

FORMATION AND INHIBITION OF HETEROCYCLIC AMINES IN MEAT PRODUCTS

by

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AN ABSTRACT OF A DISSERTATION

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## ABSTRACT

Heterocyclic amines (HCAs) are produced in meats cooked at high temperature, which are potent mutagens and a risk factor for human cancers. Occurrence of HCAs in ready-to-eat (RTE) meat products and cooked meat products based on prevalence of various cooking methods that are preferred among U.S. meat consumers were investigated. The primary HCAs detected in samples were PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine), MeIQx (2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline), and DiMeIQx (2-amino-3,4,8-trimethyl-imidazo [4,5-*f*]quinoxaline). RTE meat products were ranked in the following order of increasing total HCA content: pepperoni (0.05 ng/g) < hot dogs and deli meat products (0.5 ng/g) < fully cooked bacon (1.1 ng/g) < rotisserie chicken meat (1.9 ng/g) < rotisserie chicken skin (16.3 ng/g). In cooked meat products, high levels of total HCAs were found in fried pork (13.91 ng/g), fried fish (14.91 ng/g), and fried bacon (17.91 ng/g).

Inhibition of HCAs by rosemary extracts, which were extracted with different solvents, were evaluated in cooked beef patties. Five rosemary extracts were 100W (100% water), 10E (10% ethanol), 20E (20% ethanol), 30E (30% ethanol), and 40E (40% ethanol). Rosemary extract 20E containing a mixture of rosmarinic acid (27.3 mg/g), carnosol (72.9 mg/g), and carnosic acid (4.2 mg/g) showed the greatest inhibition of MeIQx (up to 91.7%) and PhIP (up to 85.3%).

The effect of enhancement and marination on HCA formation in meat products was investigated. The addition of salt and phosphate greatly improved the water-holding capacity and decreased HCA formation (up to 58%) in enhanced fresh meat products. An greater reduction of HCAs (up to 79%) was found in marinated fresh meat; the enhancement solution for this meat contained ingredients that exhibited good antioxidant properties.

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Approved by:  
Major Professor  
J. Scott Smith

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## PART I. REVIEW OF LITERATURE

### INTRODUCTION

Since the late 1970s when Japanese scientists associated potent mutagenic activity with *Salmonella typhimurium* T98 in the charred surface of broiled fish, the number of studies examining cooking-induced mutagens and carcinogens has dramatically increased. Cooking-derived mutagens and carcinogens were later isolated and characterized as heterocyclic amines (HCAs). Further studies showed that HCAs are mainly formed in muscle foods, mainly meat and fish, via the Maillard reaction with creati(ni)ne, amino acids, and sugars as the precursors (Sugimura and others 2004). More than 25 HCAs have been isolated from different cooked muscle foods. These HCAs contain two to five of the condensed aromatic cycles with one or more nitrogen atoms coupled with one exocyclic amino group in their ring systems (Alaejos and others 2008). HCAs can be classified into two groups, non-polar and polar HCAs, based on their chemical structure. Non-polar HCAs occur infrequently and are detected at low levels in normal cooked foods because they only form above normal cooking temperatures. On the other hand, polar HCAs can be easily formed at normal cooking temperature; therefore, they have received much more attention than non-polar HCAs (Knize and Felton 2005).

The common HCAs found in cooked muscle foods are 2-amino-3-methylimidazo [4,5-*f*]quinoline (IQ), 2-amino-3-methylimidazo [4,5-*f*]quinoxaline (IQx), 2-amino-3,4-dimethylimidazo [4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline (MeIQx), and 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP) (Knize and others 1994). Four of these HCAs (IQ, MeIQ, MeIQx and PhIP) are listed in the U.S. Department of

Health and Human Services's 11th Report of Carcinogens (2005) as compounds "reasonably anticipated to be a human carcinogen". The International Agency for Research on Cancer [IARC] (1993) categorize MeIQ, MeIQx, and PhIP as "reasonably anticipated to be human carcinogens" and IQ as a "probable human carcinogen". The presence of HCAs in foods ranges from 0 to 100 part per billion (nanogram per gram). Cooking methods that use temperature above 150 °C, such as frying, grilling, and roasting, contained high amount of HCAs. The HCA levels in foods depend on the cooking time and temperature, cooking method, and concentration of the precursors in raw meat (Knize and Felton 2005).

As with many chemical mutagens and carcinogens, HCAs have the capability to form DNA adducts. Some of them have been demonstrated to exhibit strong mutagenicity based on the Ames *Salmonella*/mutagenicity test (Sugimura and others 2004). The epidemiological studies over the past 25 years have shown that the high intake of well-done meat and high exposure to meat carcinogens, particularly HCAs, increase the risk of human cancers: colon, pancreas, prostate, and breast (Zheng and Lee 2009). The public considers cancer a potential life-threatening disease affecting people of all ages (Lynch and others 1995), and after cardiovascular diseases, cancer has been the main cause of death worldwide (Oliveira and others 2007). The total HCA-induced cancer risk mainly depends on the amount of HCAs present in the diet and the frequency of consumption.

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

formed through the free radicals in the Maillard reaction (Vitaglione and Fogliano 2004). Because of concerns about the toxicological safety of synthetic antioxidants, consumers prefer to use natural antioxidants (Pokorný 2007). Therefore, the search for natural sources of antioxidants has greatly intensified in recent years.

Over the past 30 years, extensive studies regarding HCAs have been conducted. They can be broadly classified into three categories: (1) those examining the kinetics and mechanism of HCA formation and possible mechanisms of their mutagenic/carcinogenic activities, (2) those isolating, characterizing, and quantifying the HCAs in foods, and (3) those developing the strategies to inhibit HCA formation in foods (Cheng and others 2006). This literature review will cover all aspects of HCAs, including the formation mechanism of HCAs, HCAs and human health, the occurrence of HCA in foods, the human intake of HCAs, the factors affecting HCA formation, and the inhibition of HCA formation.

## **CLASSIFICATION AND FORMATION OF HETEROCYCLIC AMINES**

To date, more than 20 HCAs have been isolated and characterized from both various cooked meat products and meat model systems. All the HCAs have at least one aromatic and one heterocyclic structure (Cheng and others 2006). Based on their chemical structure, HCAs can be classified into two groups: non-polar and polar HCAs. The chemical names and abbreviations of these HCAs are given in Table 1.

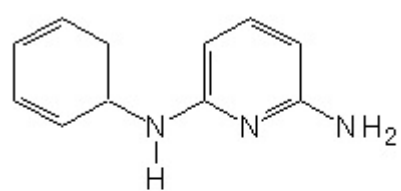
Table 1. Chemical names and abbreviations of the non-polar and polar heterocyclic amines (Sugimura and others 2004)

<b>Non-Polar (Amino-carbolines)</b>		
$\alpha$ -amino-carbolines	A $\alpha$ C	2-Amino-9H-dipyrido[2,3- <i>b</i> ]indole
	MeA $\alpha$ C	2-Amino-3-methyl-9H-dipyrido[2,3- <i>b</i> ]indole
$\beta$ -amino-carbolines	NorHarman	9H-pyrido[3,4- <i>b</i> ]indole
	Harman	1-methyl-9H-pyrido[3,4- <i>b</i> ]indole
$\gamma$ -amino-carbolines	Trp-P-1	3-Amino-1,4-dimethyl-5H-pyrido[4,3- <i>b</i> ]indole
	Trp-P-2	3-Amino-1-methyl-5H-pyrido[4,3- <i>b</i> ]indole
$\delta$ -amino-carbolines	Glu-P-1	2-Amino-6-methyl-dipyrido[1,2- <i>a</i> :3',2'- <i>d</i> ]imidazole
	Glu-P-2	2-Amino-dipyrido[1,2- <i>a</i> :3',2'- <i>d</i> ]imidazole
<b>Polar (Amino-imidazo-azaarenes)</b>		
Quinolines	IQ	2-Amino-3-methylimidazo[4,5- <i>f</i> ]quinoline
	MeIQ	2-Amino-3,4-dimethylimidazo[4,5- <i>f</i> ]quinoline
Quinoxalines	IQx	2-Amino-3-methylimidazo[4,5- <i>f</i> ]quinoxalines
	MeIQx	2-Amino-3,8-dimethylimidazo[4,5- <i>f</i> ]quinoxaline
	4,8-DiMeIQx	2-Amino-3,4,8-trimethylimidazo[4,5- <i>f</i> ]quinoxaline
	7,8- DiMeIQx	2-Amino-3,7,8-trimethylimidazo[4,5- <i>f</i> ]quinoxaline
	4,7,8- TriMeIQx	2-Amino-3,4,7,8-tetramethylimidazo [4,5- <i>f</i> ]quinoxaline
Pyridines	PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5- <i>b</i> ]pyridine
	DMIP	Dimethylimidazopyridine
	TMIP	Trimethylimidazopyridine

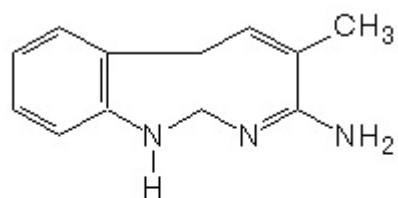
### **Non-polar heterocyclic amines**

The structure of non-polar HCAs (also known as amino-carboline HCAs) include the five-membered heterocyclic aromatic ring sandwiched between two-six membered aromatic rings (Cheng and others 2006) (Figure 1). Non-polar HCAs can be further divided into  $\alpha$ -subgroup (e.g. A $\alpha$ C and MeA $\alpha$ C),  $\beta$ -subgroup (e.g. norharman, harman),  $\gamma$ -subgroup (e.g. Trp-P-1, Trp-P-2), and  $\delta$ -subgroup (e.g. Glu-P-1, Glu-P-2). Unlike other HCAs,  $\beta$ -amino-carbolines (e.g. norharman and harman) lack the exocyclic amino group that cause them to be nonmutagenic in the Ames *Salmonella* mutagenicity test (Cheng and others 2006).

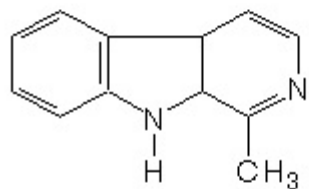
The main precursors of non-polar HCA formation are amino acids or protein, with formation independent on the presence of creati(ni)ne (Murkovic 2004, Cheng and others 2006). These HCAs form only at temperatures above 300 °C via amino acids or protein pyrolysis, sometimes referred to as pyrolytic HCAs (Jägerstad and others 1998). The amino acids and proteins that are involved in this reaction are tryptophan, glutamic acid, lysine, phenylalanine, ornithine, creatine, or pyrolysed proteins (e.g. casein, albumin, gluten or soybean globulin) (Jägerstad and others 1998). Since they are formed under such extremely high temperatures, they are not detectable or occur infrequently in normal cooked foods. The possible mechanisms of amino-carbolines formation postulated by Skog and others (2000) is via free radical reactions, which produce many reactive fragments and then condense to form amino-carboline HCAs.



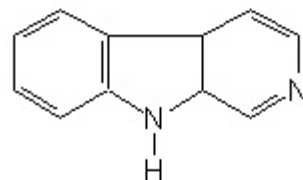
**AaC**



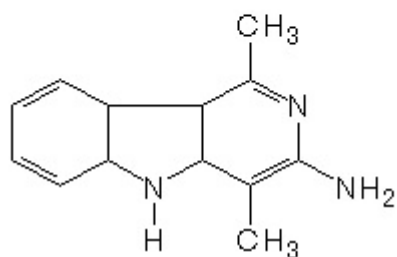
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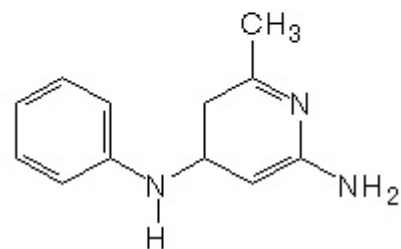
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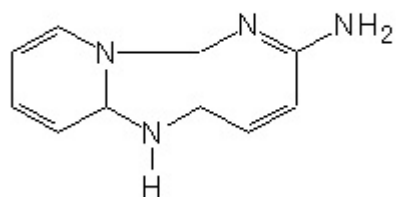
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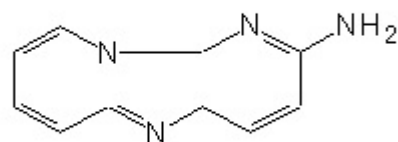
**Trp-P-1**



**Trp-P-2**



**Glu-P-1**

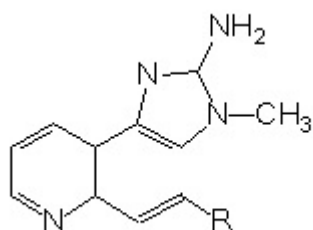


**Glu-P-2**

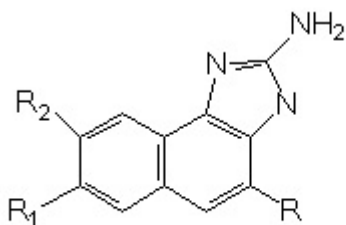
Figure 1. Chemical structure of non-polar heterocyclic amines (Murkovic 2004).

## Polar heterocyclic amines

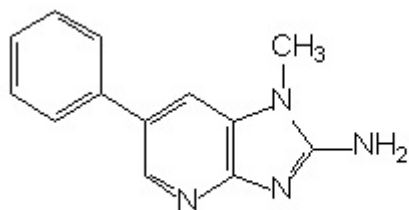
The structure of polar amines (also known as amino-imidazo-azaarenes) have 2-amino-imidazo moiety and a methyl group attached to one of the nitrogens in the imidazo ring (Figure 2). They all have molecular weights in the range of 180-250, and boiling points below 200-300 °C (Murkovic 2004). Polar HCAs can be divided into three subgroups depending on the groups that are attached to the 2-amino-imidazo. These subgroups are quinolines, quinoxalines, and pyridines. Quinolines are called IQ-type (e.g. IQ and MeIQ) while quinoxalines are called IQx-type (e.g. IQx, MeIQx and DiMeIQx). The important HCAs in the pyridine subgroup are PhIP, DMIP, and TMIP (Cheng and others 2006).



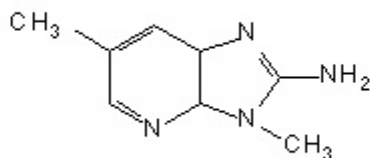
**IQ:** R = H  
**MeIQ:** R = CH<sub>3</sub>



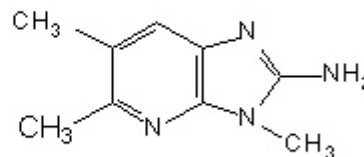
**IQx:** R = H, R<sub>1</sub> = H, R<sub>2</sub> = H  
**MeIQx:** R = H, R<sub>1</sub> = H, R<sub>2</sub> = CH<sub>3</sub>  
**DiMeIQx:** R = CH<sub>3</sub>, R<sub>1</sub> = H, R<sub>2</sub> = CH<sub>3</sub>



**PhIP**



**DMIP**



**TMIP**

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



Polar HCAs can be easily formed at the temperature used for normal cooking, such as frying, broiling and baking, which is below 300 °C. Therefore, they have received much more attention than non-polar HCAs. The three main precursors leading to the formation of polar HCAs are creatine/creatinine, sugars, and amino acids originally found in muscle foods (Knize and Felton 2005). Experiments using  $^{14}\text{C}$ -labeled glucose in a model system with threonine and creatinine showed that the  $^{14}\text{C}$ -labeled glucose was incorporated to IQ- and IQx- type HCAs; it has been suggested that the carbon atoms of pyridine/pyrazine moiety of HCAs originate from the glucose molecule. In addition, the C-4 of IQ- and the IQx-type HCAs are derived from the C-2 of the amino acid (Figure 3).

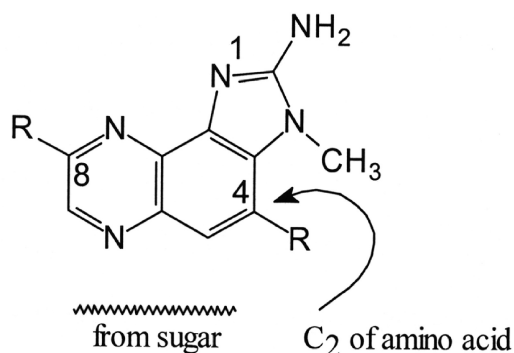


Figure 3. Origin of carbon atom of IQ and IQx type heterocyclic amines (Murkovic 2004).

Pearson and others (1992), Jägerstad and others (1998), and Skog and others (1998) postulated that the IQ-type and IQx-type HCAs form through the Maillard reaction as shown in Figure 4. The mechanism, glycol-aldehyde alkylimine (enol type) being produced by a reverse-aldol reaction from sugar and amino acid. Then the Maillard reaction may follow to yield the different types of HCAs. The first pathway produces IQx-type amine through the biomolecular

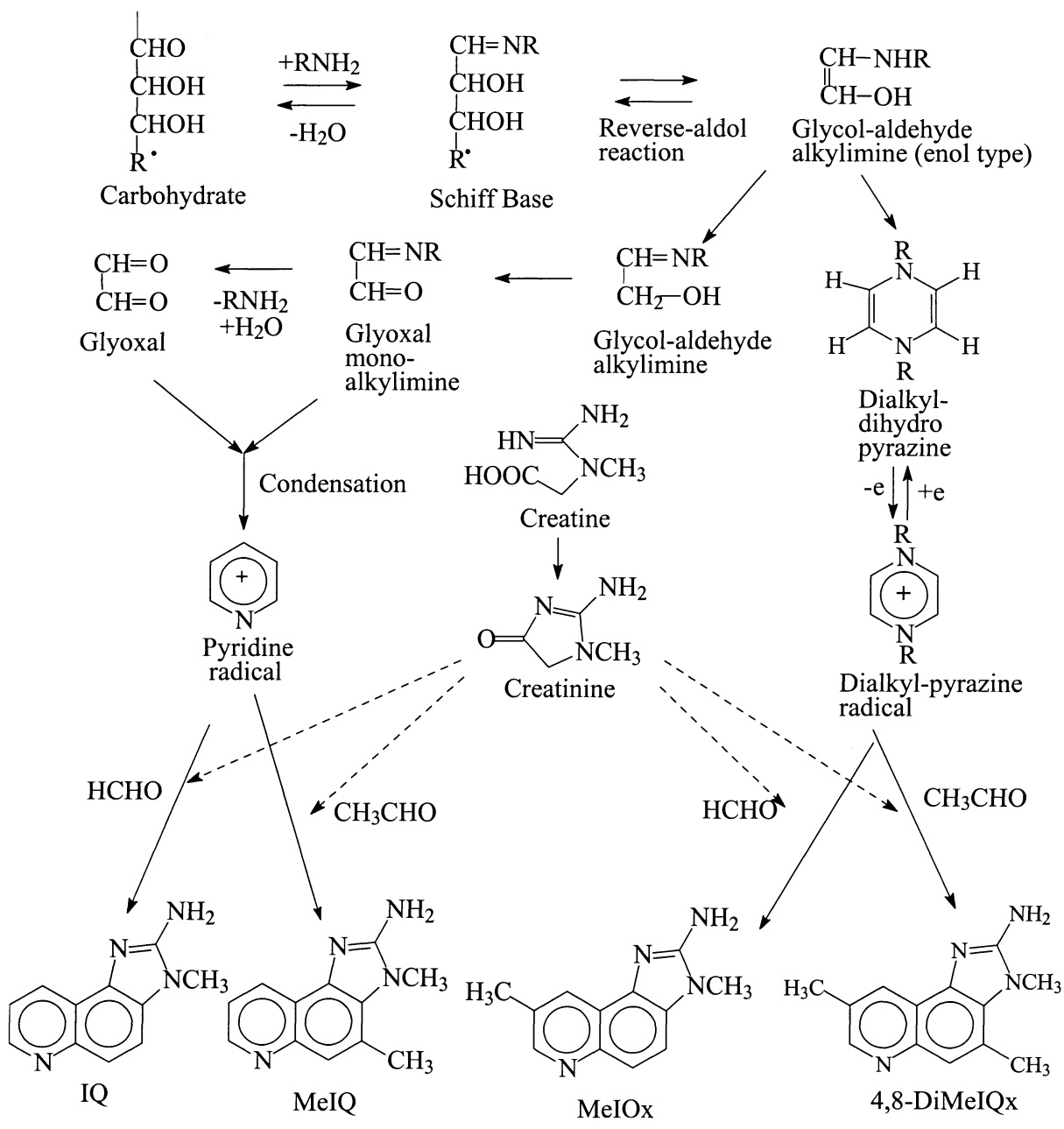


Figure 4. Formation of IQ-type and IQx-type heterocyclic amines involving pyridine and pyrazine intermediates (Vitaglione and Fogliano 2004).

ring formation of glycol-aldehyde alkylimine to produce the dialkyldihydropyrazine, and then later after the loss of one electron, and the dialkyl-pyrazine radicals formed. The second pathway producing IQ-type amines begins with the formation of glycol-aldehyde alkylimine followed by oxidation to produce glyoxal monoalkylimine, which can be reduced to glyoxal. The molecules of glyoxal and glyoxal monoalkylimine may condense and form pyridine radicals. The free radicals, dialkyl-pyrazine and pyridine, produced in both pathways then react with the aldehyde and creatinine originally found in muscle foods, and form HCAs as final products. The type of HCA that is formed depends on the radicals and aldehyde involved in the reaction.

It has been reported that the formation of the dialkylpyrazine radical occur much more rapidly than the formation of the pyridine radical. This helps to explain why the levels of IQx-type amines in cooked meat products are higher than the levels of IQ-type amines (Murkovic 2004). The formation of the glyoxal monoalkylamine is readily reversed under acidic conditions to yield glycol aldehyde alkylimine, and this is why an acidic pH can inhibit the IQ-type HCA formation (Cheng and others 2006).

The mechanism of PhIP formation (Figure 5) is different from the mechanism that forms of other polar HCAs. The PhIP mechanism starts with the reaction of two precursors, phenylalanine and creatinine, which forms phenylacetaldehyde. Then, the aldol condensation of phenylacetaldehyde with creatinine occurs and PhIP is produced as a final product. The nitrogen-forming pyridine moiety in the PhIP molecule is originally derived from either an amino group of creatinine, or an amino group of phenylalanine or free ammonia. Experiments using <sup>13</sup>C-labeled phenylalanine in a model system with glucose and creatinine showed that <sup>13</sup>C-labeled phenylalanine was incorporated to C-5, C-6, and C-7 of the PhIP molecule (Figure 5).

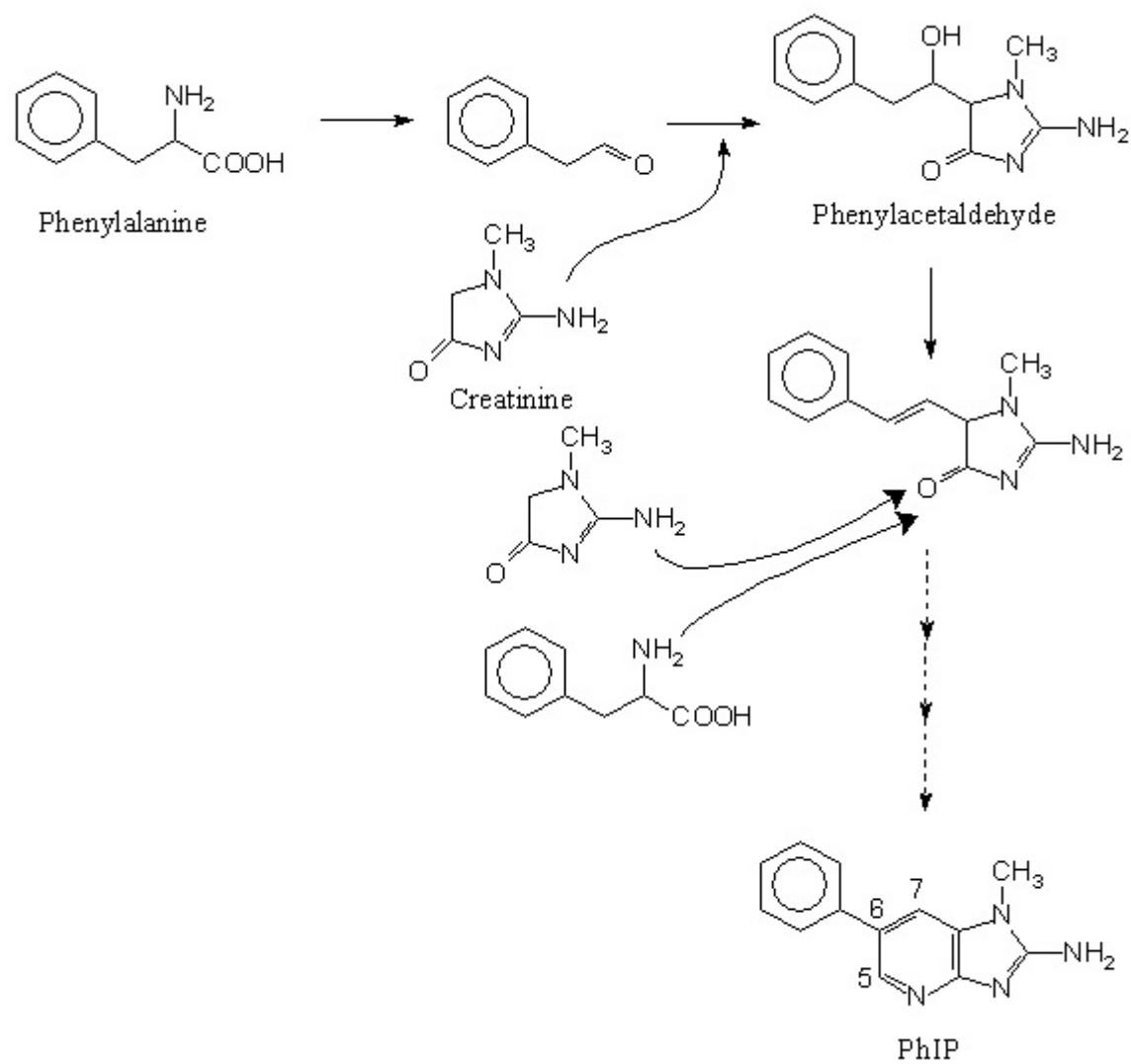


Figure 5. Formation of PhIP with identified intermediate reaction products (Murkovic 2004).

## **HETEROCYCLIC AMINES AND HUMAN HEALTH**

Cancer is a leading cause of death in human populations. Approximately 13% or 7.5 million of the world's deaths are attributed to cancer every year. A total of 1,529,560 new cancer cases and 569,420 deaths from cancer are projected to occur in the U.S. in 2010 (Jemal and others 2010). Several studies have shown that consuming meat increased the incidence of cancers. HCAs are carcinogenic compounds occurring in foods consumed under normal lifestyle conditions are believed to increase the risk of human cancers (Sugimura 2002).

### **Epidemiological studies**

Epidemiological studies provide the most direct link between HCA genotoxicity and human health risks in the long term. Several epidemiological studies have evaluated the evidence for a potential role of cooked meat intake and dietary exposure to HCAs in the etiology of human cancers (Sinha and Rothman 1997). The epidemiological studies over the past 10 years have shown that high intake of well-done meat and high exposure to meat carcinogens, particularly HCAs, may increase the risk of human cancers (Zheng and Lee 2009). Table 2 summarized the epidemiological studies evaluating the association of each HCA exposure with cancer risk in 1996-2008. Among the HCAs identified to date, PhIP, which is the most abundant of the HCAs found in human diets, has been shown to have the most positive association with cancer risk in a dose-response relationship (Zheng and Lee 2009). Four major cancers that have been reported to be associated with meat intake are colorectal, breast, prostate, and pancreatic cancers.

Table 2. Summary of epidemiological studies evaluating the association of HCA exposure with cancer risk in 1996-2008 (Zheng and Lee 2009)

Exposure	No. of studies evaluated	No. of studies reporting positive associations (%)
Mutagenicity or total HCAs	10	7 (70.0%)
PhIP	13	8 (61.5%)
MeIQx	12	6 (50.0%)
DiMeIQx	11	6 (54.5%)

### *Colorectal cancer*

Colorectal cancer (cancer of the colon and rectum) is the fourth leading cancer killer in the U.S. In 2006, 139,127 people were diagnosed with colorectal cancer, and 53,196 people died from it (Centers for Disease Control and Prevention [CDC] 2006). Kampman and others (1999) and Butler and others (2003) conducted population-based case-control studies in the U.S. and reported the positive associations of colorectal polyps with the intake of well-done meat and/or exposure to HCAs. Nowell and others (2002) reported that higher exposure to HCAs was strongly associated with colorectal cancer risk. The increased consumption of meats cooked well done or very well done produced comparable odds ratios (OR) for colorectal cancer risk (OR = 4.36, 95% confidence interval 2.08–9.60) for the highest quartile of exposure. Merchand and others (2002) also found a dose-dependent association between the HCA intake and colorectal cancer, with a two- to three-fold increase in risk and this association was strongest for MeIQx.

### *Breast cancer*

Breast cancer is the second leading cancer killer in the U.S. among women. In 2006, 191,410 women were diagnosed with breast cancer, and 40,820 women died from the disease (CDC 2006). DeStefani and others (1997) and Delfino and others (2000) conducted hospital-based case-control studies in Uruguay and the U.S. and reported the positive associations of breast cancer with well-done meat intake and HCA exposure. The increase in the risk of breast cancers was statistically significant for PhIP, but not for MeIQx and DiMeIQx (Zheng and others 2002). Furthermore, it has been suggested that the association between the risk of breast cancer incidence and well-done meat intake may be modified by genetic polymorphisms in some genes that encode metabolizing enzymes for HCA activation or detoxification (Zheng and others 1999 and 2002).

### *Prostate cancer*

Prostate cancer is the most common cancer among men. In 2006, 203,415 men were diagnosed with prostate cancer, and 28,372 men died from this disease (CDC 2006). Koutros and others (2008) conducted a cohort study in the U.S. and found a positive association between prostate cancer incidence and the intake of well or very well-done meat. Norrish and others (1999) reported that higher exposure to HCAs through consuming well-done beef steak was strongly associated with the prostate cancer risk (relative risk = 1.68; 95% confidence interval = 1.02-2.77).

### *Pancreatic cancer*

Approximately, 33,730 people developed pancreatic cancer, and 32,300 people died from the disease in 2006 (CDC, 2006). Pancreatic cancer has a median survival period of less than 6 months and a dismal 5-year survival rate of 4.6% (Sarkar and others 2007). Positive associations between the pancreatic cancer risk and the intake of grilled or barbequed red meat (Anderson and others 2002) and the high exposure of HCAs have also been observed (Li and others 2007). Stolzenberg-Solomon and others (2007) reported that high intakes of red meat, high temperature cooked meat, well-done meat, and very well-done meat were found to be associated with an increased risk of pancreatic cancer among men but not among women. At the ages 50 to 70, men showed a significant 50% increased risk from consuming grilled, barbecued, and broiled meat. The reported incidence of pancreatic cancer is higher among men than women and higher among African-Americans than Whites. The last observation is attributed to the African-Americans preference for cooking meat products by grilling, which produces high levels of HCAs. On the other hand, Whites prefer to fry meats (Keating and Bogen 2004).

### *Uncertainties associated to epidemiological results*

Numerous studies have clearly demonstrated that the risk of cancer increases with HCA exposure; however, several studies used small sample sizes, which limited the statistical power of the studies in a way that could lead to the data being misinterpreted. Therefore, additional research is needed to improve exposure assessment and quantity in testing the association between HCA exposure and cancer risk (Zheng and Lee 2009).



### **Bioavailability of heterocyclic amines in humans**

Several studies have been reported on the bioavailability of HCAs in humans. Lynch and others (1992) measured the excretion of unchanged forms of PhIP, MeIQx, and DiMeIQx in the urine of volunteers within 12 h after ingestion of fried beef containing known amount of HCAs. The percentage of ingested HCAs excreted in the unchanged form were 1.1% of PhIP, 2.1% of MeIQx, and non-quantifiable level of DiMeIQx. Their results agreed with another study of humans by Reistad and others (1997), which showed that about 0.5-2% of ingested PhIP and 1-6% of ingested MeIQx were recovered in unchanged forms from the urine 0-24 h post-meal. In contrast, studies at higher dose in rodents and monkeys reported 50-70% excretion of unchanged PhIP and MeIQx in the urine (Turesky and others 1991, Watkins and others 1991). It is possible that the dramatically increased amount of excretion of unchanged HCAs at the higher doses was caused by the saturation of the metabolism pathways. According to these bioavailability studies of HCAs, when low doses are applied, very small amounts of unchanged HCAs are excreted in the urine, which means approximate 98% of the ingested HCAs are likely to be extensively metabolized in the human body (Gooderham and others 2001).

### **Metabolic bioactivation of heterocyclic amines**

Most HCAs in their native forms are not mutagenic or carcinogenic, but after metabolic activation, they have the ability to form DNA adducts (Gooderham and others 2001 ). The major metabolic activation pathway of HCAs consists of phase I and phase II bioactivations. For phase I bioactivation, HCAs are activated via *N*-hydroxylation catalyzed at the exocyclic amino group by means of hepatic cytochrome CYP1A2 and form *N*-hydroxy-HCA derivatives (Gooderham

and others 2001). Apart from CYP1A2, other cytochrome P450s, including CYP1A1, CYP3A4, CYP2C9/10, CYP2A3 and CYP1B1, are capable of metabolizing HCAs, but they have much lower capacity than CYP1A2 (Gooderham and others 2001). Although these metabolites could directly react with DNA, they do so infrequently. Most of these *N*-hydroxy-HCA derivatives usually follow phase II bioactivation when four cytosolic enzymes including *N*-acetyltransferase (NAT) produce *N*-acetoxy ester derivatives, when sulfotransferase produces sulfonyloxy ester derivatives, when prolyl tRNA synthetase produces *N*-prolyloxy ester derivatives, and when phosphorylase produces *N*-phosphatyl ester derivatives at the exocyclic amino groups (Alaejos and others 2008). These reactive esters then undergo heterolytic cleavage to produce the reactive nitrenium ion-acetate anion pair, which is able to react with DNA to form DNA adduct, or with proteins and other cellular constituents (Turesky and Vouros 2004). Figure 6 shows the metabolic activation of MeIQx by CYP1A2 followed by NAT to form *N*-acetoxy-MeIQx, which then generates the nitrenium ion-acetate anion with a positive charge delocalized at the exocyclic amino group and the C-5 atom of MeIQx. This reactive nitrenium ion intermediate is then able to bind to DNA base and form DNA adducts (Turesky and Vouros 2004). Many studies confirm that HCA-DNA formation is greatly enhanced by phase II bioactivation of the *N*-hydroxy-HCA derivatives. For example, Synderwine and others (1988) reported that NAT-mediated metabolism of *N*-hydroxy-IQ to *N*-acetoxy-IQ produced a 30-fold increase in IQ-DNA adducts levels.

Among four cytosolic enzymes in phase II bioactivation, NAT appears to play the most important role. NAT is found predominantly in the liver and is expressed both in humans and rodents (Alaejos and others 2008). Although the liver is the largest organ in which HCA

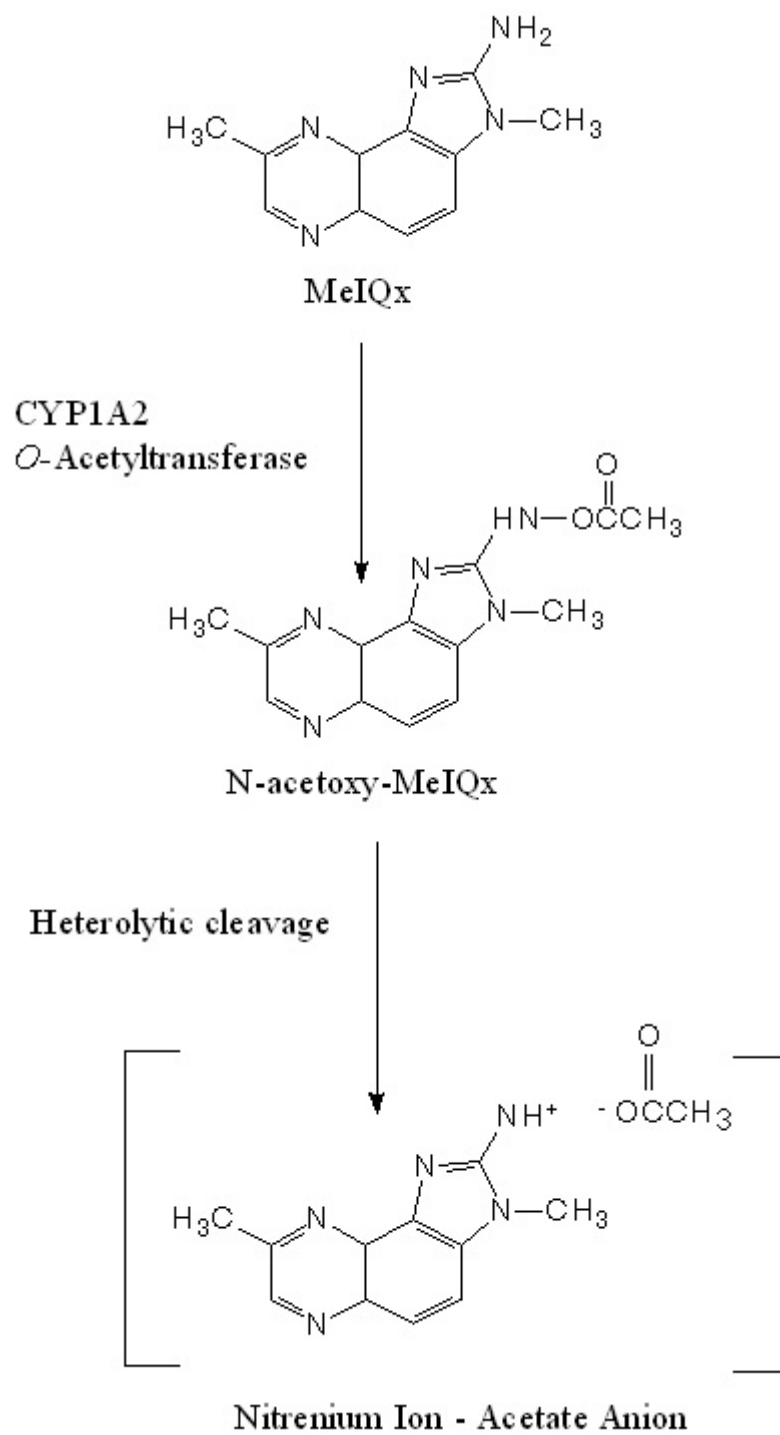


Figure 6. Metabolic activation of MeIQx (Turesky and Vouros 2004).

metabolisms occurs, the other organs, including the lung, kidney, mammary gland, colon, and pancreas have been also reported to have a role in HCAs metabolic activation (Cheng and others 2006). For example, when PhIP was given to rats, PhIP-DNA adducts were detected in the blood, mammary glands, and colon, all of which lack those cytosolic enzymes (Kaderlik and others 1994).

