancer causing compounds formed during the cooking of meat. Previous studies show that incorporating antioxidant spices into meat as well as marinating meat with antioxidant spices reduces formation of HCAs. The purpose of this study was to determine if commercially available spices applied to the surface of meat could effectively reduce HCA formation. Two commercially available spice blends and one blend of spices with known quantities of antioxidant spices were sprinkled onto the surface of beef just prior to pan-frying. The quantities of spices used were based on the amounts customarily consumed in typical Western cooking. The results of direct application were then compared to marinating with the same types and amounts of spices. The antioxidant potential of the spices was analyzed using DPPH and total phenolics methods. Results indicated that the spices would be effective antioxidants. Low recovery rates and problems during the extraction process made results inconclusive, but suggest that further research may find that applying spices directly to the surface of meat in consumer acceptable quantities may be as effective as marinating at reducing the formation of HCAs.

Introduction

Heterocyclic amines (HCAs), also known as heterocyclic aromatic amines, are cancer causing compounds formed on the surface of meat during cooking (Murkovic 2007). Cancer being the second most common cause of death in the United States (National Vital Statistics System, National Center for Health Statistics, CDC 2007), causes a need for limiting the exposure to carcinogenic compounds. The association between cancer and food is not a new phenomenon, the earliest studies date back to 1939 by Widmark and current estimates suggest that one-third of all cancer is related to food (Dashwood 2002; Sugimura 2002). HCAs are not the only compounds in food which cause cancer, but estimates of exposure to HCAs suggest that they could be a contributing factor to human cancer.

The ten most common 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).

While not all HCAs are known to be carcinogens, specific HCAs have been listed by the United States as well as internationally. The 12th Report on Carcinogens declares MeIQ, MeIQx, IQ, and PhIP *reasonably anticipated to be human carcinogens* (NTP, U.S. Department of Health and Human Services, Public Health Service 2011). The International Agency for Research on Cancer lists IQ as *probably carcinogenic to humans* and nine other HCAs as *possibly carcinogenic to humans* (IARC 1993). A review of research by the World Cancer Research

Fund/ American Institute for Cancer Research found convincing evidence for an association between colorectum cancer and consumption of red meat and processed meat (World Cancer Research Fund / American Institute for Cancer Research 2007).

The HCAs of concern in cooked meat are categorized as amino-imidazo-azaarenes; polar compounds formed at typical cooking temperatures. The precursors of HCAs are amino acids, creat(in)ine, and carbohydrates in the presence of heat (Murkovic 2004). The exact mechanisms of formation are yet unknown, but multiple pathways have been suggested (Pearson and others 1992; Murkovic 2004).

The formation of HCAs is dependent on not only the precursors, but also the temperature and time of cooking. Arvidsson and others (1997) found that increasing the temperature and the length of cooking time significantly increased the amount of HCAs formed (Arvidsson and others 1997). Other factors which also influence HCA formation include cooking method (Murkovic and others 1998; Shin 2005), frequency of turning meat (Balogh and others 2000), and final internal temperature (Knize and Felton 2005).

While the simplest way to reduce exposure to HCAs is to limit consumption of meat; the prevalence of meat in the western diet suggests this is not a plausible solution. Other methods for reducing exposure to HCAs have been proposed which include optimizing meat cooking conditions and applying ingredients to block formation of HCAs. Optimizing meat cooking methods have been shown to be as simple as turning meat more frequently (Salmon and others 2000) or precooking meat in a microwave (Felton and others 1994).

Blocking formation of HCAs includes adding antioxidant spices, or other materials, to meat to reduce HCA formation. The methods of adding antioxidant spices to meat include incorporation into meat, surface application, and marinating. Studies find that mixing antioxidant

materials, such as spices, into meat effectively reduces HCA formation (Balogh and others 2000; Tsen and others 2006; Cheng and others 2007b).

Other methods of adding inhibitory ingredients include surface application and marinating. Reduction in HCA formation has been associated with surface application of green tea (Weisburger and others 2002), antioxidant spices (Murkovic and others 1998) and Vitamin E (Balogh and others 2000). Marinades which include antioxidants such hibiscus, garlic, and onion, (Gibis 2007; Gibis and Weiss 2010), green tea (Quelhas and others 2010), as well as commercially available marinades (Smith and others 2008), are capable of reducing HCAs.

While all these methods of reducing HCA formation appear plausible, it is important that, no matter the method, the consumer preferences and trends be considered. While a method may be very effective at reducing HCA formation, if the resulting product is not palatable then the change is likely to not be accepted by the consumer. Taking all these factors into consideration, this study aims to determine if the amount, type, and application method of antioxidant spices influences HCA formation. Specifically, direct application of spices by sprinkling on to the surface of beef will be studied for effective types of spice, quantity of spices, and if direct application is as effective as marinating with antioxidant spices.

Materials and Methods

Quantification of HCAs is a multi-step process including solid phase extraction and analysis with high performance liquid chromatography. The meat is first cooked then solid phase extraction is used to remove the HCAs from the meat. Finally high performance liquid chromatography separates and quantifies the compounds. The original procedure by Gross and Gruter (1992) was optimized by Gibis (2009) to decrease HPLC run time and improve separation (Gross and Gruter 1992; Gibis 2009).

Materials

Ethyl acetate, hydrochloric acid, methanol (HPLC grade), ammonium acetate, ammonium hydroxide, phosphoric acid, sodium carbonate, and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Fairlawn, New Jersey, U.S.A.). Deionized water was processed by a Sybron/Barnstead PCS unit (Barnstead/Thermolyne, Inc.; Dubuque, Iowa, U.S.A.). Triethylamine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin- Ciocalteu's reagent, and gallic acid were purchased from Sigma Chemicals (St. Louis, Missouri, U.S.A.). Triethylamine was stored in the refrigerator for no longer than six months; the prepared solution of triethylamine was used for a period no longer than two days. Ethanol, 190 proof, was purchased from Decon Lab Inc. (King of Prussia, Pennsylvania, U.S.A.). The HCA 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), PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine) were obtained from Toronto Research Chemicals (Ontario, Canada).

Sample Preparation and Cooking

Eye round steaks, *semitendinosus*, were purchased from a local grocery store in Manhattan, Kansas, U.S.A. The grocery store cut the steaks to 1 inch thick. The steaks were placed in individual freezer bags and frozen until use; freezer time did not exceed four weeks. The steaks were partially thawed in the refrigerator at 4 °C then cut to size using an 8 cm x 5 cm template and then weighed. The steaks then fully thawed in a refrigerator overnight at 4 °C. Five samples of meat were submitted to the Analytical Services Lab, Kansas State University (Manhattan, Kansas, U.S.A.) for analysis of fat, moisture, protein, and ash. A CEM Smart Trac system (CEM Corporation, Matthews, North Carolina, U.S.A.) using rapid magnetic drying and

nuclear magnetic resonance was used for fat and moisture. Protein was determined using a LECO FP-2000 protein analyzer (LECO Corp., St. Joseph, Michigan, U.S.A.). The results of composition analysis are shown in Table 9.

Table 9. Fat, moisture, protein, and ash analysis for beef samples (n = 5)

	Fat	Moisture	Protein	Ash
Mean ± standard deviation	4.59 ± 1.61	70.01 ± 0.96	23.71 ± 0.61	1.16 ± 0.03

Commercial spices were obtained from a local grocery store in Manhattan, Kansas, U.S.A. The spices consisted of a Montreal Steak blend and Italian blend. The suggested serving size for the Montreal Steak blend was 2.5 g (¾ t.). The suggested serving size for the Italian was 0.2 g (¼ t). Standardized individual spices were donated by the McCormick Science Institute. A blend of standardized spices was prepared using 25% thyme, 25% rosemary, 25% oregano, and 25% sage; further information, including ORAC and Total Phenolic values, are presented in Appendix A - McCormick Spice Standards. These spices were chosen based on the main spices in the commercial Italian blend. It should be noted that the particle size of the standardized spices was dramatically smaller than the commercial blends. The treatment amount for each spice was determined based on low, medium, and high amounts of the suggested serving sizes (Table 10). While sensory analysis studies of statistical relevance were not completed, a small group of people tasted sample steaks prepared and cooked under the same conditions as the research samples. The small tasting panel suggested that treatment amounts or kinds of spice were not objectionable.

For direct application of spice treatment, steaks were removed from the refrigerator 30 min prior to cooking. Low, medium, high, or none (control) amounts of spice were sprinkled directly on to each side of the steak 5 min prior to cooking.

Table 10. Type and amount of spices for each treatment

Spice	Low (g ± 0.005)	Medium (g ± 0.005)	High (g ± 0.005)
Blend	0.105	0.215	0.325
Italian	0.095	0.195	0.295
Montreal	0.365	0.725	1.085

For marinade treatment, steaks were removed from the refrigerator 30 min prior to cooking. The high amount of spice was mixed with 15 mL each of water, vinegar, and peanut oil. Five minutes prior to cooking, both sides of the steak were dipped in the marinade. The steak was flipped continusously during the five minute marinating period to ensure coverage of the treatment.

Directly prior to cooking the internal temperature of the steak was recorded as well as the weight of the treated sample. Steaks were cooked 10 min per side at 204 °C (400 °F) on a preheated, non-stick electric griddle (Oster, Sunbean Corp., Boca Raton, Florida). The surface temperature of the griddle was set at 400°F and was monitored using a direct-contact thermometer (Branant Company, Barrington, Illinios). Prior to cooking, the griddle was wiped with a small amount of peanut oil, purchased from a local grocery store in Manhattan, KS. A thermocouple thermometer (Branant Company, Barrington, Illinois) was used to monitor the

initial temperature, temperature at mid-point, and final temperature. The steaks were allowed to cool at least 30 min before measurement of cooked weight and preparation for extraction.