It is important to note that not every reactive nitrenium ion-acetate anion pair that is generated from metabolic activation process can form DNA adducts. Some metabolites can be conjugated by sulphation by means of glutathione *S*-transferase and by glucuronidation by means of UDP-glucosyltransferase and produce the polar compounds, which are excreted through the bile or urine (Turesky and Vouros 2004).

### **DNA adduct formation**

DNA-adduct formation is considered a biomarker for assessing the mutagenic and carcinogenic potential of genotoxic chemicals (Schut and Snyderwine 1999). The measurement of HCA-DNA adducts in human tissues can be achieved by the use of a <sup>32</sup>P-postlabeling method, ultraviolet/visible spectrophotometry (UV/VIS), mass spectrometry (MS), or nuclear magnetic resonance (NMR) (Turesky and Vouros 2004). The *in vivo* and *in vitro* studies have confirmed that HCAs are capable of forming DNA adducts. The chemical structures of IQ-, MeIQx-, and PhIP-adducts are shown in Figure 7. The mechanism is believed to be mediated through on electrophilic attack at the C-8 position of guanine base of the DNA molecules by the reactive HCA nitrenium ions (dG-C8-HCA), and the damage to the DNA depends on the parent HCAs (Schut and Snyderwine 1999). In the case of IQ and MeIQx, adducts can be formed at the N-2

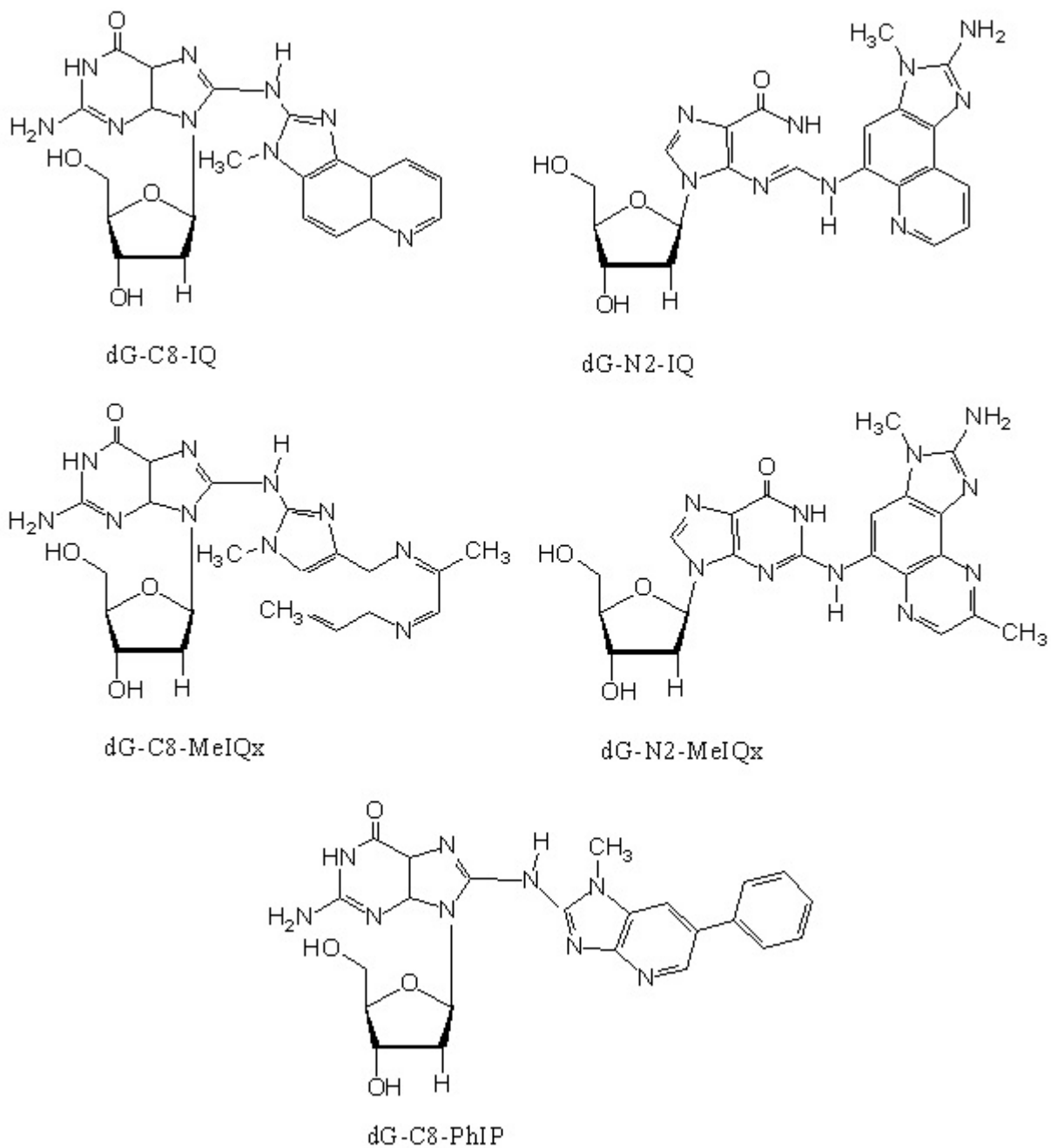


Figure 7. Chemical structures of HCA-DNA guanine adducts (Turesky and Vouros 2004).

position of the guanine base as well; however, the amounts of adducts are lower than those that occur at the C-8 position by approximately 5-10 folds (Schut and Snyderwine 1999). Lynch and others (1995) reported that the C8-guanine adduct is the major adduct formed by IQ, MeIQx and PhIP and accounts for 35-45% of the total DNA adducts in the colon, pancreas, heart, lung and liver in rats and accounts 47-68% of the total DNA adducts in the liver of non-human primates.

In addition, once DNA adducts are formed, they can undergo DNA repair; the 3' cutting step of nucleotide excision repair appears to play an important role in repair of DNA damaged by HCAs (Felton and others 2007). The activity of DNA repair depends greatly on the rate of cell cycling. If cell cycling is enhanced, as it is often as a result of cell damage from the action of exogenous toxicants such as carcinogens, mutagens, or of organ-specific promoters, DNA damage can not be repaired. The mutated DNA is a likely precursor of tumor formation. On the other hand, if cell cycling is decreased, as is observed with some phytochemicals like the tea polyphenols, raspberry polyphenols or carotenoids, DNA damage can be repaired (Weisburger 2002).

### **Carcinogenicity**

The carcinogenicity of HCAs has been well documented in a wide range of organs/tissues in long-term animal studies. Sugimura and others (2004) collected studies involved in the carcinogenicity of HCAs in rats and mice and their results are shown in Table 3. The International Agency for Research on Cancer (1993) classifies HCAs into 2 groups based on carcinogenicity studies: MeIQ, MeIQx, PhIP, A $\alpha$ C, MeA $\alpha$ C, Trp-P-1, Trp-P-2, and Glu-P-2 as possible human carcinogens (group 2B), and IQ as a probable human carcinogen (group 2A). IQ is classified as

Table 3. Carcinogenicity of heterocyclic amines in rats and mice (Sugimura and others 2004)

HCA	Animal	Strain	Concentration in diet (ppm)	Experimental period (weeks)	Target organs
Trp-P-1	Rat	F344	150	52	Liver
	Mouse	CDF <sub>1</sub>	200	89	Liver
Trp-P-2	Rat	F344	100	112	Liver, Urinary bladder
	Mouse	CDF <sub>1</sub>	200	89	Liver
Glu-P-1	Rat	F344	500	64	Liver, small/large intestine, zymbal gland
	Mouse	CDF <sub>1</sub>	500	57	Liver, blood vessels
Glu-P-2	Rat	F344	500	104	Liver, small/large intestine, zymbal gland
	Mouse	CDF <sub>1</sub>	500	84	Liver, blood vessels
AαC	Rat	F344	800	104	No tumors
	Mouse	CDF <sub>1</sub>	800	98	Liver, blood vessels
MeAαC	Rat	F344	100	100	Liver
	Mouse	CDF <sub>1</sub>	800	84	Liver, blood vessels
IQ	Rat	F344	300	55-72	Liver, small/large intestine, zymbal gland, skin
	Mouse	CDF <sub>1</sub>	300	96	Liver, forestomach, lung
MeIQ	Rat	F344	300	40	Large intestine, zymbal gland, skin, oral cavity, mammary gland
	Mouse	CDF <sub>1</sub>	400, 100	91	Liver, forestomach
MeIQx	Rat	F344	400	61	Liver, zymbal gland, skin
	Mouse	CDF <sub>1</sub>	600	84	Liver, lung
PhIP	Rat	F344	400	52	Large intestine, mammary gland, prostate, lymphoid tissue
	Mouse	CDF <sub>1</sub>	400	82	Lymphoid tissue

probable human carcinogen due to its capability to induce hepatocellular carcinoma in 55% of nonhuman primates at the low dose (10 mg/kg of IQ) and in 95% at the high dose (20 mg/kg of IQ) (Adamson and others 1994).

The liver has been shown to be susceptible to carcinogenesis induced by many HCAs due to its predominant role in the metabolic activation of HCAs (Schut and Snyderwine 1999). This is supported by evidence that shows the carcinogenicity is correlated with HCA levels of metabolites and/or DNA-adducts in corresponding organs/tissues (Cheng and others 2006). However, other organs and tissues that have been reported to have tumors induced by HCAs are the small and large intestine, zymal gland, skin, forestomach, lung, mammary gland, prostate, and lymphoid tissue (Sugimura and others 2004).

### **Mutagenicity**

The Ames mutagenicity assay developed in the 1970s was usually used to assess the mutagenicity of genotoxic compounds. In this assay, if a mutagen is present, the number of relevant colonies found increase in a dose-responder manner in the absence of an external histidine source (Maron and Ames 1983). Thirty years ago the discovery that cooked fish and beef showed highly mutagenic potency was detected by the Ames mutagenicity assay and has drawn the attention of scientists around the world to search for the mutagens present in these foods. To date, the assay has already confirmed that HCAs are promutagens that require metabolic activation for DNA adduct formation (Sugimura and others 2004). Table 4 also shows the mutagenicity level of different HCAs in *Salmonella enteritica* sv. Typhimurium TA98 and TA 100 with S9 mix. The mutagenicity of the polar HCAs in *Salmonella* TA98 and TA1538 are in the following order:



Table 4. Mutagenicity of heterocyclic amines in *Salmonella enteritica* sv. TyphimuriumTA98 and TA 100 with S9 mix (Sugimura and others 2004).

HCA	Revertants/ $\mu$ g	
	TA98	TA100
MeIQ	661,000	30,000
IQ	433,000	7000
4,8-DiMeIQx	183,300	8000
7,8-DiMeIQx	163,300	9900
MeIQx	145,000	14,000
Trp-P-2	104,200	1800
IQx	75,400	1500
Glu-P-1	49,000	3200
Trp-P-1	39,000	1700
Glu-P-2	1900	1200
PhIP	1800	120
A $\alpha$ C	300	20
MeA $\alpha$ C	200	120

MeIQ > IQ > DiMeIQx > MeIQx > PhIP (Felton and Knize 1991). The level of potential mutagenicity of HCAs is directly related to their chemical structure, e.g. the number of fused aromatic rings, the number of nitrogen atoms in the ring, the presence of methyl substituent group on a nitrogen within an imidazo ring, and the number and location of the exocyclic amino group (Hatch and others 1991). Also, a linear relationship between the levels of DNA adducts and mutagenicity exists for several HCAs, meaning that when the level of DNA adducts is higher, the

mutagen is more potent (Kerdar and others 1993).

It has been reported that HCA-DNA adducts can drive mutations in different ways, e.g. by single base deletion, base substitution or base transversion. For example, in their bacterial assays, Fuscoe and others (1988) reported that IQ, MeIQ, and PhIP showed a single GC deletion in the *hisD* gene of *Salmonella* TA1538. Koch and others (1998) found that PhIP induced predominantly GC →TA transversion, which was believed to involve the dG-C8-phIP adduct. In addition, in mammalian cells, PhIP- and IQ- DNA adducts induced G →T transversion mutations in guanine nucleotides adjacent to either guanine or adenine nucleotide, and also induced base substitution at guanine and deletion of a guanine base adjacent to guanine and/or adenine (Wu and others 1995, Yadollahi-Fardani and others 1996).

### **Chemopreventive of heterocyclic amines in human**

Many compounds, mainly from plant sources, have been reported as being able to reduce the mutagenicity and carcinogenicity of HCAs in human. Chlorophyllin and fibers from sorghum, wheat bran, cabbages, burdock, radish, carrots, and green pepper have all been shown to have strong binding affinities for HCAs by Van der Waals or electrostatic interactions which result in reduced HCA uptake from the gut and increased elimination of parent compounds in the urine and feces (Dashwood 1992, Dashwood 2002, Vikse and others 1992). Indole-3-carbinol, a compound in cabbage, cauliflower and broccoli, and tea polyphenols have been shown to serve as chemopreventive agents by inhibiting the activation steps of HCAs mediated by cytochrome P450 1A2 and other cytochromes (Stavric and others 1996, Xu and others 1997, Dashwood 2002). Tea polyphenols have been shown to have the capability to interact with the reactive nitrenium ion-

acetate anion pair, which is able to react with DNA forming DNA adducts (Hernaes and others 1997). Vanillin, coumarin, and caffeine have been shown to act as bioantimutagens in that they repair the damaged DNA, leading to decrease in mutation frequency (Sanyal and others 1997, Dashwood 2002).

### **Occurrence of heterocyclic amines in foods**

HCAAs found in cooked meat and fish products mainly contribute to HCA intake, since the levels of HCA found in other foods or in the environment are extremely low. The amount of HCAAs formed depends on the type of meat, cooking method, and cooking time and temperature. Table 5 summarizes the levels of major HCAAs found in cooked meat and fish products. The PhIP levels in cooked muscle foods are normally higher than MeIQx and DiMeIQx. The large amounts of PhIP may occur because PhIP is formed by a different mechanism, which allows them to form more easily than other polar HCAAs (Knize and Felton 1995). IQ and MeIQ are seldom detected in meat samples (Knize and Felton 1995).

Cooked offal products, such as chicken liver, beef liver, beef tongue, and lamb kidney contain very low or non-detectable levels of HCAAs (Solyakov and Skog 2002, Khan and others 2009); this may be due to the low levels of creatine and creatinine present in these raw products (Khan and others 2009). The creatine level detected in raw chicken liver was 2  $\mu\text{mol/g}$  wet weight, which was approximately 10-fold less than the amount in chicken muscle (Solyakov and Skog 2002).

Processed food flavors are commercially simulated meat flavors frequently added to many processed foods as flavor enhancers. These processed food flavors are produced by exposing raw

Table 5. Estimated MeIQx, DiMeIQx, and PhIP concentrations in cooked meat products by meat type and cooking method (Skog and others 1997, Knize and others 1999, Solyakov and Skog 2002, Busquets and others 2004, Ni and others 2008, Iwasaki and others 2010)

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

nd = not detected

meat (e.g., beef, chicken, pork) to prolonged heating at higher than normal cooking temperatures (Starvic and others 1997). Although most have undetectable levels of HCAs, some samples contain as much as 5 to 20 ng/g of total HCAs, especially IQ (Starvic and others 1997, Knize and

Felton 2005). However, since processed food flavors are not used as such for direct consumption, only tiny amounts are added to foods; therefore, they should not play a significant role in the human intake of HCAs (Knize and Felton 2005).

Pan residues seem to be an important source of HCAs and make a significant contribution to the intake of HCAs. Gravies prepared from pan residues are very common (Johansson and Jägerstad 1994). The amounts of HCAs in gravies have been reported to be comparable or higher than the amounts in the corresponding beef, chicken, turkey, pork and fish that produced the pan residues (Janoszka and others 2009, Sinha and others 1998). Janoszka and others (2009) estimated that the consumption of gravy that is formed when meat is fried in 100 g portions may increase the exposure by 1.02-1.51  $\mu\text{g}/\text{day}/\text{person}$ , which corresponds to an increase by 82 to 142%. Also, grill scrapings from grilling of the meat have been reported to have higher amounts of MeIQx, DiMeIQx, and PhIP compared with the meat that was grilled, however, grill scrapings are not commonly consumed (Gross and others 1993).

## **HUMAN INTAKE OF HETEROCYCLIC AMINES**

Generally, the HCA intake for meat is estimated by taking into account the frequency of consumption, cooking method, degree of doneness, and HCA content in the foods (Sinha and others 2005). The estimates of daily intake for the U.S. population have been reported at 6.3 ng/kg per day (Bryne and others 1998), 20.1 ng/kg per day (Layton and others 1995), and 2.3-6.6 ng/kg per day (Keating and Bogen 2004), while the estimates for European populations have been reported at 2.3 ng/kg per day (Augustsson and others 1997) and 6.6 ng/kg per day (Zimmerli and others 2001). The daily consumption of meat was estimated to range from 80 to 160 g/day in the

United States, Sweden, New Zealand, and Japan (Augustsson and others 1997, Kobayashi and others 2002, Layton and others 1995).

Keating and Bogen (2004) studied the effect of race-ethnicity on HCA intake for the U.S. population. Table 6, shows little difference in the HCA daily intake based on age (child vs adult) or gender (male and female); however, race had great effect on HCA intake. African-American males were estimated to consume almost twice as much HCA as White males. PhIP was the most predominant HCA in the diets of both groups (70% of total HCAs), especially in pan-fried and grilled chickens. This may be due to the different cooking methods used by African-Americans and Whites; for example, African-Americans prefer to cook their hamburgers by grilling which yields a higher rate of formation of HCAs than deep frying or pan frying, cooking methods which are preferred by Whites (Keating and Bogen 2004).

The estimates of HCA daily intake for the Chinese population has been reported as 50.0 ng/day; however, the range was very wide, with the 90<sup>th</sup> percentile of intake at 95.8 ng/day and the 10<sup>th</sup> percentile of the intake at 14.0 ng/day (Wong and others 2005), which is comparable to the HCA intake for the Japanese population (in Okinawa) reported at 52.8 ng/day in men (91.2g meat intake/day) and 50.6 ng/day in women (82.6 g meat intake/day) (Kobayashi and others 2002). Wong and others (2005) show that the younger age group (20-39 years) had a 1.5 fold greater dietary intake of HCAs compared to the older age group (40-59 years). Deep-fried chicken, pan-fried pork and grilled beef contributed the most to the HCA intake in the younger age group.

Table 6. Estimated heterocyclic amine intake of African-American and White males and females in the U.S. (Keating and Bogen 2004)

Age	Race	Gender	<i>n</i>	HCA concentration (ng/g)			
				PhIP	MeIQx	DiMeIQx	Total
Child (15 years)	White	Male	2478	7.7	1.3	0.27	11.0
		Female	2233	8.1	1.2	0.28	11.4
	African-American	Male	520	14.7	1.8	0.67	19.9
		Female	608	10.8	1.5	0.42	14.8
Adult (> 30 years)	White	Male	4734	9.2	1.4	0.28	13.4
		Female	4955	9.2	1.4	0.28	13.6
	African-American	Male	546	12.6	2.1	0.47	18.4
		Female	858	12.7	1.6	0.41	18.4

The different cooking methods may play a role in dietary HCA intake of each country. In the U.S., pan frying was the most common method for cooking chicken (56%) and fish (54%) while broiling and grilling was common for cooking steak (34%) and hamburgers (63%) (Keating and Bogen 2004). In the Sweden, the common cooking methods for meat were frying (61%), baking or roasting (20%), boiling (15%), and grilling (4%) (Augustsson and others 1997). In the China and Singapore, the most common cooking methods were boiling and steaming, whereas grilling and roasting were used less often (Koh and others 2005).

It must be emphasized that considerable uncertainties are related to the estimation of HCA intake. Some challenges in estimating human exposure to HCAs are due to there being limited data on the HCA concentration in meats cooked at home, in commercial meat products, and in

meats cooked at fast-food restaurants. Absent more completed information, experimentally derived HCA concentrations provide the best available data with which to estimate HCA intake in most studies (Skog 2002).

## **FACTORS AFFECTING HETEROCYCLIC AMINE FORMATION**

To study the factors affecting HCA formation, a model system may be used. A model system was recently developed and applied to kinetic studies investigating and confirming the mechanism for HCA formation (Knize and Felton 2005). The most common chemicals used in the model system are the main precursors for HCA formation, including creat(in)ine, amino acids, and carbohydrates. A mixture of these compounds may be heated in a dry powder form or a dissolved form in diethylene glycol/water. Lyophilized meat or meat juice are used for the model systems to simulate the chemical environment in the meat, which gives more relevant results than using the chemicals; however, the meanings of the results can be complicated due to the presence of impurities (Knize and Felton 2005).

### **Temperature and time of cooking**

Cooking time and temperature are the most important factors in the formation of HCAs. Both time and temperature were found to have a strong impact on the formation of HCAs (Knize and Felton 2005). Each HCA in a complex meat system is formed differently at certain temperature and time points. Activation enthalpy ( $\Delta H^*$ ) is approximately equal to the activation energy ( $E_a$ ) which means the amount of energy that must be supplied in order to initiate reactions. The activation enthalpy of each HCA is shown in Table 7. IQx and 7,8-DiMeIQx had activation



Table 7. The calculated activation enthalpy  $\Delta H^\ddagger$  ( $\approx E_a$ ) of heterocyclic amines (Arvidsson and others 1997)

HCA	activation enthalpy (KJ/mol)
IQx	-110.1
MeIQx	-54.8
7,8-DiMeIQx	-131.5
4,8-DiMeIQx	-67.2
PhIP	19.8

enthalpies that were less than those for MeIQx and 4,8-DiMeIQx while PhIP had the highest activation enthalpy. This is why the formation of PhIP has so often been reported at low temperature. Bordas and others (2004) examined the way heating time affected the formation of HCAs by heating the lyophilized meat extract with water at temperature 175 °C. When heating times increased from 1 h to 2 h, the amounts of HCAs increased, especially for PhIP, which increased from 9.3 ng/g to 19.5 ng/g. Figure 8 shows the relation between cooking temperature and levels of total HCAs (ng/g) in fried beef patties cooked at different cooking temperatures until they reached the same internal temperature at 70 °C. Fried beef patties cooked at 140 °C had very low amounts of HCAs, and the levels of HCAs increased gradually when cooking temperature rose higher from 140 to 250 °C (Knize and Felton 2005).

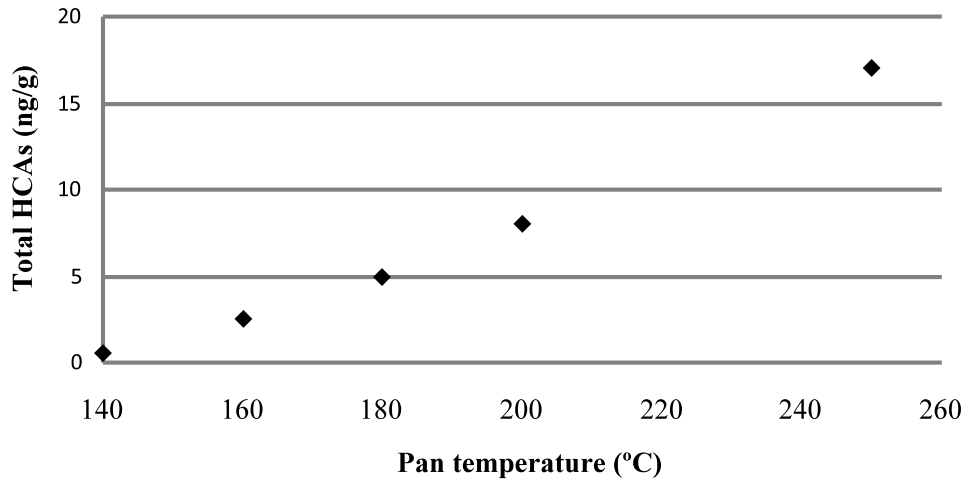


Figure 8. Formation of heterocyclic amines in beef patties after cooking to an internal temperature of 70 °C at different frying pan temperatures (Knize and others 2005).

Arvidsson and others (1997) examined the effect of heating time and temperature on HCA formations by heating a mixture of creatine, glucose, and amino acids at 150 to 225 °C for 0.5-120 min, and the results showed that HCA formation was a time and temperature function (Figure 9). At 200 °C, the yield of HCAs increased very rapidly and reached a maximum level after only 5 to 10 min of heating. However, at 225 °C, some HCA yields (e.g., MeIQx and 7,8-DiMeIQx) decreased after they reached their maximum. This suggests that an excess of temperature and time may cause the degradation of the HCA, which agrees with the results obtained by other authors of model system studies (Jackson and Hargraves 1995, Bordas and others 2004). Chiu and Chen (2000) reported that the degradation of HCAs during heating increases as both heating temperature and time increase, and the degradation of HCAs fits a first-order model. PhIP is the least stable, while IQx is the most stable. Their finding agrees with Arvidsson and others (1997),

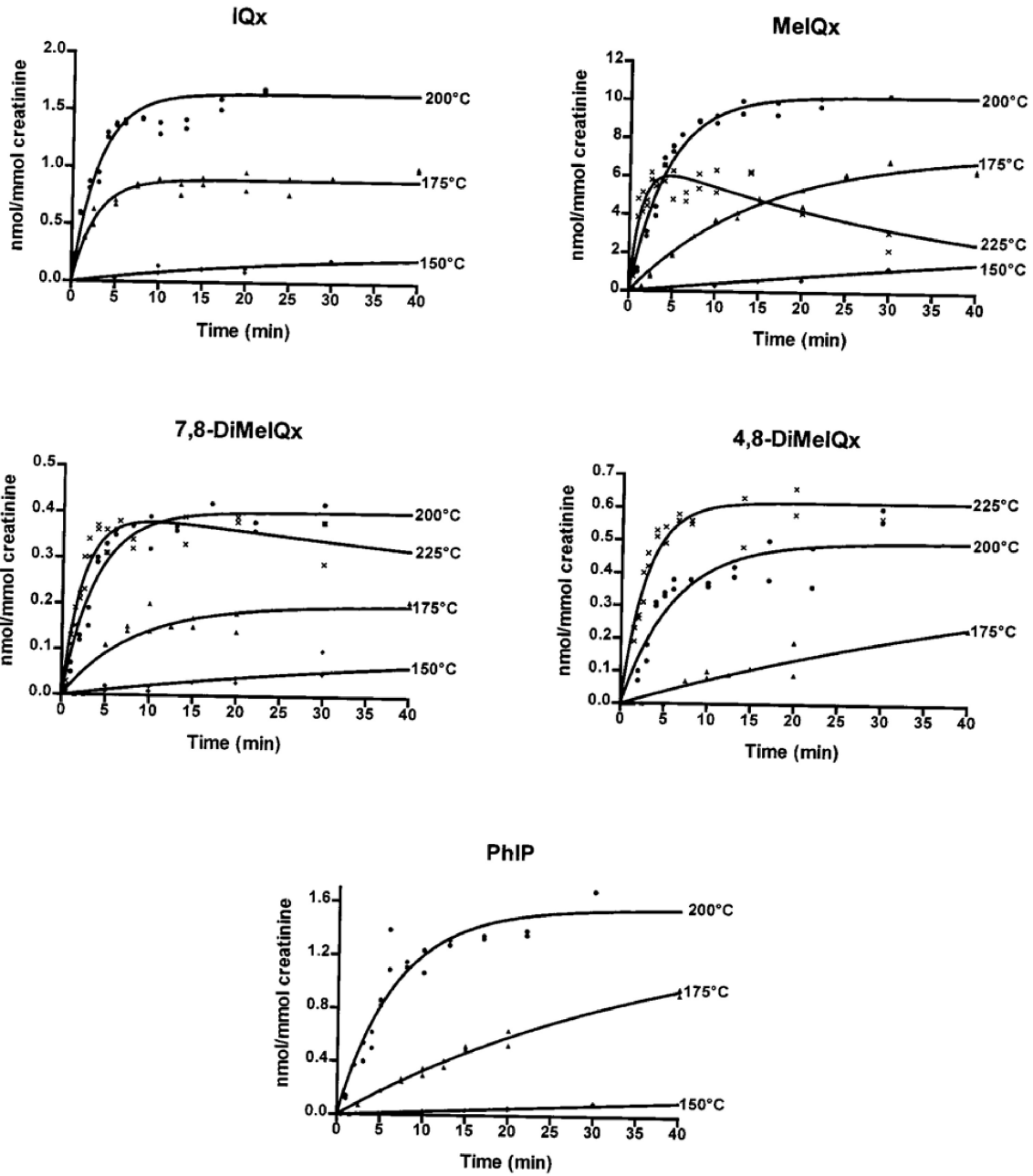


Figure 9. Formation of HCAs at 150, 175, 200, and 225 °C (Arvidsson and others 1997).

whose studies reported that PhIP was the most susceptible to being degraded at 225 °C followed by 7,8-DiMeIQ<sub>x</sub>, 4,8-DiMeIQ<sub>x</sub>, and IQ<sub>x</sub>. The instability of PhIP during heating may be a consequence of PhIP containing more conjugated carbon-carbon double bonds or perhaps PhIP containing a benzene ring attached to a single bond of a side chain, which make it free to rotate (Chiu and Chen 2000).

### **Cooking methods**

The cooking method is believed to affect HCA formation and involves heat transfer and water loss (Skog and others 1995). Pan-frying, grilling, and broiling are high-temperature cooking methods, in which food is heated by radiative and conductive processes. These methods have been reported to produce high concentrations of HCAs (Chen and Chiu 1998). Oven-roasting and baking use medium or medium-high heat levels to cook foods by indirect convection. These methods have been reported to produce low or intermediate levels of HCAs (Skog and others 2003). Skog and others (2003) demonstrated that the mutagenicity activity of chicken fillets cooked in convection ovens depended on air temperature, air velocity, and the presence of steam. The mutagenicity activity was higher when air temperature and air velocity were increased; the presence of steam; however, reduced the mutagenicity activity, probably due to the surface temperature being lower. Boiling and steaming are normally achieved at temperature below 100 °C (212 °F) and usually produce low or undetectable level of HCAs (Chen and Chiu 1998).

At the same internal temperature of 88 °C, total HCAs in pan-fried pork patties (17.2 ng/g) were significantly higher than those in oven-broiled pork patties (5.0 ng/g) and boiled pork patties (1.0 ng/g) (Shin 2005). When chicken breasts were cooked in various ways at a temperature of

180 °C, charcoal-grilled chicken had the highest total HCAs (35.77 ng/g), followed with pan-fried chicken (22.97 ng/g) and deep-fried chicken (3.31 ng/g) (Liao and others 2010). When fish, rainbow trout, were cooked in various ways to a well-done level, cooking by microwave for 9 min (8.19 ng/g) had the highest total HCA levels, followed by barbecuing for 9 min (5.22 ng/g), pan-frying at 200 °C for 15 min (3.77 ng/g), cooking on a hot-plate at 200 °C for 15 min (1.59 ng/g), and cooking in the oven at 200 °C for 15 min (1.44 ng/g) (Oz and others 2010). Brazillian-barbecuing, also called churrasco, is cooking meat or fish using indirect heat or low-level direct radiant heat from charcoals or embers, at lower temperatures and over longer cooking times than those for normal barbecue. Iwasaki and others (2010) reported that the total HCAs in chicken cooked by Brazillian-barbecueing (50.82 ng/g) was much higher than chicken cooked by normal barbecueing (32.22 ng/g) and pan-frying (3.78 ng/g) to the same doneness level (internal temperature of 90 °C).

Smoking has been reported to produce significant amounts of HCAs. Knize and Felton (2005) used smoking process for 4-6 hours at 80 to 85 °C and noted that the water activity in the surface of product decreased during the smoking process. The formation of HCAs may have been favored during the smoking process because of combination of an optimum low level of water activity (0.4-0.6) for the Maillard reaction and a moderately high temperature for several hours.

### **Concentration of precursors**

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

system.

### *Creatine and creatinine*

The addition of a 5-fold increase in glucose to a lyophilized bovine meat juice at 200 °C for 30 min enhanced the formation of MeIQx but inhibited the formation of PhIP (Skog and others 2000). This contrasted with a study by Bordas and others (2004), which reported that the addition of 5-fold increase in creatinine led to the generation of IQ, MeIQx, and PhIP in relatively high amounts. However, when creatinine is increased up to a 25-fold, the amount of PhIP increased but the amounts of IQ and MeIQx decreased, showing that excess creatinine was not favorable for generating IQ- and IQx-type compounds.

### *Sugar*

The addition of a 2.5-fold increase in glucose to a dry lyophilized meat extract at 200 °C for 30 min did not increase the formation of HCAs. However, when glucose was increased 5-fold in this model system, IQ was not detected and the amounts of MeIQx, DiMeIQx, and PhIP decreased. This suggests that glucose at high concentration has an inhibitory effect on the formation of HCAs (Skog and Jägerstad 1990, Skog and others 1998, Skog and others 2000). When sugar was added in different concentrations (9%, 14%, and 19% w/w); the HCA formation in fried fish fiber increased when the sugar levels increased up to 14%; however when a 19% sugar concentration was added, the HCA levels declined by 43% (Tai and others 2001).

By heating creatinine, glucose and amino acids in proportions similar to those in bovine meat, Arvidson and others (1997) measured the decay of these precursors. Figure 10 shows that

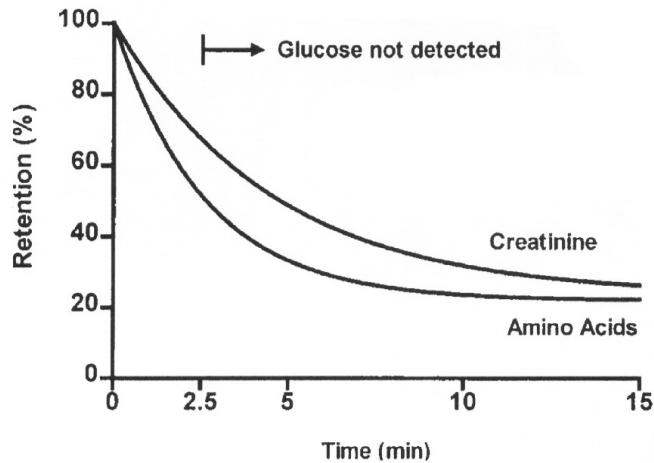


Figure 10. Decay of creatine, amino acids, and glucose during heating at 200 °C for 15 min (Arvidsson and others 1997).

after 15 min creatinine and amino acids remained at more than 20% in the system while all glucose had been depleted after only 2.5 min, meaning that glucose was the limiting precursor; however, adding glucose did not increase the production of HCAs.

#### *Amino acids*

The addition of glycine, alanine, and phenylalanine in 50-fold amounts to lyophilized meat extract, increased the formation of MeIQx and PhIP (Skog and others 1998). Phenylalanine, leucine, isoleucine, and tyrosine have been reported to be precursor amino acids for the formation of PhIP (Pais and others 1999).

#### **Moisture contents**

In a meat system, water is very important for the transport of water-soluble precursors

from the center to the high temperature surface of meat during cooking, where HCAs are formed (Persson and others 2008). It has been shown that the formation of IQx compounds in a model system was promoted at a water activity of 0.44 (Jägerstad and others 1998). The presence of water clearly affects the type and amount of HCAs. The formation of pyridine amines (PhIP, TMIP, IFP) are favored by dry conditions while the formation of IQ- and IQx-type amines are favored by wet conditions (Skog and others 2000).

PhIP formation that increased dramatically in the dry model system is usually found in the surface of meat (crust) during cooking. Cooking conditions that generate a high rate of drip loss are most favorable for the formation for PhIP (Messner and Murkovic 2004). PhIP has been detected at very high levels (up to 480 ng/g) at the surface of pan-fried, oven-broiled or grilled/barbecued chicken breast (Sinha and others 2005).

## **Lipid**

The effect of lipids is not well understood because they have been shown to have both inhibitory and enhancement effects on HCA formation. The inhibition effect of lipids on HCA formation may be due to the dilution of the precursors in the meat (Hwang and Ngadi 2003). In contrast, the enhancement effects on HCA formation may be due to lipids conduct heat more efficiently into the food (Johansson and Jägerstad 1994, Hwang and Ngadi 2002). Lipids may be involved chemically in the formation of HCAs by their generation of free radicals via lipid oxidation, which enhances the formation of certain Maillard reaction products (pyridines, pyrazines, and strecker aldehydes) in the Maillard reaction (Barnes and others 1983). It is also possible that a higher lipid content might inhibit thermal degradation of HCA once it has been



formed (Johansson and Jägerstad 1994). Johansson and Jägerstad (1993) studied the effects of oxidized lipids on the HCA formation using a model system. Even though they found that the degree of oxidation of the lipid (created by adding oxygen and hydrogen peroxide) slightly increased the formation of MeIQx in the model system, they did not observe a consistent trend; therefore, they did not reach a conclusion on the effect of lipid oxidation on HCA formation.

### **Meat characteristics**

Meat characteristics, including animal species, muscle type, aging, and meat quality, have been reported to play a significant role on the endogenous concentrations of precursors in the muscle affecting the formation of HCAs in cooked meat products.

Meat portions from different species, cooked in the same way, exhibit large variations in the formation of HCAs. The RN<sup>-</sup> allele of pig is a major gene that has been shown to significantly influence production and pork meat quality (Johansson and others 2002). The meat of the carrier of the RN<sup>-</sup> allele exhibits a lower level of pH, has less intramuscular fat and fewer free amino acids, and has considerably greater amounts of reducing sugars and residual glycogen (e.g., glycogen, glucose, and glucose-6-phosphate) compared to the pork of non-carriers of the gene (Johansson and others 2002, Olsson and others 2002). Fried-pork from carriers of the RN<sup>-</sup> allele obtain darker crust color than meat from noncarriers, and contain lower levels of HCAs than fried-pork from non-carriers (Olsson and others 2002 and 2005). Since previous studies have shown that high concentrations of reducing sugars in relation to other precursors (creatine and fatty acid) can reduce the formation of HCAs in both the meat and model system, the high residual glycogen content of the meat of the carrier of the RN<sup>-</sup> allele most probably explains the reduced HCA levels

(Olsson and others 2005).

In pan-roasted mackerel, the HCA levels in ordinary muscle were higher than those in dark muscle and skin (Gu and others 2002). This may be due to higher levels of moisture and creatine in ordinary muscle than what exists in dark muscle. These higher moisture levels promote the formation of HCAs. No creatine is present in the skin; skin contains mucus that is composed mostly of glycoproteins (Gu and others 2002).

Aging (conditioning) of meat, especially beef, is a normal procedure that increases in the tenderness and flavor due to the protein proteolysis stages (protein breakdown and peptide formation) (Polak and others 2008 and 2009). The levels of HCAs (MeIQx and PhIP) have been observed to increase in cooked beef when the beef has been aged longer (Polak and others 2008), but not significantly increase in cooked pork that has been aged longer (Polak and others 2009). The HCA levels of cooked aged beef are believed to increase because aging over a period of 1 to 10 days was shown to increase the creatinine, total free amino acids and glucose (Polak and others 2008).

Compared to normal meat quality, PSE (pale, soft and exudative) pork muscles have a low pH ( $\text{pH} < 6.0$ ), which favors the denaturation of muscle proteins, particularly myosin. Polak and others (2009) reported that the MeIQx contents of grilled PSE pork was 22% higher than that for the normal pork; this is probably due to the differences in the microstructure of normal and PSE pork.

### **Presence of other compounds**

The addition of iron ( $\text{Fe}^{2+}$ ) at 5-fold levels to aqueous lyophilized meat extract, which was heated at 175 °C for 10 min, was shown to enhance the formation of MeIQx but inhibited the formation of PhIP (Skog and others 2000). Murkovic and others (1998) found that MeIQx and PhIP levels doubled when myoglobin containing iron ( $\text{Fe}^{2+}$ ) was applied to the surface of meat before it was fried, but the addition of copper ( $\text{Cu}^{2+}$ ) did not affect the HCA yield. It has been suggested that the catalytic effect of iron ions is related to their capacity to accelerate free radical reactions via the Fenton reaction (Johansson and Jägerstad 1993). During the cooking of meat, there are two possible iron sources: heme-iron that is released through the denaturation of myoglobin present in animal muscle and iron that is leaching from the cooking equipment (Johansson and Jägerstad 1993).

The total amount of HCAs in cooked fried-fish fiber increased when the level of monosodium glutamate increased from 0 to 1.5% (Tai and others 2001). In contrast, the addition of sodium nitrite (150 ppm) in ground pork reduced the total HCA formation by 47% during cooking compared to a control; this appears to be due to the antioxidant effect of nitrite (Shin 2005).

### **INHIBITION OF HETEROCYCLIC AMINE FORMATION**

Epidemiological studies have shown that the dietary intake of HCAs through the consumption of cooked meat products increases the risk of human cancers. Consequently, finding ways to decrease HCA formation in foods is desirable.

### **Modifying cooking methods**

As mentioned earlier, cooking time and temperature are the most important factors in the HCA formation. HCA concentrations increase as both cooking time and temperature increase. Therefore, cooking at lower temperatures for less time, and/or changing the cooking methods are the most practical ways to reduce HCA formation (Chen and Chiu 1998, Skog and others 2003). Flipping foods over during cooking every minute until they reach the desired internal temperature has also been reported to reduce HCA levels (Knize and Felton 2005).

Microwave pretreatment is one practical way to reduce the HCA level in meat products. Felton and others (1994) investigated the effect of microwave pretreatment on the reduction of HCAs in fried beef patties. In their experiment, beef patties were microwaved for 0 to 3 min before being fried at 250 °C for 6 min on each side. Their results showed that fried beef patties that were microwaved for 3 min before being fried resulted in 3- to 9-fold decrease of MeIQx, IQ, DiMeIQx, and PhIP compared to non-microwaved beef patties, and also a decrease in mutagenicity of up to 95%. The reduction effect was believed to be due to the elimination of the water soluble precursors of HCA (creatine, sugar, and amino acids) by approximate 30%, during the microwaving process (Felton and others 1994).

### **Improving water-holding capacity**

As mentioned earlier, water plays a significant role on the transport of water-soluble precursors in the formation of HCAs at the surface of meat during cooking. Therefore, the addition of water-binding compounds may restrict the transport of precursors from the inner parts of the food to the surface, leading to a decrease in HCA formation at the surface (Knize and

Felton 2005).

Enhancement can be defined as an application in which adding a mixture solution, mainly containing water, salt, and sodium phosphates, to the meat usually adds 8-12% of original weight to improve the cooked meat's eating quality (juiciness, tenderness, and flavor) (Baczwaski and Mandigo 2003). Persson and others (2003) demonstrated that the addition of a solution of 1.5% sodium chloride and 0.3% sodium tripolyphosphate to beef burgers improved their water-holding capacity and was an effective way of reducing cooking loss, which also resulted in the reduction of the formation of HCAs in fried beef burgers by 38% compared to controls.

Carbohydrates (e.g., starch, gum, fiber) are increasingly being used to improve the textural characteristics such as tenderness, juiciness, and cooking loss as well as taste and aroma of meat products, especially low-fat products (Desmond and others 1998). The addition of some carbohydrates have been reported to reduce HCA formation in foods (Knize and Felton 2005). Persson and others (2004) demonstrated that adding 1.5% NaCl and 0.3% tripolyphosphate with 1.5% of certain carbohydrates (potato starch, potato fiber, and wheat bran) to beef burgers affected both weight loss and the formation of HCAs during cooking; raw potato starch inhibited the formation of HCAs by 70% compared to control.

## **Marination**

Marination is the process of immersing foods in a liquid marinade before cooking to allow the penetration of the meat by the marinade through diffusion over a period that may last a few hours or even days (Alvarado and Mckee 2007). Marination has been recognized as a means of enhancing the quality and palatability of meats in terms of tenderization and flavor enhancement

and also increases the water-binding capacity of meats, thus reducing cooking losses and improving the meat's juiciness (Baczwaski and Mandigo 2003, Alvarado and McKee 2007). At home, people marinate their meats by using simple ingredients: e.g. spices, vinegars, lemon juice, wine, soy sauce, salt, and sugar. Nowadays people also can buy marinade in ready-to-use forms (e.g. powders, liquid marinades) which require simple preparation at home (Yusop and others 2010). Most commercial liquid-based marinades consist of water, oil, phosphate, salt, spices, functional additives (e.g. xanthan gum or guar gum), antimicrobial agents (e.g. sorbate or benzoate), and organic acids (acetic, lactic, or citric acid) (Miller 1998).

Several studies have been performed on the effect of marination on HCA formation in meat products. Tikkanen and others (1996) found a reduction in PhIP formation and the mutagenic activity of grilled chicken marinated with mixture of spices and flavonoids compared to unmarinated chicken. Nerurkar and others (1999) showed that barbecued beef steaks marinated with commercial teriyaki sauce and turmeric-garlic sauce had 40-60% lower PhIP and MeIQx levels than unmarinated samples; however, marinating with barbecue sauce caused up to a 5-fold increase in PhIP and MeIQx levels, which could be due to their use of high fructose corn syrup and honey in the sauce. Red wine marinades were found to reduce the formation of the PhIP up to 88% in fried chicken breast (Busquets and others 2006). In another study, the application of oil marinades with optimum amounts of garlic, onion, and lemon juice achieved a reduction of MeIQx and PhIP formation in fried beef patties, and garlic was found to be the most active ingredient to have HCA inhibitory effect (Gibis 2007). Recently, marinating beef in green tea has been shown to significantly decrease levels of PhIP up to 74% in pan-fried beef, but decreases for MeIQx and DiMeIQx were not observed (Quelhas and others 2010). Marinating meats with

hibiscus extracts (*Hibicus sabdariffa*) have been shown to reduce PhIP and MeIQx levels by 40-50% in fried beef patties without negatively impacting the product's sensory characteristics (Gibis and Weiss 2010).

### **Addition of Antioxidants**

The addition of antioxidants is considered a promising and consistently effective treatment for limiting the formation of HCAs through radical quenchers and free radical scavengers because of the hypothetical free radical pathway (pyridine and pyrazine radicals) leading to HCA formation (Pearson and others 1992, Vitaglione and Fogliano 2004).

#### *Synthetic Antioxidants*

The synthetic antioxidants that have been reported to inhibit the HCA formation are butylated hydroxyanisole (BHA), propyl gallate (PG), tert-butylhydroquinone (TBHQ), and 1-O-hexyl-2,3,5-trimethylhydroquinone (HTHQ) (Hirose and others 1999, Vitaglione and Fogliano 2004). Barnes and others (1983) showed that IQ can be reduced by 40% in fried beef patties, when 50 mmol of BHA is added to 100 g meat. The amount of MeIQx in a fried beef patty was reported to decrease by 56% when BHA was added, 71% when PG was added, and 76% when TBHQ was added (as cited in Johansson and Jägerstad 1996). These synthetic antioxidants contain quinone-like compounds acting as potent free-radical scavengers blocking the reaction with the HCA precursors, thus preventing HCA formation (Pearson and others 1992, Vitaglione and Fogliano 2004). In contrast, butylated hydroxytoluene (BHT) has been shown to increase the formation of MeIQx and DiMeIQx in a heated mixture containing HCA chemical precursors; perhaps become

the methyl group at the para position of the BHT molecule may react with the dialkylpyrazine radical and creatinine and thus form MeIQx and DiMeIQx (Pearson and others 1992). Even though synthetic antioxidants have been shown to have the HCA formation, some synthetic antioxidants are believed to have cytotoxic and carcinogenic activity (Ito and others 1983). Due to concerns about the toxicological safety of synthetic antioxidants, consumers prefer to use natural antioxidants (Pokorný 2007).

### *Natural Antioxidants*

Natural antioxidants, such as phenolic, polyphenol, and flavonoids from plants, have been studied extensively for their inhibitory effects on HCAs (Vitaglione and Fogliano 2004). Based on previous studies, the natural antioxidants that affect HCAs can be divided into three groups: pure compounds, food extracts, and whole food.

Many studies have been performed to investigate the effect of pure antioxidant compounds on the inhibition of HCAs both in model meat systems and meat systems. For example, in meat model systems, the addition of green tea polyphenols (catechins and epigallocatechin gallate), flavonoids (quercetin and luteolin), and caffeic acid (0.2 mmol each) were found to clearly suppress the formation of both MeIQx and PhIP up to 75% of the level found in the controls (Oguri and others 1998). The addition of 0.67 mmol of diallyl disulfide and dipropyl disulfide, organosulfur compounds found mostly in garlic and onion, in a meat model system inhibited the formation of IQx, MeIQx, and PhIP in the range of 20 to 84 % compared to the control. In a meat system, Balogh and others (2000) reported that the reduction of the levels of IQ, MeIQ, MeIQx, DiMeIQx and PhIP in fried beef patties was achieved when vitamin E at the 1% level (fat basis)



was added to the patties before frying. Lan and others (2004) also reported that the addition of  $\alpha$ -tocopherol at a concentration of 0.2% to ground pork (cooked at 98 °C for 32 hours in a closed pan in the presence of water, soy sauce, and sugar) produced a reduction of HCAs of up to 30% compared to the control. The addition of naringenin, a flavonoid found in many citrus fruits, at the level of 0.1% to ground beef before frying reduced the amounts of MeIQx, DiMeIQx, and PhIP found after cooking in the range of 63-74% (Cheng and others 2007).

Food extracts, especially from fruit and vegetables, have been reported to inhibit HCA formation in cooked meat products. Using tomato extracts contained carotenoids (lycopene,  $\beta$ -carotene, phytoene, and phytofluene) at a concentration of 1,000 ppm inhibited the formation of MeIQx by 12% and DiMeIQx by 5% in a meat juice model system (Vitaglione and others 2002). Rosemary extracts were found to effectively decrease the formation of HCAs in beef patties by up to 69% (Tsen and others 2006). Four fruit extracts (apple, elderberry, grape seed, and pineapple) rich in polyphenols were found to significantly reduce the amount of MeIQx, DiMeIQx, and PhIP formed in fried beef patties in a range from 15 to 71% (Cheng and others 2007).

The use of antioxidants in the form of whole food to inhibit HCA formation has also been studied. Britt and others (1998) reported that the formation of the HCAs was reduced by 79% by the addition of tart cherry tissue at an 11.5% level. Frying beef patties in virgin olive oil, which naturally contains strong antioxidant polyphenols named secoiridoids (approximately 1,200 mg/kg oil), reduced the formation of MeIQx and PhIP up to 40% compared to the use of refined olive oil (Persson and others 2003). Many herbs and spices, usually used to flavor dishes, are an excellent source of phenolic compounds, which have been reported to show high antioxidant activity. The application of dried rosemary, thyme, sage, and garlic on the surface of ground beef patties before

cooking significantly reduced HCA levels to below 60% of the amount found in control (Murkovic and others 1998).

## **ANALYSIS OF HETEROCYCLIC AMINES IN FOODS**

The analysis of HCAs in foods is a challenge for the scientists due to their being present at low levels (0.1-50 ng/g) in a complex sample matrix; the challenge has been to develop an effective purification method and a sensitive and selective analytical method.

### **Extraction and clean-up**

The most common extraction and clean-up method was developed by Gröss and Grüter (1992) and became widely accepted. In this method, the sample is first saponified with sodium hydroxide and mixed with inert solid e.g. diatomaceous earth to avoid formation of a very stable emulsion during the extraction of the saponified liquid (Murkovic 2007), and macromolecules, e.g. proteins and carbohydrates, remain adsorbed on the inert material (Melo and others 2008). Then the subsequent extraction and two-step solid-phase extraction are performed. The solvents used in the extraction step are ethyl acetate, dichloromethane, or diethylether (Alaejos and others 2008). Of these solvents, ethyl acetate has been shown to slightly improve the recoveries of some HCAs in meat samples (Alaejos and others 2008). The two-step solid phase extraction is conducted by means of a strong cation exchanger (e.g. propylsulfonyl silica gel, PRS) and an octadecylsilane (C18) (Meester 1998, Toriobio and others 2000a). In the normal-phase elution mode of PRS, HCA molecules are retained through non-specific adsorption on the polar sodium propylsulfonate residues as well as on the residual silanol groups. After that, the normal-phase

elution is switched to a reversed-phase elution by rinsing PRS with hydrochloric acid to allow interaction with the ion-exchange sites on PRS (Gross 1990). After that, the interferences are removed by using a large volume of methanol-hydrochloric acid at a high flow rate while HCA molecules remain attached in the PRS cartridge. This clean-up step is a crucial step to rid interferences that may overlap with the HCA peaks (Gross 1990). After that, the HCA molecules in the PRS cartridge are eluted to C18 cartridge by using an alkaline aqueous buffer solution, e.g. ammonium acetate pH 8.0, to concentrate the HCAs and remove the buffer salt (Gross 1990). The HCA molecules are then eluted from C18 cartridge by a MeOH-ammonium hydroxide solution (Gross 1990). This method produces recoveries of HCAs are in range of 50-80% (Gross 1990, Toribio and others 1999). Instead of two-step solid phase extraction, a single step solid phase extraction has also been developed by using an Oasis MCX LP SPE cartridge whose active surface is a copolymer of N-divinylbenzene and N-vinylpyrrolidone with sulfonic acid substituents on the phenyl groups (Turesky and others 2005).

A solid-phase microextraction (SPME) was also developed by Cárdenes and others (2004) to extract HCAs from the food matrix. Carbowax-templated resin fiber was recommended due to its high efficiency. The fiber was directly immersed in the meat extract to extract HCAs; then the HCAs were eluted from the fiber with methanol and separated on HPLC column coupled with fluorescence and UV diode array detections. This method produced recoveries of HCAs ranging between 57.4 and 82.4%. This method also allows to the clean-up steps to be simplified and the amounts of time and organic solvents needed to be reduced. Other methods that have been used for the extraction and purification of HCAs are extraction with blue cotton, blue rayon, and blue chitin (Bang and others 2002; Skog 2004) and immunoaffinity purification (Vanderlaan and

others 1993).

### **Identification and quantitation**

The identification and quantitation of HCAs has been commonly carried out with chromatographic or related techniques in different detection systems. Frequently, high pressure liquid chromatography (HPLC) is used for the determination of HCAs because they consist of the highly polar, non-volatile, and thermal unstable compounds (Alaejos and others 2008). Each HCA compound is separated on the column with a base-inactivated reversed phase containing the aminoimidazo moiety (Murkovic 2007). The TSK Gel ODS-80TS column is most commonly used due to its ability to detect low limits, its high injection volume, short equilibration time (Murkovic 2007), peak symmetry, and separation efficiency (Melo and others 2008). A better separation has been achieved by using a ternary mobile phase composed of triethylamine at pH 3.2, triethylamine at pH 3.6, and acetonitrile. Less polar HCAs and PhIP are typically identified with fluorescence detection whose high sensitivity provides clean chromatograms (Ristic and others 2004). In contrast, the IQ- and IQx-type amines have no fluorescence characteristics, therefore they can be measured with ultraviolet (UV) detection. Although the sensitivity of UV detection is not high, around 100-400 times lower than that of fluorescence, it allows the confirmations of the chromatographic peaks of samples with the reference, which is a very crucial step in HCAs analysis at the low concentrations present in samples, and for this reason, the detection method most commonly used is diode array detection (DAD) recording at a wavelength of 264 and 273 nm. Enhanced selectivity and sensitivity of the detection of HPLC have been reported using the following: electrochemical detection (HPLC-ED) (Van Dyck and others 1995);

ion-exchange chromatography (Van Dyck and others 1995); tandem mass spectrometry (HPLC-ESI-MS-MS) (Richling and others 1997); atmospheric pressure chemical ionisation (HPLC-APCI) (Guy and others 2000, Toribio and others 2000b); mass spectrometry (LC-MS) (Santos and others 2004); electrospray (LC-ESP) (Barceló-Barrachina and others 2004b, Ni and others 2008); thermospray (LC-TSP); coulometric electrode array (Krach and Sontag 2000, Gerbl and others 2004); time-of-flight mass spectrometry (LC-TOF-MS) (Barceló-Barrachina and others 2004a); and electrophoresis, either with mass spectrometry (CE-MS), ultra-violet (CE-UV), or electrochemical (CE-ED) with high detection limits having been obtained (Puignou and others 1997).

Other methods of detection that have been used are gas chromatography (GC) coupled a with nitrogen-phosphorus selective detector (NPD), which has the advantage of a high sensitivity to HCAs because of the presence of the nitrogen atoms in the structure of the HCAs. Gas chromatography-mass spectrometry (GC-MS) has very high selectivity and a relatively high sensitivity of MS for HCAs (Casal and others 2004, Warzecha and others 2004). However, HCAs are polar and non-volatile compounds; therefore, a derivatization step is required. The derivatizing agents that have been tested for the analysis of HCAs are acetic, trifluoroacetic, pentafluoropropionic and heptafluorobutyric anhydrides; pentafluorobenzyl bromide; 3,5-bistrifluoromethylbenzyl bromide; and 3,5-bistrifluoromethylbenzoyl chloride (Casal and others 2004, Barceló-Barrachina and others 2005). Also, ELISA (enzyme-linked immunosorbent assay) methods have been proposed for HCAs analysis because of their high sensitivity, high selectivity, and low sample preparation requirements (Vanderlaan and others 1993).

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## PART II. HETEROCYCLIC AMINE CONTENT IN COMMERCIAL READY TO EAT MEAT PRODUCTS <sup>1</sup>

### ABSTRACT

Heterocyclic amines (HCAs) are produced in meats cooked at high temperature, which are potent mutagens and a risk factor for human cancers. The aim of this study was to estimate the amount of HCAs in some commonly consumed ready-to-eat (RTE) meat products. The RTE products were purchased from a local grocery store, and HCA were analyzed using an analytical method based on solid-phase extraction followed by HPLC. The primary HCAs in these samples were PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine) (not detected-7.9 ng/g) and MeIQx (2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline) (not detected-3.6 ng/g). Products ranked in order of increasing total HCA content: pepperoni (0.05 ng/g) < hot dogs and deli meat products (0.5 ng/g) < fully cooked bacon (1.1 ng/g) < rotisserie chicken meat (1.9 ng/g) < rotisserie chicken skin (16.3 ng/g). We believed that cooking conditions and ingredients influenced the level of HCAs in these RTE products and concluded that consumption of RTE meat products contributes very little to HCA intake. Results from this study can be used in risk assessment study to estimate human exposure to HCAs due to food consumption.

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<sup>1</sup>: Priyadarshini Gadgil, Kanithaporn Puangsombat, J. Scott Smith, Heterocyclic amines in selected ready to eat type meat products. Institute of Food Technologists Annual Meeting & Food Expo. Anaheim, CA. June 06-10, 2009. # 09-A-2704.

## INTRODUCTION

Ready-to-eat (RTE) products are defined in CFR Title 9 Part 430 (2005a) as “A meat or poultry that is in a form that is edible without additional preparation to achieve food safety and may receive additional preparation for palatability or aesthetic, epicurean, gastronomic, or culinary purposes. RTE product is not required to bear a safe-handling instruction (as required for non-RTE products by 9 CFR 317.2(I) and 381.125(b)) or other labeling that directs that the product must be cooked or otherwise treated for safety, and can include frozen and poultry products”. Demand for RTE meat products has increased over the years and these are widely consumed in modern society because of their convenience and variety.

Evaluating the risk of HCAs in terms of human cancer development requires determining exposure levels from specific amounts of HCAs in food and frequencies of exposure to HCAs from food (Sinha 2002). Most studies have concentrated on investigating the influence of different cooking conditions on HCA formation and finding strategies to limit HCA formation in cooked meat products. Few studies have reported the HCA content in foods from restaurants, fast-food outlets, and RTE meat products.

The objective of this study was to estimate the amount of HCAs in commonly consumed RTE meat products including hot dogs, deli meat products, fully-cooked bacon, pepperoni, and rotisserie chicken. Hot dogs are comminuted, semisolid sausages prepared from one or more kinds of raw skeletal muscle meat or raw skeletal muscle meat and raw or cooked poultry meat, and seasoned and cured, and they may or may not be smoked (CFR Title 9 part 319.180 2005b). Deli meat (cold cut) products are sliced, either in an official establishment or after distribution from an

official establishment, and typically assembled in a sandwich for consumption (CFR Title 9 part 430 2005a). Sliced deli meat is available in vacuum packs and also can be purchased at a deli counter. The most popular deli meats are deli turkey, deli ham and deli beef (Xiong and Mikel 2001). Thermal processing of hot dogs and deli meat is usually carried out by cooking at 74 to 80 °C to obtain an internal temperature of 70 to 72 °C (Fiener 2006). Fully cooked bacon was developed and introduced about 10 years ago (Xiong and Mikel 2001). This product is an alternative for consumers who want to prepare bacon without messy preparation and clean up. Pepperoni is a fermented sausage made from beef and pork or pork only. The final pH of pepperoni typically ranges from 4.8 to 5.2. Smoke is applied for 1 to 3 hr at 30 to 35 °C, and thermal treatment of pepperoni is typically conducted at 74 to 78 °C with steam or dry heat until an internal temperature of 70 °C is obtained (Xiong and Mikel 2001).

## **MATERIALS AND METHODS**

### **Materials**

Eight types of RTE meat products were purchased from a local grocery store: hot dogs (beef and beef-pork-turkey), deli meat (roast beef, ham and turkey), fully cooked bacon, pepperoni as removed from a frozen pizza, and rotisserie chicken (Table 8).

### **Chemicals**

The HCA standards IQ (2-amino-3-methyl-imidazo [4,5-*f*]quinoline), IQx (2-amino-3-methyl-imidazo [4,5-*f*]quinoxaline), MeIQ (2-amino-3,4-dimethyl-imidazo [4,5-*f*]quinoline), MeIQx (2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline), 4,8-DiMeIQx (2-amino-3,4,8-

Table 8. Description and ingredients in selected ready-to-eat meat products

<b>Sample</b>	<b>Description</b>	<b>Ingredients</b>
Hot dog beef	N/A	beef, water, corn syrup, contain 2% or less of salt, potassium lactate, partially hydrolyzed beef stock, sodium phosphate, flavorings, sodium diacetate, ascorbic acid, sodium nitrate, extractives of paprika
Hot dog turkey-pork-beef	N/A	turkey, pork, water, beef, corn syrup, contain 2% or less of salt, potassium lactate, sodium phosphate, flavorings, partially hydrolyzed beef stock, sodium diacetate, ascorbic acid, sodium nitrite, extractives of paprika
Deli roast beef	Top round roast beef coated with seasonings and caramel color added	beef, water, less than 2% sodium lactate, sodium phosphate, sodium diacetate, salt, hydrolyzed corn protein, flavorings
Deli ham	Ham with natural juices and naturally smoked	pork, water, salt, sugar, dextrose, sodium phosphate, honey, sodium erythorbate, sodium nitrite
Deli turkey	Turkey breast with broth browned with caramel color and oven roasted	turkey breast meat, turkey broth, 2% or less of dextrose, modified food starch, salt, sodium phosphate, acidified calcium sulfate
Pepperoni	Original	pork and beef, salt, water, dextrose, spices, lactic acid and starter culture, oleoresin of paprika, flavoring, sodium nitrite, BHA, BHT, citric acid
Fully cooked bacon	Fully cooked bacon with naturally smoked	pork, water, salt, sugar, sodium phosphate, sodium erythorbate, sodium nitrite
Rotisserie chicken	Slow-roasted, traditional style	N/A

N/A - not available.

trimethyl-imidazo [4,5-*f*]quinoxaline), TriMeIQx (2-amino-3,4,7,8-tetramethyl-imidazo [4,5-*f*]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine) were obtained from Toronto Research Chemicals (Toronto, Canada). Ammonium acetate and triethylamine were purchased from Aldrich Chemicals (Milwaukee, WI, USA). Phosphoric acid was obtained from Sigma Chemicals (St. Louis, MO, USA). Deionized water was processed by a Sybron/Branstead PCS unit (Barnstead/Thermolyne, Dubuque, IA, USA). The solid-phase extraction Extrelut NT 20 columns and diatomaceous earth refill material were purchased from VWR International (Bristol, CT, USA). Bond Elut propyl-sulfonic acid (PRS) cartridges, C-18 cartridges, and the coupling adaptors were purchased from Varian Sample Preparation (Harbor City, CA, USA).

Trichloroacetic acid, diacetyl, 1-naphthol, and sodium hydroxide were obtained from Sigma Aldrich (St. Louis, MO, USA). Solvents and chemicals such as acetonitrile (HPLC grade), methanol (HPLC grade), and sodium hydroxide (ACS-grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA).

### **Sample preparation methods**

Two hot dogs of each kind were heated in a microwave (1000 W) on high power according to package directions (35 s wrapped in a paper towel). The fully-cooked bacon was heated in a microwave (1000 W) on high for 30 and 60 s as per package directions. Pepperoni taken from the top of the frozen pizza was analyzed as unheated pepperoni. Oven-cooked pepperoni was taken from pizzas that had been cooked for approximately 23 min in an oven at 204 °C (400 °F). Microwave-cooked pepperoni was taken from pizzas cooked in a microwave (1000 W) on high for approximately 4 min per package directions. For the rotisserie chicken, skin

and meat were separated before analysis. Deli roast beef, deli ham, and deli turkey were used as obtained. All samples were chopped before analysis. Figure 11 shows the pictures of RTE meat products in this present study.

### **Chemical analyses**

The samples pH was measured according to method by Jang and others (2008). Five grams of chopped sample was added to 45 mL of distilled water and blended for 30 s at medium speed in a Waring blender (Waring Laboratory, Torrington, CT, USA). The pH of each sample was measured with an Accumet AP115 portable pH meter (Fisher, Pittsburgh, PA, USA).

Fat and moisture for each sample were determined by rapid microwave drying and nuclear magnetic resonance using the CEM Smart Trac system (CEM Corporation, Matthews, NC, USA). Crude protein was determined with a LECO FP-2000 protein analyzer (Leco Corp, St Joseph, MI, USA).

Creatine content was determined according to the method described by Polak and others (2009) with modifications. A 0.25-g finely ground sample was homogenized for 5 min at 9500 rpm (IKA, Ultra-Turrax T18) (Wilmington, NC., USA) in 100 mL trichoroacetic acid (30 g/L in distilled water), and then the samples were filtered through Whatman #4 filter paper. Twenty milliliters of the filtrate was defatted with 10 mL diethylether, and then samples were shaken vigorously and allowed to stand 10 min to separate the phases. After the phases were separated, 4 mL of defatted extract (bottom layer) was mixed with 2 mL of diacetyl (0.2 g/L in distilled water) and 2 mL of 1-naphthol (25 g/L in 20 g/L of sodium hydroxide solution).



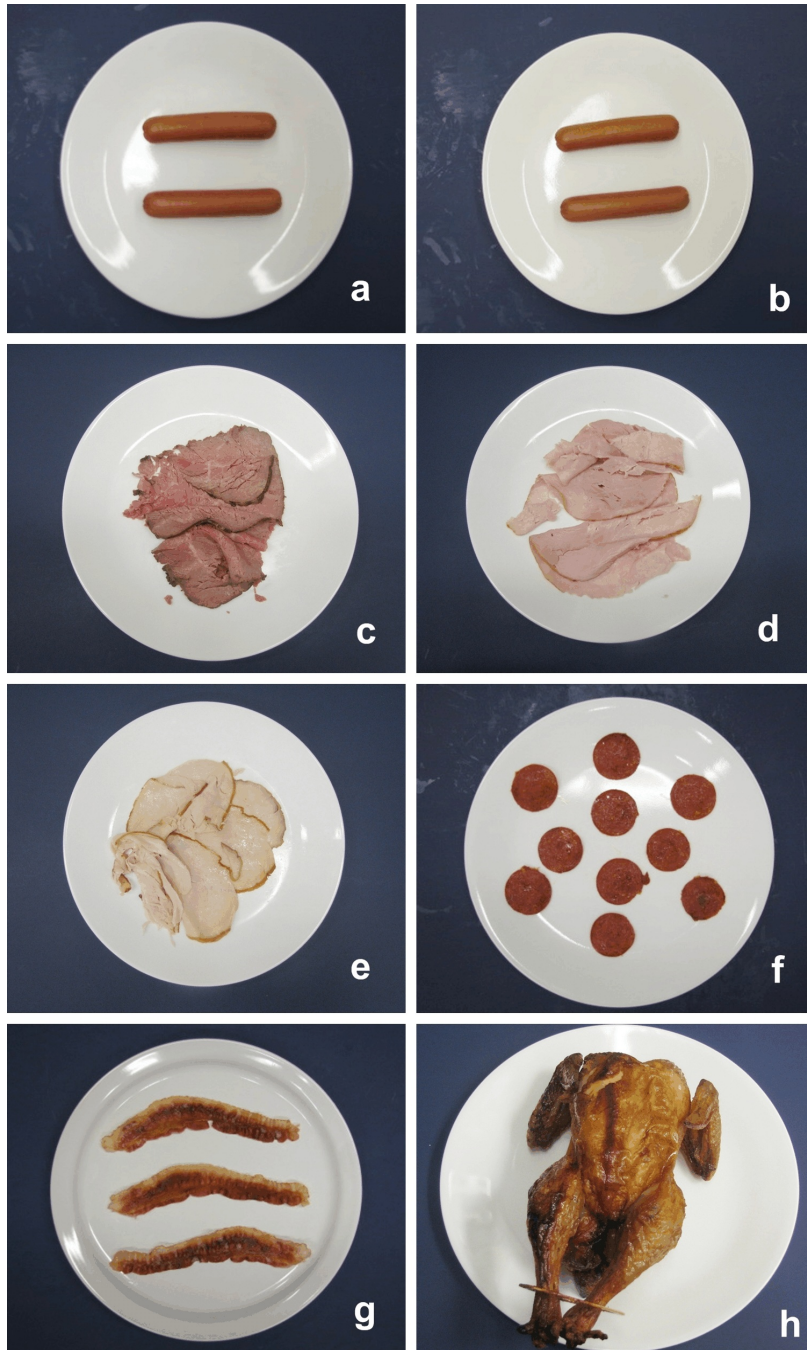


Figure 11. RTE meat products: hot dog beef heated in microwave for 35 s (a), hot dog turkey-pork-beef heated in microwave for 35 s (b), deli roast beef (c), deli ham (d), deli turkey (e), pepperoni heated in an oven for 23 min at 204 °C (f), and bacon heated in microwave for 30 s (g), and rotisserie chicken (h).

The mixture heated for 5 min at 40 °C. Each sample's absorbance was measured at 520 nm against a reagent blank. The creatine content was expressed as milligram per gram of meat sample.

### **Extraction and analysis of HCAs**

The HCAs were extracted and purified from meat using the method described by Gross and Grüter (1992) except that ethyl acetate was used as the extraction solvent (Santos and others 2004, Smith and others 2008). Each chopped RTE meat sample (3 g) was homogenized with 12 mL of 1 M NaOH in a commercial Waring blender (Fisher, Pittsburgh, PA, USA). The homogenate was then mixed with 24 g of Extrelut refill material (Merck, Darmstadt, Germany) and poured into an empty Extrelut 20 column. For determination of recovery, selected homogenate samples were spiked with 50 ng of each of the HCA standards. The HCAs were eluted from the Extrelut columns with 60 mL ethyl acetate into a PRS cartridge conditioned with 7 mL of ethyl acetate. The PRS cartridge was then rinsed with 6 mL of 0.1 M HCl, 15 mL of methanol/0.1 M HCl (45:55 v/v), and 2 mL of distilled water to wash out the nonpolar HCAs and other impurities. The HCAs were eluted from the PRS cartridge with 20 mL of 0.5 M ammonium acetate pH 8.5 into 100-mg C-18 cartridges preconditioned with 5 mL of methanol followed by 5 mL of distilled water. The HCAs were then eluted from the C-18 cartridge with 1 mL of methanol/ammonium hydroxide (9:1, v/v) into the vial. The HCA extract was concentrated until dry under a stream of nitrogen and dissolved in 25 µl of methanol before it was injected into the HPLC. The HCAs were analyzed on a HP1090A Series II HPLC (Agilent Technologies) coupled with a photodiode array UV-visible detector (HP 1040) and an HP 1046A programmable

fluorescence detector. The HCAs separation was performed on a reversed-phase TSK gel ODS-80 TM column (25 cm × 4.6 mm, 5 μm, 80 Å, Tosohass, Montgomeryville, PA, USA) with a mobile phase of 0.01 M triethylamine pH 3.6 (A) and acetonitrile (B). The HCAs separation was achieved using a linear gradient that started with 95% A, and 5% B, and changed to 75% A and 25% B in 30 min at a flow rate of 1 mL/min and a column temperature of 40 °C. After 30 min, the mobile phase returned to its original ratio (95% A, 5 % B) for 10 min to allow the column to reequilibrate before the next injection. The UV detector was set at 252 nm for IQ, IQx, MeIQ, MeIQx, and DimeIQx, and the fluorescence detector was programmed accordingly to the excitation/emission wavelengths of 229 and 437 for PhIP. Data were analyzed with an HP 9000 series 300 Chemstation. The identities of HCAs peaks were confirmed by comparing the retention times and the UV absorbance spectrum of each peak with library spectra acquired from standard solutions.

### **Quantitation, recovery, and spectral matching**

The HCA concentrations were quantitated by the internal standard method (Lindsay 1992). A known amount of TriMeIQx (used as internal standard) was added to samples before they were injected into the HPLC. The relative responses (R) of HCA standards were calculated using the following equation:

$$R = (C/A)/(C_s/A_s)$$

where

C	=	Concentration of HCA standards
A	=	Peak area for HCA standards
C <sub>s</sub>	=	Concentration of internal standard

$A_s$  = Peak area of internal standard

The HCA concentrations in samples were calculated using the following equation:

$C_u = A_u \times R \times (C_s' / A_s')$

where  $C_u$  = Concentration of HCAs in sample

$A_u$  = Peak area of HCAs in sample

$C_s'$  = Concentration of internal standard in sample

$A_s'$  = Peak area of internal standard in sample

The limits of detection (LOD) and limits of quantification (LOQ) for the HCAs were 0.15 ng/g and 0.45 ng/g for IQ, IQx, MeIQ, MeIQx, DiMeIQx and PhIP. The HCA identities were verified in the cooked meat extracts by online UV spectral matching to a spectral library made from pure standards. Match factors typically were observed at 95% or greater (Abdulkarim and Smith 1998). Average recoveries for the HCAs were 72% for IQx, 61% for IQ, 63% for MeIQ, 68% for MeIQx, 60% for DiMeIQx, and 65% for PhIP. The recoveries of MeIQx and PhIP are in agreement with previous reports from this laboratory (Tsen and others 2006, Smith and others 2008) and from Persson and others (2003) and Cheng and others (2007).