Extraction and Analysis of HCAs

The outer 2 mm of the cooled steak was sliced off using a rotary slicer (Cabela's by Chef's Choice Commercial Grade Slicer, Sidney, Nebraska, U.S.A). The weight of the trim was recorded. The trim was then coarsely ground in a food processor (Kitchen Aid, model KFP 750). A 3.0 (± 0.05) g sample was weighed in a Waring blender bowl and 9 ml of 1 M sodium hydroxide (NaOH) added. The internal standard, TriMeIQx, was added prior to blending and extraction (150 µl of 250 ppb TriMeIQx). The sample was blended in a Waring blender (ThermoFisher Scientific Co., Pittsburgh, PA, U.S.A.) for 3.0 min on speed 4. The ground sample was transferred to 13 g of Isolute HM-N diatomaceous earth (Biotage, Charolotte, North Carolina). To aid in complete transfer from the chopper bowl, 3 ml more of NaOH were used to clean the bowl and added to the Isolute. The mixture was then tapped into extraction columns (Merck, Darmstadt, Germany) fitted with fiber glass filters. The Isolute filled columns were held under refrigeration conditions up to 24 h prior to extraction.

For extraction, Bond Elute solid phase extraction 500 mg propyl-sulfonic acid (PRS) cartridges and 100 mg C-18 cartridges were obtained from Varian (Agilent, Lake Forest, California). PRS cartridges were pre-conditioned with 5 ml ethyl acetate with vacuum suction plus 2 ml ethyl acetate without pressure. Prepared Isolute columns were then attached to PRS cartridges using coupling adapters (Varian Inc., Harbor City, CA, U.S.A.). HCAs were extracted from the Isolute column on to the PRS cartridge using 60 ml ethyl acetate in accordance with the procedure by Borgen and others (2001b). The PRS cartridge was then vacuum dried 4 min at 15 mm Hg. The PRS cartridge was then washed to remove the nonpolar HCAs with 6 ml 0.1 M

HCl, 15 ml MeOH/0.1 M HCl (45 : 55, v/v), and 2 ml distilled water. The C-18 cartridges were then attached to the vacuum chamber and conditioned with 5 ml MeOH and 5 ml distilled water. The PRS cartridges were attached to the C-18 cartridges and HCAs extracted using 20 ml 0.5 M ammonium acetate, adjusted to pH 8.0 or greater with NH_4OH. 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. HCAs were eluted into vials from the C-18 cartridges with 1 ml MeOH/ NH_4OH (9:1, v/v). Samples were taken to dryness with N_2 pressure and held at 0°C for no more than 1 week.

The HCAs were measured by reverse phase chromatography with a Hewlett Packard Series II 1090 Liquid Chromatograph (Agilent Technologies) fitted with a photodiode array ultraviolet- visible detector and Hewlett Packard 1046A programmable fluorescence detector. Ultraviolet detection was from 262 nm to 435 nm. The fluorescence detector was programmed at wavelengths of 229 nm for excitation and 437 nm for emission. Following the Gibis method, with slight modifications (Gibis 2009), the mobile phase was 0.01 M triethylamine, pH 3.0, 0.01 M triethylamine, pH 4.0, and acetonitrile. The gradient is shown in Table 11. Adjustments to the pH were made using phosphoric acid. Separation was achieved with a TSK-gel Super ODS, reversed-phase C-18 column (10 cm x 4.6 mm I.D., 2 μm particle size) (Tosoh Bioscience, Stuttgart, Germany) at 40 °C. Injections were made by first hydrating each sample with 25 μL of starting mobile phase; 20 μL of the mixture was then injected into the HPLC.

Table 11. HPLC elution gradient for sample analysis

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

Standard Curves and Spectral Matching

MeIQx, DiMeIQx, TriMeIQx, and PhIP standards were prepared by creating a 30 ppm solution of the individual standards using 3.0 mg of standard with 100 ml of 100% methanol. These stock solutions were used to make serial dilutions for obtaining a standard curve of 25ppb, 50 ppb, 100 ppb, 250 ppb, 500 ppb, and 1000 ppb using the peak area from the HPLC analysis. Dixon's Q-test (Miller and Miller 2005) was performed to determine if outliers existed in the data, specifically noting MeIQx. One sample, 25 ppb MeIQx, was deleted based on a 90 % confidence interval. All standard curves had a coefficient of determination (R^2) of 0.98 or greater. The standard curves are presented in Appendix A.

HCA identification was based on the online UV spectral matching to a spectral library made from pure standards with a match factor of no less than 950.

Recovery

Percent recovery was calculated by following the procedures for extraction and analysis; however, instead of adding meat to the diatomaceous earth, 300 µl of 250 ppb mixed HCAs (MeIQx, DiMeIQx, TriMeIQx, and PhIP) was mixed directly with 12 ml of 1 M sodium hydroxide (NaOH) and then mixed with the Isolute. Four recovery extractions were completed; however, one sample did not extract properly, and was therefore discarded. The remaining samples were injected into the HPLC. To calculate the percent recovery, the area counts from the chromatograms were compared to the standard curves. The recoveries were determined to be 62.0 % MeIQx, 48.9 % DiMeIQx, 28.3 % TriMeIQx, and 26.8 % PhIP. These values are low compared to Borgen and others (2001) whom found 70 – 100 % recovery for MeIQx and DiMeIQx (Borgen and others 2001a). However, the majority of articles related to HCAs do not specify percent recovery and those that do, seldom report how recovery was determined.

Numerous experiments were conducted to troubleshoot the reason behind such low recovery rates. While the sample sizes in the troubleshooting experiments were too low to obtain statistically significant results, these tests indicate areas of further study to improve analysis of HCAs. The areas of analysis included hydration volume prior to injection, hydration solvent prior to injection, diatomaceous earth source, and step-by-step extraction analysis.

One point in the procedure which was suspected to influence the percent recovery was the method in which the dried sample was injected into the HPLC. The method by Puangsombat and Smith (2010) hydrated the dried sample with 25 µL methanol then injected 20 µL of the mixture into the HPLC (Puangsombat and Smith 2010). While the use of methanol appeared to effectively transport the HCAs from the dried sample container to the HPLC, noise was noticed on the HPLC as the methanol interacted with the mobile phase. To combat this, the starting

mobile phase was used, at the same volume, to hydrate the sample. Hydrating the sample with the starting mobile phase greatly reduced the initial interference caused by methanol on the chromatograms (See Appendix C). Increasing the amount of starting mobile phase used to hydrate the sample was also examined; however no improvement in spectral matching or recovery was observed.

From preliminary studies to data collection for statistical analysis the source of diatomaceous earth changed due to the original product becoming unavailable. The preliminary trials for the project used Extrulet columns and diatomaceous earth refill material supplied by VWR International (Bristol, CT, U.S.A.). Prior to beginning data collection for statistical analysis, samples of Extrulet, and the new source, Isolute, were compared. TriMeIQx was the only HCA analyzed. No dramatic difference in Extrulet and Isolute percent recovery for TriMeIQx was observed. During data collection for statistical analysis however, it was noted that ethyl acetate did not pass through Isolute during the first phase of extraction consistently; 60 mL of ethyl acetate was added to each column but the volume out of the column was not always the same. For the majority of the samples the volume was close to 50 mL, but a small number of the extractions had a volume closer to 30 mL. While this was noted for Isolute it is not possible to rule-out the possibility of the same occurrence happening with the Extrulet; however, no such issues were observed.

Step-by-step analysis of the extraction procedure was performed to determine when loss in HCAs was occurring. One analysis, point A in Figure 10, looked at the material coming off of the PRS column. Findings suggest that the PRS column effectively ‘holds’ on to the HCAs when ethyl acetate is the solvent, a desirable characteristic. Another analysis, point B in Figure 10, looked at the HCA concentration in the ethyl acetate which passed through the Isolute, but

caught the sample prior to the PRS column. The recovery in these samples was very similar to the amount when extraction was carried out in full. While the sample size was too low to obtain statistically significant differences, the results indicate that the percent recovery is dependent upon the amount of HCAs extracted in the first phase of extraction. Increasing the amount of ethyl acetate used in the first phase of extraction was also examined; however, increasing ethyl acetate did not result in higher recovery rates.

Another factor which may result in lower recovery was the chemical used in the initial phase of extraction. Studies indicate that dichloromethane, instead of ethyl acetate, is a better extraction solvent (Borgen and others 2001b; Kondjoyan and others 2010). Dichloromethane was not used for any portion of this research project due to its cost and its possible carcinogenicity. The recent Report on Carcinogens (NTP, 2011) has classified it as *reasonably anticipated to be a human carcinogen* and thus there are certain risks associated with its use.