### **Statistical analyses**

The experimental design was a randomized complete block with repeated measurements, and each experiment was replicated three times. Duplicate measurements taken on the same experimental unit were averaged for statistical analysis. All statistical significance tests were analyzed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA, 2002). Data were examined by analysis of variance (ANOVA) followed by Tukey's multiple comparison test, and means were considered significant at  $p < 0.05$ .

## RESULTS AND DISCUSSION

The pH and composition of each RTE sample are shown in Table 9. The pH of most RTE samples ranged from 5.0 to 6.5; pepperoni, which is a fermented meat product had a low pH level (pH 4.78). Both types of hot dogs had a low amount of creatine (less than 1 mg/g). Creatine in the deli meat ranged from 1.9 to 2.3 mg/g, and pepperoni contained 1.37 mg/g creatine. Bacon had the highest amount of creatine at 3 mg/g. The creatine values of some RTE samples in our study agreed with values reported by Campo and others (1998) who showed creatine levels of 2.31 to 3.25 mg/g in ham and 0.81 to 2.74 mg/g in frankfurters. Deli meat products had high moisture levels (69 to 76 %) followed by hot dogs (47 to 50%). Pepperoni and bacon had low moisture levels (24.4% for pepperoni and 15.3% for bacon). Deli meat contained low levels of fat (less than 10 %), and hot dogs contained approximately 30% fat. Bacon and pepperoni had high fat contents (37.9 % for bacon and 44.5% for pepperoni). Protein content was lowest for hot dogs (10 %), intermediate for deli meat products (20 %), and highest for bacon (42.8 %).

There was considerable variation in moisture, protein, and fat levels among four replications of rotisserie chicken (Table 10). This was the reason that the standard deviations of moisture, fat and protein levels in chickens were very high, especially in the chicken skin. These four chickens were bought on different visits to the same store. Factors that may have affected the amount of heat absorbed into the chicken include weight of chicken, placement of chicken in the oven, cooking time and temperature, and the number of chickens in the oven during each cooking cycle. The chickens weighed between 850 and 1,040 g. Chicken in replication 1 had the lowest weight (850 g) and the most brown color and shrinkage among the four chickens (Figure 12). The lighter weight chicken tends to absorb heat more than a heavier weight chicken, leading to a

Table 9. pH and composition of ready-to-eat meat products

<b>Sample</b>	<b>pH</b>	<b>creatine (mg/g)</b>	<b>moisture (%)</b>	<b>fat (%)</b>	<b>protein (%)</b>
Hot dog beef	6.17 ± 0.03	0.75 ± 0.08	47.40 ± 0.44	30.78 ± 0.17	10.53 ± 0.17
Hot dog turkey-beef-pork	6.39 ± 0.11	0.57 ± 0.06	49.86 ± 0.71	28.54 ± 0.61	10.61 ± 0.15
Deli roast beef	5.47 ± 0.04	2.23 ± 0.13	69.41 ± 0.65	5.67 ± 1.09	21.33 ± 0.68
Deli ham	6.40 ± 0.04	2.02 ± 0.28	71.63 ± 1.69	4.24 ± 0.61	19.20 ± 1.41
Deli turkey	6.32 ± 0.02	1.95 ± 0.12	75.18 ± 0.48	1.74 ± 0.21	18.28 ± 1.92
Pepperoni (unheated)	4.78 ± 0.20	1.37 ± 0.10	24.40 ± 0.42	44.52 ± 1.17	21.15 ± 1.05
Bacon (unheated)	6.44 ± 0.73	3.00 ± 0.61	15.31 ± 0.82	37.86 ± 1.37	42.79 ± 1.69

Each value is expressed as mean ± standard deviation ( $n = 3$ ).

Table 10. The contents of moisture, fat, protein, and heterocyclic amines (IQ, IQx, MeIQx, DiMeIQx, PhIP and total) of rotisserie chicken meat and skin in each replication

replication		moisture (%)	fat (%)	protein (%)	HCAs (ng/g)					
					IQ	IQx	MeIQx	DiMeIQx	PhIP	Total
Meat	1	64.46	2.43	31.88	2.91	0.09	0.21	0.06	1.31	4.58
	2	67.49	2.39	29.43	0.01	0.25	0.08	nd	0.08	0.42
	3	70.89	2.78	26.03	0.04	0.41	0.23	nd	0.07	0.75
	4	70.00	2.80	27.45	0.03	0.22	0.15	nd	0.09	0.49
	mean	68.21	2.60	28.70	0.75	0.24	0.17	0.02	0.39	1.56
	SD	2.88	0.22	2.54	1.44	0.13	0.07	0.03	0.62	2.02
Skin	1	21.80	42.42	33.80	1.11	0.34	9.98	2.30	27.24	40.97
	2	30.37	40.20	25.37	0.05	0.49	1.56	0.63	2.33	5.06
	3	39.60	31.21	24.27	0.07	0.38	1.42	0.15	0.73	2.74
	4	38.20	38.49	23.59	0.04	0.34	1.50	0.36	1.25	3.53
	mean	32.49	38.08	26.76	0.32	0.39	3.62	0.86	7.89	13.07
	SD	8.20	4.85	4.75	0.53	0.07	4.24	0.98	12.92	18.63

nd = not detected

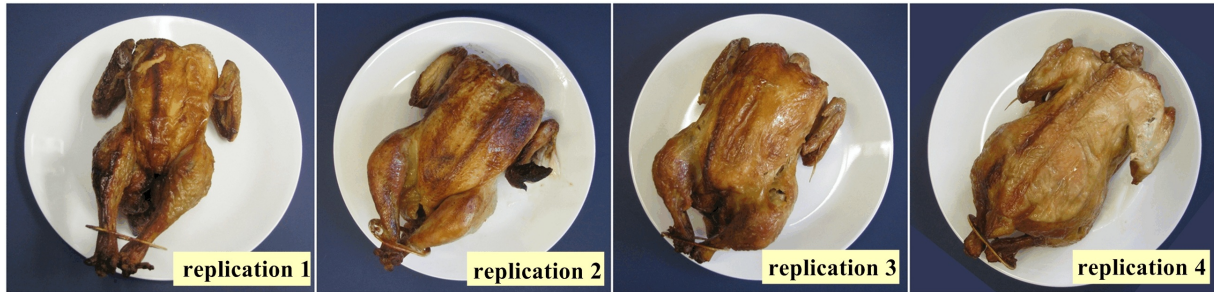


Figure 12. Physical appearances of rotisserie chicken for replication 1, 2, 3, and 4.

greater loss of moisture (Murkovic 2004). Chicken replication 1 had the lowest moisture content, followed by chicken replication 2, replication 3, and replication 4. Chicken replication 1 also had more fat and protein than the other three replications.

Table 11 summarizes the result of HCA quantitative determinations in the eight selected RTE products. The amount of HCAs was calculated as nanogram per gram (ng/g) of sample; reported value are the average of four sample determinations. Total contents of the five determine HCAs (IQ, IQx, MeIQx, DiMeIQx, and PhIP) of RTE products ranged from 0.05 to 13.07 ng/g. Hot dogs, deli meat products, and pepperoni generally had relatively low levels of total HCAs. Bacon and rotisserie chicken, especially the skin, had high HCA levels. The dominating HCAs in these RTE samples were PhIP (not detected to 7.89 ng/g) and MeIQx (not detected to 3.62 ng/g). The other HCAs were present at low concentrations: less than 0.8 ng/g for IQ, less than 0.4 ng/g for IQx, and less than 1.0 ng/g for DiMeIQx. IQx was found only in bacon and chicken, and DiMeIQx was found only in chicken. IQ and MeIQx were found in all RTE samples except pepperoni. PhIP was found in all RTE samples. Except for rotisserie chicken, the HCA levels of most RTE samples did not vary much between replications.



Table 11. Heterocyclic amine contents (IQ, IQx, MeIQx, DiMeIQx, PhIP, and total) of ready-to-eat meat products

Sample	HCAs (ng/g)					
	IQ	IQx	MeIQX	Di MeIQX	PhIP	Total
Hot dog beef	0.31 ± 0.09 <sup>a</sup>	nd	0.07 ± 0.02 <sup>b</sup>	nd	0.06 ± 0.01 <sup>b</sup>	0.44 ± 0.08 <sup>b</sup>
Hot dog turkey-beef-pork	0.28 ± 0.10 <sup>a</sup>	nd	0.07 ± 0.03 <sup>b</sup>	nd	0.07 ± 0.03 <sup>b</sup>	0.42 ± 0.10 <sup>b</sup>
Deli roast beef	0.20 ± 0.09 <sup>a</sup>	nd	0.08 ± 0.03 <sup>b</sup>	nd	0.15 ± 0.15 <sup>ab</sup>	0.44 ± 0.19 <sup>b</sup>
Deli ham	0.29 ± 0.13 <sup>a</sup>	nd	0.03 ± 0.01 <sup>b</sup>	nd	0.14 ± 0.08 <sup>ab</sup>	0.46 ± 0.06 <sup>b</sup>
Deli turkey	0.22 ± 0.10 <sup>a</sup>	nd	0.13 ± 0.07 <sup>b</sup>	nd	0.09 ± 0.01 <sup>ab</sup>	0.44 ± 0.11 <sup>b</sup>
Pepperoni	nd	nd	nd	nd	0.05 ± 0.01 <sup>b</sup>	0.05 ± 0.01 <sup>b</sup>
Bacon, microwaved 30 s.	0.60 ± 0.05 <sup>a</sup>	0.04 ± 0.03 <sup>a</sup>	0.14 ± 0.02 <sup>a</sup>	nd	0.14 ± 0.03 <sup>a</sup>	0.91 ± 0.06 <sup>a</sup>
Rotisserie chicken meat	0.75 ± 1.44 <sup>a</sup>	0.24 ± 0.13 <sup>a</sup>	0.17 ± 0.07 <sup>b</sup>	0.02 ± 0.03 <sup>b</sup>	0.39 ± 0.62 <sup>ab</sup>	1.56 ± 2.02 <sup>ab</sup>
Rotisserie chicken skin	0.32 ± 0.53 <sup>a</sup>	0.39 ± 0.07 <sup>a</sup>	3.62 ± 4.24 <sup>a</sup>	0.86 ± 0.98 <sup>a</sup>	7.89 ± 12.92 <sup>a</sup>	13.07 ± 18.63 <sup>a</sup>

Each value is represented as mean ± standard deviation ( $n = 4$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

nd = not detected

The measured HCAs in hot dog samples were IQ < 0.3 ng/g, MeIQx < 0.07 ng/g, and PhIP < 0.07 ng/g. There was no statistical difference ( $p < 0.05$ ) between beef and turkey-beef-pork hot dogs. Sinha and others (1998) reported no MeIQx and PhIP in pan-fried, oven-broiled, grilled/barbecued, and boiled hot dog samples. For the deli meat products, the detected HCAs were present at low levels (IQ < 0.3 ng/g, MeIQx < 0.13 ng/g, and PhIP < 0.15 ng/g) (Table 11). Abdulkarim and Smith (1998) reported no detectable MeIQx and PhIP in precooked meat products including ham and bologna. In the present study, IQ was the HCA found at the highest levels in both hot dogs and deli meat products, but previous studies did not report IQ in hot dogs and deli meat products (Abdulkarim and Smith 1998, Sinha and others 1998). It is possible that the IQ detected in hot dogs and deli meat products were a result of smoking process. Kataoka and others (1998) tested nine HCAs and showed that IQ was detected most in combustion smoked of wood chips, black pepper, and semi-dried fish, whereas MeIQx and DiMeIQx were not detectable.

Overall, hot dogs and deli meat products had low levels of HCAs. This may be due to the low-temperature manufacturing process of these products, which require heating at a temperature of 74 to 80 °C (Fiener 2006). The low HCA amounts found in hot dogs and deli meat products also could be due to the presence of ingredients and additives that inhibit HCA formations during the cooking process. Most of these RTE products contain salt and sodium phosphate (Table 8), which are believed to confer better water-holding capacity which reduces the transport of HCA precursors toward the surface during cooking and results in less HCA formation (Persson and others 2003). Sodium chloride and sodium phosphate are often added to meat products to improve water-holding capacity and sensory quality. These RTE meat products also contained modified starch that has been reported to improve water-holding capacity of meat products by providing

starch-water systems as a polymer matrix during gelatinization when meat products are cooked (Aktas and others 2006). However, a decrease of HCA formation by adding modified starch to the meat products has not yet been reported. Ascorbic acid and sodium nitrite, which are ingredients in hot dogs, may have a role in HCA inhibition because of their antioxidant properties. Murkovic and others (1998) reported that sodium nitrite, which is used as a curing agent in cured meat products, showed inhibited MeIQx by 15%, IQ by 14%, MeIQ by 51%, and PhIP by 34% in fried beef patties. Ascorbic acid is known to exert antioxidant or prooxidant effects depending on the concentration, therefore it may decrease or increase HCA formation. Lan and others (2004) reported that although incorporation of ascorbic acid inhibited HCA in marinated food, the effect was very minor. Johansson and Jägerstad (1996) found that a high concentration of ascorbic acid (1000 ppm) reduced MeIQx formation by 84%, but low concentrations (10 and 100 ppm) had no effect.

Among the RTE products in this study, only chicken skin and breast meat contained all five HCAs. PhIP in chicken skin was very high, especially in replication 1 (27.27 ng/g total HCAs) (Table 10). This agrees with results of Liao and others (2010) who found that PhIP can form easily in cooked chicken. As mentioned previously, the skin of chicken replication 1 had more fat and protein and less moisture than other replications. Consequently, the total amount of HCAs in the skin of chicken replication 1 (41 ng/g) was much higher than that in the other replications (2 to 5 ng/g); PhIP and MeIQx were most abundant (Table 10). This result agrees with other research that showed an increase in HCA levels as moisture content decreased (Murkovic 2004). The amount of HCAs in rotisserie chickens in the present study are in the same range as amounts reported by Knize and others (1998), who showed 0.45 ng/g of MeIQx and 0.75

ng/g of PhIP in white chicken meat and 0.40 ng/g of MeIQx and 0.59 ng/g of PhIP in dark chicken meat; however, they did not report HCAs in rotisserie chicken skin. In this study, total HCAs were 1.56 ng/g in rotisserie chicken meat and 13.08 ng/g in the skin. This findings indicate that HCA exposure can be reduced by not eating chicken skin.

Frozen pepperoni pizzas were used in this study to investigate the effect of cooking method on HCA formation in pepperoni. The HCAs in uncooked, microwave-cooked, and oven-cooked pepperoni are shown in Table 12. Cooking loss was higher for microwave-cooked pepperoni (39.1%) than for oven-cooked pepperoni (37.4%). All three pepperoni sample types had very low levels of total HCAs, and there were no statistically significant differences in HCAs among types. PhIP was the only HCA found in the pepperoni samples. The pepperoni contained spices, oleoresin of paprika, sodium nitrite, lactic acid starter culture, BHA, and BHT, which can act as antioxidants (Johansson and Jägerstad 1994, Shin and others 2002, Perucka and Materska 2003, Lan and others 2004, Fiener 2006, Gibis 2007, Janoszka 2010). This may explain why the pepperoni had low levels of HCAs (0.02 to 0.05 ng/g) relative to the other products. Some of these components, alone or in combination, may inhibit HCA formation. Common spices used for pepperoni are cayenne, anise seeds, garlic, mustard seeds, and black peppers (Fiener 2006). Garlic (fresh, dried powder, and extract) contains organosulfur compounds such as diallyl disulfide, dipropyl disulfide, cysteine, and cystine which have been reported to have inhibitory effects on HCA formation in meat model systems and meat products (Shin and others 2002, Gibis 2007, Janoszka, 2010). Paprika, which is commonly added to give a touch of red color to the pepperoni, is a spice made from ground, dried fruits of *Capsicum annuum* (e.g., bell peppers and chilli peppers) (Feiner 2006). Paprika has been reported to have an antioxidant property that is due to

the presence of total phenolics and carotenoids (e.g.,  $\beta$ -carotene and  $\beta$ -cryptoxanthine) (Perucka and Materska 2003). BHA and BHT are synthetic antioxidants and also have been reported to have an inhibitory effect on HCA formation in meat model systems and meat products (Johansson and Jägerstad, 1996, Lan and others 2004).

The package directions recommended heating the fully-cooked bacon for 30 s in the microwave, but some consumers may heat the product for a longer time to produce crispier bacon. Therefore, we investigated the effect of heating time on HCA formation. The HCAs in unheated bacon, and bacon heated for 30 and 60 s are shown in Table 13. As expected, the cooking loss of bacon heated for 60 s (24.2%) was higher than that of bacon heated for 30 s (20.9%), and the bacon heated for 60 s was crispier and darker than the bacon heated for 30 s. The physical appearance of unheated bacon and bacon heated for 30 and 60 s is shown in Figure 16. Bacon heated for 30 or 60 s had significantly higher levels of IQ, MeIQx, and PhIP than unheated bacon. The increased amount of HCAs after microwave heating may be due to the loss of water during heating, which could lead to more concentrated HCAs or formation of more HCAs. The amount of HCAs in bacon heated for 30 s was not statistically different from that in bacon heated for 60 s. Therefore, we conclude that microwave heating time of precooked bacon did not affect the amount of HCAs in heated bacon. It was interesting that the total amount of HCAs in fully cooked bacon in our study was less than that in cooked fresh bacon in other studies. We believed that the low amount of HCAs in fully cooked bacon is due to the precooking process. Industrial fully cooked bacon is cooked at low temperature (162 °C) in presence of steam induced high humidity either by using a continuous microwave oven or a continuous linear circulating air oven. Sinha and others (1998) detected 1.5 ng/g of MeIQx and 3.1 ng/g of PhIP in microwave-cooked fresh

Table 12. Heterocyclic amines (IQ, IQx, MeIQx, DiMeIQx, PhIP, and total) in unheated pepperoni, oven-cooked pepperoni, and microwave-cooked pepperoni

Sample	Cooking loss (%)	HCAs (ng/g)					
		IQ	IQx	MeIQX	Di MeIQX	PhIP	Total
Unheated pepperoni		nd	nd	nd	nd	0.03 ± 0.02 <sup>a</sup>	0.03 ± 0.02 <sup>a</sup>
Oven-cooked pepperoni	37.41 ± 2.63	nd	nd	nd	nd	0.05 ± 0.01 <sup>a</sup>	0.05 ± 0.01 <sup>a</sup>
Microwave-cooked pepperoni	39.06 ± 1.86	nd	nd	nd	nd	0.01 ± 0.01 <sup>a</sup>	0.01 ± 0.01 <sup>a</sup>

Each value is represented as mean ± standard deviation ( $n = 4$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

nd = not detected

Table 13. Cooking loss and heterocyclic amines (IQ, IQx, MeIQx, DiMeIQx, PhIP, and total) in fully cooked bacon heated for 0 (unheated), 30 and 60 s

Sample	Cooking loss (%)	HCAs (ng/g)					
		IQ	IQx	MeIQX	Di MeIQX	PhIP	Total
Unheated bacon		0.33 ± 0.07 <sup>b</sup>	0.00 ± 0.00 <sup>a</sup>	0.09 ± 0.06 <sup>b</sup>	nd	0.10 ± 0.02 <sup>b</sup>	0.53 ± 0.11 <sup>b</sup>
Bacon heated for 30 s	20.91 ± 0.68	0.60 ± 0.05 <sup>a</sup>	0.04 ± 0.03 <sup>a</sup>	0.14 ± 0.02 <sup>a</sup>	nd	0.14 ± 0.03 <sup>a</sup>	0.91 ± 0.06 <sup>a</sup>
Bacon heated for 60 s	24.23 ± 0.32	0.52 ± 0.03 <sup>a</sup>	0.03 ± 0.02 <sup>a</sup>	0.36 ± 0.15 <sup>a</sup>	nd	0.18 ± 0.00 <sup>a</sup>	1.10 ± 0.14 <sup>a</sup>

Each value is represented as mean ± standard deviation ( $n = 4$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

nd = not detected

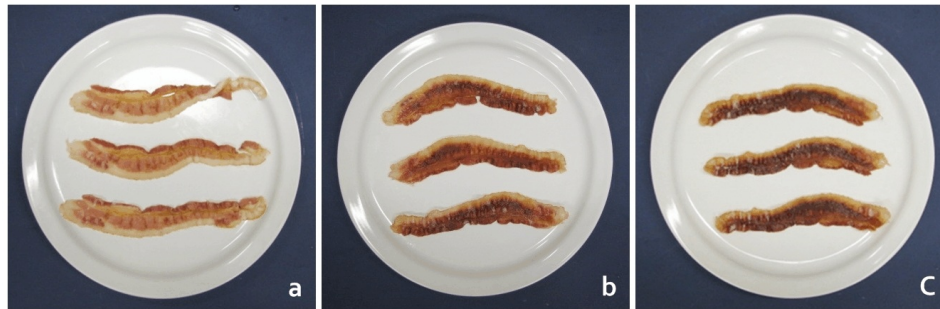


Figure 13. Physical appearances of fully cooked bacon heated for 0 (unheated) (a), 30 (b), and 60 s (c).

bacon and 4.3 ng/g of MeIQx and 4.8 ng/g of PhIP in pan-fried fresh bacon. Johansson and Jägerstad (1994) detected 10.2 ng/g of total HCAs (3.8 ng/g IQ, 2.8 mg/g MeIQx, 3.4 ng/g DiMeIQx, and 0.2 ng/g PhIP) in bacon fried at 150 °C for 2.5 min per side and 16.7 ng/g of total HCAs (10.5 ng/g IQ, 1.7 ng/g MeIQ, 2.5 ng/g MeIQx, and 1 ng/g DiMeIQx and PhIP) in bacon fried at 150 °C for 5 min per side.

Overall, the amounts of HCAs in RTE products in our study were much lower than those in cooked meat products in other studies. Other studies have shown the concentrations up to 35 ng/g in cooked beef, 330 ng/g in cooked poultry, and 15 ng/g in cooked pork and fish (Busquets and others 2004, Keating and Bogen 2004, Murkovic 2004, Iwasaki and others 2010).

## **CONCLUSIONS**

Our results indicate that HCA levels in RTE meat products are generally low, but some items (e.g. rotisserie chicken) may contain elevated amounts of HCAs. Thus, we conclude that consumption of RTE meat products contributes very little to HCA intake. Taken together, our results show that cooking conditions and ingredients influence HCA levels in RTE meat products. These results can be used along with dietary assessments to estimate HCA exposure due to consumption of RTE meat products.



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## PART III. OCCURRENCE OF HETEROCYCLIC AMINES IN COOKED MEAT PRODUCTS <sup>1</sup>

### ABSTRACT

Heterocyclic amines (HCAs), which are potent mutagens and a risk factor for human cancers, are produced in meats cooked at high temperature. The aim of this study was to determine the HCA content in cooked meat products (beef, chicken, pork, fish) prepared by various cooking methods (pan frying, oven broiling, and oven baking at 170 to 230 °C) that are preferred by U.S. meat consumers. HCAs were analyzed using an analytical method based on solid-phase extraction followed by HPLC. The primary HCAs in these samples were PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine) (1.49-10.89 ng/g), MeIQx (2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline) (not detected-4.0 ng/g), and DiMeIQx (2-amino-3,4,8-trimethyl-imidazo [4,5-*f*]quinoxaline) (not detected-3.57 ng/g). High levels of total HCAs were found in fried pork (13.91 ng/g), fried fish (14.91 ng/g), and fried bacon (17.91 ng/g). These data can be along used with food consumption survey data to estimate human exposure to HCAs due to meat consumption.

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<sup>1</sup> Kanithaporn Puangsombat, Terry A. Houser, Melvin C. Hunt, J. Scott Smith. Occurrence of heterocyclic amines in fresh meat products. Institute of Food Technologists Annual Meeting & Food Expo. Chicago, IL, CA. July 17-21, 2010 # 289-03.

## INTRODUCTION

The major HCAs formed in cooked meat and fish are PhIP, MeIQx and DiMeIQx (2-amino-3,4,8-trimethyl-imidazo [4,5-*f*]quinoxaline) (Pais and others 1999, Janoszka and others 2009). The concentration and type of HCAs formed in thermally treated meat and fish depend on many factors including cooking method, cooking time and temperature, the concentration of precursors, and presence of water and fat in the raw product (Janoszka and others 2009). The amount of HCAs formed increases with increasing temperature and time (Knize and others 1994). High cooking loss is related to the formation of large amounts of HCAs (Knize and others 1994, Skog and others 1995), and the amount of cooking loss during cooking depends on several factors including the muscle tension and direction of muscle fibers (Pais and others 1999). Many cooking methods, including frying, roasting, smoking, broiling, and baking have been reported to induce HCA formation, and the type HCAs formed can be different for various cooking methods (Chen and Chiu 1998). For example, IQ, MeIQx, PhIP were detected in broiled beef, whereas MeIQx and DiMeIQx were detected in fried ground beef (Starvic 1994).

The studies on HCA levels in cooked meat products have yielded inconsistent results, and there are gaps in the available HCA data. It is difficult to directly compare results between studies because of the differences in food items, cooking procedures, and food preparation. In some previous studies, samples were cooked at high temperature or for a long time; these cooking conditions exceed those needed to produce acceptable cooked meat products (Murkovic and others 1997, Pais and others 1999). Reports from some previous studies did not include the information on internal temperature of the cooked samples (Murkovic and others 1997, Oz and others 2007, Jo and others 2008, Janoszka and others 2009). Internal temperature is usually used to evaluate the safety of cooked meat products. Collecting this type of data would allow

researchers to better monitor HCA levels in meat products cooked under normal household conditions and develop more accurate estimates of human HCA exposure. The main objective of the study was to determine HCA contents of the major categories of cooked meat products prepared with various cooking methods that are preferred by U.S. meat consumers. These data can be combined with food consumption survey data to estimate exposure to HCAs due to meat consumption.

## **MATERIALS AND METHODS**

### **Chemicals**

The HCA standards IQ (2-amino-3-methyl-imidazo [4,5-*f*]quinoline), IQx (2-amino-3-methyl-imidazo [4,5-*f*]quinoxaline), MeIQ (2-amino-3,4-dimethyl-imidazo [4,5-*f*]quinoline), MeIQx (2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline), 4,8-DiMeIQx (2-amino-3,4,8-trimethyl-imidazo [4,5-*f*]quinoxaline), TriMeIQx (2-amino-3,4,7,8-tetramethyl-imidazo [4,5-*f*]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine) were obtained from Toronto Research Chemicals (Toronto, Canada). Ammonium acetate and triethylamine were purchased from Aldrich Chemicals (Milwaukee, WI, USA). Phosphoric acid was obtained from Sigma Chemicals (St. Louis, MO, USA). Deionized water was processed by a Sybron/Branstead PCS unit (Barnstead/Thermolyne, Dubuque, IA, USA). The solid-phase extraction Extrelut NT 20 columns and diatomaceous earth refill material were purchased from VWR International (Bristol, CT, USA). Bond Elut propyl-sulfonic acid (PRS) cartridges, C-18 cartridges, and coupling adaptors were purchased from Varian Sample Preparation (Harbor City, CA, USA). Trichloroacetic acid, diacetyl, 1-naphthol, and NaOH were obtained from Sigma Aldrich (St. Louis, MO, USA). Solvents and chemicals such as acetonitrile (HPLC grade), methanol (HPLC grade),

and sodium hydroxide (ACS-grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA).

### **Fresh meat samples**

The following fresh meat samples were purchased from local grocery stores: consisting of beef (top loin, round tip, and ground beef), pork (top loin, ground pork, and bacon), chicken (breast without skin, breast with skin, thigh without skin, and thigh with skin), and fish (catfish, salmon, and tilapia).

### **Chemical analyses**

The pH was measured according to the method of Jang and others (2008). Five grams of fine ground sample was added to 45 mL of distilled water and blended for 30 s at medium speed in a Waring blender (Waring Laboratory, Torrington, CT, USA). The pH of each sample was measured with an Accumet AP115 portable pH meter (Fisher, Pittsburgh, PA, USA).

Fat and moisture for each sample were determined by rapid microwave drying and nuclear magnetic resonance using the CEM Smart Trac system (CEM Corporation, Matthews, NC, USA). Crude protein was determined with a LECO FP-2000 protein analyzer (Leco Corp, St Joseph, MI, USA).

Creatine content was determined according to the method described by Polak and others (2009) with modifications. A 0.25-g finely ground sample was homogenized for 5 min at 9500 rpm (IKA, Ultra-Turrax T18, Wilmington, NC, USA) in 100 mL trichoroacetic acid (30 g/L in distilled water), and then the samples were filtered through Whatman #4 filter paper. Twenty milliliters of the filtrate was defatted with 10 mL diethylether, and then samples were shaken

vigorously and allowed to stand for 10 min to separate the phases. After the phases were separated, 4 mL of defatted extract (bottom layer) was mixed with 2 mL of diacetyl (0.2 g/L in distilled water) and 2 mL of 1-naphthol (25 g/L in 20 g/L of sodium hydroxide solution). The mixture heated for 5 min at 40 °C. Each sample's absorbance was measured at 520 nm against a reagent blank. The creatine content was expressed as milligrams per gram of meat sample. The chemical analyses of raw meat samples are summarized in Table 14.

### **Cooking procedure and cooking loss**

Fresh meat products were removed from refrigerator and allowed to approach room temperature before they were cooked (Infrared thermometer model ST20XE, Raytek, Santa Cruz, CA, USA). Spear-point thermocouple temperature probes were inserted horizontally to the midpoint of samples, and temperature was monitored with a data logger (USB-TC model, Measurement Computing, Norton, MA, USA). Each meat sample was cooked as described in Table 15. All meats were cooked one sample at a time, except for fried bacon, which was cooked three slices at a time. No salt, spice, food additive, or oil were used in the cooking procedures. Cooked samples were allowed to cool at room temperature for approximately 30 min, and then cooking loss was determined using the following equation:

$$\% \text{ cooking loss} = [(\text{before cook weight} - \text{after cook weight}) / \text{before cook weight}] \times 100$$



Table 14. Chemical analyses of pH, moisture, fat, protein, and creatine in uncooked meat samples

<b>Sample</b>		<b>pH</b>	<b>Moisture (%)</b>	<b>Fat (%)</b>	<b>Protein (%)</b>	<b>Creatine (mg/g)</b>
Beef	Top loin	5.62 ± 0.05	69.32 ± 0.83	7.25 ± 0.01	21.29 ± 0.18	2.93 ± 0.06
	Round tip	5.47 ± 0.04	71.21 ± 1.22	4.61 ± 2.27	22.50 ± 0.64	2.95 ± 0.23
	Ground beef	5.89 ± 0.04	69.79 ± 0.79	9.22 ± 1.46	19.66 ± 0.54	2.53 ± 0.09
Pork	Top loin	6.01 ± 0.36	75.07 ± 0.45	7.73 ± 0.32	20.90 ± 1.01	1.88 ± 0.69
	Ground pork	6.23 ± 0.08	60.12 ± 1.15	21.42 ± 0.59	15.48 ± 0.10	1.79 ± 0.14
	Bacon	6.71 ± 0.05	37.74 ± 2.14	47.99 ± 0.16	11.83 ± 1.15	1.23 ± 0.33
Chicken	Breast meat	6.19 ± 0.16	74.63 ± 0.62	4.87 ± 0.71	23.37 ± 0.25	2.21 ± 0.17
	Breast skin	6.35 ± 0.07	37.06 ± 3.56	53.67 ± 4.36	11.04 ± 1.56	1.02 ± 0.16
	Thigh meat	6.70 ± 0.10	74.44 ± 1.47	4.58 ± 0.64	19.85 ± 0.54	2.51 ± 0.07
	Thigh skin	6.64 ± 0.04	37.36 ± 2.47	52.98 ± 3.80	9.04 ± 1.29	1.18 ± 0.22
Fish	Catfish	6.94 ± 0.11	77.98 ± 0.39	4.99 ± 0.13	15.49 ± 0.13	2.81 ± 0.15
	Salmon	6.80 ± 0.05	78.66 ± 2.91	1.12 ± 1.11	18.21 ± 2.71	2.66 ± 0.23
	Tilapia	7.91 ± 0.14	82.03 ± 2.64	1.07 ± 1.02	15.72 ± 1.01	1.80 ± 0.12

Each value is represented as mean ± standard deviation ( $n = 3$ ).

Table 15. Cooking description

<b>Cooking</b>	<b>Food item</b>	<b>Description</b>
<b>Method</b>		
Frying	Beef, pork, chicken, fish	Meat was fried in a Teflon-coated frying pan without adding oil at a surface temperature of 204 °C. Meat was fried, turned once, and removed from the pan when the desired temperature was reached.
Broiling	Beef	Oven was preheated to 232 °C (monitored with oven thermometer). The meat was placed on a broiler pan to keep the broiled beef out of the drippings. The meat was removed when a final internal temperature was achieved.
Baking	Beef, Pork, Fish	Oven was preheated to 177 °C (monitored with oven thermometer). The meat was placed on a baking pan. The meat was removed when a final internal temperature was achieved.

The cooking information for each type of meat consists of the data for uncooked meat (weight of raw meat, thickness of raw meat), and cooking data (desired cooking internal temperature, cooking temperature, surface cooking time, and cooking loss); this information is summarized in Table 16. After samples were cooled at room temperature, they were refrigerated overnight. For the samples of chicken with skin, the skin was removed from the muscle. Approximately 2 mm of the surface was removed from chilled meats with a commercial-grade meat slicer (Cebela’s commercial grade slicer, 1/3 hp, Sidney, NE, USA). The meat surface was ground and homogenized with a food processor (KitchenAid, model KFP 750), and refrigerated at 4 °C, and extraction of HCAs in meat samples was performed on the next day.

Table 16. Cooking conditions and cooking loss in cooked meat samples

Meat type	Types/cuts of meat	Type of cooking	Raw meat (g)	Thickness, raw meat (cm)	Desired internal temperature (°C)	Surface cooking temperature (°C)	Cooking time (min per side)	Cooking loss (%)
Beef	Top loin	Frying (medium rare)	350-400	3.8	57	204	6	17.50 ± 1.84
	Top loin	Frying (well done)	350-400	3.8	71	204	12	31.86 ± 1.66
	Top loin	Broiling (medium rare)	350-400	3.8	57	232	5	23.57 ± 1.66
	Top Loin	Broiling (well done)	350-400	3.8	71	232	10	33.81 ± 2.19
	Round tip	Baking (well done)	650-680	9.0	71	177	80 (total)	30.75 ± 3.75
	Ground beef	Frying	140-160	2.3	71	204	6	35.30 ± 2.35
Pork	Top loin	Frying	230-250	2.3	71	204	8	26.12 ± 1.70
	Top loin	Baking	650-680	9.0	71	177	70 (total)	26.48 ± 2.24
	Ground pork	Frying	130-135	2.3	71	204	6	22.20 ± 1.93
	Bacon	Frying	18-25	0.3	-	172	3	71.94 ± 1.26

Table 16. Continued

<b>Meat type</b>	<b>Types/cuts of meat</b>	<b>Type of cooking</b>	<b>Raw meat (g)</b>	<b>Thickness, raw meat (cm)</b>	<b>Desired internal temperature (°C)</b>	<b>Surface cooking temperature (°C)</b>	<b>Cooking time (min per side)</b>	<b>Cooking loss (%)</b>
Chicken	Breast without skin	Frying	250-280	2.5	74	204	10	27.88 ± 1.26
	Breast with skin	Frying	280-310	2.5	74	204	10	24.39 ± 4.71
	Thigh without skin (bone-in)	Frying	140-180	2.5	74	204	7	24.96 ± 3.09
	Thigh with skin (bone-in)	Frying	150-200	2.5	74	204	7	26.74 ± 2.99
Fish	Catfish	Frying	170-190	1.8	63	204	6	27.28 ± 2.46
	Salmon	Frying	180-200	1.8	63	204	6	21.60 ± 2.39
	Tilapia	Frying	140-160	1.5	63	204	6	23.65 ± 1.84
	Catfish	Baking	170-190	1.8	63	177	15 (total)	20.68 ± 2.45
	Salmon	Baking	180-200	1.8	63	177	14 (total)	18.41 ± 2.24
	Tilapia	Baking	140-160	1.5	63	177	12 (total)	18.55 ± 3.73

## Extraction and analysis of HCAs

The HCAs were extracted from meat samples and purified using the method described by Gross and Grüter (1992) except that ethyl acetate was used as the extraction solvent (Santos and others 2004, Smith and others 2008). Each sample (3 g) was homogenized with 12 mL of 1 M NaOH in a commercial Waring blender (Fisher, Pittsburgh, PA, USA). The homogenate was then mixed with 24 g of Extrelut refill material (Merck, Darmstadt, Germany) and poured into an empty Extrelut 20 column. For determination of recovery, selected homogenate samples were spiked with 50 ng of each of the HCA standards. The HCAs were eluted from the Extrelut columns with 60 mL ethyl acetate into a PRS cartridge conditioned with 7 mL of ethyl acetate. The PRS cartridge was then rinsed with 6 mL of 0.1 M HCl, 15 mL of methanol/0.1 M HCl (45:55 v/v), and 2 mL of distilled water to wash out the nonpolar HCAs and other impurities. The HCAs were eluted from the PRS cartridge with 20 mL of 0.5 M ammonium acetate pH 8.5 into 100-mg C-18 cartridges preconditioned with 5 mL of methanol followed by 5 mL of distilled water. The HCAs were then eluted from the C-18 cartridge with 1 mL of methanol/ammonium hydroxide (9:1, v/v) into the vial. The HCA extract was concentrated until dry under a stream of nitrogen and dissolved in 25  $\mu$ L of methanol before it was injected into the HPLC. The HCAs were analyzed on an HP1090A Series II HPLC (Agilent Technologies, Santa Clara, CA, USA) coupled with a photodiode array UV-visible detector (HP 1040) and an HP 1046A programmable fluorescence detector. The HCA separation was performed on a reversed-phase TSK gel ODS-80 TM column (25 cm  $\times$  4.6 mm, 5  $\mu$ m, 80 Å, Tosohass, Montgomeryville, PA, USA) with a mobile phase of 0.01 M triethylamine pH 3.6 (A) and acetonitrile (B). The HCA separation was achieved using a linear gradient that started with 95% A and 5% B and changed to 75% A and 25% B in 30 min at

a flow rate of 1 mL/min and a column temperature of 40 °C. After 30 min, the mobile phase returned to its original ratio (95 % A, 5 % B) for 10 min to allow the column to reequilibrate before the next injection. The UV detector was set at 252 nm for IQ, IQx, MeIQ, MeIQx, and DiMeIQx, and the fluorescence detector was programmed accordingly to the excitation/emission wavelengths of 229 and 437 for PhIP. Data were analyzed with an HP 9000 series 300 Chemstation. The identities of HCA peaks were confirmed by comparing the retention times and the UV absorbance spectrum of each peak with library spectra acquired from standard solutions.

### **Quantitation, recovery, and spectral matching**

The HCA concentrations were quantitated by the internal standard method (Lindsay 1992). A known amount of TriMeIQx (used as internal standard) was added to samples before they were injected into the HPLC. The relative responses (R) of HCA standards were calculated using the following equation:

$$R = (C/A)/(C_s/A_s)$$

where

C	=	Concentration of HCA standards
A	=	Peak area for HCA standards
C <sub>s</sub>	=	Concentration of internal standard
A <sub>s</sub>	=	Peak area of internal standard

The HCA concentrations in samples were calculated using the following equation:

$$C_u = A_u \times R \times (C_s'/A_s')$$

where

C <sub>u</sub>	=	Concentration of HCAs in sample
A <sub>u</sub>	=	Peak area of HCAs in sample
C <sub>s</sub> '	=	Concentration of internal standard in sample

$A_s'$  = Peak area of internal standard in sample

The limits of detection (LOD) and limits of quantification (LOQ) for the HCAs were 0.15 ng/g and 0.45 ng/g for IQ, IQx, MeIQ, MeIQx, DiMeIQx and PhIP. The HCA identities were verified in the cooked meat extracts by online UV spectral matching to a spectral library made from pure standards. Match factors typically were observed at 95% or greater (Puangsombat and Smith 2010). Average recoveries for the HCAs were 72% for IQx, 61% for IQ, 63% for MeIQ, 68% for MeIQx, 60% for DiMeIQx, and 65% for PhIP. The recoveries of MeIQx and PhIP are in agreement with previous reports from this laboratory (Tsen and others 2006, Smith and others 2008) and from Cheng and others (2007).

### **Statistical analyses**

The experimental design was a randomized complete block with repeated measurements, and each experiment was replicated four times. Duplicate measurements taken on the same experimental unit were averaged for statistical analysis. All statistical significance tests were analyzed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Data were examined by analysis of variance (ANOVA) followed by Tukey's multiple comparison test (Tukey 1993), and means were considered significant at  $p < 0.05$ .

## **RESULTS AND DISCUSSION**

The choice of meat samples in our study was based on a previous internet-based survey of U.S. consumers' preference for method of cooking and degree of doneness of meat and fish. The survey was conducted by Exponent, Inc. developed to assess customer's HCA intake (unpublished data). Meat samples selected for the present study included beef (fried beef and broiled beef

cooked to medium-rare and well-done, baked beef, and fried beef patty), pork (fried pork, baked pork, fried pork patty, and fried bacon), chicken (fried-chicken breast and fried-chicken thigh with skin and without skin), and fish (fried and baked catfish, salmon, and tilapia).

### **Chemical analyses**

Table 14 summarizes the results of chemical analyses in the selected fresh meat products. The pH of beef samples (5.47 to 5.89) was lower than that of pork samples (6.01 to 6.71) and chicken samples (6.19 to 6.70); fish samples had the highest pH (6.94 to 7.91). The moisture level of fresh meat products ranged between 60 to 82%, except in the high fat parts (bacon, breast skin, and thigh skin), which contained low moisture levels (approximately 37%). The fat levels of raw meat samples ranged from 1.07 to 53.67%; tilapia contained the lowest amount of fat and skin of chicken breast contained the highest amounts of fat. The protein levels of raw meat samples ranged from 9.04 to 23.37%; chicken thigh skin contained the lowest amount of protein, and skin of chicken breast contained the highest amount of protein. Creatine in the uncooked meat samples ranged from 1.02 to 2.95 mg/g. There was not much difference in creatine level among these samples.

### **Identification and quantification of HCAs**

Because consumption of undercooked meat and fish has been linked epidemiologically to foodborne outbreaks, the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS 1998) has established guidelines for both consumers and the food service industry for safe handling and preparation of cooked meat and fish. USDA-FSIS recommends a minimum



instantaneous internal cooking temperature of 63 °C (145 °F) for beef steak and fish, 71 °C (160 °F) for pork and ground beef, and 74 °C (165 °F) for chicken. According to the survey by Exponent Inc. (unpublished data), people mostly consume meat and fish that are cooked until their internal temperatures reach the minimum temperature recommended by USDA-FSIS, except for steak and bacon. Two doneness levels of steak, medium-rare and well-done, are most often consumed. In our study, all samples except beef steak and bacon were cooked until their internal temperature reached the temperatures recommended by USDA-FSIS. Fried and broiled beef steak samples were cooked until their internal temperature reached 57 °C (135 °F) for medium-rare and 71 °C (160 °F) for well-done. Fried bacon was cooked at 172 °C for 3 min on each side (three slices at a time) as recommended to minimize carcinogenic nitrosamine level (Ikins and others 1986). We investigated the presence of six HCAs (IQ, IQx, MeIQ, MeIQx, DiMeIQx and PhIP) that can be found in cooked meat products and have been commonly studied and reported in many papers. Level of HCAs in each sample was analyzed the outer layer (2 mm) of meat samples to increase analysis sensitivity because HCAs are mainly present on the outer surface (Busquets and others 2004). The amount of HCAs on the surface was then used to calculate the amount of HCAs in the whole cooked meat samples (Busquets and others 2004). The values were corrected for incomplete recovery. To our knowledge, this is the first report of HCA contents in food samples commonly consumed in the U.S. and prepared by domestic cooking procedures to internal temperatures recommended by USDA-FSIS to eliminate foodborne illness.

All cooked meat products in the present study are shown in Figures 14 to 17. The quantitative analyses of HCAs in cooked meat products are summarized in Tables 17 to 21. The total amount of HCAs ranged from 1.72 ng/g (medium-rare broiled beef) to 17.59 ng/g (fried

bacon). In all meat samples, PhIP was found at the highest level (1.49 to 10.89 ng/g), followed by MeIQx (not detected to 4.00 ng/g), DiMeIQx (not detected to 3.57 ng/g), and IQx (not detected to 3.11 ng/g); neither IQ nor MeIQ was found in any sample. The highest level of PhIP was found in fried tilapia (10.89 ng/g), followed by fried pork (9.20 ng/g). IQx was not found except in fried bacon (3.11 ng/g) and baked fish (0.38 to 0.85 ng/g).

We investigated the effect of HCA formation for three types of fried meat (beef, pork, chicken) and the results of the HCA quantitative determinations are summarized in Table 17. All of these samples contained MeIQx, DiMeIQx, and PhIP. Although the target internal temperatures of the meat samples fried at 204 °C were slightly different, total HCAs in fried pork (13.91 ng/g, PhIP accounting for 9.20 ng/g) were significantly higher than those in fried beef (8.92 ng/g, PhIP accounting for 5.27 ng/g) and fried chicken (7.06 ng/g, PhIP accounting for 6.06 ng/g). There was no significant differences in total HCAs between fried beef and fried chicken ( $p > 0.05$ ). This is in agreement with results of Skog and others (1997) who reported higher amounts of total HCAs in fried pork (21.3 ng/g) than fried chicken breast (10.7 ng/g) when cooked at 225 °C. However, our result is not in agreement with data from Pais and others (1999), who reported that total HCAs were higher for chicken (38.2 ng/g) than for pork (8.6 ng/g) and beef (2.83 ng/g) when cooked at 275 °C for 30 min. In contrast, Iwasaki and others (2010) reported a lower amount of total HCAs in fried chicken (1.01 ng/g), fried pork (0.5 ng/g), and fried beef (0.1 ng/g) when cooked to well-done (internal temperature 75 °C for chicken, 88 °C for pork, and 78 °C for beef). The inconsistent results could be due to different cooking methods, different weight/thickness of meat

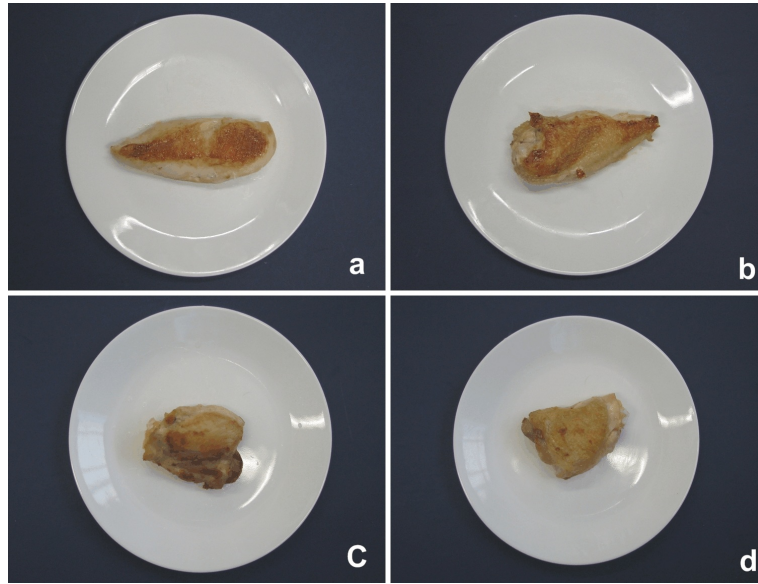


Figure 14. Fried breast without skin (a), fried breast with skin (b), fried thigh without skin (c), and fried thigh with skin (d).

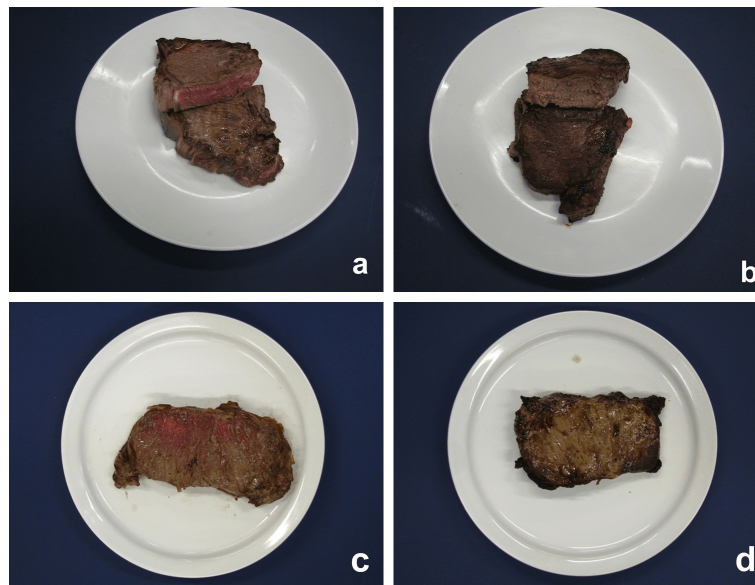


Figure 15. Medium-rare fried beef (a), well-done fried beef (b), medium-rare broiled beef (c), and well-done broiled beef (d).

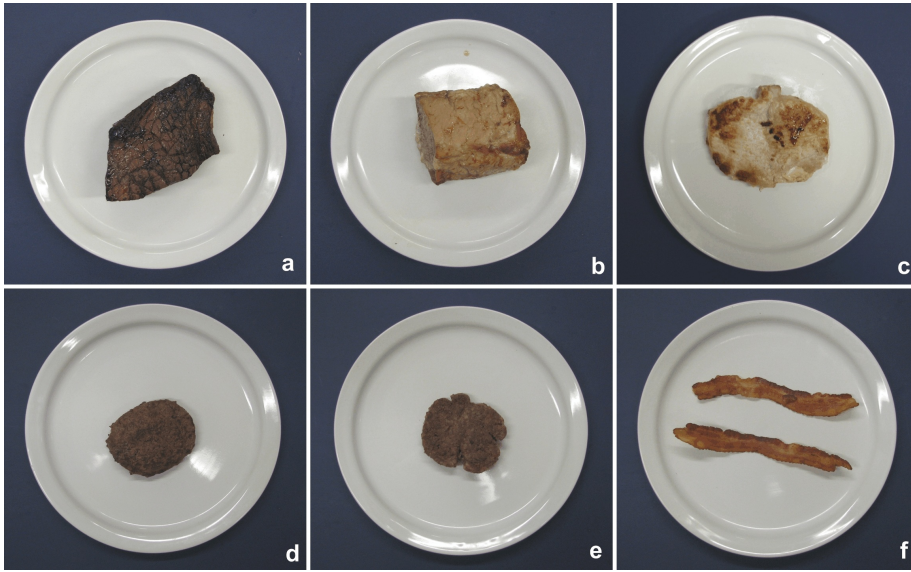


Figure 16. Oven baked beef (a), oven baked pork (b), fried pork (c), fried beef patty (d), fried pork patty (e), and fried bacon (f).

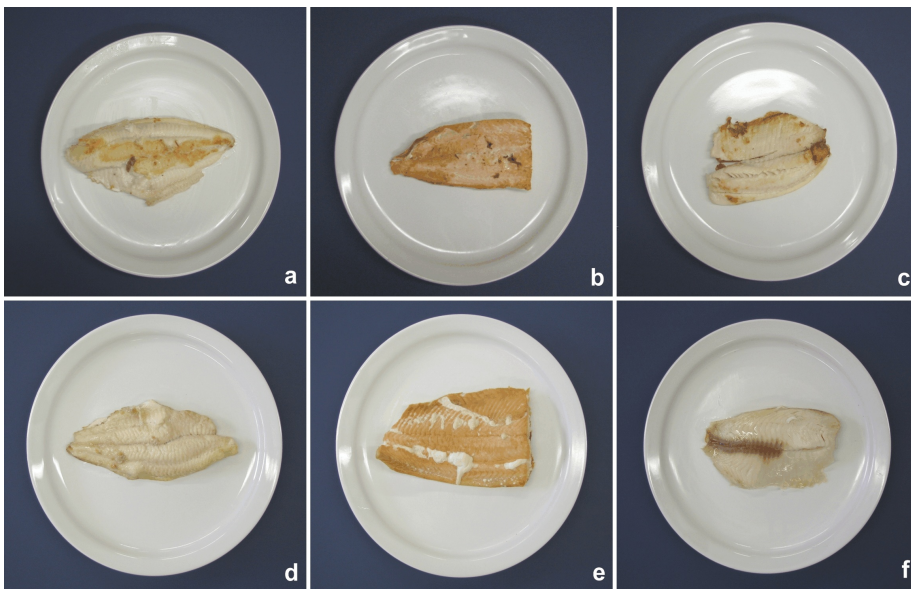


Figure 17. Fried catfish (a), fried salmon (b), fried tilapia (c), baked catfish (d), baked salmon (e), and baked tilapia (f).

Table 17. Heterocyclic amine content (MeIQx, DiMeIQx, PhIP, and total) of fried meat samples

Cooked itmes	Internal temperature (°C)	Heterocyclic amines (ng/g)			
		MeIQx	DiMeIQx	PhIP	Total
Fried beef (well done)	71	3.33 ± 0.38	0.33 ± 0.38	5.27 ± 0.81	8.92 ± 1.08 <sup>b</sup>
Fried pork	71	2.39 ± 0.50	2.33 ± 0.52	9.20 ± 1.20	13.91 ± 1.81 <sup>a</sup>
Fried chicken (breast without skin)	74	0.46 ± 0.34	0.54 ± 0.19	6.06 ± 0.10	7.06 ± 0.56 <sup>b</sup>

Each value is represented as mean ± standard deviation ( $n = 4$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

Table 18. Heterocyclic amine content (MeIQx, DiMeIQx, PhIP, and total) of fried chicken samples

Cooked items			Heterocyclic Amines (ng/g)			
			MeIQx	DiMeIQx	PhIP	Total
Breast	without skin	meat	0.46 ± 0.34	0.54 ± 0.19	6.06 ± 0.10	7.06 ± 0.56 <sup>a</sup>
	with skin	meat	0.23 ± 0.15	0.05 ± 0.01	2.61 ± 0.63	2.89 ± 0.72 <sup>b</sup>
		skin	1.61 ± 0.72	0.93 ± 0.50	4.52 ± 0.37	7.06 ± 1.43 <sup>a</sup>
		meat and skin	0.31 ± 0.15	0.10 ± 0.02	2.72 ± 0.60	3.13 ± 0.67 <sup>b</sup>
Thigh	without skin	meat	0.09 ± 0.05	0.06 ± 0.04	5.43 ± 0.43	5.58 ± 0.38 <sup>a</sup>
	with skin	meat	nd	nd	2.06 ± 0.04	2.06 ± 0.04 <sup>b</sup>
		skin	0.47 ± 0.18	0.24 ± 0.14	4.16 ± 0.42	4.87 ± 0.65 <sup>a</sup>
		meat and skin	0.05 ± 0.03	0.02 ± 0.02	2.25 ± 0.10	2.32 ± 0.14 <sup>b</sup>

Each value is represented as mean ± standard deviation ( $n = 4$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

nd = not detected

Table 19. Heterocyclic amine content (MeIQx, DiMeIQx, PhIP, and total) of fried beef and broiled beef

Cooked items		Heterocyclic amines (ng/g)			
		MeIQx	DiMeIQx	PhIP	Total
Fried beef	Medium rare	1.75 ± 1.43	0.04 ± 0.07	0.94 ± 0.70	2.73 ± 2.01 <sup>c</sup>
	Well done	3.33 ± 0.38	0.33 ± 0.38	5.27 ± 0.81	8.92 ± 1.08 <sup>a</sup>
Broiled beef	Medium rare	0.08 ± 0.07	0.06 ± 0.04	1.58 ± 0.36	1.72 ± 0.43 <sup>c</sup>
	Well done	0.12 ± 0.07	0.11 ± 0.02	5.63 ± 0.95	6.04 ± 0.97 <sup>b</sup>

Each value is represented as mean ± standard deviation ( $n = 4$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

nd = not detected

Table 20. Heterocyclic amine content (IQx, MeIQx, DiMeIQx, PhIP, and total) of fried beef and pork patties, baked beef and pork, and fried bacon

Cooked items	Heterocyclic amines (ng/g)				
	IQx	MeIQx	DiMeIQx	PhIP	Total
Fried beef patty	nd	3.11 ± 0.69	nd	2.35 ± 0.30	5.46 ± 0.78
Fried pork patty	nd	1.09 ± 0.16	1.24 ± 0.75	1.80 ± 0.10	4.12 ± 0.72
Baked beef	nd	0.33 ± 0.05	0.53 ± 0.12	1.49 ± 0.10	2.34 ± 0.11
Baked pork	nd	0.23 ± 0.06	0.86 ± 0.24	2.20 ± 0.12	3.29 ± 0.36
Fried bacon	3.11 ± 1.38	4.00 ± 1.46	3.57 ± 1.12	6.91 ± 2.06	17.59 ± 5.18

Each value is represented as mean ± standard deviation ( $n = 4$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

nd = not detected

samples, and different ways of preparing meat before cooking, as well as the efficiencies of heat transfer. The high level of HCAs in the fried pork in the present study is an important finding because of the three meats studied, the consumption of pork is growing the fastest (1.6 % annually) (FAPRI 2010 U.S. and world agricultural outlook 2010).

Table 18 shows the HCA levels of cooked chicken samples. MeIQx, DiMeIQx, and PhIP were detected in all chicken samples and the values were in agreement with the HCA found in fried chicken reported by Solyakov and Skog (2002) and Liao and others (2010). All chicken samples had more PhIP than MeIQx and DiMeIQx. For the chicken samples without skin, total HCA levels in the chicken breasts (7.06 ng/g) were higher than those in the chicken thighs (5.58 ng/g); this may be because the weight of raw chicken breast (250 to 280 g) was higher than



Table 21. Heterocyclic amine content (IQx, MeIQx, DiMeIQx, PhIP, and total) of fried and baked fish

Cooked items		Heterocyclic amines (ng/g)				
		IQx	MeIQx	DiMeIQx	PhIP	Total
Fried	Catfish	nd	2.31 ± 0.10	2.72 ± 0.08	10.31 ± 0.83	15.35 ± 0.78 <sup>a</sup>
	Salmon	nd	2.05 ± 0.50	1.93 ± 0.12	9.11 ± 1.25	13.09 ± 0.90 <sup>a</sup>
	Tilapia	nd	3.11 ± 0.42	2.29 ± 0.35	10.89 ± 1.35	16.29 ± 1.98 <sup>a</sup>
Baked	Catfish	0.85 ± 0.45	2.95 ± 0.70	0.51 ± 0.03	4.40 ± 0.64	8.70 ± 1.61 <sup>b</sup>
	Salmon	0.38 ± 0.19	2.03 ± 0.85	1.66 ± 0.77	4.34 ± 0.48	8.41 ± 1.09 <sup>b</sup>
	Tilapia	0.52 ± 0.21	1.27 ± 0.16	0.29 ± 0.23	5.67 ± 0.44	7.85 ± 0.65 <sup>b</sup>

Each value is represented as mean ± standard deviation ( $n = 4$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .  
nd = not detected

that of raw chicken thigh (140 to 180 g), therefore, a longer cooking time was needed for chicken breast to reach the same internal temperature of 74 °C, leading to higher cooking loss (Table 16) and increasing the level of HCAs. This result is in agreement with data by Pais and others (1999), who reported that chicken breast had more total HCAs (38.2 ng/g) than chicken thigh (8.07 ng/g). For the chicken samples with skin (both breasts and thighs), the meat and skin were analyzed separately. MeIQx, DiMeIQx, and PhIP levels in the skin were much higher than the levels detected in the muscle ( $p < 0.05$ ). Total HCAs were 7.07 ng/g for skin and 2.89 ng/g for meat in chicken breast and 4.87 ng/g for skin and 2.07 ng/g for meat in chicken thigh. The cooking loss of chicken breast with skin (24.39%) was lower than that of chicken breast without skin (27.88%); the cooking loss of chicken thigh with skin (26.74%) was lower than that of chicken thigh without skin (24.96%) (Table 16). This suggests that the skin present at the surface acts as an insulating

layer for the meat and can help retain moisture during frying, thus decreasing HCA formation. This is in agreement with results of Chiu and others (1998) and Solyakov and Skog (2002). The high level of HCAs in the skin can be explained by the direct exposure to the cooking surface. In addition, the higher fat content in skin might affect HCA formation. Lipids are known to conduct heat more efficiently into the product, which favors the formation of HCAs (Johansson and Jägerstad 1994). It is possible that lipids, and perhaps lipid oxidation, form products that may enhance the formation of certain Maillard reaction products and lead to an increased amount of HCA formation (Barnes and others 1983, Johansson and Jägerstad 1994). Removing the skin portion before consumption could significantly reduce total HCA levels from 7.06 to 2.89 ng/g in chicken breast ( $p < 0.05$ ), and from 5.58 to 2.07 ng/g in chicken thigh ( $p < 0.05$ ). Although chicken skin contains a high level of HCAs, the weight of skin portion is much less than meat portion. The total HCA levels in meat cooked with skin (3.13 ng/g in breast and 2.33 ng/g in thigh) were still significantly lower than the levels in meat cooked without skin (7.06 ng/g in breast and 5.58 ng/g in thigh) ( $p < 0.05$ ). These results agree with studies by Gašperlin and others (2009), who reported a lower amount of total HCAs in chicken with skin (3.49 ng/g) than in chicken without skin (4.75 ng/g) grilled at a temperature of 220 °C to an internal temperature of 82 °C, and Chiu et al. (1998), who reported a lower amount of total HCAs in chicken with skin (6.67 ng/g) than in chicken without skin (12.71 ng/g) fried at 200 °C for 15 min. Taken together, these results indicated that presence of skin reduces HCA formation. However, it is still best to remove skin before consuming chicken to minimize HCA intake.

Table 19 shows the HCA levels of fried beef and broiled beef cooked to medium-rare (internal temperature 57 °C) and well-done (internal temperature 71 °C). MeIQx, DiMeIQx, and

PhIP were detected in fried and broiled beef. There was a dramatic increase in total HCAs (approximately 3.5-fold) for both fried beef and broiled beef with the increase in cooking time (degree of doneness) from medium-rare to well-done (from 2.73 ng/g to 8.92 ng/g for fried beef and from 1.72 ng/g to 6.04 ng/g for broiled beef) ( $p < 0.05$ ). We observed approximate increases of 2-fold for MeIQx level, 8-fold for DiMeIQx, and 6-fold for PhIP when comparing medium-rare with well-done fried beef. This result is in agreement with studies by Skog and others (1997) and Janoszka and others (2009). When cooking time increases, more proteins are denatured, pressing more water, which contains water-soluble HCA precursors, out of the protein network to the meat surface. Thus more of these precursors are transferred to the surface for HCA formation (Skog and others 1997, Persson and others 2008). Different cooking methods affected total HCA formation.

Total HCAs in fried beef (2.73 ng/g) were slightly higher than those in broiled beef (1.72 ng/g) for medium-rare samples, but the difference was not significant ( $p > 0.05$ ). Total HCAs of fried beef (8.92 ng/g) were significantly higher than those of broiled beef (6.04 ng/g) for well-done samples ( $p < 0.05$ ). Cooking time may have more influence on HCA formation than cooking temperature because the cooking temperature used for broiling (232 °C) was higher than that used for frying (204 °C); however, the cooking time used for broiling was less than that used for frying. Also, in oven broiling, the heat is transferred to the meat by air, this produces fewer HCAs than frying, in which the meat is in direct contact with a heated pan (Skog and others 1997). This result clearly indicates that controlling cooking temperature is a way to minimize HCA formation.

The HCA quantitative determination in fried beef and pork patties, baked beef and pork, and fried bacon is summarized in Table 20. The level of total HCAs did not differ much between fried beef patty and fried pork patty, and between baked beef and baked pork. Baked beef had a

lower amount of HCAs than fried and broiled beef because baking is done at a lower temperature and because a higher weight of raw beef was used for baking. The level of HCAs in fried bacon was the highest of all meat samples in the present study. The total amount of HCAs in fried bacon was 17.59 ng/g (6.91 ng/g PhIP, 4.00 ng/g MeIQx, 3.57 ng/g DiMeIQx, and 3.11 ng/g IQx). The amount of HCAs in fried bacon in the present study was much higher than that of fully cooked bacon, ready-to-eat meat product included in our previous studies. Fully cooked bacon (heated in a microwave for 30 s according to package direction) had only 0.91 ng/g HCAs (0.14 ng/g PhIP, 0.14 ng/g MeIQx, 0.60 ng/g IQ, and 0.04 ng/g IQx). Cooking loss of fried bacon in the present study (71.94%) was much higher than that of fully cooked bacon (20.91%). This may explain the high level of HCAs, especially PhIP, in fried bacon compared with fully cooked bacon. PhIP formation increases dramatically in the cooking conditions that generate high cooking loss (Messner and Murkovic 2004). The amount of cooking loss and total HCAs of fried bacon in the present study agree with results of a study by Johansson and Jägerstad (1994), who reported 50.3 to 71.4% cooking loss and 16.7 ng/g total HCAs of bacon fried at 150 °C for 5 min per side.

Table 21 summarizes the results of HCA quantitative determination in fried and baked fish (catfish, salmon, and tilapia). There was no significant difference in amount of HCAs among the three fish species ( $p > 0.05$ ). Concentrations of MeIQx, DiMeIQx, and PhIP in fried fish (catfish, salmon, and tilapia) were similar to those reported earlier for fried mackerel (Gu and others 2002) and fried salmon (Iwasaki and others 2010). For all three fish species, total HCAs in fried fish (13.09 to 16.29 ng/g) were significantly higher than those in baked fish (7.85 to 8.70 ng/g) ( $p < 0.05$ ); however, the small amounts of IQx (0.38 to 0.85 ng/g) were detected only in baked fish samples.

The total amount of HCAs can be used to order these cooked meat products from low to high. Low levels of total HCAs (less than 5 ng/g) were found in baked beef (2.34 ng/g), fried chicken thigh with skin (2.33 ng/g), medium-rare fried beef (2.73 ng/g), fried chicken breast with skin (3.13 ng/g), baked pork (3.29 ng/g), and fried pork patty (4.12 ng/g). Intermediate levels of total HCAs (5 to 10 ng/g) were found in fried beef patty (5.46 ng/g), fried chicken thigh without skin (5.58 ng/g), well-done broiled beef (6.04 ng/g), fried chicken breast without skin (7.06 ng/g), baked fish (8.32 ng/g), and well-done fried beef (8.92 ng/g). High levels of total HCAs (higher than 10 ng/g) were found in fried pork (13.91 ng/g), fried fish (14.91 ng/g), and fried bacon (17.59 ng/g). The high levels of HCAs in some cooked meat products in the present study raises several interesting issues related to HCA intake and cancer aetiology. Data from the National Health and Nutrition Examination Survey 2003-2006 (unpublished data), which estimated meat consumption of U.S. populations, indicated that chicken breast without skin was the most frequently consumed meat item in the U.S. (9.57 g/day), followed by beef steak (8.52 g/day), pork chops (2.89 g/day), and bacon (1.39 g/day). Thus, according to our study, the high levels of HCAs found in fried bacon and fried pork and the intermediate levels of HCAs found in fried chicken breast without skin and well-done fried beef steak indicate that people consuming these products frequently have a high exposure to HCAs that could lead to the possibility of an increased risk of cancers.

## **CONCLUSIONS**

The HCA content in cooked meat depends on type of meat, cooking methods, and cooking time and temperature. Controlling cooking temperature can minimize the HCA formation. Our data can help food safety professionals recommend cooking methods to be used at home or in the food industry to reduce HCA formation in cooked meat products, will provide important information for use in estimating HCA exposure, and will facilitate investigation of the role of HCAs in the etiology of cancer of population in the U.S.

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**PART IV. INHIBITION OF HETEROCYCLIC AMINE FORMATION IN BEEF  
PATTIES BY ETHANOLIC EXTRACTS OF ROSEMARY <sup>1,2</sup>**

**ABSTRACT**

Heterocyclic amines (HCAs) are mutagenic compounds formed during cooking muscle foods at high temperature. Inhibition of HCAs by rosemary extracts were evaluated with beef patties cooked at 191 °C (375 °F) for 6 min each side and 204 °C (400 °F) for 5 min each side. Five rosemary extracts extracted with different solvents were used in this study: extract 100W (100% water), 10E (10% ethanol), 20E (20% ethanol), 30E (30% ethanol), and 40E (40% ethanol). The five extracts were directly added to beef patties at three levels (0.05%, 0.2%, and 0.5%) before cooking and HCA contents were extracted and quantified. All of the patties contained MeIQx and PhIP. There was no statistical difference in the inhibition of HCAs in the 0.05%, 0.2%, and 0.5% rosemary extracts. All rosemary extracts significantly decreased the levels of MeIQx and PhIP at both cooking conditions. When cooking at 204 °C (400 °F) for 5 min each side, rosemary extracts 10E and 20E were superior to rosemary extracts 100W, 30E, and 40E in inhibiting HCA formation. Rosemary extract 20E showed the greatest inhibition of MeIQx (up to 91.7%) and PhIP (up to 85.3%). The inhibiting effect of rosemary extracts on HCA formation corresponded to their antioxidant activity based on a DPPH scavenging assay. Rosemary extract 10E and 20E contain a mixture of rosmarinic acid, carnosol, and carnosic acid. It is possible that these compounds might act synergistically in inhibiting the formation of HCAs.

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1. Reproduced with permission from Puangsombat K, Smith JS. 2010. Inhibition of heterocyclic amine formation in beef patties by ethanolic extracts of rosemary. *J Food Sci* 75(2): T40-T47.

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## INTRODUCTION

Numerous strategies have been used to limit the formation of HCAs, including lowering cooking times, lowering temperatures, and precooking food in the microwave. However, the most promising and consistently effective treatments involve the use of various antioxidants, which serve as free-radical scavengers because the HCAs are formed in the Maillard reaction through the free radicals pyridine or pyrazine (Knize and others 1994). The precursors of this reaction are creatine or creatinine, free amino acids, and reducing sugars, which are commonly compounds of animal origin. Several synthetic antioxidants have been used to prevent the formation of HCAs as a result of their methoxyl group, which can change into quinone-like compounds acting as free-radical scavengers (Vitaglione and Fogliano 2004); however, some synthetic antioxidants are believed to possess cytotoxic and carcinogenic activity (Ito and others 1983). Due to concerns about toxicological safety of synthetic antioxidants, consumers prefer to use natural antioxidants (Pokorný 2007). In order to inhibit the formation of HCAs in cooked beef patties, the addition of spices (Murkovic and others 1998), spice extracts (Tsen and others, 2006), and plant extracts/tissues (Britt and others 1998; Balogh and others 2000; Vitaglione and others 2002; Cheng and others 2007) have effectively reduced the amount of total HCAs in the range between 50-93% relative to control.

Rosemary (*Rosmarinus officinalis* Labiatae) is commonly used as a spice and a flavoring agent in food processing for its desirable flavor and high antioxidant activity. The main antioxidant compounds in rosemary are phenolic diterpenes, such as carnosol, rosmanol, rosmaridiphenol, carnosic acid, and other phenolic acids, such as rosmarinic acid and caffeic acid (Madsen and Bertelsen 1995, Cuvelier and others 1994). Among the antioxidant compounds in

rosemary, carnosol and carnosic acid provide the most powerful antioxidant activities (Lo and others 2002), which are similar to that of BHA and BHT (Chen and others 1992). However, the use of rosemary as an additive is limited by the characteristic aroma; therefore, commercial rosemary extracts are produced in order to extract the components with high antioxidant activity while also eliminating the essential oils that give rosemary its distinctive aroma.

Rosemary extracts are commercially available in both water-soluble and oil-soluble products as well as in liquid and powder forms. The use of rosemary extracts and oleoresin as antioxidants in meat and meat products has been extensively studied, especially the way in which they inhibit oxidation of both lipid and myoglobin and improve desirable sensory characteristics and shelf-life of beef patties (Sánchez-Escalante, 2001). It has been shown that applying dried rosemary (Murkovic and others 1998) or oleoresin rosemary (Balogh and others 2000) on beef patties before cooking reduced 60% of the total HCAs relative to control. Tsen and others (2006) showed that adding rosemary extracts reduced the formation of MeIQx up to 69% and PhIP up to 66% in beef patties with the effects more dramatic when cooking temperature and time increased.

Solvent extraction is the method frequently used for preparing rosemary extracts. Selection of solvents or solvent mixtures is an important factor and can affect the antioxidant activities of rosemary due to the different potentials of antioxidant compounds with different polarity. While the antioxidant properties of rosemary are well known there is little information characterizing the effects of the ratios of the major phenolic compounds on the inhibition of HCA formation. The objective of this work was to compare five rosemary extracts formulated using different ratios of water and ethanol (100% water, 10% ethanol, 20% ethanol, 30% ethanol, and 40% ethanol) in inhibiting HCAs formation in cooked beef patties.

## MATERIAL AND METHODS

### Materials

Five rosemary extracts obtained from Mitsubishi-Kagaku Foods Corporation that were prepared by extracting rosemary leaves with 100% water (100W), 10% ethanol (10E), 20% ethanol (20E), 30% ethanol (30E), and 40% ethanol (40E). Dried rosemary leaves were purchased from a local supermarket.

The HCA standards IQ (2-amino-3-methylimidazo [4,5-*f*]quinoline), IQx (2-amino-3-methylimidazo [4,5-*f*]quinoxaline), MeIQ (2-amino-3,4-dimethylimidazo [4,5-*f*]quinoline), MeIQx (2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine) were obtained from Toronto Research Chemicals (Toronto, Canada). Rosmarinic acid (97%) and carnosic acid (91%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Carnosol (96%) was purchased from A.G. Scientific (San Diego, CA, USA).

Ammonium acetate and triethylamine were purchased from Aldrich Chemicals (Milwaukee, WI, USA). Phosphoric acid was obtained from Sigma Chemicals (St. Louis, MO, USA). Deionized water was processed by a Sybron/Branstead PCS unit (Barnstead/Thermolyne, Inc.; Dubuque, IA, USA). The solid-phase extraction Extrelut NT 20 columns and diatomaceous earth refill material were purchased from VWR International (Bristol Conn., USA). Bond Elut propyl-sulfonic acid (PRS) cartridges, C-18 cartridges, and the coupling adaptors were purchased from Varian Sample Preparation (Harbor City, Calif, USA). Solid phase microextraction (SPME) fibers coated with 75  $\mu\text{m}$  carboxen-polydimethylsiloxane (CAR-PDMS) were obtained from Supelco (Bellefonte, PA, USA). In addition, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-

Ciocalteu's reagent, sodium carbonate, and gallic acid were obtained from Sigma (St. Louis, MO, USA). Solvents and chemicals such as acetonitrile (HPLC grade), methanol (HPLC grade), and sodium hydroxide (ACS-grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA).