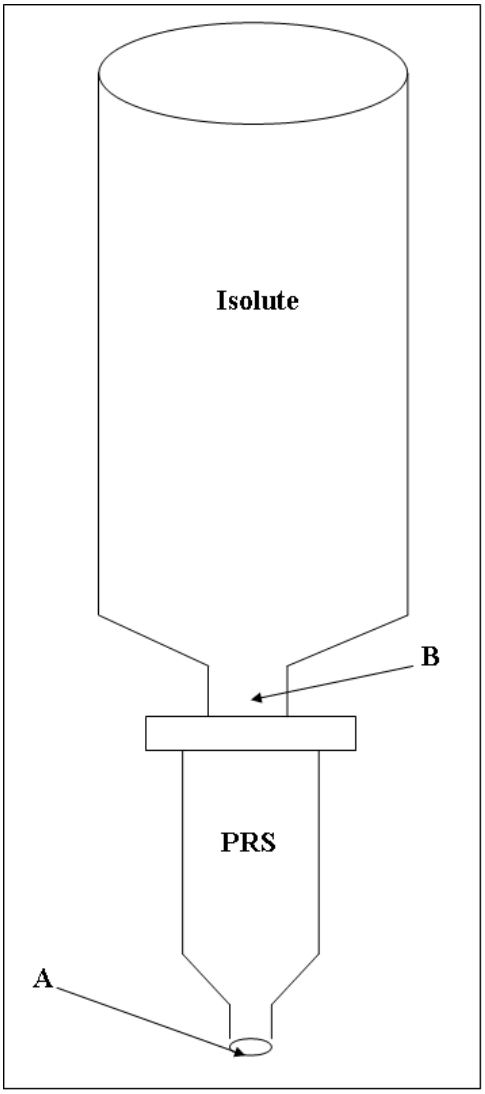

Figure 10. Step-by-step analysis of extraction procedure; theoretically, Sample A should not contain HCAs because they are held on the PRS column while Sample B should contain HCAs extracted from the meat

DPPH and Total Phenolics

For the spice mixtures of Montreal, Italian, and Blend, 3.5 g of each were mixed with 35 mL 95 % ethanol in an Erlenmeyer flask. The samples were shaken for 2 hours by a wrist-action shaker (Burrell, Pittsburgh, PA, USA) at room temperature. Each mixture was then filtered

through Whatman # 4 filter paper. The ethanol extract was used for 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Total Phenolic determination.

The DPPH radical scavenging activity of the spices was analyzed using the method by Singh and others (2002). Ethanol was used to dilute the sample 1:2, 1:5, 1:10, 1:20, 1:40, and 1:100 (v/v). DPPH powder was mixed with methanol to create a 0.1 mM DPPH methanolic solution; the solution was stored in a container which excluded light, under refrigeration conditions for no more than 2 days. 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 minutes. Controls were prepared by combining 0.1 mL of each dilution with 2.9 mL 95 % ethanol. The absorbance of samples and controls was measured at 517 nm with a UV/VIS spectrophotometer. The results were recorded as the radical scavenging activity as calculated using the following equation (Singh and others 2002):

$$\text{Radical scavenging activity (\%)} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

The procedure by Chang and others (2006) was used to measure total phenolics. Dilutions of 1:10 and 1:20 (v/v) were made to the prepared spice extracts using ethanol. For each dilution, 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 minutes before adding 1 mL of 7.5 % sodium carbonate solution and, again, vortexing. The samples were stored in the dark for 2 hours at room temperature. Then, the absorbance at 765 nm was measured using a UV/VIS spectrophotometer. The readings were compared to a standard

curve of gallic acid at a range of 25 – 250 μg / mL and reported as mg gallic acid equivalents per 1 g dried spice mixture (Chang and others 2006).

Statistical Analysis

All statistical tests were analyzed using SAS version 9.2 (SAS Institute Inc., Carry, North Carolina, U.S.A.). Analysis of Variance (ANOVA) with fixed factors only was used to examine statistically significant differences in the data; the level of significance was set at $p < 0.05$. The treatment structure was 2 x 3 factorial plus control with a Completely Randomized Design (CRD). The data was divided into two experiments. The first looked across the samples treated with the different kinds of spices at the different levels; a spice by concentration interaction. The second experiment compared the marinade treatment to the direct application treatment across the different spices at the highest treatment amount; a spice by spice application interaction. All results were reported with a 95 % level of confidence.

Results and Discussion

High variability was seen between preliminary results and results used for statistical analysis. While the preliminary data indicated that direct application of spices would reduce the HCAs of interest, the final results were less convincing. However, trends can be seen which indicate a need for further research in this area. The results for DPPH and Total Phenolics show that commercially available spice blends are sources of antioxidants, but vary greatly in antioxidant capacity.

DPPH and Total Phenolics

DPPH and Total Phenolics were analyzed to determine the antioxidant capacity of the spices. The DPPH radical scavenging results correspond to the idea that increasing the quantity

of antioxidant spices in the spice blend increases the percent inhibition. The graph of the spices with corresponding percent inhibition can be seen in Figure 11. The ingredient statement for the commercially available Montreal spice lists salt, garlic, spices (including black and red pepper), sunflower oil, onion, natural flavor, and extracts of paprika. As seen in Figure 11, Montreal had the lowest percent inhibition across all concentrations.

The Italian ingredient statement is marjoram, thyme, rosemary, savory, sage, oregano, and basil. The Blend treatment was developed based on the Italian ingredient list, but with equal parts thyme, rosemary, oregano, and sage. The spices making up the Italian mixture have been shown to have an higher antioxidant capacity than those in the Montreal mixture. While the Blend had the greatest percent inhibition, an association can be seen between Blend and Italian. Besides the difference in ingredients, a major factor which is likely to contribute to the difference in Italian and Blend is the size of the particles within the spice. Blend had dramatically smaller pieces than Italian. A smaller grind would have more surface area and therefore have more space for direct interaction with the meat.

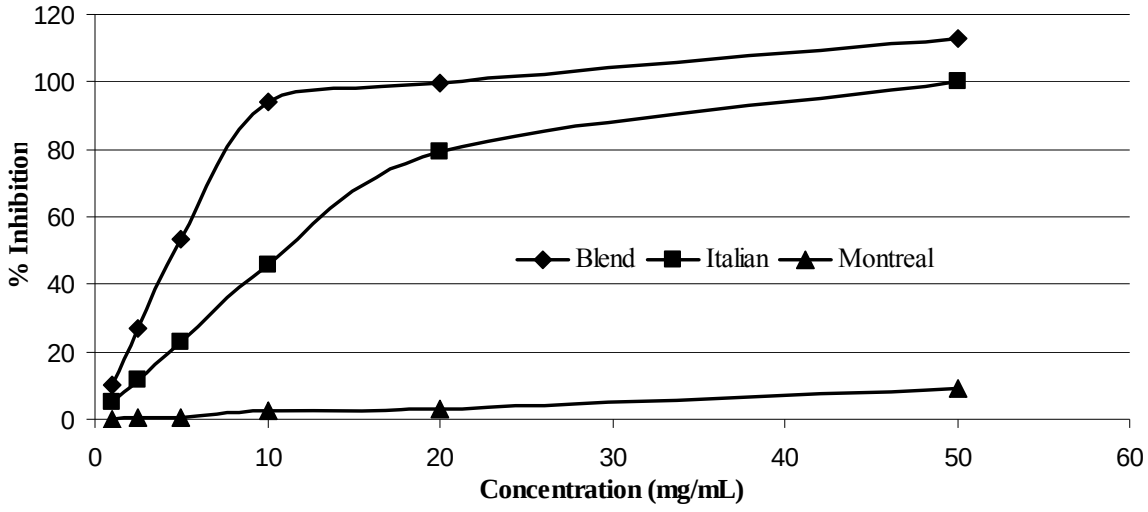

Figure 11. DPPH scavenging activity of Blend, Italian, and Montreal spice mixtures

Total phenolics, as shown in Figure 12, is another method to analyze the antioxidant potential of substances, including spices. Similar to the DPPH results, Montreal showed the lowest antioxidant potential as measured by milligrams gallic acid equivalents per 1 gram of spice; blend had the highest value.

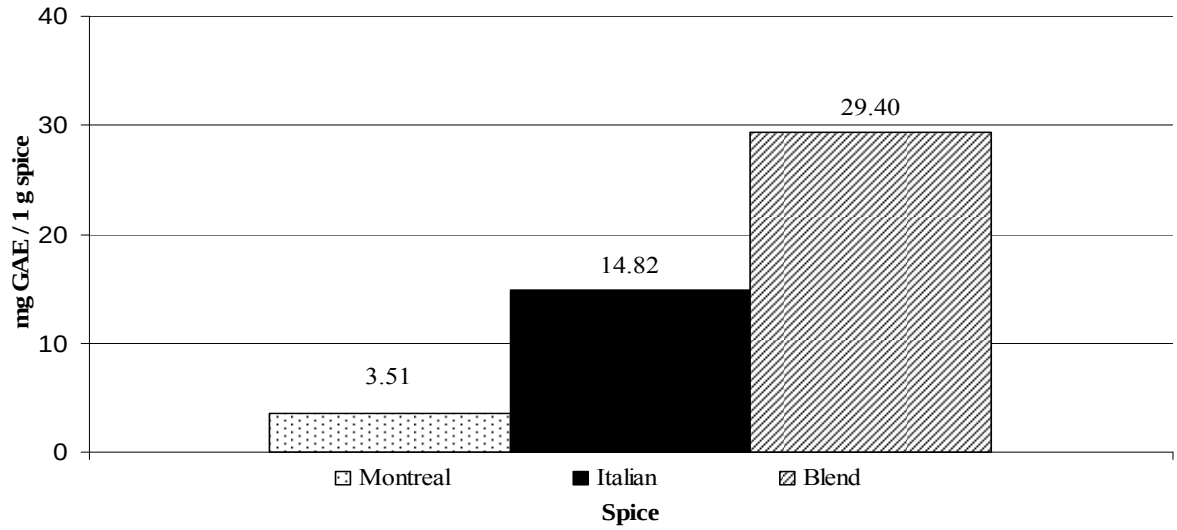

Figure 12. Total phenolics analysis of Blend, Italian, and Montreal spice mixtures

Direct Application of Spices

As previously mentioned, the results for comparing the type and amount of spice with the amount of HCAs, were inconsistent from preliminary to final results. For the data analyzed, three sub-samples were determined to be outliers based on univariant studentized residual internally, univariant studentized residual externally, and residual; those samples were deleted. Table 12 shows the results as means with standard deviations.