### **Preparation of Ground Beef Patty Samples**

Fresh ground beef (10% fat) was obtained from a local supermarket in Manhattan, KS. The five types of rosemary extract samples were added directly to 100 g ground beef at levels of 0.05%, 0.2%, and 0.5%. Control samples contained no added rosemary extracts. Each patty was formed using a Petri dish (10 cm x 1 cm) to ensure uniformity. The patties were covered with aluminum foil in plastic freezer bags and refrigerated overnight at 4 °C before frying. The patties were fried in a Teflon-coated frying pan with a temperature controller (Bernant, Barrington, IL, USA) at surface temperatures of 191 °C (375 °F) for 6 min on each side or 204 °C (400 °F) for 5 min on each side. The cooked patties were cooled at room temperature, homogenized with a food processor (KitchenAid, model KFP 750), and refrigerated at 4 °C. The extraction step was performed within 24 hours after cooking.

### **Comparison of Volatile Compounds in Dried Rosemary Leaves and Rosemary Extracts by SPME-GC-MS**

An amount of 0.1 g of rosemary extracts and crushed dried rosemary leaves were placed in 5 mL sealed vials. Each vial was incubated in a 60 °C water bath for 5 min. The extraction was carried out with a CAR-PDMS fiber. The fiber was immersed in the headspace vial for 15 min at the same temperature. After each extraction, the fiber was removed and inserted into the injection

port of the GC-MS for 15 min.

The GC-MS was performed with a HP 5890 GC (Agilent Technologies, Palo Alto, CA, USA) fitted with a HP-5 MS column (cross-linked 5% Ph Me siloxane, 30 m x 0.22 mm x 0.25  $\mu\text{m}$  film thickness), and a HP MSD 5070 detector. GC-MS conditions were as follows: oven temperature was programmed at an initial temperature of 70  $^{\circ}\text{C}$  for 3 min, followed by an increase in temperature at a rate of 7  $^{\circ}\text{C}/\text{min}$  to 180  $^{\circ}\text{C}$  and maintained for 5 min. Injector and detector temperatures were 260 and 300  $^{\circ}\text{C}$ . The carrier gas was helium at a flow rate of 1 mL/min. Headspace-SPME injections were carried out in the splitless mode. Volatile compounds were identified by comparing the spectra with those in a NIST98/Wiley spectral library (7<sup>th</sup> ed).

## **Extraction, Identification, and Quantification of Phenolic Antioxidants in Rosemary**

### **Extracts**

The phenolic antioxidants were extracted following the procedure described by Wellwood and Cole (2004) using ethanol. All the solutions passed through a 0.45  $\mu\text{m}$ , 25 mm, nylon syringe filter (Alltech, Seerfield, IL, USA) before being injected into the high-performance liquid chromatograph (HPLC). For HPLC analysis of antioxidants, a method adapted from Cuvelier and others (1996) was performed on a reversed-phase analytical C18 column (25cm x 4.6 mm x 5  $\mu\text{m}$ ) (Alltima, Alltech Assoc., Deerfield, IL, USA) using a C18 guard column. The separation was monitored by a UV diode-array detector at 284 nm. Three mobile phases were used: solvent A was 1% (v/v) acetic acid in deionized water; solvent B was methanol; and solvent C was acetonitrile. The total run time was 40 min. A gradient profile was used with a program that started with 90% A, 10% B, which were changed to 30% A, 55% B and 15% C at 20 min, and

changed to 100% B at 40 min. A 25 min post-run was used to equilibrate the column. The flow rate was 1 mL/min and the column temperature was 40 °C. The compounds were identified by comparison with the retention time and spectral matching with a UV spectral library generated from standards. The range of concentrations used for standard curves of antioxidants was between 5 to 100 ppm. Triplicate analyses were performed for each extract.

### **Determination of Total Phenolic Content in Rosemary Extracts**

The total phenolic content of the rosemary extracts was determined using Folin-Ciocalteu's reagent according to the method described by Chang and others (2006) with slight modifications. Each rosemary extract (1 g) was mixed with 10 mL of 95% ethanol and shaken on a wrist action shaker (Burrell Shaker model 75, Burrell Corp., Pittsburgh, PA, USA) for 2 hours at room temperature. The samples were filtered through Whatman #4 filter paper, then the extract was diluted 1:5 (v/v) with 95% ethanol. Each 100 µL of the diluted extract was mixed with 2 mL of DI water in a test tube followed by addition of 200 µL of Folin-Ciocalteu reagent and allowed to stand for 6 min at room temperature. Then, 1 mL of 7.5% sodium carbonate solution was added, mixed thoroughly, and stored in the dark for 2 hours at room temperature. Each sample's absorbance was measured at 765 nm using an UV/VIS spectrophotometer. The linear reading of the standard curve was from 0 to 300 µg of gallic acid per mL. The total phenolic content was expressed as mg gallic acid equivalents per 1 g of dried sample material. Triplicate analyses were performed for each extract.



### **DPPH Radical Scavenging Activity Assay**

The DPPH radical scavenging activity of the rosemary extracts was determined according to the method described by Singh and others (2002) with slight modifications. Each rosemary extract (0.2 g) was mixed with 100 mL of 95% ethanol and shaken on a wrist action shaker (Burrell Shaker model 75, Burrell Corp., Pittsburgh, PA, USA) for 2 hours at room temperature. The samples were filtered through Whatman #4 filter paper, then the extract was diluted to 0.1, 0.25, 0.50, and 1mg/mL with ethanol. Each 0.1 mL of the diluted extract was mixed with 2.9 mL of 0.1 mM freshly prepared DPPH methanolic solution and stored in the dark for 30 min at room temperature before the absorbance was measured at 517 nm. Ethanol (95%) was used as a blank. The control solution consisted of 0.1 mL of 95% ethanol and 2.9 mL of DPPH solution. The radical scavenging activity (%) was expressed as the inhibition percentage and calculated using the following equations:

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

IC<sub>50</sub> of the extract was determined from the graph of radical scavenging activity (%) against the concentration of extract (mg/mL). The IC<sub>50</sub> was defined as the concentration of an extract that was required to provide a 50% decrease of the absorbance from the blank solution. Triplicate analyses were performed for each extract.

### **Extraction and Analysis of HCAs**

HCAs were extracted and purified from meat using the method described by Gross and Grüter (1992) except that ethyl acetate was used as the extraction solvent (Borgen and others 2001; Santos and others 2004; Smith and others 2008). The cooked beef patty sample (3 g) was

homogenized with 12 mL of 1 M NaOH using a commercial Waring blender (ThermoFisher Scientific Co., Pittsburgh, PA, USA). The homogenate was then mixed with 24 g of Extrelut refill material (Merck, Darmstadt, Germany) and poured into an empty Extrelut 20 column. For determination of recovery, selected homogenate samples were spiked with 50 ng of each of the HCA standards. The HCAs were eluted from the Extrelut columns with 60 mL ethyl acetate into a PRS cartridge conditioned with 7 mL of ethyl acetate. The PRS cartridge was then dried under a stream of nitrogen and rinsed with 6 mL of 0.1 M HCl, 15 mL of methanol/0.1 M HCl (45:55 v/v) and 2 mL of distilled water to wash out the non-polar HCAs. The HCAs were eluted from PRS cartridge with 20 mL of 0.5 M ammonium acetate pH 8.0 into 100 mg C-18 cartridges preconditioned with 2 mL of water. The HCAs were then eluted from the C-18 cartridge with 1 mL of methanol/ammonium hydroxide (9:1, v/v) into the vial. The HCA extract was concentrated until dry under a stream of nitrogen and dissolved in 25  $\mu$ L of methanol before injected into the HPLC. The HCAs were analyzed on a HP1090A Series II HPLC (Agilent Technologies, Palo Alto, CA, USA) coupled with a photodiode array UV-visible detector (HP 1040) and an HP 1046A programmable fluorescence detector. The HCAs separation was performed on a reversed-phase TSK gel ODS-80 TM column (25 cm x 4.6 mm, 5  $\mu$ m, 80 Å, Tosohass, Montgomeryville, PA, USA) with a mobile phase of 0.01 M triethylamine pH 3.6 (A) and acetonitrile (B). The HCAs separation was achieved by using a linear gradient starting with 95% A, 5% B, changing to 75% A, 25% B in 30 min, flow rate of 1 mL/min at a column temperature of 40 °C. After 30 min, the mobile phase returned to its original ratio (95% A, 5 % B) for 10 min to allow the column to re-equilibrate before the next injection. The UV detector was set at 252 nm for IQ, IQx, MeIQ, and MeIQx, while the fluorescence detector was programmed accordingly to the

excitation/emission wavelengths of 229 and 437 nm for PhIP. The data were analyzed with an HP 9000 series 300 ChemStation. The identities of HCAs peaks were confirmed by comparing the retention times and the UV absorbance spectrum of each peak with library spectra acquired from standard solutions. Triplicate analyses were performed for each extract.

### **Standard Curves, Recovery, and Spectral Matching**

HCAs standards were dissolved in 100% methanol to make a solution of 1000 ng/mL (1 ppm). These stock solutions were used for making serial dilutions of each HCA to yield standards of 250 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, and 5 ng/mL. Peak area from the HPLC runs was used to establish standard curves for each HCA. All standard curves were evaluated by linear regression and had a coefficient of determination ( $R^2$ ) greater than 0.97. The limits of detection (LOD) and limits of quantification (LOQ) for the HCAs were 0.15 ng/g and 0.45 ng/g for IQ, IQx, MeIQ, MeIQx and PhIP. HCA identity was verified in the cooked meat extracts by online UV spectral matching to a spectral library made from pure standards with a match factor typically observed of 95% or greater (Abdulkarim and Smith 1998). The average recoveries for the HCAs were 72% for IQx, 61% for IQ, 62 % for MeIQ, and 65% for MeIQx and PhIP. The recoveries of MeIQx and PhIP are in agreement with previous reports from this laboratory (Tsen and others 2006; Smith and others 2008) and the study of Persson and others (2003) and Cheng and others (2007).

### **Statistical Analyses**

The experimental design was a randomized complete block with repeated measurements,

and each experiment was replicated three times. Duplicate measurements taken on the same experimental unit were averaged for statistical analysis. All statistical significance tests were analyzed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA, 2002). Data were examined by analysis of variance (ANOVA) followed by Tukey's multiple comparison test, and means were considered significant at  $p < 0.05$ .

## **RESULTS AND DISCUSSION**

### **Characterization of Phenolic Antioxidants in Rosemary Extracts**

The five rosemary extracts, 100% water (100W), 10% ethanol (10E), 20% ethanol (20E), 30% ethanol (30E) and 40% ethanol (40E), were obtained from Mitsubishi-Kagaku Foods Corporation. The composition of the phenolic antioxidants in the extract was dependent on the ratio of water and ethanol in the solvent, as shown in Table 22, and their chromatograms are shown in Figure 18. Rosmarinic acid (RA), carnosol (CL), and carnosic acid (CA) were identified and quantified in different rosemary extract samples because these phenolics are major antioxidants present in rosemary, and it might be expected that HCAs inhibition would have some relationship. The limits of detection were 0.5 mg/g for RA, and 1 mg/g for CL and CA. The rosemary extract 100W contained 51.5 mg/g RA while CL and CA were not detected; 10E contained 34.7 mg/g RA, 31.8 mg/g CL, and 6.3 mg/g CA; 20E contained 27.2 mg/g RA, 72.9 mg/g CL, and 4.2 mg/g CA; 30E contained 13.5 mg/g RA, 96.5 mg/g CL, and 5.9 mg/g CA; and 40E contained 1.7 mg/g RA, 114.6 mg/g CL, and 6.0 mg/g CA. These results indicated that water exhibited the highest extraction capacity for rosmarinic acid, which is a water soluble compound, whereas 40% ethanol extract had the strongest extraction capacity for carnosol, a non-water

Table 22. Polyphenolic antioxidants levels, total phenolic and IC<sub>50</sub> on DPPH radicals present in the water/ethanol rosemary extracts as determined by HPLC analysis of the extracts.

Treatment	Polyphenolic antioxidant compounds (mg/g)			Total Phenolic (mg gallic acid equivalents/g dry weight)	IC <sub>50</sub> on DPPH radicals (mg/mL)
	Rosmarinic Acid	Carnosol	Carnosic Acid		
<b>100 W</b>	51.51 ± 0.61 <sup>a</sup>	0.00 ± 0.00 <sup>c</sup>	0.00 ± 0.00 <sup>b</sup>	33.45 ± 1.29 <sup>b</sup>	0.57 ± 0.00 <sup>b</sup>
<b>10 E</b>	34.67 ± 0.18 <sup>b</sup>	31.82 ± 0.21 <sup>d</sup>	6.30 ± 0.48 <sup>a</sup>	34.21 ± 1.82 <sup>b</sup>	0.52 ± 0.01 <sup>d</sup>
<b>20 E</b>	27.25 ± 0.12 <sup>c</sup>	72.87 ± 0.29 <sup>c</sup>	4.19 ± 0.74 <sup>a</sup>	38.93 ± 1.25 <sup>a</sup>	0.54 ± 0.01 <sup>c</sup>
<b>30 E</b>	13.45 ± 0.13 <sup>d</sup>	96.49 ± 0.97 <sup>b</sup>	5.92 ± 0.82 <sup>a</sup>	38.06 ± 1.32 <sup>a</sup>	0.58 ± 0.01 <sup>b</sup>
<b>40 E</b>	1.70 ± 0.09 <sup>e</sup>	114.59 ± 0.25 <sup>a</sup>	5.91 ± 0.18 <sup>a</sup>	34.11 ± 0.88 <sup>b</sup>	0.69 ± 0.01 <sup>a</sup>

Each value is represented as mean ± standard deviation ( $n = 3$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

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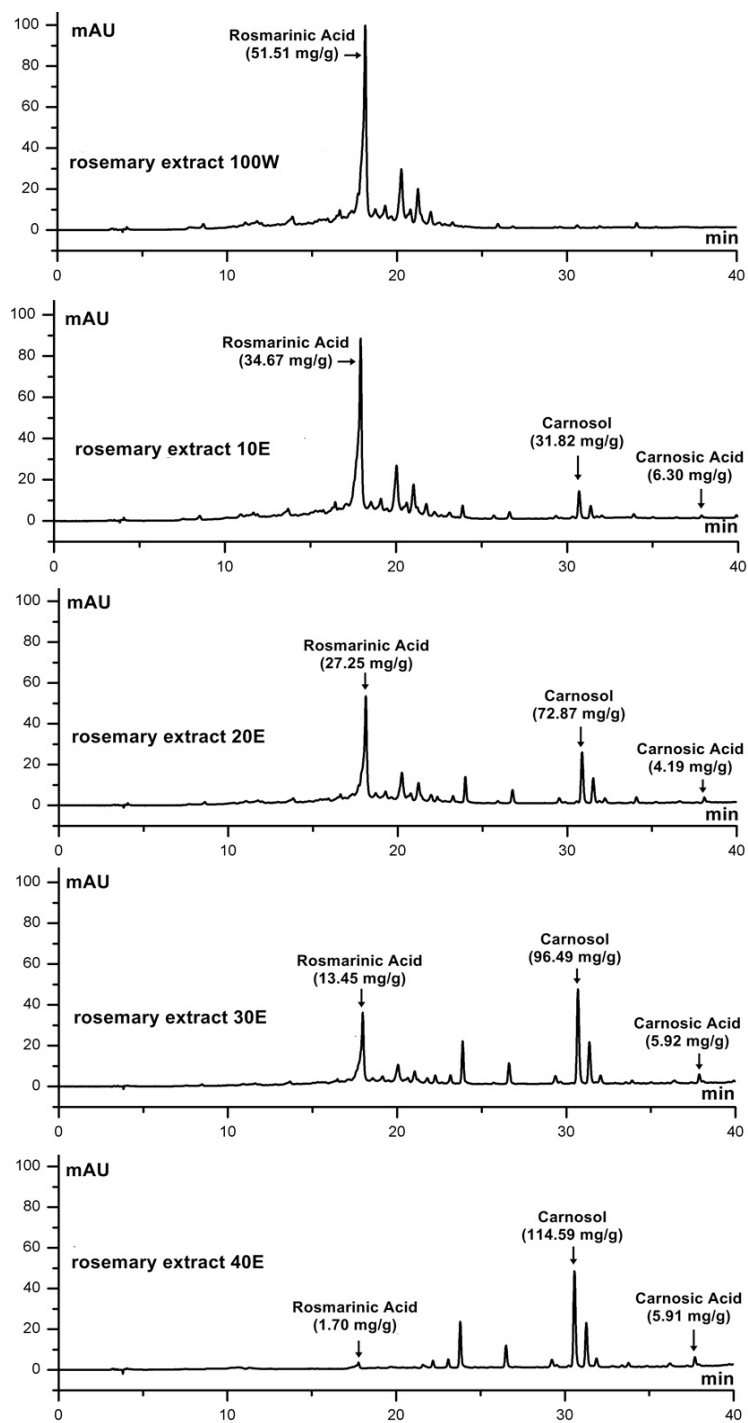


Figure 18. Chromatogram of polyphenolic antioxidants (rosmarinic acid, carnosol, and carnosic acid) present in the rosemary extracts.

soluble compound. Once the ratio of ethanol in the extraction process was increased from 10% to 40%, the level of rosmarinic acid decreased while the level of carnosol concomitantly increased. These results agree with those reported by Wada and others (2004) which showed that the water-soluble extract rosemary contained primarily a large amount of rosmarinic acid while the ethanol rosemary extract contained carnosol, carnosic acid, and other lipid-soluble phenolic diterpenes, but rarely contained rosmarinic acid. Carnosic acid was found only in rosemary extracts that used a mixture of water and ethanol as an extraction solvent, rosemary extract 10E-40E; however, the amounts are quite low and there is not a significant difference among them. This may be due to the degradation of carnosic acid during extraction process, which is the major phenolic in fresh rosemary, as it forms other phenolic diterpenes with lactone structure, such as carnosol, rosmanol, epirosmanol, and 7-methyl-epirosmanol (Schwarz and others 1992).

### **Total Phenolic Content and Antioxidant Activity**

The total phenolic content and antioxidant activity of the five rosemary extracts are presented in Table 22. Total phenolic contents are expressed as mg/gallic acid equivalent/g dry weight (dw). The total phenolic in the extracts were dependent on the extraction solvent used in the extraction. The rosemary extracts 20E and 30E contained the highest total phenolic contents (38-39 mg gallic acid equivalents/g dw), which were significantly higher than those in rosemary extract 10E, 40E, and 100 W (33-35 mg gallic acid equivalents/g dw). The antioxidant activity, expressed as  $IC_{50}$  value (mg/mL), was estimated by DPPH scavenging assay. A lower  $IC_{50}$  value corresponds with a higher antioxidant activity. Statistically significant differences were found among the five rosemary extracts. The rosemary extract 10E (0.52 mg/mL) showed the highest

antioxidant activity, followed by rosemary extract 20E (0.54 mg/mL), rosemary extract 100W (0.57 mg/mL), rosemary extract 30E (0.58 mg/mL), and rosemary extract 40E (0.69 mg/mL).

Many studies have reported a correlation between phenolic content and antioxidant activity (Erkan and others 2008; Ling and others 2009); however, according to a regression analysis of our study, there was no significant relationship between total phenolic content and antioxidant activity of five rosemary extracts (data not shown), which agreed with those reported by Kamiloğlu and others (2009). This may be explained due to the fact that each phenolic compound has different responses in the Folin-Ciocalteu method. Not only do phenolic compounds respond to the antioxidant activity in the assay, but some flavonoids in rosemary extract, luteolin and luteolin-7-glucoside, for example, may also contribute to the antioxidant activity value in the assay (Li and others 2005). Furthermore, the presence of synergistic effects among phenolics or other compounds may increase the value of antioxidant activity (Li and others 2005). Therefore, the same amount of phenolic compounds does not necessarily give the same value of antioxidant activity.

### **Comparison of Volatile Compounds in Dried Rosemary Leaves and Rosemary Extracts by SPME-GC-MS**

The most desirable rosemary extract to use as a natural antioxidant possesses high antioxidant activity and low aroma. In the extraction process, however, it is very difficult to completely remove all aroma compounds in rosemary. Due to a possibility that the residual volatile compounds in the extracts might have antioxidant activity and play a role in HCAs inhibition as well, it was necessary to characterize residual volatile compounds in the extracts. In



our study, the volatile compounds of five rosemary extracts along with dried rosemary leaves were analyzed by using SPME GC-MS. The CAR-PDMS fiber was used in our experiment because it has been reported to provide the best sensitivity to characterize rosemary fragrances (Carrillo and Tena 2006). There were 16 compounds found in dried rosemary (Figure 19); the major compounds found were  $\rho$ -cymene, eucalyptol, camphor,  $\alpha$ -terpineol, and caryophyllene. On the other hand, only 4 volatile compounds were found in rosemary extracts, namely camphor, borneol, verbenone, and bornyl acetate (Figure 20). Thus, it was confirmed that treatment of rosemary extracts obtained by water and/or ethanol extraction was effective since all of the major compounds found in dried rosemary leaves disappeared. Our result is similar to studies of López-Sebastián and others (1998) and Carrillo and Tena (2006), who investigated the volatile compounds in solid rosemary extract by using SPME GC-MS. Camphor, borneol, and bornyl acetate are oxygenated monoterpenes, whereas verbenone is a monoterpene bicyclic ketone (Bozin and others 2008). It has been reported that these compounds are found in essential oils extracted from herbal plants, for example, from the genus *Asteraceae* (*Compositae*), and they expressed similar antioxidant capacities compared to BHT, which is dose-dependent (Amiri 2007; Bozin and others 2008).

Among the five rosemary extracts, the 40% ethanolic rosemary extract (40E) contained the highest amount of these compounds. Interestingly, when the ratio of ethanol as an extraction media in the extraction step was decreased to 30%, 20%, and 10%, the amount of these 4 compounds were proportionally decreased. The water extract contained only a small amount of verbenone, bornyl acetate, and camphor, while borneol was not found. In conclusion, the

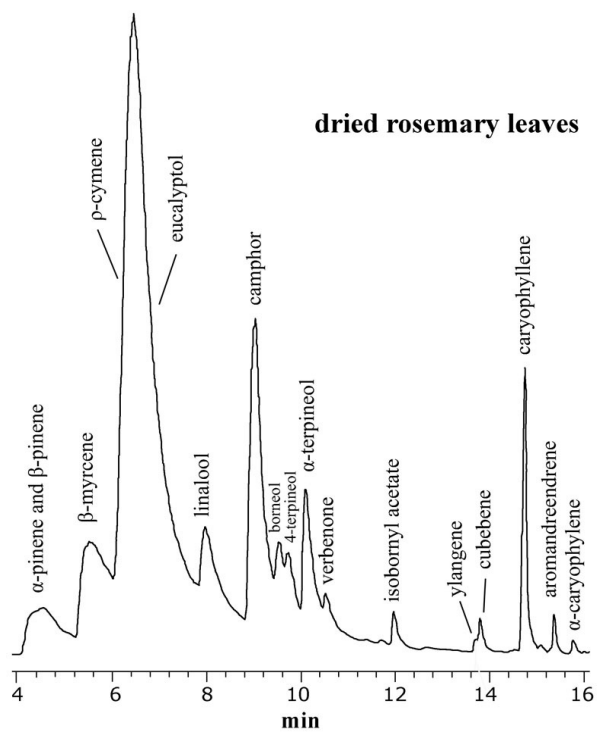


Figure 19. Chromatogram for dried rosemary leaves determined by SPME GC-MS.

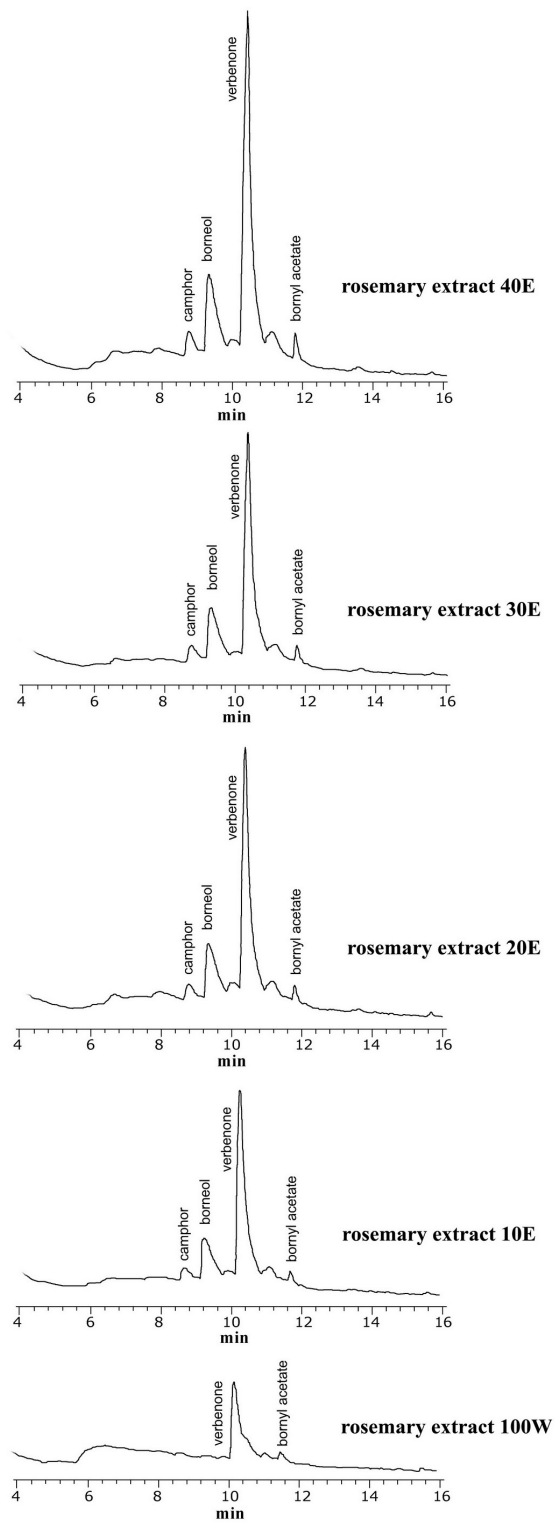


Figure 20. Chromatograms for rosemary extracts determined by headspace SPME GC-MS.

different quantities of these volatile compounds, which serve as antioxidants of each extract, may inhibit the formation of HCAs.

### **Effects of Rosemary Extracts on HCAs Formation in Cooked Beef Patties**

In our experiment, we used two cooking conditions: 191 °C (375 °F) for 6 min each side (375 °F/6min) and 204 °C (400 °F) for 5 min each side (400 °F/5min). At 375 °F/6 min, patties cooked to a final temperature of 74 to 77 °C (165 to 170 °F) produced medium done products with lightly pink internal color. At 400 °F/5min, patties cooked to a final temperature of 82 to 85 °C (180 to 185 °F) produced medium-well products with no pink internal color remaining. The internal temperature of patties at both cooking conditions was greater than 71 °C (160 °F), which is the temperature recommended by the FDA for cooking beef patties to ensure safety. Five rosemary extracts were added to the ground beef at different concentrations to evaluate their abilities to inhibit HCAs forming in the cooked beef patties. HCAs were extracted and quantitated by HPLC; the results are shown in Table 23. A typical HPLC chromatogram of HCAs obtained by UV and fluorescent detection from cooked beef patties is shown in Figure 21.

All of the patties including those without rosemary extracts (control) contained MeIQx and PhIP, while IQx, IQ, and MeIQ were not detectable in any samples. The HCA contents of control beef patties cooked at 375 °F/6 min were 4.96 ng/g MeIQx and 3.14 ng/g PhIP, while control patties cooked at 400 °F/5 min were 6.93 ng/g MeIQx and 4.89 ng/g PhIP. These results were comparable to those reported by Balogh and others 2000, Persson and others 2003, and Cheng and others 2007. As expected, the formation of HCAs was highly dependent on the cooking time and temperature. The HCA contents were increased from 4.96 to 6.93 ng/g MeIQx,

Table 23. Effect of rosemary extracts on total HCA concentrations (ng/g) and percent inhibition in cooked beef patties at 191 °C (375 °F) for 6 min each side and 204 °C (400 °F) for 5 min each side.

Treatment	Level	375 °F for 6 min each side				400 °F for 5 min each side			
		MeIQx	PhIP	Total	% inhibition	MeIQx	PhIP	Total	% inhibition
<b>control</b>		4.96 ± 0.51 <sup>a</sup>	3.14 ± 0.33 <sup>a</sup>	8.10		6.93 ± 0.52 <sup>a</sup>	4.89 ± 0.23 <sup>a</sup>	11.82	
<b>100 W</b>	<b>0.05 %</b>	1.86 ± 0.10 <sup>b</sup>	1.69 ± 0.28 <sup>bc</sup>	3.55	56.2 %	2.43 ± 0.07 <sup>bcd</sup>	2.43 ± 0.07 <sup>bcd</sup>	5.36	54.7 %
	<b>0.20 %</b>	1.41 ± 0.29 <sup>bcde</sup>	0.75 ± 0.10 <sup>ef</sup>	2.16	73.3 %	2.56 ± 0.14 <sup>bc</sup>	2.32 ± 0.13 <sup>bcde</sup>	4.89	58.7 %
	<b>0.50 %</b>	0.96 ± 0.19 <sup>cdef</sup>	0.80 ± 0.13 <sup>def</sup>	1.76	78.3 %	1.81 ± 0.21 <sup>cde</sup>	1.63 ± 0.12 <sup>def</sup>	3.43	70.9 %
<b>10 E</b>	<b>0.05 %</b>	1.18 ± 0.11 <sup>bcdef</sup>	1.48 ± 0.12 <sup>bcd</sup>	2.66	67.2 %	1.01 ± 0.10 <sup>efg</sup>	1.40 ± 0.38 <sup>ef</sup>	2.41	79.6 %
	<b>0.20 %</b>	0.84 ± 0.12 <sup>def</sup>	1.29 ± 0.06 <sup>bcdef</sup>	2.13	73.7 %	0.95 ± 0.14 <sup>efg</sup>	1.40 ± 0.03 <sup>ef</sup>	2.36	80.1 %
	<b>0.50 %</b>	0.64 ± 0.03 <sup>ef</sup>	0.85 ± 0.14 <sup>def</sup>	1.49	81.6 %	0.88 ± 0.31 <sup>fg</sup>	1.76 ± 0.42 <sup>cdef</sup>	2.64	77.6 %
<b>20 E</b>	<b>0.05 %</b>	1.70 ± 0.06 <sup>bc</sup>	1.16 ± 0.12 <sup>cdef</sup>	2.86	64.7 %	1.14 ± 0.06 <sup>efg</sup>	0.72 ± 0.06 <sup>f</sup>	1.86	84.3 %
	<b>0.20 %</b>	1.10 ± 0.04 <sup>cdef</sup>	1.96 ± 0.43 <sup>b</sup>	3.07	62.1 %	0.92 ± 0.13 <sup>efg</sup>	1.41 ± 0.97 <sup>def</sup>	2.33	80.3 %
	<b>0.50 %</b>	1.00 ± 0.18 <sup>cdef</sup>	1.40 ± 0.18 <sup>bcde</sup>	2.40	70.4 %	0.61 ± 0.07 <sup>g</sup>	1.33 ± 0.26 <sup>ef</sup>	1.94	83.6 %
<b>30 E</b>	<b>0.05 %</b>	1.24 ± 0.47 <sup>bcdef</sup>	0.89 ± 0.24 <sup>def</sup>	2.13	73.8 %	2.31 ± 0.46 <sup>bcd</sup>	2.42 ± 0.15 <sup>bcde</sup>	4.73	60.0 %
	<b>0.20 %</b>	0.61 ± 0.51 <sup>ef</sup>	0.60 ± 0.25 <sup>f</sup>	1.12	86.2 %	1.56 ± 0.23 <sup>def</sup>	2.42 ± 0.04 <sup>bcde</sup>	3.98	66.3 %
	<b>0.50 %</b>	0.46 ± 0.40 <sup>f</sup>	0.81 ± 0.42 <sup>def</sup>	1.27	84.3 %	1.70 ± 0.33 <sup>cdef</sup>	1.96 ± 0.14 <sup>cde</sup>	3.66	69.0 %
<b>40 E</b>	<b>0.05 %</b>	1.23 ± 0.20 <sup>bcdef</sup>	1.40 ± 0.13 <sup>bcde</sup>	2.63	67.6 %	3.13 ± 0.32 <sup>b</sup>	3.38 ± 0.42 <sup>b</sup>	6.51	44.9 %
	<b>0.20 %</b>	1.21 ± 0.03 <sup>bcdef</sup>	1.10 ± 0.07 <sup>cdef</sup>	2.31	71.5 %	2.96 ± 0.50 <sup>b</sup>	2.24 ± 0.14 <sup>bcde</sup>	5.20	56.0 %
	<b>0.50 %</b>	1.45 ± 0.10 <sup>bcd</sup>	1.76 ± 0.31 <sup>bc</sup>	3.21	60.4 %	2.79 ± 0.43 <sup>b</sup>	2.60 ± 0.36 <sup>bcde</sup>	5.39	54.4 %

Each value is represented as mean ± standard deviation ( $n = 3$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

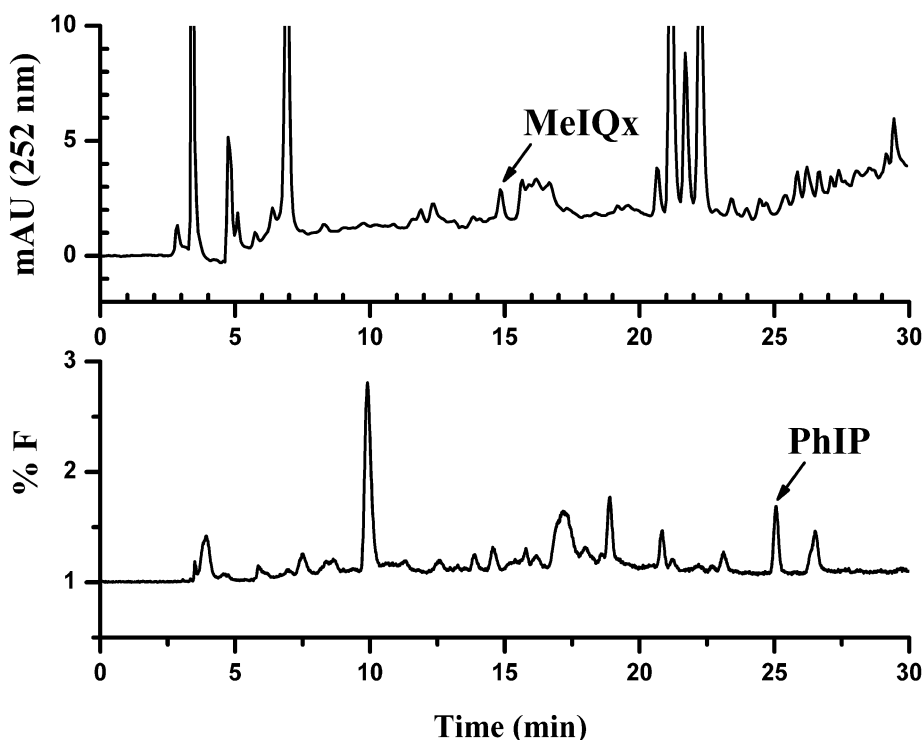


Figure 21. Typical HPLC chromatogram of heterocyclic amines detected with a UV detector (252 nm) for MeIQx, and a fluorescence detector for PhIP from cooked beef patty (excitation 229 nm, emission 437 nm).

and 3.14 to 4.89 ng/g PhIP when the cooking condition was changed from 375 °F/6min to 400 °F/5min. Similar studies by Balogh and others (2000) reported that the total amount of HCAs formed at 200 °C (392 °F)/6 min was three times greater than the total amount of HCAs formed at 175 °C (347 °F)/10 min while Tsen and others (2006) reported the amount of total HCAs formed at 204 °C (400 °F)/7.5 min were three times greater than the total HCAs formed at 191 °C (375 °F)/5 min. The high quantity of HCAs measured in beef patties grilled at higher temperatures might be explained by the mass-transport controlled phenomenon (Kovácsné and

others 2006). Using higher temperatures during cooking increases the rate of mass transfer of water containing precursors moving from the center to the high temperature surface of the patties, causing higher HCAs formation in cooked beef patties (Persson and others 2008).

Table 23 summarizes the HCAs content (MeIQx, PhIP, and total) in cooked beef patties with the addition of different types and levels of rosemary extract along with their percentage of inhibition relative to controls. All the extracts showed an inhibiting effect by significantly decreasing HCAs formation ( $p < 0.05$ ) at both cooking conditions. In our experiment, three levels of rosemary extracts were added: 0.05%, 0.2%, and 0.5%. The addition of rosemary extracts to patties at the lowest level (0.05%) seems to effectively inhibit HCAs formation; most of the results presented here statistically reveal that there is no difference in effect on the inhibition of HCAs between different concentrations. Balogh and others (2000) reported a similar reduction of HCAs formation after the addition of vitamin E at two levels, 1% and 10% level (fat basis), to beef patties before cooking; the greatest inhibition of HCAs formation in fried beef patties was observed when a 1% level of vitamin E was added.

When cooking at 375 °F/6min, all rosemary extracts significantly ( $p < 0.05$ ) decreased the levels of MeIQx and PhIP as compared to the controls. It seems that the rosemary extract 30E was responsible for the highest decrease in the total HCAs, but according to statistical analysis, most of the results showed no significant difference among them in HCAs reduction. On the other hand, when cooking at a higher temperature 400 °F/5min not only did all the extracts show a significant ( $p < 0.05$ ) decrease in the levels of MeIQx and PhIP as compared to control, but there were also significant differences among them. Apparently, rosemary extracts 10E and 20E were superior to rosemary extracts 100W, 30E, and 40E in the inhibition of HCAs

(both MeIQx and PhIP) at all three extract concentrations. Rosemary extract 20E showed the greatest inhibition of MeIQx (up to 91.7%), PhIP (up to 85.3%), and total (up to 84.3%). The variability in how much the HCAs were inhibited at different temperature levels may be due to the fact that the rise in temperature may induce changes in some antioxidant molecules, affecting a change in the mechanism of initiation and/or propagation properties, and thus improving their effectiveness as antioxidants (Chen and Ho 1997).

The rosemary extracts 10E and 20E possessed high antioxidant activity (low  $IC_{50}$  value) and showed the highest inhibition of HCAs formation among the five extracts. Interestingly, rosemary extracts 10E and 20E contain a mixture of three polyphenolic compounds (rosmarinic acid, carnosol, and carnosic acid), but rosemary extracts 100W and 40E contain only one major polyphenolic compound (rosmarinic acid for rosemary extract 100W and carnosol for rosemary extract 40E). Our results are supported by the study of Wada and others (2004), which reported that the non-water soluble rosemary extracts rich in carnosol along with the presence of a small amount of carnosic acid and other phenolic antioxidants had a greater quenching effect than the water soluble rosemary extract containing only rosmarinic acid on superoxide anion, singlet oxygen, hypochlorite anion, and linolenic acid peroxide. This phenomenon may be due to the presence of a combination of different antioxidants, which might act additively and even synergistically. Although no study regarding a synergistic antioxidant effect between the presence of single phenolic compounds in rosemary extracts have been done so far, there have been several studies reporting a synergistic effect between phenolic compounds in rosemary and other compounds. Fuhrman and others (2000) reported that the combination of rosmarinic acid and carnosic acid with lycopene, vitamin E, and glabridin effectively produced a synergistic



antioxidant effect on LDL oxidation. Romano and others (2009) reported that rosemary extract containing 30% carnosic acid, 16% carnosol, and 5% rosmarinic acid enhances the antioxidant efficiency of BHA and BHT, allowing a decrease in the amounts of the synthetic compounds used up to 17 fold.

The use of phenolic antioxidants to inhibit HCAs in cooked beef patties is well documented. The inhibitory effect of rosemary extracts on HCAs in our experiment was as good or better than that achieved by other phenolic antioxidants. Britt and others (1998) reported that total HCAs were reduced by 79% by adding tart cherry tissue at an 11.5% level. Four spices (rosemary, thyme, sage, and garlic) were already shown to effectively inhibit the content of HCAs below 60% of the amount found in the control (Murkovic and others 1998). The direct addition of vitamin E (1% based on fat content) to the surface of beef patties before frying reduced HCAs by up to 75% (Balogh and others 2000). Persson and others (2004) showed that adding small amounts of carbohydrate such as potato starch or potato fiber significantly reduced the amount of HCAs. Rosmarinic acid and rosemary extract were found to effectively decrease the formation of HCAs in beef patties by up to 69% (Tsen and others 2006). Recently, four fruit extracts (apple, elderberry, grape seed, and pineapple) rich in polyphenols were found to be significantly effective at reducing the amount of HCAs formed (Cheng and others 2007). Data showing the inhibition of HCA formation in the presence of phenolic antioxidants strongly support the hypothesis that the mechanism responsible for the formation of HCAs involves the unstable free radical Maillard intermediates reactions. This is supported by the fact that the phenolic antioxidants act as antioxidants by trapping or quenching the unstable free radicals.

## CONCLUSIONS

This study demonstrated that the use of rosemary extract as an additive can effectively inhibit the formation of HCAs in beef patties cooked at 191 °C (375 °F) for 6 min each side and 204 °C (400 °F) for 5 min each side. The inhibiting effect of the extract was affected by the extraction solvent used in the extraction process. Among five rosemary extracts, which included 100W (100% water extract), 10E (10% ethanol extract), 20E (20% ethanol extract), 30E (30% ethanol extract), and 40E (40% ethanol extract), rosemary extracts 10E and 20E showed the greatest inhibition of the formation of HCAs, which corresponded to their containing the highest level of antioxidant activity based on a DPPH scavenging assay. A synergistic antioxidant effect between phenolic compounds in rosemary may play an important role in HCAs inhibition.

The data obtained in this study suggest that the addition of rosemary extracts is an important factor in decreasing carcinogenic compounds in cooked beef patties. The use of rosemary extracts, which have less volatile compounds than natural rosemary, provides the additional advantage of not affecting the flavor or odor of meat products.

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**PART V. HETEROCYCLIC AMINE CONTENT**  
**IN ENHANCED AND MARINATED MEAT PRODUCTS**

**ABSTRACT**

Heterocyclic amines (HCAs) which are produced in meats cooked at high temperature are potent mutagens and a risk factor for human cancers. This study evaluated the effect of enhancement and marination on HCA formation in meat products. Three HCAs were identified in all samples: PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine), MeIQx (2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline), and DiMeIQx (2-amino-3,4,8-trimethylimidazo [4,5-*f*]quinoxaline). Addition of salt and phosphate greatly improved water-holding capacity and decreased HCA formation up to 58% in enhanced fresh meat products. More reduction of HCAs (up to 79%) was found in marinated fresh meat; the enhancement solution for this meat contained ingredients that exhibit good antioxidant properties. These results can be used by consumers as a guideline for making a purchase decision and by the meat industry to modify formulation processes to minimize HCA levels in meat products.

## **INTRODUCTION**

A high cooking loss has been found to be related to the formation of large amounts of HCAs (Knize and others 1994, Skog and others 1995); thus, reducing the cooking loss is likely to reduce HCA formation in meat products. Persson and others (2003) showed that addition of water-binding ingredients (1.5% sodium chloride and 0.3% sodium tripolyphosphate) to beef burgers improved the cooking loss and also decreased the formation of PhIP, MeIQx, and DiMeIQx when beefburgers were fried at 180 °C and 220 °C. Another treatment to inhibit HCA formation involves the use of various antioxidants, which serve as free-radical scavengers; this treatment is effective because the HCAs are formed in the Maillard reaction through the free radicals pyridine or pyrazine (Knize and others 1994).

Case-ready fresh meat products are defined as products that come in a packaged state from the supplier and are not repackaged at the store (Belcher 2006). The prevalence of case ready products has grown at a tremendous rate, increasing from 50% in 2002 to 64% of total fresh meat packages in 2007 (Baczwaski and Mandigo 2003, Belcher 2006), and the number is expected to rise above 70% by 2010 (Young 2009). Two technologies that are commonly used in case-ready meat products are enhancement and marination. Enhancement is the process of injecting a solution of water, salt, and sodium phosphates that typically adds 7 to 15% addition to the beginning weight of fresh meat to improve the eating quality (juiciness, tenderness, and flavor) of the final product (Baczwaski and Mandigo 2003, Sheard and Tali 2004, Knock and others 2006). Marination expands the solution by using ingredients with additional flavor and texture profiles. A marinade typically contains the same ingredients as an enhancement solution plus color and flavor components such as caramel coloring and dressings with spices



(Baczwaski and Mandigo 2003). The Sealed Air Corporation, the National Cattlemen's Beef Association, and the National Pork Board assessed case-ready meat products across the country. Within enhanced meat products, pork had the greatest number of products (35%), followed by chicken (19%), beef (13%), and turkey (6%); within marinated meat products, pork also had the greatest number of products (42%), followed by beef (30%), chicken (16%), and turkey (12%) ("Today's Retail Meat Case" 2007)

Non-meat ingredients used in enhanced and marinated meat products play various roles. Water is used mainly to dissolve other non-meat ingredients and increase yield; it also contribute juiciness and tenderness (Miller 1998). Salt and phosphate are sometimes used alone but often are used in combination to provide a synergistic action (Sheard and Tali 2004). Salt is added at low levels (0.1 to 3.0%) to increase water-holding capacity, improve texture by solubilizing of meat myofibrillar proteins, and improve flavor (Miller 1998, Baczwaski and Mandigo 2003). Salt concentration is not a regulated ingredient but is self-limiting because high concentrations will negatively affect palatability of the products (Alvarado and Mckee 2007). Sodium phosphates increase water-holding capacity by increasing meat pH and also by interacting with meat myofibrillar proteins to increase their ability to hold water inside the meat during cooking, resulting in increased juiciness and tenderness of cooked meat products (Miller 1998, Baczwaski and Mandigo 2003, Dušek and others 2003). There are different forms of sodium phosphates available, such as sodium hexametaphosphate, sodium tripolyphosphate, and tetrasodium pyrophosphate. Sodium tripolyphosphate was reported to have the best effect in retaining the additional water associated with solution enhancement in meat products (Baublits and others 2006). When phosphates are used to increase water-holding capacity properties of

meat, USDA requires that phosphate concentrations are no higher than 0.5% of the finished product weight (Alvarado and Mckee 2007).

Although some research has been conducted regarding methods to minimize HCA formation in cooked meat products, details of the effects of increasing water-holding capacity of fresh meats by means of enhancement and marination on HCA formation are still lacking. The aim of this study was to study the effect of enhancement and marination on HCA formation in cooked meat products. In the first experiment, commercially available enhanced and marinated products were selected and HCA levels were analyzed after the products were cooked. In second experiment, pork loin samples were manufactured in our meat laboratory to study the effect of the addition of salt/phosphate and rosemary extract on HCA formation. Results from our study can help consumers make a purchase decision that will minimize their exposure to HCAs. These results also can be used as guidelines for the meat industry on how to modify a formulation process to minimize HCA formation in cooked meat products.

## **MATERIALS AND METHODS**

### **Chemicals**

The HCA standards IQ (2-amino-3-methyl-imidazo [4,5-*f*]quinoline), IQx (2-amino-3-methyl-imidazo [4,5-*f*]quinoxaline), MeIQ (2-amino-3,4-dimethyl-imidazo [4,5-*f*]quinoline), MeIQx (2-amino-3,8-dimthylimidazo [4,5-*f*]quinoxaline), 4,8-DiMeIQx (2-amino-3,4,8-trimethyl-imidazo [4,5-*f*]quinoxaline), TriMeIQx (2-amino-3,4,7,8-tetramethyl-imidazo [4,5-*f*]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine) were obtained from Toronto Research Chemicals (Toronto, Canada). Ammonium acetate and triethylamine

were purchased from Aldrich Chemicals (Milwaukee, WI, USA). Phosphoric acid was obtained from Sigma Chemicals (St. Louis, MO, USA). Deionized water was processed by a Sybron/Branstead PCS unit (Barnstead/Thermolyne, Dubuque, IA, USA). The solid-phase extraction Extrelut NT 20 columns and diatomaceous earth refill material were purchased from VWR International (Bristol, CT, USA). Bond Elut propyl-sulfonic acid (PRS) cartridges, C-18 cartridges, and the coupling adaptors were purchased from Varian Sample Preparation (Harbor City, CA, USA). Trichloroacetic acid, diacetyl, 1-naphthol, Folin-Ciocalteu's reagent, 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), sodium carbonate, gallic acid, and sodium hydroxide were obtained from Sigma Aldrich (St. Louis, MO). Solvents and chemicals such as acetonitrile (high-performance liquid chromatography [HPLC] grade), methanol (HPLC grade), and sodium hydroxide (ACS-grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Rosemary extract (FORTIUM<sup>®</sup> R-WS10 LIQUID) was obtained from Kemin Industries, Inc (Des Moines, IA, USA).

### **Commercial meat products**

Three types of commercial products (non-enhanced/marinated products, enhanced products, and marinated products) in each meat species (beef, chicken, and pork) were purchased from local grocery stores. Selected beef products consisted of non-enhanced/marinated beef (control), 12% enhanced beef, and peppercorn-marinated beef. Selected pork products consisted of non-enhanced/marinated pork (control), 12% and 30% enhanced pork, peppercorn-marinated pork, and apple bourbon-marinated pork. Selected chicken products consisted of non enhanced/marinated chicken breast without skin (control),

15% enhanced chicken breast without skin, and BBQ-marinated chicken breast without skin. The description and ingredients of each product are shown in Table 24.

### **Experimental enhanced pork samples**

Enhanced pork samples were prepared at the Kansas State University meat laboratory by using a multi-needle brine injector. Two individual loins were selected, and each was divided into four sections. Each of section was randomly assigned to one of four treatments: (1) no injection, (2) injection with 12% water, (3) injection with 12% enhancement brine (0.4% sodium chloride and 0.35% sodium tripolyphosphate), or (4) injection with 12% enhancement brine (0.075% rosemary extract). After pumping, loins were vacuum packed and held for 72 h at 4 °C to allow of the injected solution to equilibrate throughout the loins. The experiment was replicated three times.

### **Sample preparation**

The following commercial products were used as obtained: control and 12% enhanced beef, control and 12% enhanced pork, and control, 15% enhanced, and BBQ-marinated chicken. Peppercorn-marinated beef, 30% enhanced pork, peppercorn-marinated pork, and apple bourbon-marinated pork were manually sliced at a thickness of 2 cm and then stored at 4 °C. For the enhanced pork that was prepared in our meat laboratory, after the equilibration, loins were sliced at a thickness of 2 cm with a meat slicer (Cabela's commercial grade slicer, 1/3 hp, Sidney, NE, USA) and then stored at 4 °C before cooking. The samples used for chemical analyses were further chopped and ground with a food processor (KitchenAid, model KFP 750)

Table 24. Description and ingredients in selected non-enhanced, enhanced, and marinated meat products.

<b>Product</b>	<b>Description</b>	<b>Ingredients</b>
Non-enhanced beef	Beef loin steak boneless	-
12% enhanced beef	Beef loin New York strip steak	Water, sodium chloride, sodium phosphates
Peppercorn-marinated beef (30% enhancement)	Extra beef shoulder filets	Water, potassium lactate, partially hydrogenated soybean oil, salt, butter flavoring (maltodextrin, butter flavor, annatto and turmeric color), sugar, spice, sodium phosphates, dehydrated parsley, sodium diacetate, flavoring
Non-enhanced pork	Pork center cut loin filet	-
12% enhanced pork	Pork tenderloin	Water, potassium lactate, sodium phosphates, salt, sodium diacetate
30% enhanced pork	Pork center cut loin filet	<b>Solution ingredients:</b> pork broth, potassium lactate, partially hydrogenated soybean oil, cornstarch, sodium phosphates, sodium bicarbonate, salt, autolyzed yeast, sodium diacetate <b>Sauce ingredients:</b> water, corn starch, xanthan gum
Peppercorn-marinated pork (30% enhanced)	Extra lean pork tenderloin	<b>Solution ingredients:</b> water, potassium lactate, partially hydrogenated soybean oil, black pepper, sodium phosphates, caramel color, flavoring, white vinegar, sodium diacetate <b>Sauce ingredients:</b> water, cornstarch, xanthan gum

Table 24. Continued

<b>Product</b>	<b>Description</b>	<b>Ingredients</b>
Apple bourbon-marinated pork (30% enhanced)	Extra lean pork tenderloin	<b>Solution ingredients:</b> water, seasoning (brown sugar, tomato powder, dextrose, salt, sugar, maltodextrin, vinegar, onion powder, garlic powder, natural and artificial flavor spices, modified food starch, sodium diacetate, malic acid, mustard seed, partially hydrogenated soybean oil, turmeric), potassium lactate, sodium phosphates, applewood smoke flavoring, sodium diacetate, partially hydrogenated soybean oil, bourbon flavor (natural flavors, glycerin, propylene glycol, ethyl alcohol) <b>Sauce ingredients:</b> water, cornstarch, xanthan gum
Non-enhanced chicken	Boneless skinless chicken breast	-
15% enhanced chicken	Boneless skinless chicken breast	Natural chicken broth
BBQ-marinated chicken	Boneless skinless chicken breast	Sugar, salt, paprika and other spices, monosodium glutamate, dehydrated garlic and onion, wheat flour, natural flavor, dehydrated orange peel, dehydrated bell pepper, caramel color, hydrolyzed vegetable protein, citric acid, dehydrated molasses, extractive of paprika and other spices, tamarinds

and refrigerated at 4 °C before analysis.

## **Chemical analyses**

### *pH measurement*

The pH of sample was measured according to the method of Jang and others (2008) with slight modifications. Five grams of fine ground sample was added to 45 mL of distilled water and blended for 30 s at medium speed in a Waring blender (Waring Laboratory, Torrington, CT, USA). The pH of each sample was measured with an Accumet AP115 portable pH meter (Fisher, Pittsburgh, PA, USA).

### *Creatine determination*

Creatine content was determined according to the method described by Polak and others (2009) with slight modifications. A 0.25-g finely ground sample was homogenized for 5 min at 9500 rpm (IKA, Ultra-Turrax T18) (Wilmington, NC, USA) in 100 mL trichoroacetic acid (30 g/L in distilled water), and then the samples were filtered through Whatman #4 filter paper. Twenty milliliters of the filtrate was defatted with 10 mL diethylether, and then samples were shaken vigorously and allowed to stand for 10 min to separate the phases. After the phases were separated, 4 mL of defatted extract (bottom layer) was mixed with 2 mL of diacetyl (0.2 g/L in distilled water) and 2 mL of 1-naphthol (25 g/L in 20 g/L of sodium hydroxide solution). The mixture heated for 5 min at 40 °C. Each sample's absorbance was measured at 520 nm against a reagent blank. The creatine content was expressed as milligram per gram of meat sample.

### *Total phenolic content determination*

The total phenolic content was determined using Folin-Ciocalteu's reagent according to the method described by Jang and others (2008) with modifications. A 5-g sample of finely ground meat in 80% ethanol (100 mL) was homogenized for 2 min at 9500 rpm (IKA, Ultra-Turrax T18) (Wilmington, NC, USA). The samples were filtered through Whatman #1 filter paper. Two hundred milliliters of filtrate was mixed with 2 mL of deionized water in a test tube, 200  $\mu$ L of Folin-Ciocalteu reagent was added, and the tubes were allowed to stand for 6 min at room temperature. Then 1 mL of 7.5% sodium carbonate solution was added and mixed thoroughly. The mixture was stored in the dark for 2 h at room temperature. Each sample's absorbance was measured at 765 nm with a UV/VIS spectrophotometer. A standard curve was evaluated from 0 to 100  $\mu$ g of gallic acid per milliliter. The total phenolic content was expressed as milligram gallic acid equivalents per one gram of meat sample.

### *DPPH radical scavenging activity*

DPPH scavenging activity was determined using the method of Jang and others (2008) with modifications. A 5-g sample of finely ground meat in 80% ethanol (100 mL) was homogenized for 2 min at 9500 rpm (IKA, Ultra-Turrax T18) (Wilmington, NC, USA). The samples were filtered through Whatman #1 filter paper. Each 500  $\mu$ L of filtrate was mixed with 2.5 mL of 0.1 mM freshly prepared DPPH methanolic solution and stored in the dark for 30 min at room temperature before absorbance was measured at 517 nm. Ethanol (95%) was used as a blank. The control solution consisted of 0.1 mL of 95% ethanol and 2.9 mL of DPPH solution. The radical scavenging activity was expressed as the inhibition percentage and calculated using



the following equation:

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

### **Cooking procedure and cooking loss**

Sliced commercial meat products and manufactured pork loins were removed from refrigerator and allowed to approach room temperature before they were cooked (Infrared thermometer model ST20XE, Raytek, Santa Cruz, CA, USA). Spear point thermocouple temperature probes were inserted horizontally to the midpoint of samples, and temperature was monitored with a data logger (USB-TC model, Measurement Computing, Norton, MA, USA). Each meat sample was cooked in a Teflon-coated frying pan at surface temperature 204 °C (400 °F) to an internal core temperature of 77 °C (170 °F). The cooked sample was allowed to cool at room temperature for approximately 30 min, and then cooking loss was determined using the following equation:

$$\% \text{ cooking loss} = [(\text{before cook weight} - \text{after cook weight}) / \text{before cook weight}] \times 100$$

### **Extraction and analysis of HCAs**

The HCAs were extracted from meat samples and purified using the method described by Gross and Grüter (1992) except that ethyl acetate was used as the extraction solvent (Santos and others 2004, Smith and others 2008). Each sample (3 g) was homogenized with 12 mL of 1 M NaOH in a commercial Waring blender (Fisher, Pittsburgh, PA, USA). The homogenate was then mixed with 24 g of Extrelut refill material (Merck, Darmstadt, Germany) and poured into an empty Extrelut 20 column. For determination of recovery, selected homogenate samples were

spiked with 50 ng of each of the HCA standards. The HCAs were eluted from the Extrelut columns with 60 mL ethyl acetate into a PRS cartridge conditioned with 7 mL of ethyl acetate. The PRS cartridge was then rinsed with 6 mL of 0.1 M HCl, 15 mL of methanol/0.1 M HCl (45:55 v/v), and 2 mL of distilled water to wash out the nonpolar HCAs and other impurities. The HCAs were eluted from the PRS cartridge with 20 mL of 0.5 M ammonium acetate pH 8.5 into 100-mg C-18 cartridges preconditioned with 5 mL of methanol followed by 5 mL of distilled water. The HCAs were then eluted from the C-18 cartridge with 1 mL of methanol/ammonium hydroxide (9:1, v/v) into the vial. The HCA extract was concentrated until dry under a stream of nitrogen and dissolved in 25  $\mu$ L of methanol before it was injected into the HPLC. The HCAs were analyzed on an HP1090A Series II HPLC (Agilent Technologies) coupled with a photodiode array UV-visible detector (HP 1040) and an HP 1046A programmable fluorescence detector. The HCA separation was performed on a reversed-phase TSK gel ODS-80 TM column (25 cm  $\times$  4.6 mm, 5  $\mu$ m, 80  $\text{Å}$ , Tosohass, Montgomeryville, PA., USA) with a mobile phase of 0.01 M triethylamine pH 3.6 (A) and acetonitrile (B). The HCA separation was achieved using a linear gradient that started with 95% A and 5% B and changed to 75% A and 25% B in 30 min at a flow rate of 1 mL/min and a column temperature of 40  $^{\circ}$ C. After 30 min, the mobile phase returned to its original ratio (95% A, 5 % B) for 10 min to allow the column to reequilibrate before the next injection. The UV detector was set at 252 nm for IQ, IQx, MeIQ, MeIQx, and DiMeIQx, and the fluorescence detector was programmed accordingly to the excitation/emission wavelengths of 229 and 437 for PhIP. Data were analyzed with an HP 9000 series 300 Chemstation. The identities of HCA peaks were confirmed by comparing the

retention times and the UV absorbance spectrum of each peak with library spectra acquired from standard solutions.

### Quantitation, recovery, and spectral matching

The HCA concentrations were quantitated by the internal standard method (Lindsay 1992). A known amount of TriMeIQx (used as internal standard) was added to samples before they were injected into the HPLC. The relative responses (R) of HCA standards were calculated using the following equation:

$$R = (C/A)/(C_s/A_s)$$

where C = Concentration of HCA standards

A = Peak area for HCA standards

C<sub>s</sub> = Concentration of internal standard

A<sub>s</sub> = Peak area of internal standard

The HCA concentrations in samples were calculated using the following equation:

$$C_u = A_u \times R \times (C_s'/A_s')$$

where C<sub>u</sub> = Concentration of HCAs in sample

A<sub>u</sub> = Peak area of HCAs in sample

C<sub>s</sub>' = Concentration of internal standard in sample

A<sub>s</sub>' = Peak area of internal standard in sample

The limits of detection (LOD) and limits of quantification (LOQ) for the HCAs were 0.15 ng/g and 0.45 ng/g for IQ, IQx, MeIQ, MeIQx, DiMeIQx and PhIP. The HCA identities were verified in the cooked meat extracts by online UV spectral matching to a spectral library

made from pure standards. Match factors typically were observed at 95% or greater (Tsen and others 2006). Average recoveries for the HCAs were 72% for IQx, 61% for IQ, 63% for MeIQ, 68% for MeIQx, 60% for DiMeIQx, and 65% for PhIP. The recoveries of MeIQx and PhIP are in agreement with previous reports from this laboratory (Tsen and others 2006, Smith and others 2008) and from Cheng and others (2007).

### **Statistical analyses**

The experimental design was a randomized complete block with repeated measurements, and each experiment was replicated three times. Duplicate measurements taken on the same experimental unit were averaged for statistical analysis. All statistical significance tests were analyzed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA, 2002). Data were examined by analysis of variance (ANOVA) followed by Tukey's multiple comparison test, and means were considered significant at  $p < 0.05$ .

## **RESULTS AND DISCUSSION**

### **Commercial meat products**

#### *Beef products*

There were many enhanced fresh beef products available at the local grocery stores; however, only a few marinated beef products were available (e.g., peppercorn-marinated beef filet and teriyaki-marinated beef filet). The commercial non-enhanced/marinated (control), 12% enhanced, and peppercorn-marinated (30% enhancement) beef products were selected for this

study. Salt and sodium phosphate are ingredients in both the 12% enhanced and marinated beef products. Table 25 summarizes the results of chemical analyses and HCA quantitative determinations in the selected beef products. The pH of peppercorn-marinated beef was higher than that of control and 12% enhanced beef. The higher pH of peppercorn marinated beef may be due to the presence of potassium lactate, sodium diacetate, or other ingredients (Table 25). Cooking loss in peppercorn-marinated beef, which was injected with a 30% solution, was the highest among the three products, and cooking loss of 12% enhanced beef was higher than that of the control. There was small but significant difference ( $p < 0.05$ ) in total phenolic content of the peppercorn-marinated beef in compared with the control and 12% enhanced beef. In contrast, DPPH radical scavenging activity was slightly higher for peppercorn-marinated beef than for control and 12% enhanced beef. Levels of HCAs in control, 12% enhanced, and peppercorn-marinated beef products are shown in Table 25. Total HCAs were higher for control than for 12% enhanced beef and peppercorn-marinated beef, and there were no statistically significant differences between 12% enhanced and peppercorn-marinated beef products. In all three products, PhIP was found at the highest levels, followed by MeIQx and DiMeIQx. There were no statistically differences in MeIQx and PhIP among these three products; DiMeIQx was higher in control than in 12% enhanced beef and was not detected in peppercorn-marinated beef.

Taken together the results for beef products indicate that enhancement and marination of beef could reduce HCA formation in cooked products up to 20% compared with control products. In the present study, adding spices (peppercorn) did not help reduce HCA formation.

Table 25. Chemical analyses (pH, total phenolic, and antioxidant activity), cooking loss, and HCA contents in non-enhanced, enhanced, and marinated commercial beef products.

Product		pH	Cooking loss (%)	Total phenolic (mg GAE/g sample)	DPPH radical scavenging activity (%)	Heterocyclic amines (ng/g)			
						MeIQx	DiMeIQx	PhIP	Total
Non-enhanced beef	Mean	5.52 <sup>b</sup>	32.53 <sup>c</sup>	1.33 <sup>a</sup>	11.27 <sup>b</sup>	3.87 <sup>a</sup>	1.28 <sup>a</sup>	6.60 <sup>a</sup>	11.74 <sup>a</sup>
	SD	0.04	0.95	0.02	0.60	0.31	0.25	0.27	0.51
12% enhanced beef	Mean	5.70 <sup>b</sup>	36.37 <sup>b</sup>	1.35 <sup>a</sup>	7.68 <sup>c</sup>	2.59 <sup>a</sup>	0.67 <sup>b</sup>	6.03 <sup>a</sup>	9.30 <sup>b</sup>
	SD	0.07	1.17	0.01	0.40	1.15	0.11	0.72	0.37
Peppercorn-marinated beef (30% enhancement)	Mean	6.15 <sup>a</sup>	42.98 <sup>a</sup>	1.22 <sup>b</sup>	13.33 <sup>a</sup>	3.13 <sup>a</sup>	0.00 <sup>c</sup>	6.16 <sup>a</sup>	9.29 <sup>b</sup>
	SD	0.28	2.15	0.01	0.19	0.33	0.00	0.28	0.07

Each value is represented as mean  $\pm$  standard deviation ( $n = 3$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

### *Pork products*

At local grocery stores that we visited, there were more enhanced fresh pork products available than non-enhanced fresh pork products. Within meat species, enhanced pork products are more available in the market than enhanced beef and chicken products. Most of the major pork manufacturers offer case-ready enhanced pork products that are available in modified-atmosphere packaging and vacuum packaged; typical enhancement levels are 12 to 30%. Loin and tenderloin are used the most for manufacturing marinated pork products with high enhancement level (up to 30%) because they contain less fat and are very easy to dry out during cooking. Many types of marinated pork products are available in the market (e.g., peppercorn, teriyaki, lemon garlic, apple bourbon, masquite barbecue, honey mustard, teriyaki, and onion and garlic roast), which allows retailers to satisfy unique customer requests and meet demand from ethnic populations. Interestingly, for high-enhancement-level products (30%), cornstarch and xanthan gum were added to these products as sauce ingredients; which are believed to improve cooking loss and retain the flavor solution inside the products during cooking (Baczwaski and Mandigo 2003, Somboonpanyakul and others 2007). Potassium lactate and sodium diacetate, which increase shelf life, were also found in these products (Blom and others 1997, Baczwaski and Mandigo 2003).

The non-enhanced (control), 15% and 30% enhanced, and peppercorn- and apple bourbon-marinated pork products were selected for this study. The 12% and 30% enhanced pork products were selected to determine the effect of enhancement level on HCA formation. The two marinated pork products, peppercorn and apple bourbon, were selected because of their different ingredients. Apple bourbon-marinated pork contains more types of ingredients than

peppercorn-marinated pork, so we expected to see the different effects of these ingredients on HCA formation when the products were cooked. Table 26 summarizes the results of chemical analyses and HCA quantitative determinations in the selected pork products. The pH was lower for apple bourbon-marinated pork than for other products; this may be due to the presence of vinegar, tomato powder, and malic acid (Table 26). There was no significant difference in cooking loss ( $p > 0.05$ ) between 30% enhanced pork and the two marinated porks, which also were enhanced at the level of 30%. However, the cooking loss of these three products was higher than that of control and 12% enhanced pork. There was not much difference in total phenolic content among the five pork products. The DPPH radical scavenging activity was higher for both marinated pork products than for control and 12% and 30% enhanced porks. Surprisingly, apple bourbon-marinated pork had a very high level of DPPH radical scavenging activity (58.8%) compared with other products (6.2 to 13.7%). Apple bourbon-marinated pork contained several ingredients that have been reported to have antioxidative effects, including tomato powder (George and others 2004, Toor and Savage 2005), onion powder (Nuutila and others 2003, Roldán and others 2008), garlic powder (Bozin and others 2008, Queiroz and others 2009), and turmeric (Kumar and others 2006, Cousins and others 2007). The level of HCAs in control, 12% and 30% enhanced, and marinated pork products are shown in Table 26. There was no significance difference in total HCA levels between 12% and 30% enhanced porks. Both marinated pork products had significantly lower total HCA levels than control and both enhanced pork products ( $p < 0.05$ ); total HCAs were lowest for apple bourbon-marinated pork. Antioxidant compounds in some ingredients (e.g., tomato powder, onion powder, garlic



Table 26. Chemical analyses (pH, total phenolic, and antioxidant activity), cooking loss, and HCA contents in non-enhanced, enhanced, and marinated commercial pork products

Product		pH	cooking loss (%)	total phenolic (mg GAE/g sample)	DPPH radical scavenging activity (%)	Heterocyclic amines (ng/g)			
						MeIQx	DiMeIQx	PhIP	Total
Non-enhanced pork	Mean	6.07 <sup>a</sup>	33.54 <sup>d</sup>	1.50 <sup>a</sup>	10.44 <sup>c</sup>	3.20 <sup>a</sup>	1.07 <sup>b</sup>	13.31 <sup>a</sup>	17.58 <sup>a</sup>
	SD	0.09	0.18	0.02	0.82	1.74	0.27	0.94	2.94
12% enhanced pork	Mean	6.11 <sup>a</sup>	34.92 <sup>cd</sup>	1.45 <sup>a</sup>	11.21 <sup>c</sup>	1.71 <sup>a</sup>	0.75 <sup>b</sup>	9.60 <sup>b</sup>	12.06 <sup>b</sup>
	SD	0.14	2.96	0.02	0.76	0.80	0.38	1.19	0.46
30% enhanced pork	Mean	6.21 <sup>a</sup>	40.47 <sup>ab</sup>	1.52 <sup>a</sup>	6.23 <sup>d</sup>	2.89 <sup>a</sup>	1.80 <sup>a</sup>	9.85 <sup>b</sup>	14.54 <sup>b</sup>
	SD	0.22	0.81	0.03	0.38	0.46	0.34	0.83	1.34
Peppercorn-marinated pork (30% enhancement)	Mean	5.84 <sup>a</sup>	42.78 <sup>a</sup>	1.30 <sup>b</sup>	13.67 <sup>b</sup>	0.88 <sup>a</sup>	0.60 <sup>b</sup>	5.83 <sup>c</sup>	7.32 <sup>c</sup>
	SD	0.18	0.11	0.02	0.49	0.39	0.03	0.27	0.57
Apple-bourbon marinated pork (30% enhancement)	Mean	5.39 <sup>b</sup>	40.75 <sup>ab</sup>	1.45 <sup>a</sup>	58.78 <sup>a</sup>	0.90 <sup>a</sup>	0.61 <sup>b</sup>	0.46 <sup>d</sup>	1.96 <sup>d</sup>
	SD	0.04	0.57	0.04	1.53	0.20	0.14	0.15	0.07

Each value is represented as mean ± standard deviation ( $n = 3$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

powder, turmeric, and mustard seed) of apple bourbon marinated pork are believed to play a role in inhibiting HCAs (Shishu and Kaur 2008, Janoszka 2010).

Taken together the results for pork products indicate that enhancement and marination of pork could reduce HCA formation in cooked products up to 30% compared with control products. Increasing the level of enhancement from 12% to 30% was unable to reduce HCA formation. Perhaps the meat could not retain all of the solution that was introduced into product; it is possible that most of the injected solution may be lost in the package or during the cooking process. Cooked marinated porks, in particular apple bourbon-marinated pork, contained the lowest levels of HCAs. We believe that these effects are caused by the presence of antioxidant compounds in the ingredients of these products. Many studies have reported that antioxidants successfully reduce the formation of HCAs in meat products because of their superior radical scavenging capability. Antioxidants that inhibit HCAs are formed through the condensation of creatinine and the Strecker degradation radicals (pyridines and pyrazines) generated from the reaction of glucose and amino acids during the Maillard reaction (Vitaglione and Fogliano 2004).

### *Chicken products*

At local grocery stores that we visited, enhanced fresh chicken products were more common than non-enhanced fresh chicken products. Chicken products marinated with different ingredients are available in case-ready packages, and also can be purchased at a deli counter. The commercial non-enhanced/marinated (control), 15% enhanced, and BBQ-marinated boneless/skinless chicken breasts were selected for this study. Interestingly, most of the

enhanced chicken products were injected with only natural chicken broth; salt and phosphate were not used as ingredients in the enhancement process. As indicated on the label, BBQ-marinated chicken was prepared with a dry rub method; however, we do not know if non-enhanced chicken or enhanced chicken was used in the preparation process. Table 27 summarizes the results of chemical analyses and HCA quantitative determinations in the selected chicken products. The pH of marinated chicken was higher than that of control and 15% enhanced chicken; this might be due to the presence of ingredients such as monosodium glutamate. The cooking loss of 15% enhanced chicken was higher than that of the control; however, there was no significance difference ( $p > 0.05$ ) between 15% enhanced and marinated chickens. Total phenolic content and DPPH radical scavenging activity were higher for marinated chicken than for control and 15% enhanced chicken. The high level of DPPH radical scavenging activity may be caused by the presence of paprika and its extract, dehydrated garlic and onion, and other spices in marinated chicken, which have been reported to have antioxidant capacities (Nuutila and others 2003, Materska and Perucka 2005, Bozin and others 2008, Roldán and others 2008, Queiroz and others 2009). The HCAs in control, 15% enhanced, and marinated chicken are shown in Table 27. There were no statistically significant differences in MeIQ<sub>x</sub>, DiMeIQ<sub>x</sub>, and PhIP levels among the three chicken products. There was no difference ( $p > 0.05$ ) in total HCAs between control and 15% enhanced chickens or between control and marinated chicken.

Table 27. Chemical analyses (pH, total phenolic, and antioxidant activity), cooking loss, and HCA contents in non-enhanced, enhanced, and marinated commercial chicken products.

Product		pH	cooking loss (%)	total phenolic (mg GAE/g sample)	DPPH radical scavenging activity (%)	Heterocyclic amines (ng/g)			
						MeIQx	DiMeIQx	PhIP	Total
non-enhanced chicken	mean	5.77 <sup>c</sup>	27.08 <sup>b</sup>	1.35 <sup>b</sup>	12.88 <sup>b</sup>	1.20 <sup>a</sup>	0.64 <sup>a</sup>	5.35 <sup>a</sup>	7.20 <sup>ab</sup>
	SD	0.01	1.35	0.02	0.84	0.25	0.11	0.76	0.86
15% enhanced chicken	mean	5.92 <sup>b</sup>	33.74 <sup>a</sup>	1.36 <sup>b</sup>	12.95 <sup>b</sup>	2.65 <sup>a</sup>	1.13 <sup>a</sup>	5.01 <sup>a</sup>	8.80 <sup>a</sup>
	SD	0.01	3.27	0.06	1.42	1.01	0.35	0.54	0.71
BBQ-marinated chicken	mean	6.00 <sup>a</sup>	28.47 <sup>ab</sup>	1.48 <sup>a</sup>	17.06 <sup>a</sup>	1.87 <sup>a</sup>	0.96 <sup>a</sup>	4.14 <sup>a</sup>	6.97 <sup>b</sup>
	SD	0.04	2.64	0.02	1.85	0.22	0.47	0.51	0.20

Each value is represented as mean  $\pm$  standard deviation ( $n = 3$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

Taken together the results for chicken products suggest that enhancement of chicken with only water (natural chicken broth) could not reduce HCA formation compared with control products. The higher level of DPPH radical scavenging activity of marinated chicken had no effect on HCA reduction compared with control and enhanced chicken; this may be because only a small amount of paprika and spices were put on the marinated products or because the dry rub method did not allow spices to penetrate into the meat. Injecting the solution and spices into marinated chicken may be more effective than the dry rub method.