The highest amount of spice for both Italian and Montreal significantly reduced all HCAs of interest. However, these were the only treatments which significantly reduced the formation of PhIP. It should be noted that the lowest percent recovery was for PhIP which is likely to contribute to the lack of significant differences. Excluding PhIP, the other HCAs of interest, namely MeIQx and DiMeIQx, were significantly reduced by Italian at all amounts of treatment and Blend at the lowest amount of treatment. It is surprising that only the lowest amount of Blend showed significant reduction in these two HCAs, but this is likely related to the low recovery rates influencing the needed difference to show significance. This is also likely to be true across the remainder of the data. Across Blend treatment amounts as well as across Montreal treatment amounts, significant differences from the control are observed, but not in a consistent manner. As an example of this, the mean values of MeIQx for Blend at low amount and Blend at high amount are significantly different from the control; however, Blend at medium amount for MeIQx is not significantly different from the control.

While trends are not easily seen across the data, it should be noted that increasing the *p*-value from 0.05 to 0.1 dramatically changes the results. With $p < 0.1$ for MeIQx, all treatments are significantly different from the control except for Montreal at the lowest treatment amount. For DiMeIQx, all treatments are significantly different from the control except for Montreal at the medium amount of spice and Blend at the highest amount of spice. However for PhIP, changing the level of significance does not have as dramatic of an affect. The only significantly different values are Blend at the lowest amount, Italian at the highest, Montreal at the highest, and Montreal at the lowest.

Table 12. Amount of MeIQx, DiMeIQx, and PhIP as a result of direct application of Montreal, Italian, and Blend at three amounts to the surface of meat

Treatment	MeIQx (ng/g)	DiMeIQx (ng/g)	PhIP (ng/g)
Control	0.534 ± 0.06^{a}	0.165 ± 0.06^{a}	0.100 ± 0.01^{a}
Montreal High	0.210 ± 0.02^{c}	0.038 ± 0.02^{b}	0.036 ± 0.01^{b}
Montreal Medium	0.218 ± 0.13^{c}	0.108 ± 0.16^{ab}	0.071 ± 0.09^{ab}
Montreal Low	0.522 ± 0.04^{ab}	0.066 ± 0.02^{b}	0.054 ± 0.01^{ab}
Italian High	0.282 ± 0.10^{c}	0.067 ± 0.01^{b}	0.051 ± 0.01^{b}
Italian Medium	0.288 ± 0.01^{c}	0.047 ± 0.02^{b}	0.077 ± 0.01^{ab}
Italian Low	0.361 ± 0.13^{bc}	0.055 ± 0.02^{b}	0.067 ± 0.04^{ab}
Blend High	0.317 ± 0.13^{c}	0.105 ± 0.02^{ab}	0.084 ± 0.03^{ab}
Blend Medium	0.356 ± 0.09^{abc}	0.059 ± 0.02^{b}	0.072 ± 0.01^{ab}
Blend Low	0.293 ± 0.10^{c}	0.030 ± 0.02^{b}	0.054 ± 0.00^{ab}

Values are shown as mean ± standard deviation of three replicates with two sub-samples each. Symbols bearing different letters in the same column are significantly different ($p < 0.05$).

Spice Application Comparison

The comparison of spice application method was anticipated to give a base-point for determining if direct application of spices was as effective as marinating with spices. However, the results obtained did not correspond with previous research. Inconsistency was seen from previous studies which stated that marinades containing antioxidant spices would reduce the amount of HCAs formed (Gibis 2007; Smith and others 2008; Gibis and Weiss 2010). The data obtained did not show a significant difference between the overall marinate treatment and the marinate control in any of the HCAs of interest. This indicates that it is not appropriate to use marinate treatment as a base-point for comparing application efficacy. Sources of error are likely related to extraction procedures as well as possibly linked to the marinating technique.

One finding of interest is that the analysis of the results found a significant difference between the direct application control and the marinate control. This suggests the marinate method may influence the formation of HCAs. The findings of Smith and others (2008) indicate that the spice is the component which most significantly influences the formation of HCAs, but the results from this study also show that the bases of the marinade, all the components of the marinade except for the spice, also were significantly different from the control in certain instances.

For the overall comparison of spice application method, a significant difference was not seen between marinating and direct application of spices. This finding suggests that, while the data is likely skewed due to sources of error, further research may find that there is not a significant difference between application methods.

Conclusion

The results from DPPH and total phenolics analysis indicated that Blend and Italian would be the most effective at limiting HCA formation. However, Montreal at high treatment amount and Italian at high treatment amount were the only values which significantly reduced all HCAs of interest. While it is notable that the highest amounts of spice showed the greatest reduction in HCAs, it is likely that other factors contributed to the lack of continuity from DPPH and total phenolic results to amount of HCAs. Potential sources of error include spice size uniformity, extraction materials and reagents, percent recovery, HPLC analysis, spice application and cooking method, and/or human error. While the results are inconclusive, further research may indicate that the formation of HCAs, specifically MeIQx, DiMeIQx, and PhIP, may be reduced through the direct application of spices to the surface of beef.

Similarly, the spice application experiment did not have conclusive results; however this is also an area which merits further research. The application of this research is ultimately at the consumer level; consumers making the decision to use cooking methods which result in fewer HCAs. As cooking trends indicate (Sloan 2009), flavor as well as convenience are very important factors for consumers. As with the study by Gibis (2007) which looked not only at the efficacy of marinade treatment but also at the flavor acceptability of the marinades, future research must take consumer trends into consideration (Gibis 2007). The impact of 'convenient' foods indicates the importance of understanding cooking techniques applicable to reducing HCA formation.

Future research in this area should focus on ensuring the results are applicable to consumers, both in technique and palatability. The methods used to analyze HCA formation and reduction must be improved to reduce inconsistency of results and improve the percent recovery during extraction of HCAs. While findings from this study are inconclusive, the results indicate the potential for finding new methods for reducing the formation of HCAs.

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Appendix - Table of Contents

Appendix A - McCormick Spice Standards

Table A-1. McCormick Science Institute standardized spice information (McCormick 2009)

Spice	Origin	Total ORAC (μmol TE / g)	Total Phenolics (mg GAE / g)
Black Pepper	Blend	424	8.2
Cinnamon	Indonesia	1900	105.3
Cloves	Asia/Pacific	2996	217.8
Cumin	Blend	229	8.6
Ginger	India	264	10.2
Nutmeg	Indonesia	511	23.8
Oregano Blend	Mexico	1683	74.5
Oregano Blend	**Mediterranean**	**2657**	**63.6**
Paprika	USA	214	14.2
Red Pepper	India	123	11.3
Rosemary	**Blend**	**1704**	**49.8**
Sage	**Albania**	**2401**	**45.2**
Thyme	**Polish**	**1637**	**44.7**
Turmeric	India	1832	33.9

*Bold text indicates spices used in Blend treatment

Appendix B - Standard Curves

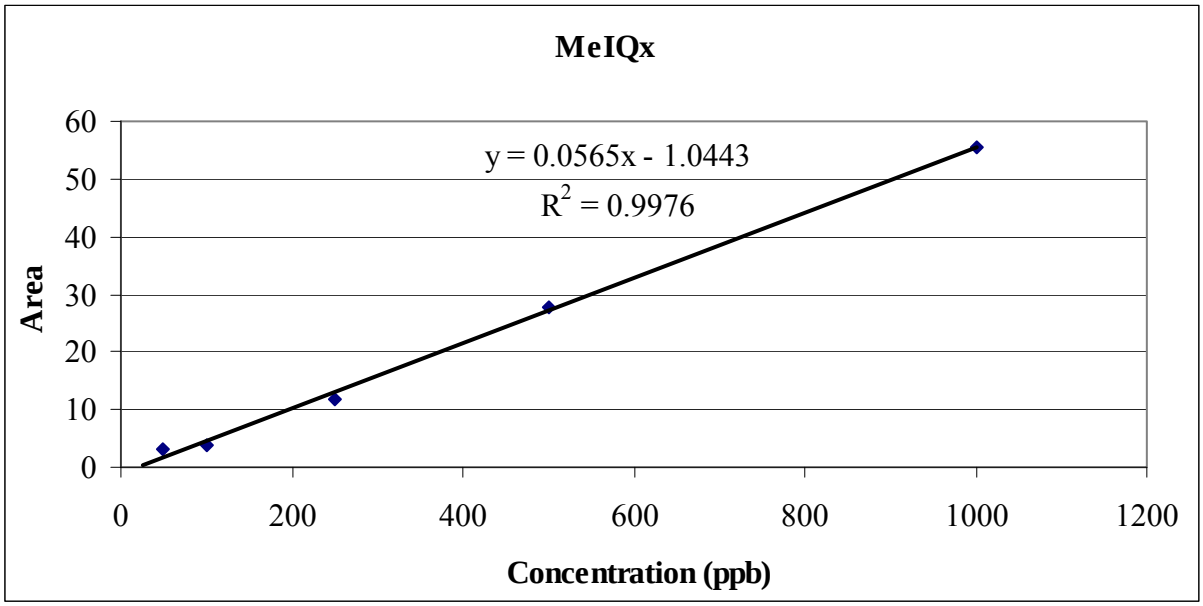

Figure B-1. Standard curve obtained by UV detection for MeIQx at 252 nm.

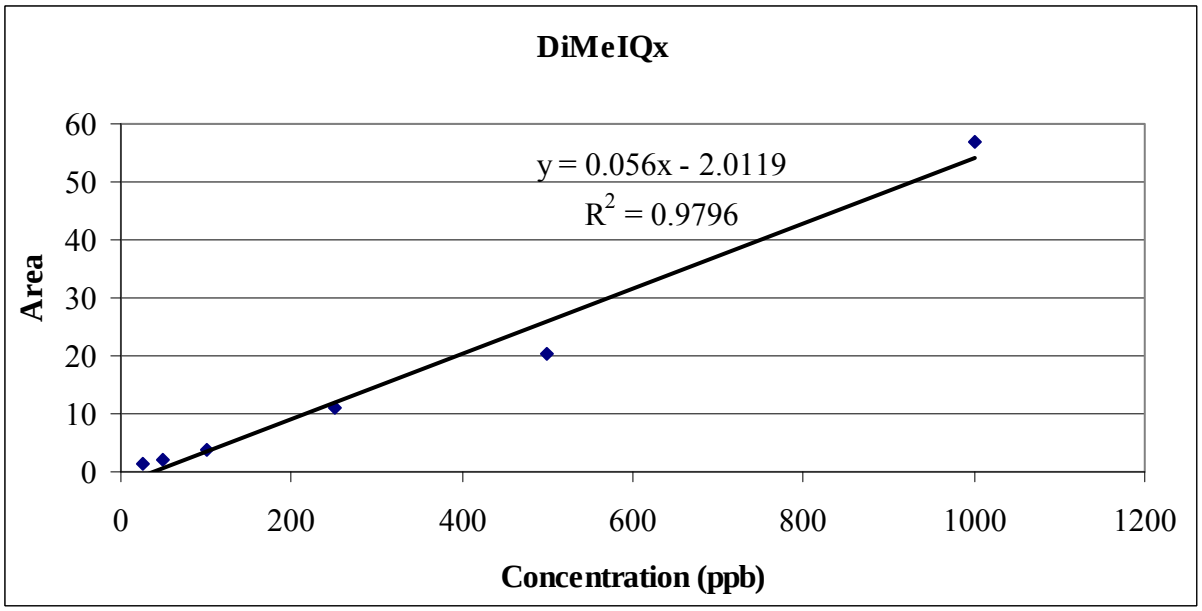

Figure B-2. Standard curve obtained by UV detection for DiMeIQx at 252 nm.

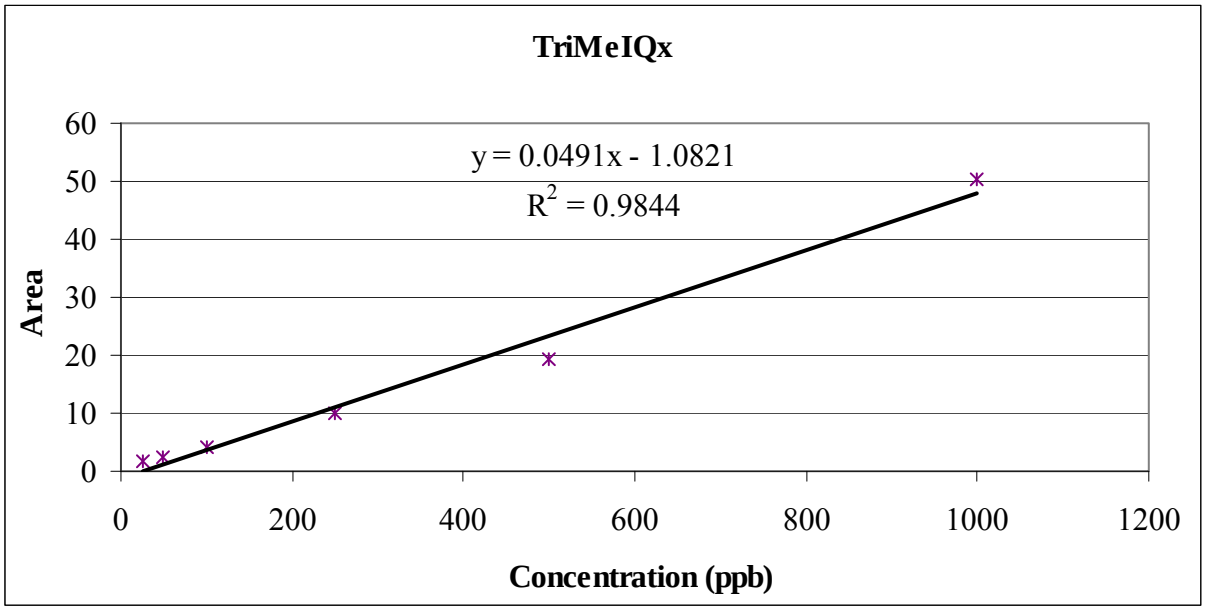

Figure B-3. Standard curve obtained by UV detection for TriMeIQx at 252 nm.

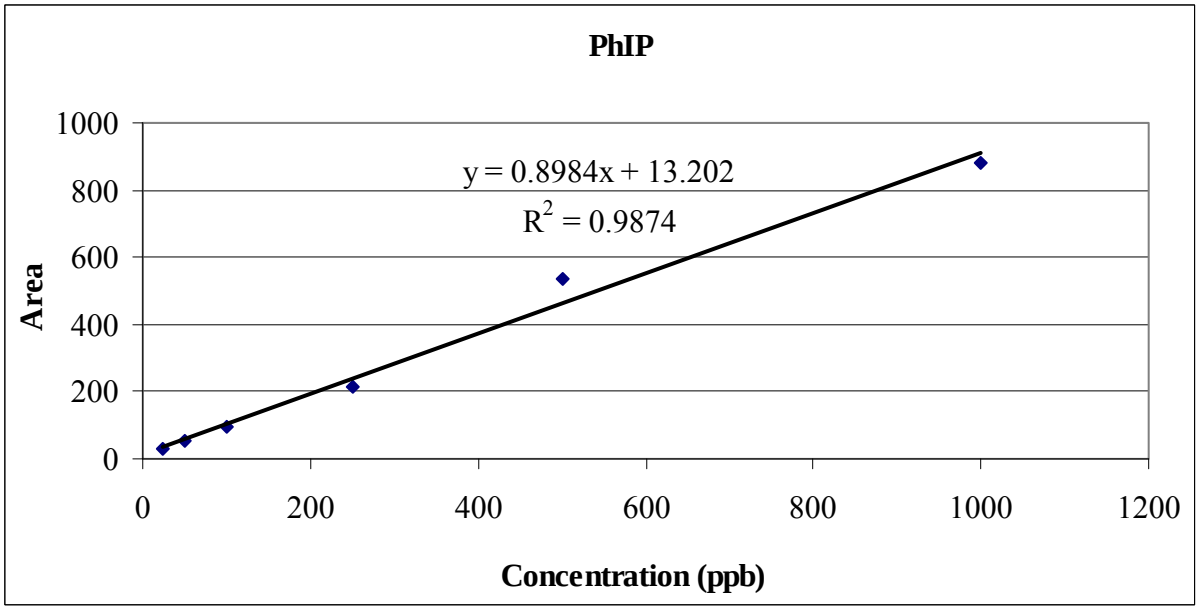

Figure B-4. Standard curve obtained by UV detection for PhIP at 252 nm.

Appendix C - Chromatograms

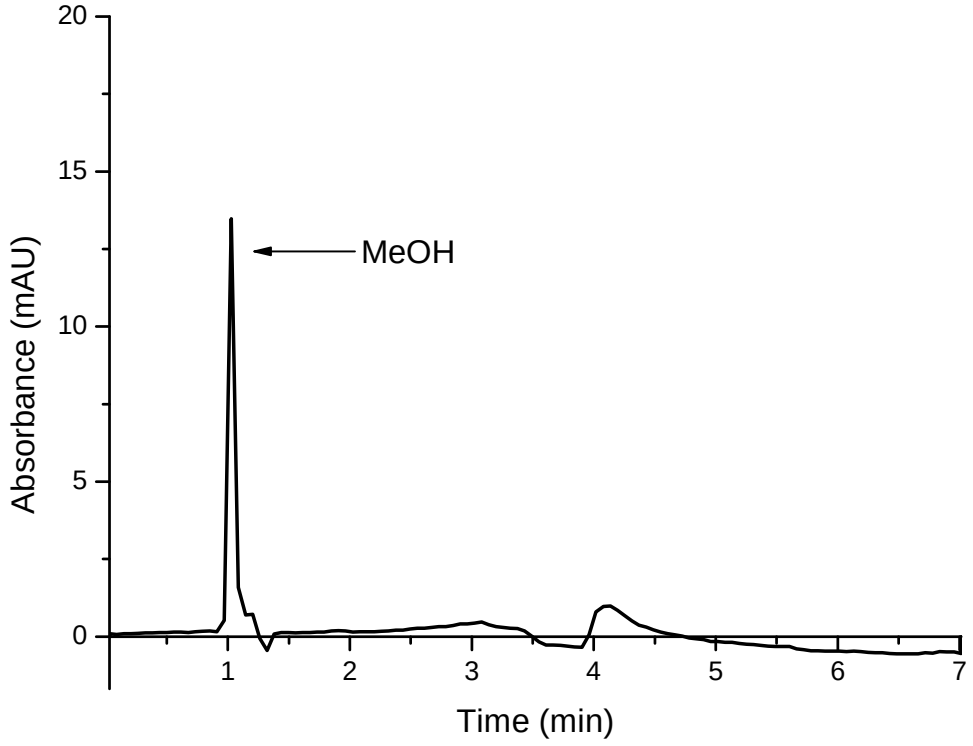

Figure C-1. UV spectra of interference caused by methanol interacting with starting mobile phase when running a blank. The starting mobile phase was used to hydrate and inject samples, rather than methanol, to avoid this interference.

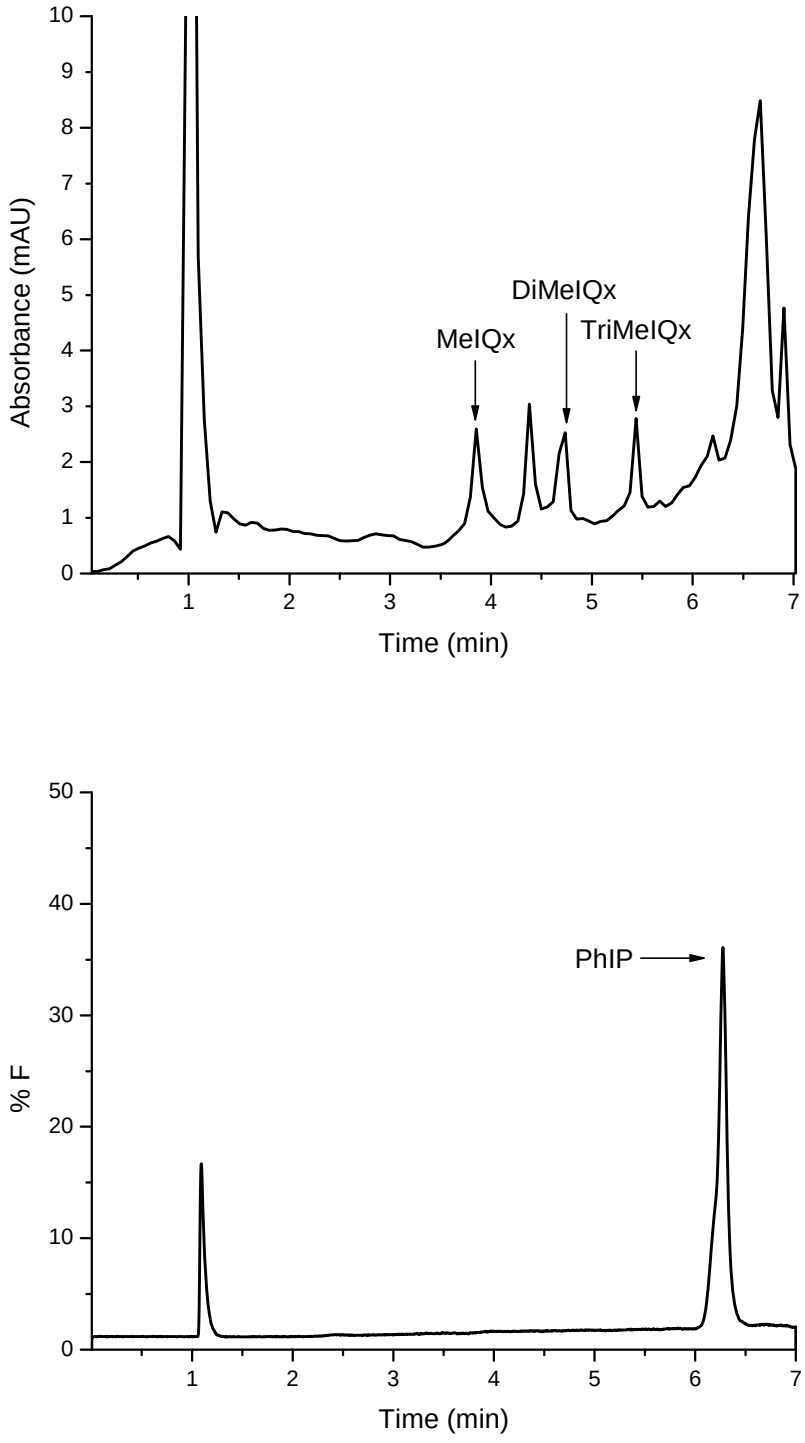

Figure C-2. UV and Florescence spectra for 250 ppb standard mixed HCAs (MeIQx, DiMeIQx, TriMeIQx, and PhIP)

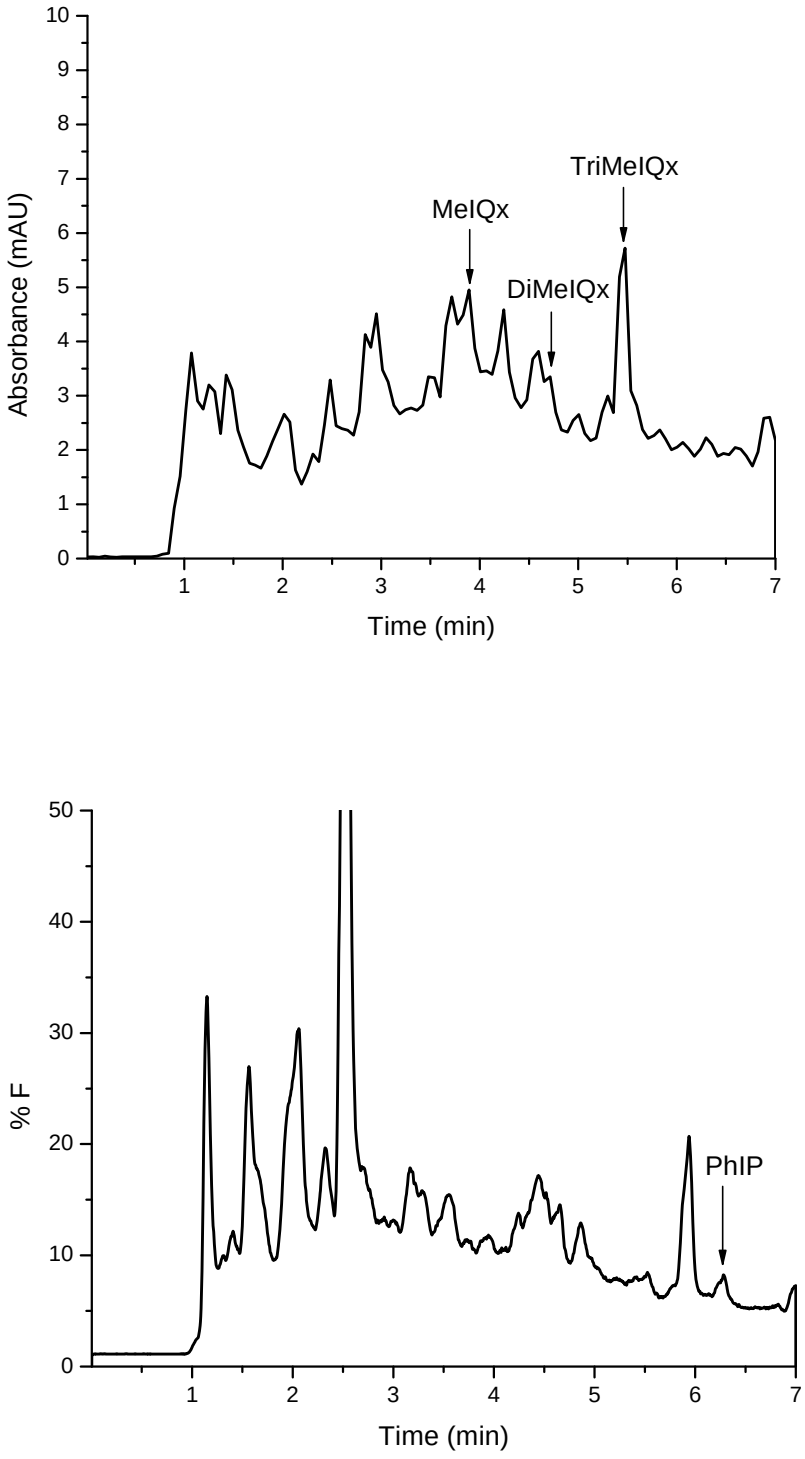

Figure C-3. UV and Florescence spectra for MeIQx, DiMeIQx, TriMeIQx, and PhIP for Montreal at high treatment amount

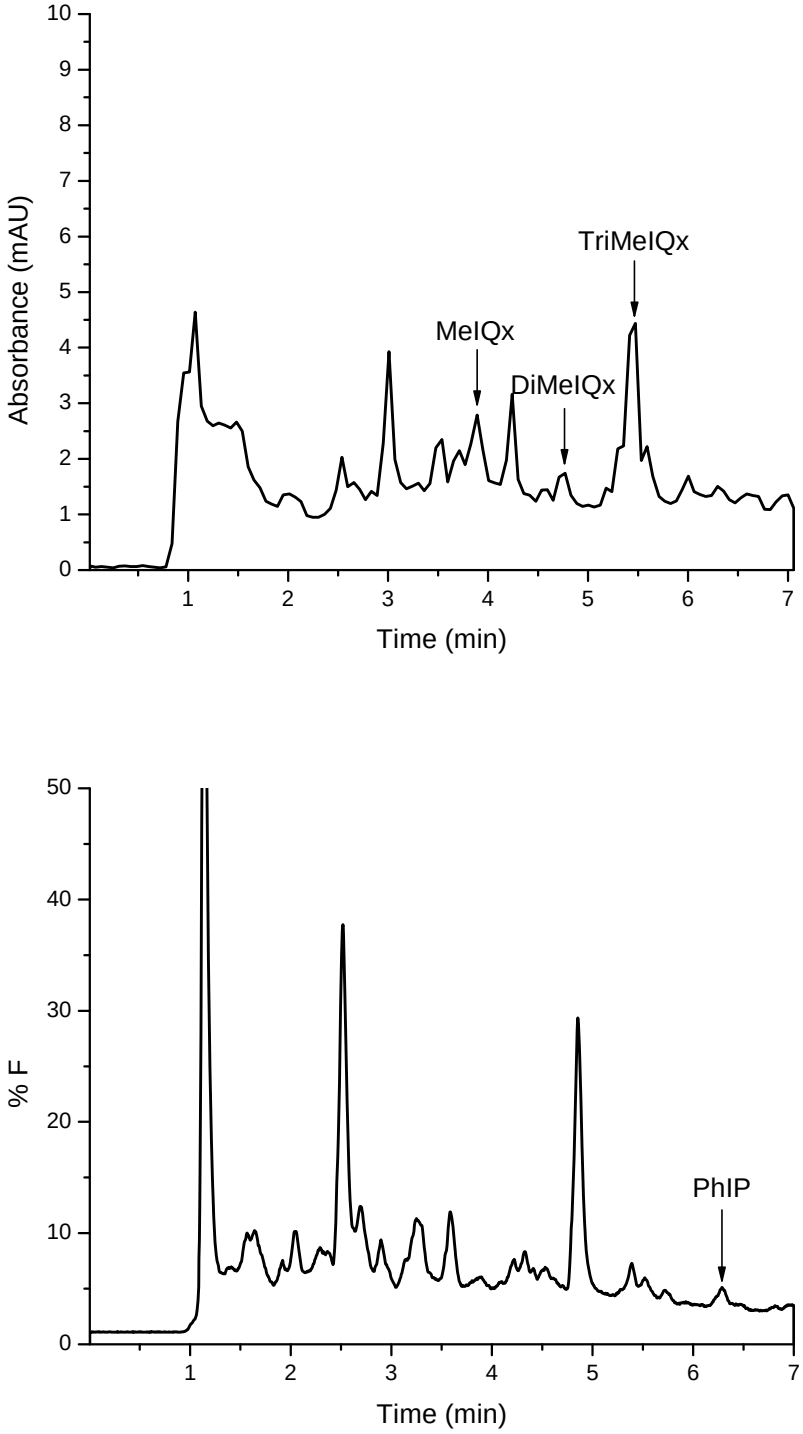

Figure C-4. UV and Florescence spectra for MeIQx, DiMeIQx, TriMeIQx, and PhIP for Italian at high treatment amount

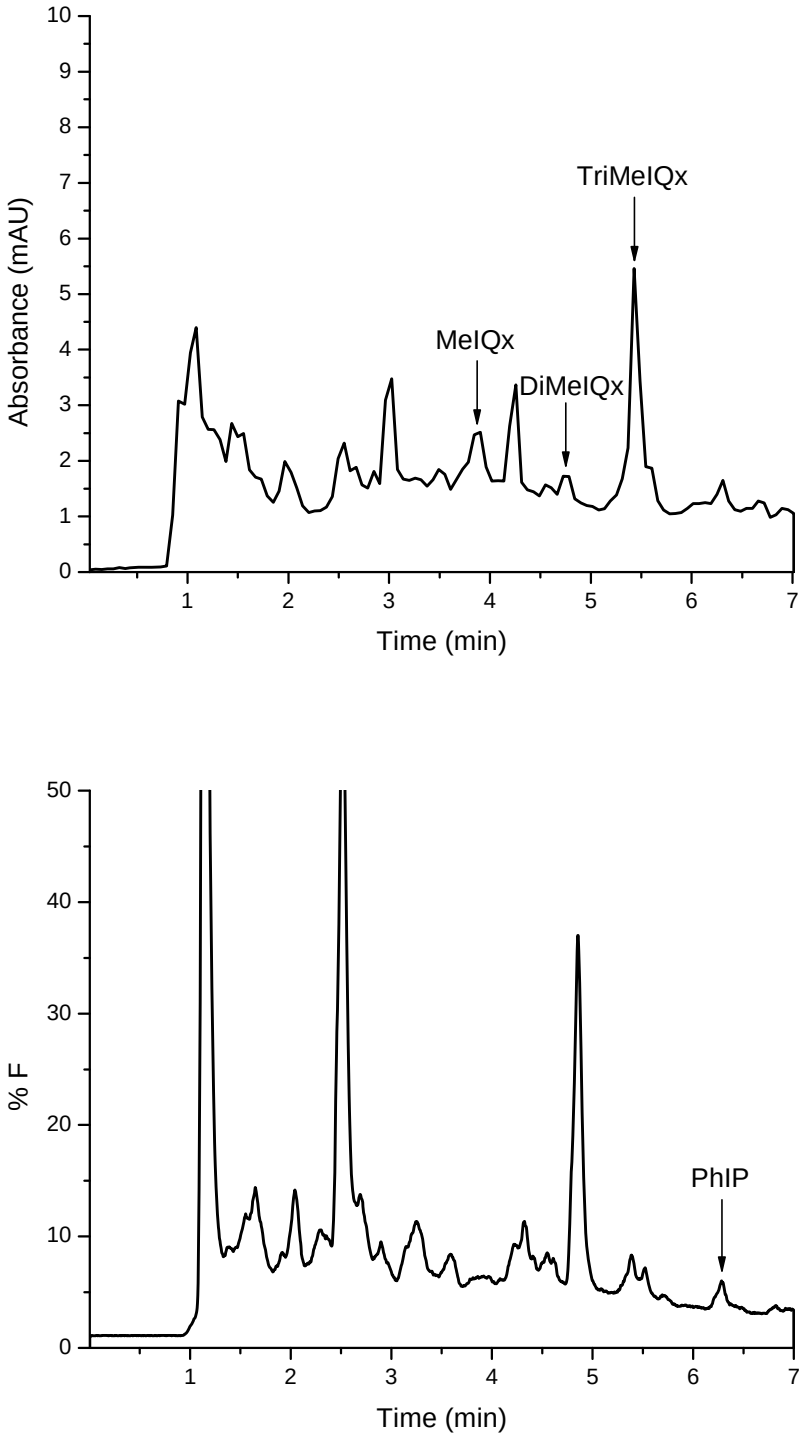

Figure C-5. UV and Florescence spectra for MeIQx, DiMeIQx, TriMeIQx, and PhIP for Blend at high treatment amount

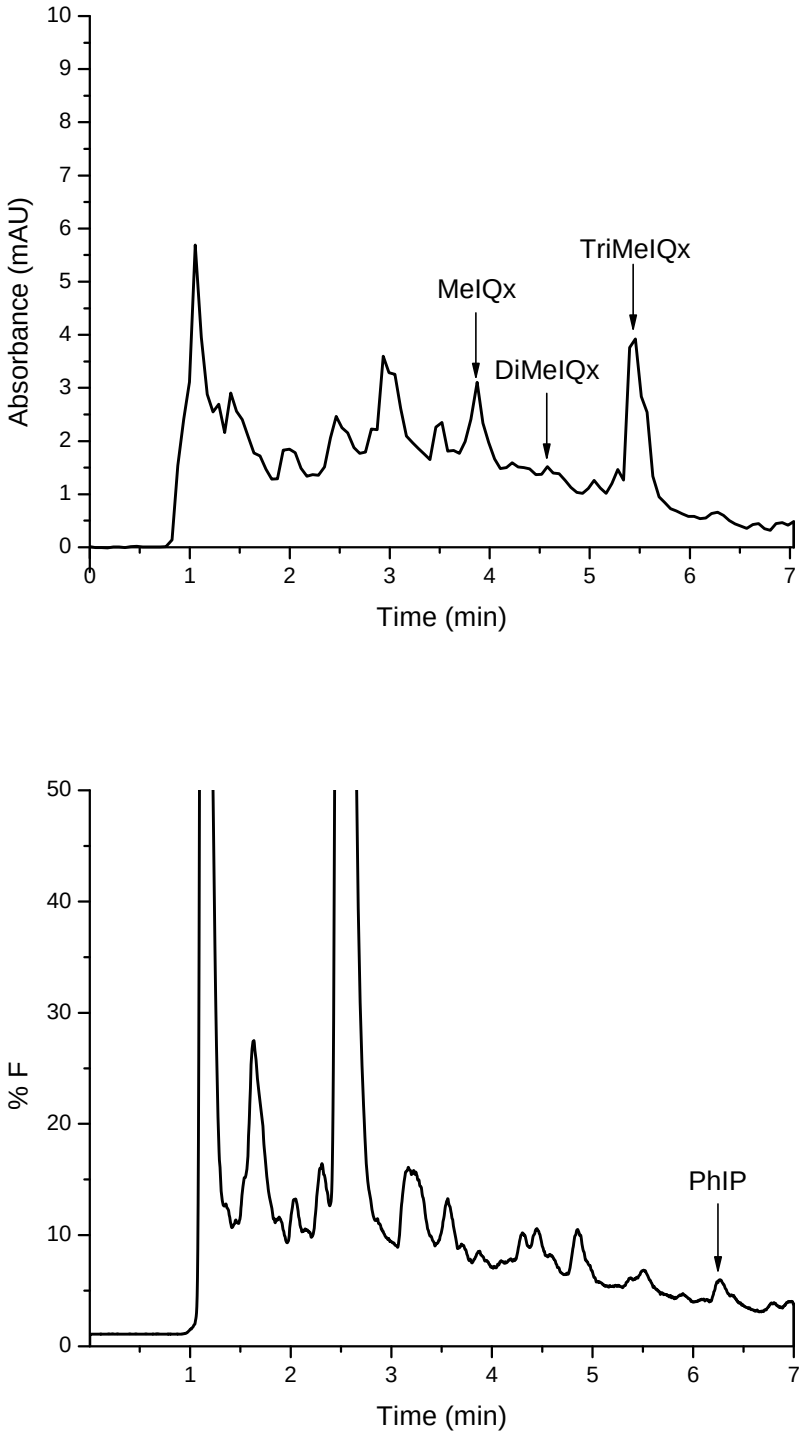

Figure C-6. UV and Florescence spectra for MeIQx, DiMeIQx, TriMeIQx, and PhIP for Montreal with marinate treatment

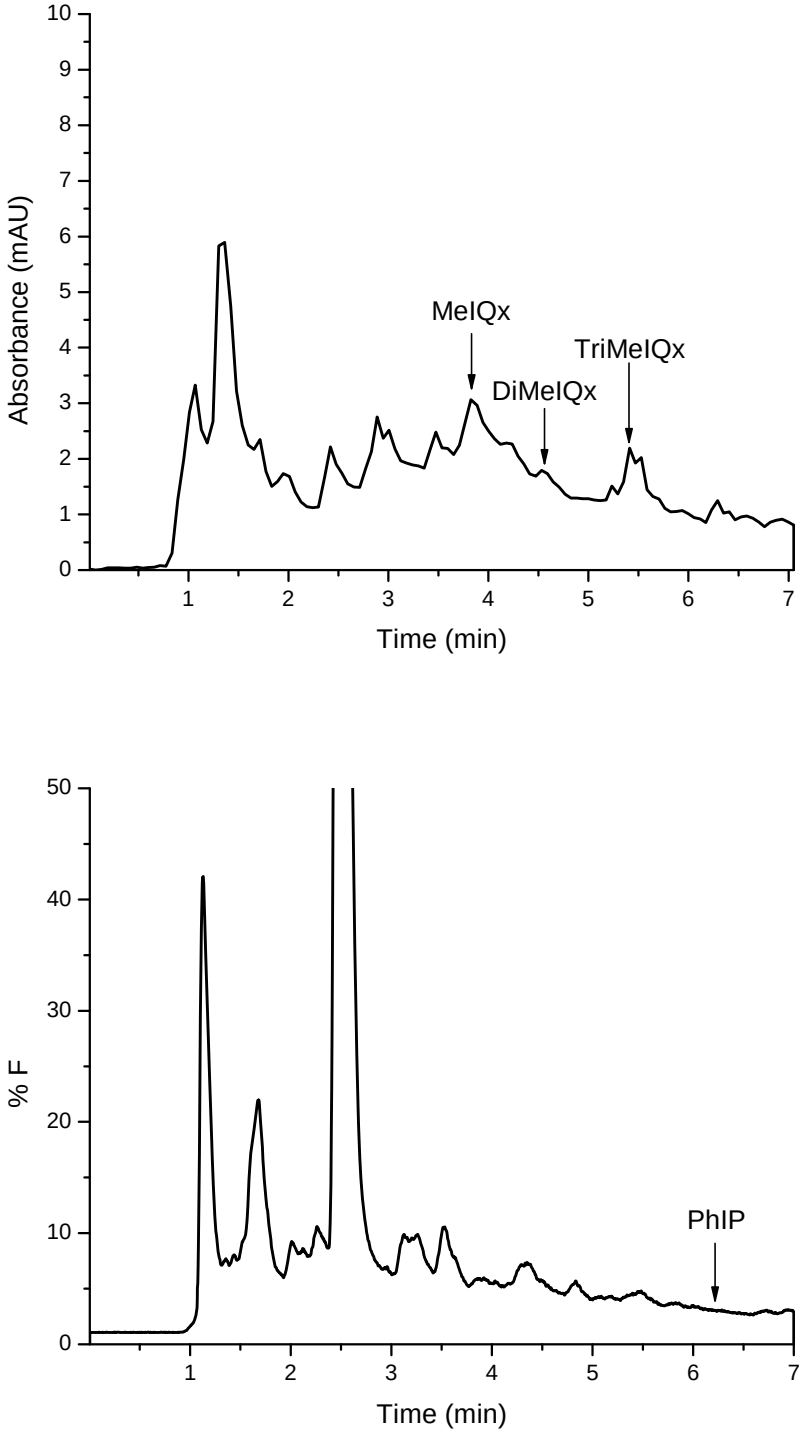

Figure C-7. UV and Florescence spectra for MeIQx, DiMeIQx, TriMeIQx, and PhIP for Italian with marinate treatment

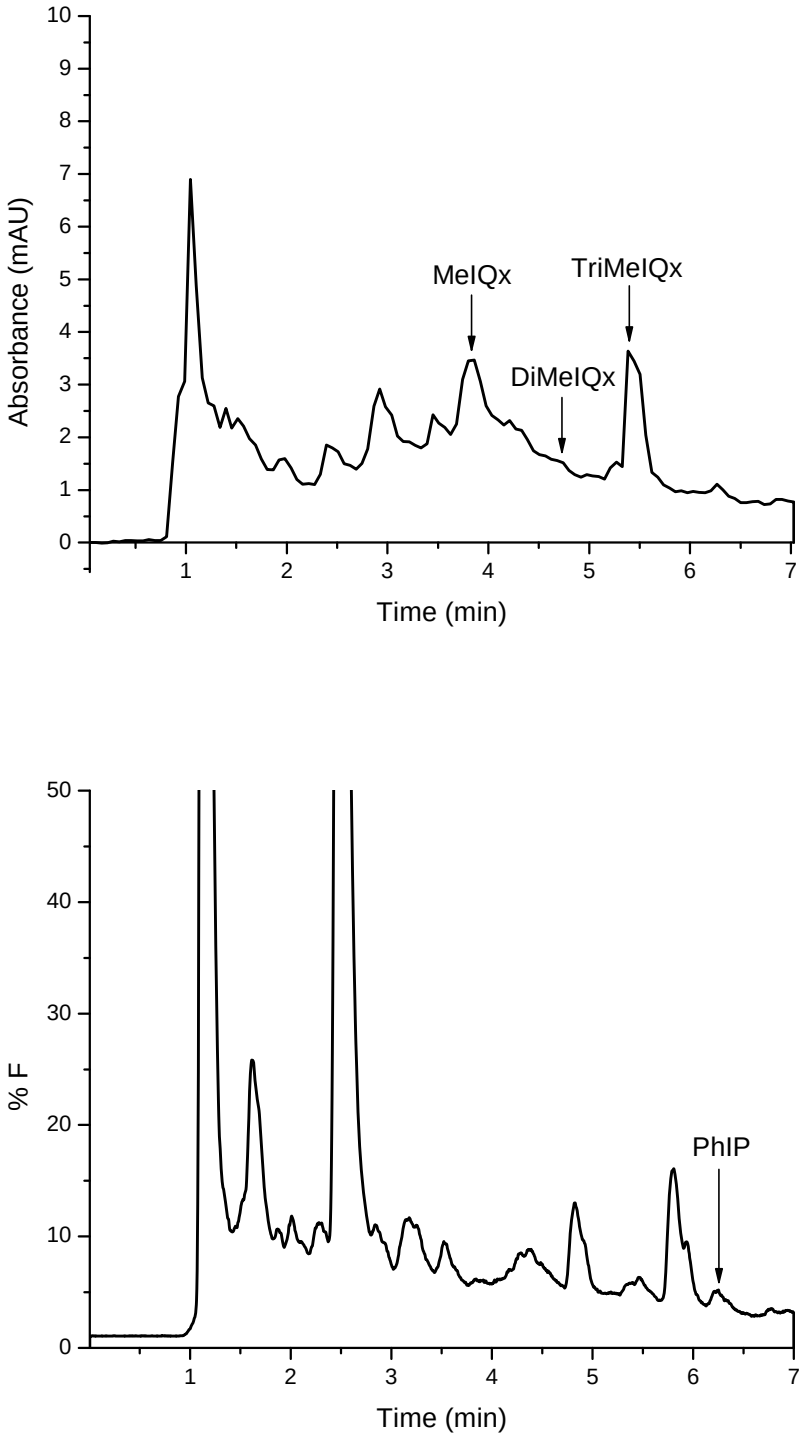

Figure C-8. UV and Florescence spectra for MeIQx, DiMeIQx, TriMeIQx, and PhIP for Blend with marinate treatment