The results for these commercial products indicate that enhancing meat products with water, salt, and phosphate can help meat retain water which results in a lower amount of HCA formation (as seen in enhanced beef and pork). Meat enhanced with only water without the addition of salt and phosphate (as seen in enhanced chicken) could not hold water very well; this increased the cooking loss and led to an increase in the amount of HCAs. However, this conclusion is based on the meat species represented in commercial products; meat type and other ingredients may influence HCA formation. Therefore, to gain a better understanding of the effect of the enhancement process on HCA formation, we decided to prepare and test enhanced pork loin product in our meat laboratory. We prepared three treatments: (1) no injection (control), (2) injection with water alone, (3) injection with a combination of water, salt, and phosphate, and (4) injection with a combination of water and rosemary extract.

## **Experimental enhanced products**

### *Chemical analyses*

The pH was significantly ( $p < 0.05$ ) higher for salt/phosphate-injected loin (pH 6.07) than for water-injected loin (pH 5.67), rosemary extract-injected loin (pH 5.71), and the control (pH 5.70) (Table 28). Creatine content of loins injected with water (4.60 mg/g), salt/phosphate (4.28 mg/g) and rosemary extract (4.30 mg/g) was slightly lower than that of the control (5.0 mg/g) (Table 28); perhaps the injected solution caused little dilution of the creatine level originally present in the loins.

Table 28. The pH and creatine levels in manufactured pork loins

<b>Treatment</b>	<b>pH</b>	<b>Creatine</b>
Control (no injection)	5.70 ± 0.12 <sup>b</sup>	5.00 ± 0.29 <sup>a</sup>
Water injection	5.67 ± 0.20 <sup>b</sup>	4.60 ± 0.34 <sup>b</sup>
Salt/phosphate injection	6.07 ± 0.17 <sup>a</sup>	4.28 ± 0.25 <sup>b</sup>
Rosemary extract injection	5.71 ± 0.13 <sup>b</sup>	4.30 ± 0.43 <sup>b</sup>

Each value is represented as mean ± standard deviation ( $n = 3$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

### *Cooking loss*

Cooking loss was defined as the weight difference of the loins before and after cooking. There was no significance difference ( $p > 0.05$ ) in cooking loss between salt/phosphate-injected loin (26.4%) and the control (25.2%). This is in agreement with studies by Sheard and others (1999) and Hayes and others (2006). In contrast, cooking loss of water-injected loin (34.7%) and rosemary extract-injected loin (33.4%) was much higher than that of the control ( $p < 0.05$ ). We

concluded that salt and phosphate had a significant effect on cooking loss by helping meat retain water that was introduced into the product and, therefore, improving the cooking loss.

#### *Heterocyclic amine formation in cooked manufactured products*

Table 29 summarizes the results of HCA quantitative determinations in control, water-injected loin, salt/phosphate-injected loin, and rosemary extract-injected loin, and their HPLC chromatograms are shown in Figures 22 and 23. All of the cooked pork loins contained MeIQx, DiMeIQx, and PhIP, but IQ, IQx and MeIQ were not detectable in any samples. Total content of the three HCAs (MeIQx, DiMeIQx, and PhIP) present in all loins ranged from 9.50 to 22.34 ng/g. The predominant HCA in these loins was PhIP (7.54 to 15.05 ng/g), followed by MeIQx (1.57 to 8.21 ng/g) and DiMeIQx (0.40 to 2.71 ng/g). The amounts of PhIP, MeIQx, and DiMeIQx in water-injected loin were slightly higher than those in the control, but the differences were not statistically significant ( $p > 0.05$ ). In contrast, the amounts of PhIP, MeIQx, and DiMeIQx in salt/phosphate-injected loin were significantly lower than those in the control and water-injected loin ( $p < 0.05$ ). Injection of water with a combination of water and rosemary extract had lower HCA contents than injection of water alone ( $p < 0.05$ ). This is due to the presence of polyphenol compounds in rosemary extract that have been reported to have strong antioxidant effects, e.g. rosmarinic acid, carnosol and carnosic acid (Tsen and others 2006). Although amounts of total phenolic were not different among treatments, but the scavenging effect on DPPH radicals of rosemary extract-injected loin was significantly higher than that

Table 29. Heterocyclic amines (MeIQx, DiMeIQx, PhIP, and total) in manufactured pork loins

Treatment	Cooking loss <sup>1</sup> (%)	Heterocyclic amines (ng/g)			
		MeIQx	DiMeIQx	PhIP	Total
control (no injection)	25.23 ± 1.02 <sup>b</sup>	7.59 ± 0.77 <sup>a</sup>	1.64 ± 0.31 <sup>a</sup>	13.12 ± 0.68 <sup>a</sup>	22.34 ± 1.33 <sup>ab</sup>
water injection	34.69 ± 4.24 <sup>a</sup>	8.21 ± 1.61 <sup>a</sup>	2.71 ± 1.62 <sup>a</sup>	15.05 ± 1.35 <sup>a</sup>	25.98 ± 4.09 <sup>a</sup>
salt/phosphate injection	26.39 ± 2.63 <sup>b</sup>	1.57 ± 0.44 <sup>c</sup>	0.40 ± 0.06 <sup>b</sup>	7.54 ± 2.67 <sup>b</sup>	9.50 ± 2.48 <sup>c</sup>
rosemary injection	33.42 ± 3.11 <sup>a</sup>	5.33 ± 1.24 <sup>b</sup>	0.88 ± 0.32 <sup>b</sup>	12.15 ± 1.98 <sup>a</sup>	18.35 ± 3.08 <sup>b</sup>

Each value is represented as mean ± standard deviation ( $n = 3$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

<sup>1</sup> Cooking loss of samples was calculated based on weight of sample after the solution was injected and before cooking.



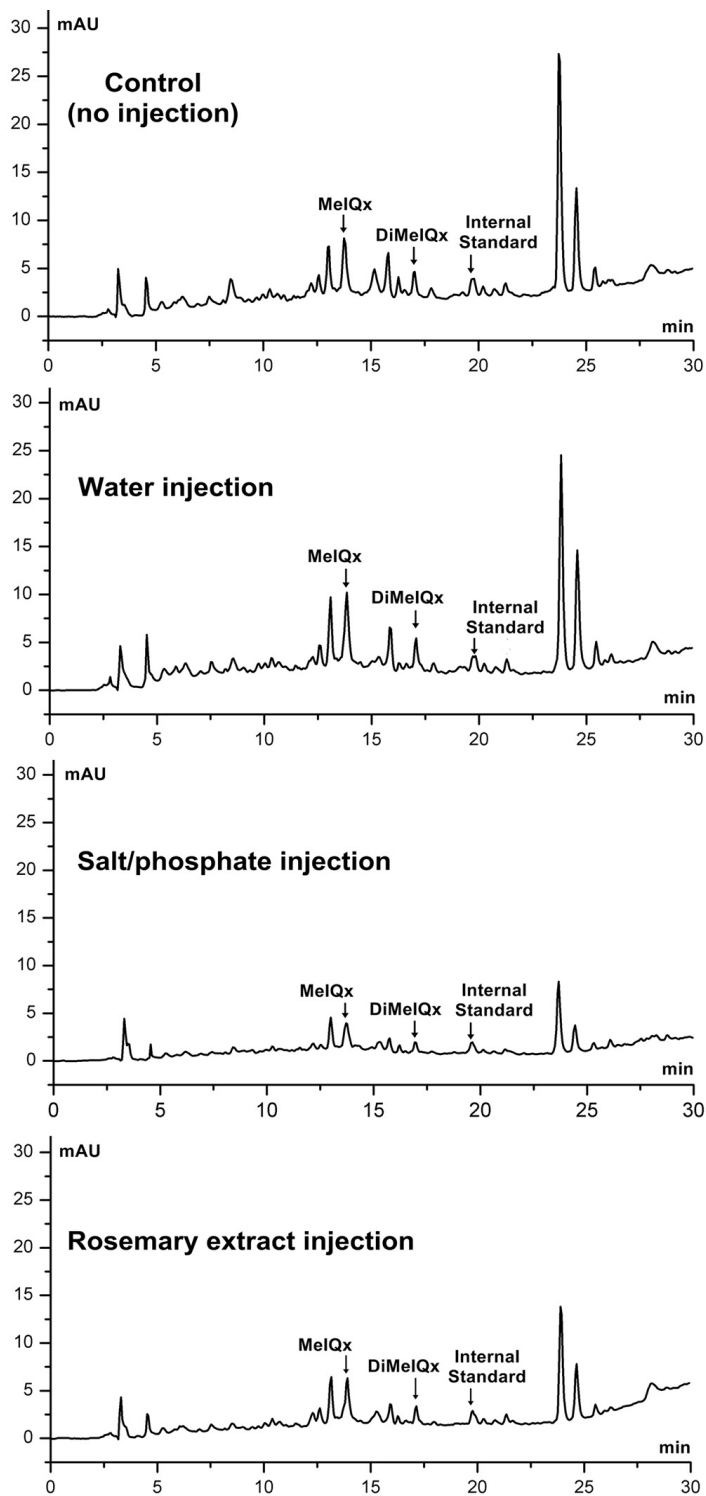


Figure 22. UV chromatogram of HCAs (MeIQx, DiMeIQx) in control, water-injected loin, and salt/phosphate-injected loin. TriMeIQx used as internal standard.

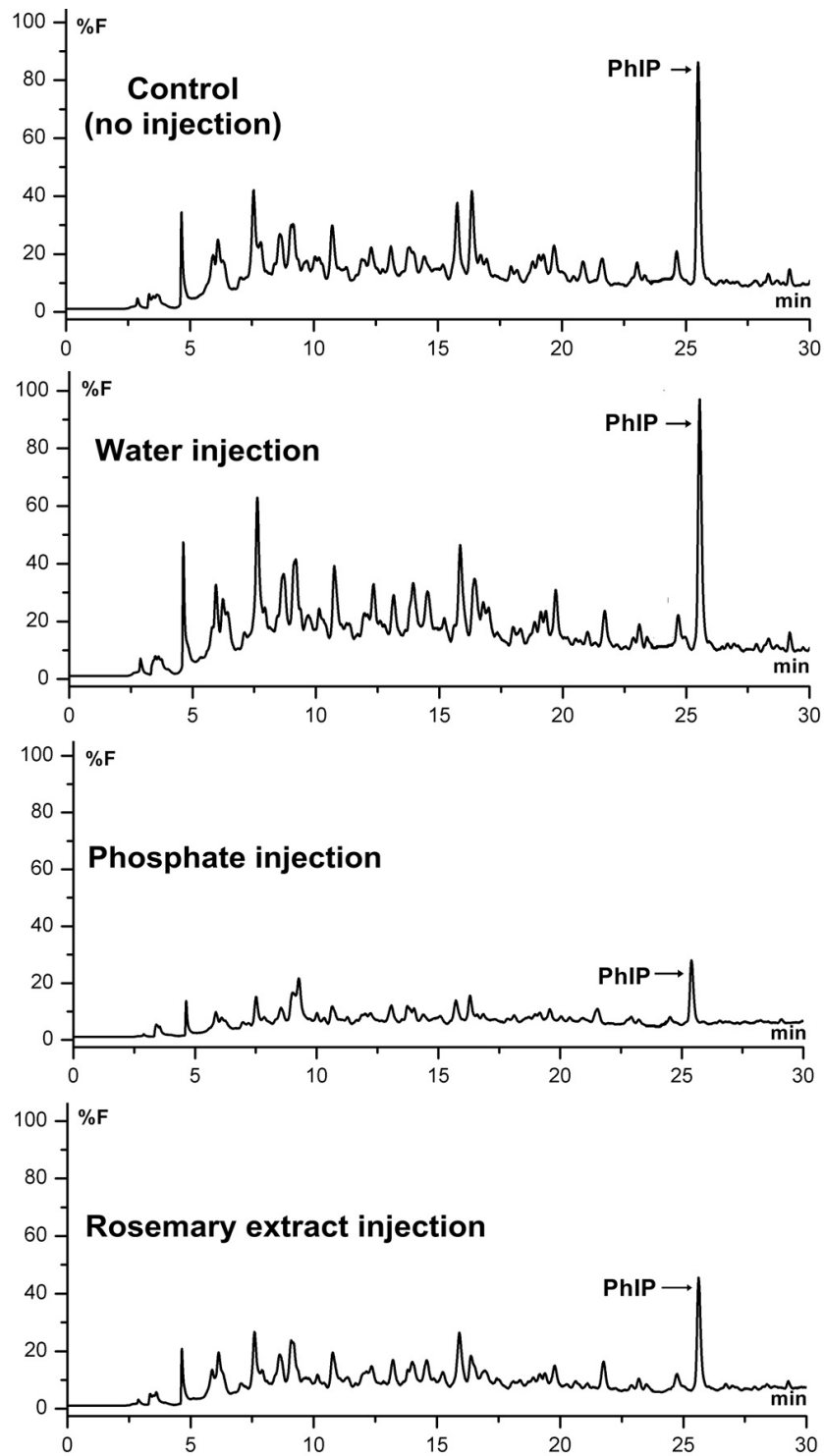


Figure 23. FLD chromatogram of HCAs (MeIQx, DiMeIQx) in control, water-injected loin, and salt/phosphate-injected loin. TriMeIQx used as internal standard.

in control, water-injected loin, and salt/phosphate-injected loin ( $p < 0.05$ ) (Table 30). Injection of water with salt and phosphate showed more reduction of HCA formation than injection of water with rosemary extract ( $p < 0.05$ ). This may be due to rosemary-extract injected loin was not able to hold injected water inside the product very well, thus some of water-soluble antioxidant compounds present in rosemary extract may be lost along with water during storage and cooking.

Table 30. Total phenolic and antioxidant activity in manufactured pork loins

<b>Treatment</b>	<b>Total phenolic (mg GAE/g sample)</b>	<b>DPPH radical scavenging activity (%)</b>
Control (no injection)	1.23 ± 0.05 <sup>a</sup>	9.12 ± 1.88 <sup>b</sup>
Water injection	1.13 ± 0.08 <sup>ab</sup>	7.70 ± 0.91 <sup>b</sup>
Salt/phosphate injection	1.10 ± 0.07 <sup>b</sup>	8.38 ± 1.21 <sup>b</sup>
Rosemary extract injection	1.11 ± 0.06 <sup>b</sup>	12.12 ± 1.83 <sup>a</sup>

Each value is represented as mean ± standard deviation ( $n = 3$ ). Means with different superscript letters within the same column are significantly different at  $p < 0.05$ .

Injection of salt/phosphate reduced the level of PhIP by 42.5% (reduce from 13.12 to 7.54 ng/g), MeIQx by 79.0% (reduce from 7.59 to 1.57 ng/g), and DiMeIQx by 75.6% (reduce from 1.64 to 0.40 ng/g) compared with the control. This is in agreement with a study by Persson and others (2003) who reported that addition of 1.5% sodium chloride and 0.3% sodium tripolyphosphate to beefburgers decreased the formation of PhIP (up to 38%), MeIQx (up to 38%), and DiMeIQx (up to 12.5%) when beefburgers were fried at 180 °C and 220 °C. In

addition, Persson and others (2003) stated that addition of salt and phosphate may inhibit the conversion of creatine to creatinine during cooking, resulting in reduced HCA formation. Because creatine is less water soluble than creatinine, inhibiting the conversion of creatine to creatinine means that fewer HCA precursors are transported to the meat surface during cooking. Therefore, addition of salt and phosphate has a great impact on improving water-holding capacity and reducing HCA formation in pork loins. It may be that injected salt and phosphate can hold the water in the sample, thus decreasing the transport of water and water-soluble precursors (creatine/creatinine, glucose, and amino acids) to the surface. Salt (sodium chloride) is added to meat products to improve their binding and water-holding properties. Chloride ions tend to penetrate into the myofilaments filaments in the meat, causing them to swell by increasing the electrostatic repulsive force between similarly charged groups, which decreases cohesion between filament proteins of muscle. Decreased cohesion produces an enlarged myofibrillar volume that can retain more water (Offer and Trinick 1983, Alvarado and Mckee 2007, Puolanne and Halonen, 2010). Phosphates improve water-holding capacity by shifting the pH further away from the isoelectric point of the myofibrillar proteins by unfolding muscle proteins, thus exposing more charged sites for water binding. In addition, phosphates have the capability to cleave the bonds between myofilaments, allowing more space for water to enter the network and bind to charged groups on proteins (Aberle and others 2001).

## **CONCLUSIONS**

Overall, the results of this study show that enhancing fresh meat products by adding salt and phosphate improves the water-holding capacity, reduces cooking loss, and is an effective strategy to reduce HCA formation in cooked products. Enhancement with water alone did not reduce HCA formation because the meat did not retain injected water. Although enhancement with a combination of water and rosemary extract could reduce amounts of HCA compared with enhancement with water alone; however, it did not produce a different result than control. We believed that salt and phosphate may need to be added to the rosemary extract injected-loin. Additional reduction of HCA levels was observed in marinated product that was prepared by adding additional ingredients, such as spices and spice/plant extracts, that exhibit good antioxidant properties in the enhancement solution.

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## SUMMARY

Heterocyclic amines (HCAs), potent mutagenic and carcinogenic compounds, are formed during the cooking of meat and fish. The major HCAs found in cooked meat and fish are PhIP, MeIQx, and DiMeIQx. Most RTE meat products contain very low HCA levels, except for rotisserie chicken that contain elevated amounts of HCAs. We concluded that consumption of RTE meat products contributes very little to HCA intake. In cooked meat products (beef, chicken and fish) prepared by various cooking methods (pan frying, oven broiling, and oven baking), high levels of total HCAs were found in fried pork, fried fish, and fried bacon. The formation of HCAs in cooked samples is highly dependent on the method and level of cooking, and the content of HCAs in cooked meat and fish will be low if an appropriate cooking procedure is selected.

Because of the increasing evidence of the risk of cancers, it is necessary to reduce the exposure to HCAs. Although, it is impossible to prevent the HCA formation completely, a reduction of the HCA levels in cooked meat and fish can be achieved by several methods. The addition of rosemary extracts in ground beef inhibited the formation of HCAs. The capabilities of rosemary extracts on the HCA inhibition depends on the ratio of water and ethanol used in the extraction process. Enhancement and marination were found to reduced the amount of HCAs formed in cooked meat products. Addition of salt and phosphate greatly improved water-holding capacity and decreased HCA formation in enhanced fresh meat products. An even greater reduction of HCAs was found in marinated fresh meat; the enhancement solution for this meat contained ingredients that exhibited food antioxidant properties.

These data can be used to recommend cooking methods to be used at home or in the

food industry, or used as guidelines for meat industry on how to modify a formulation process to minimize HCA formation in cooked meat products. Also these data will provide important information for use in estimating HCA exposure, and will facilitate investigation of the role of HCAs in the etiology of cancer of population in the U.S.

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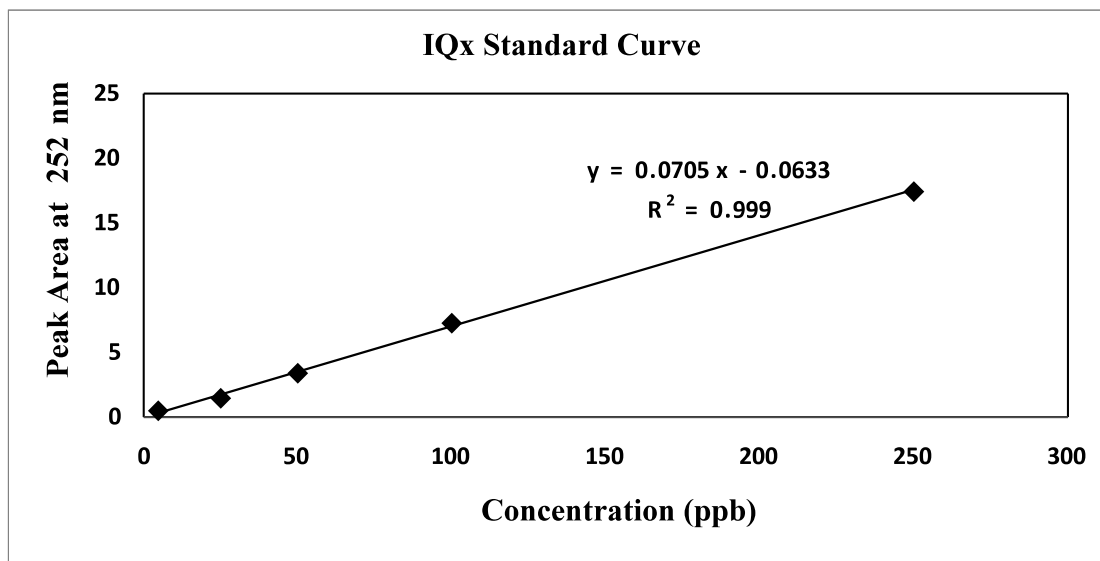
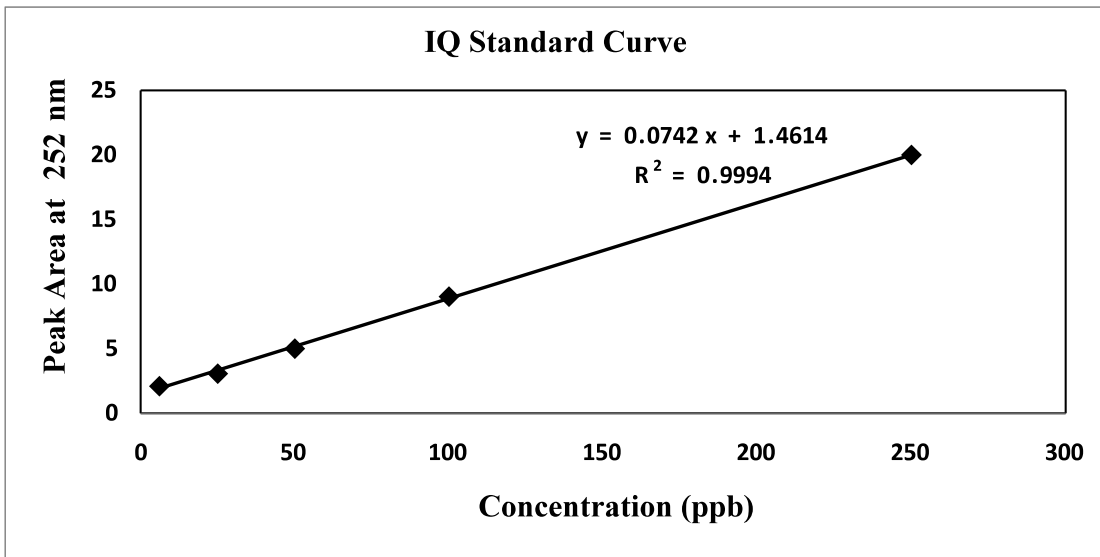


Figure A-1. Standard curves obtained by UV detection for IQ and IQx at 252 nm.

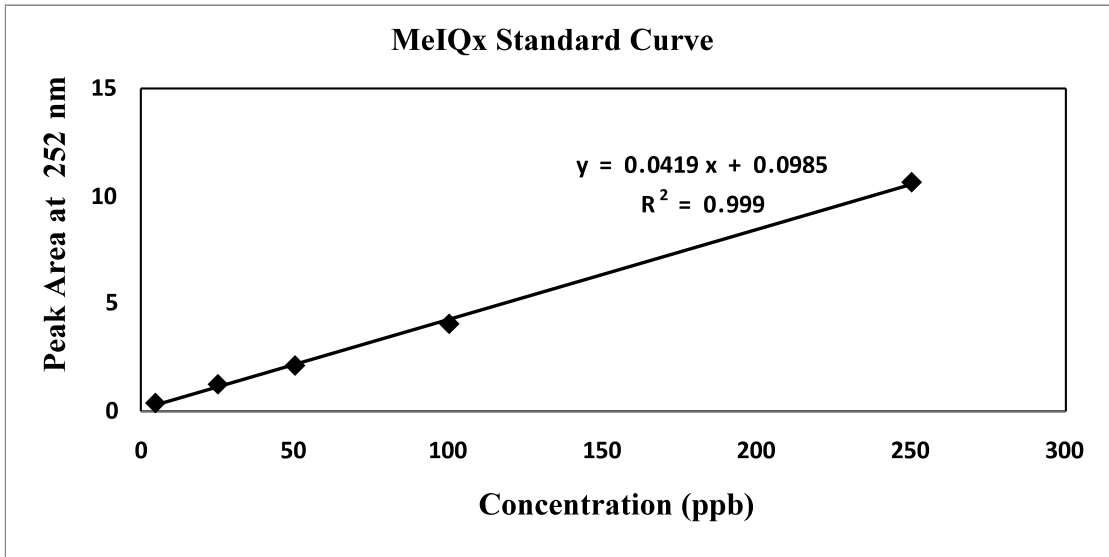
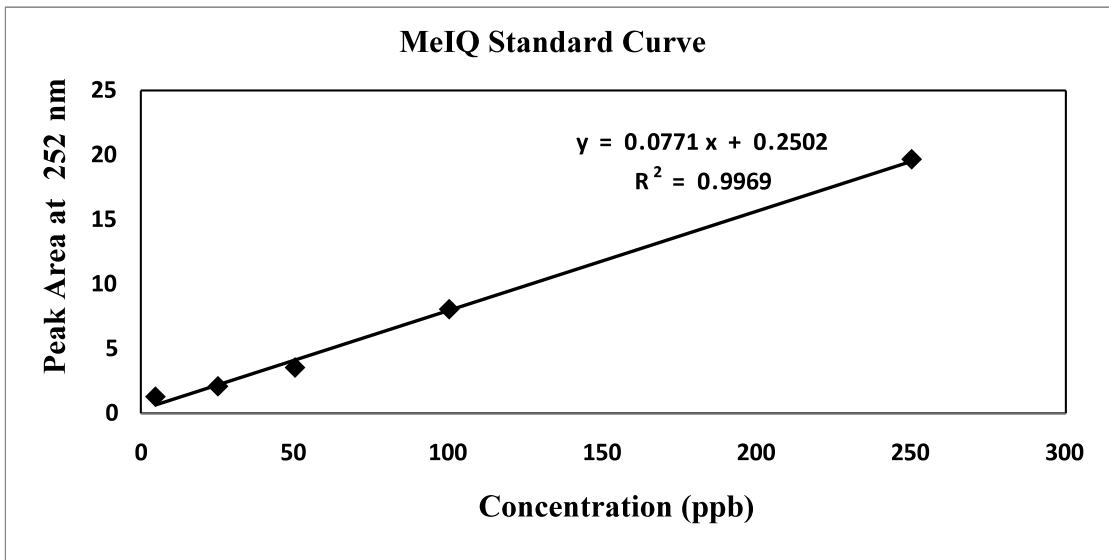


Figure A-2. Standard curves obtained by UV detection for MeIQ and MeIQx at 252 nm.



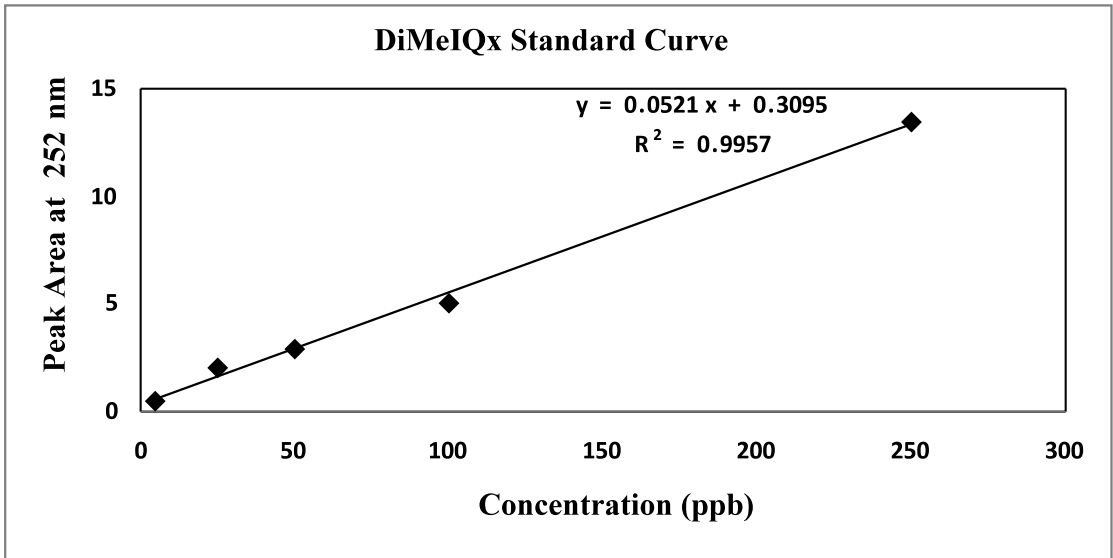


Figure A-3. Standard curves obtained by UV detection for DiMeIQx at 252 nm and by FLD detection for PhIP.

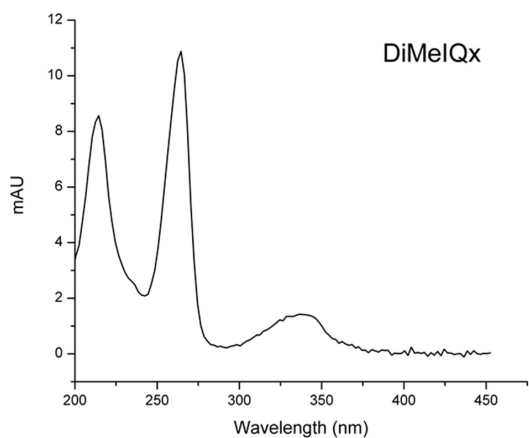
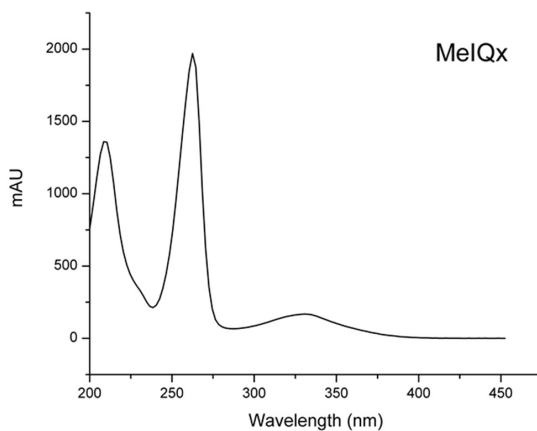
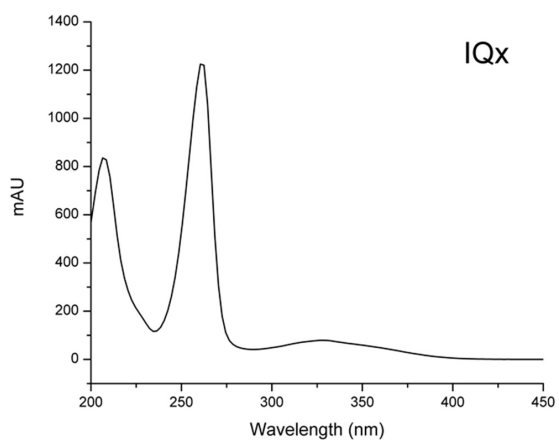
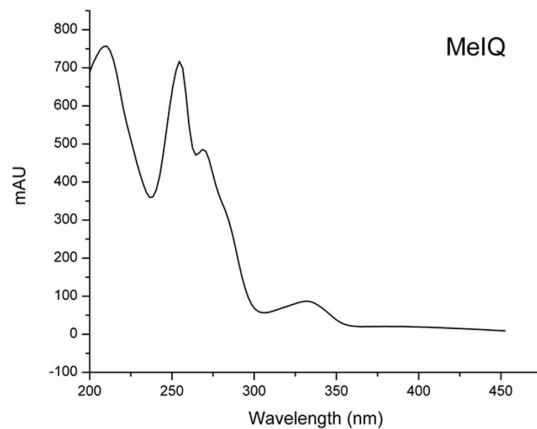
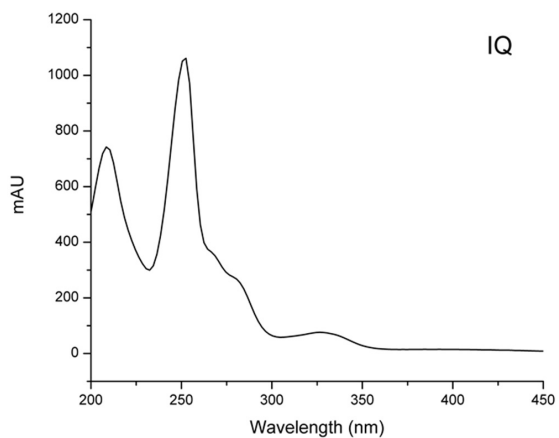


Figure A-4. UV spectra recorded for IQ, IQx, MeIQ, MeIQx, and DiMeIQx standards.

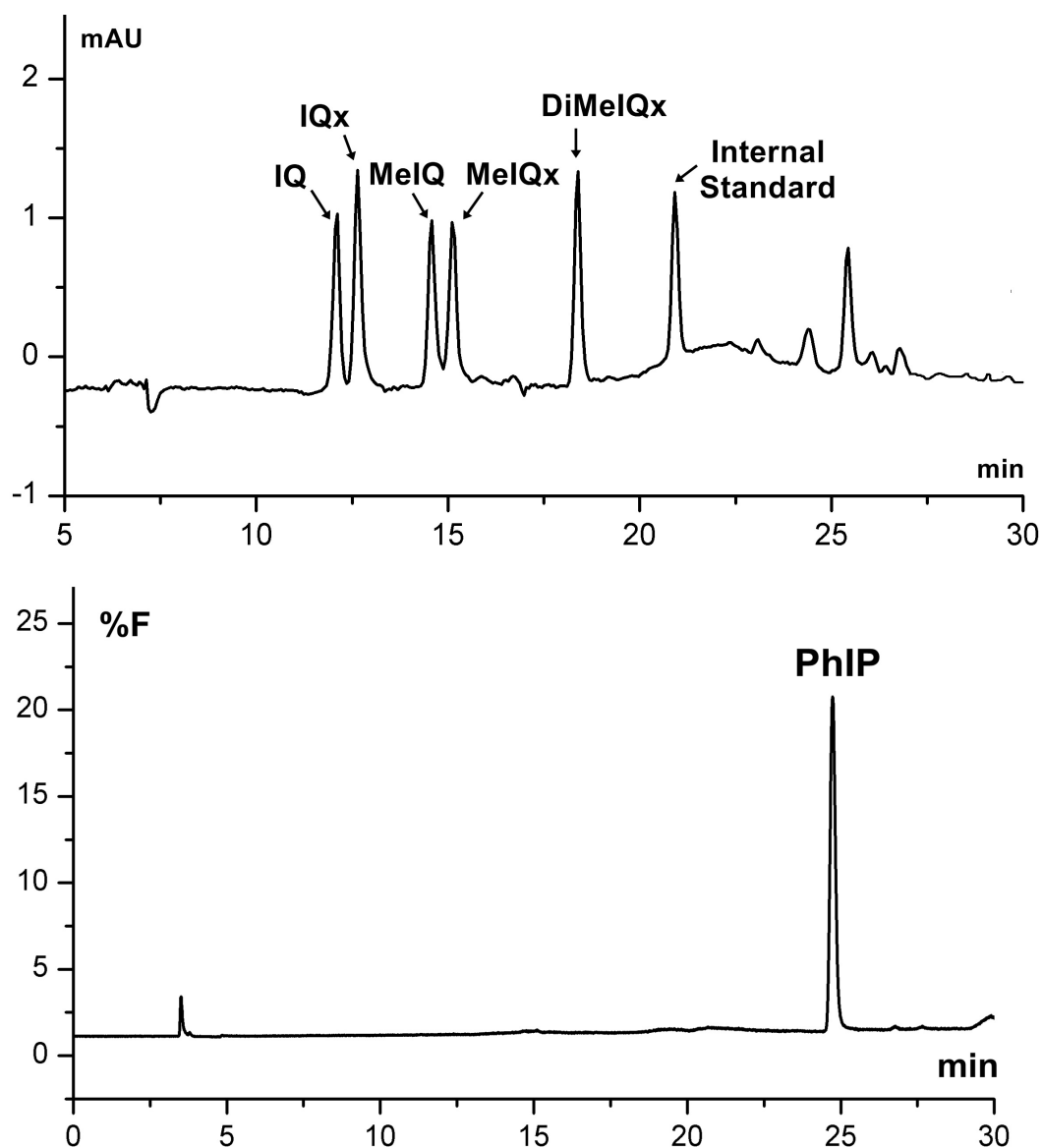


Figure A-5. Chromatogram of HCA standards at 250 ppb (20  $\mu$ L injection) using UV and FLD detection. The UV detector was set at 252 nm for IQ, IQx, MeIQ, MeIQx, and DiMeIQx detections, and The fluorescence detector was programmed accordingly to the excitation/emission wavelengths of 229 and 437 for PhIP. TriMeIQx (250 ppb) was used as internal standard.

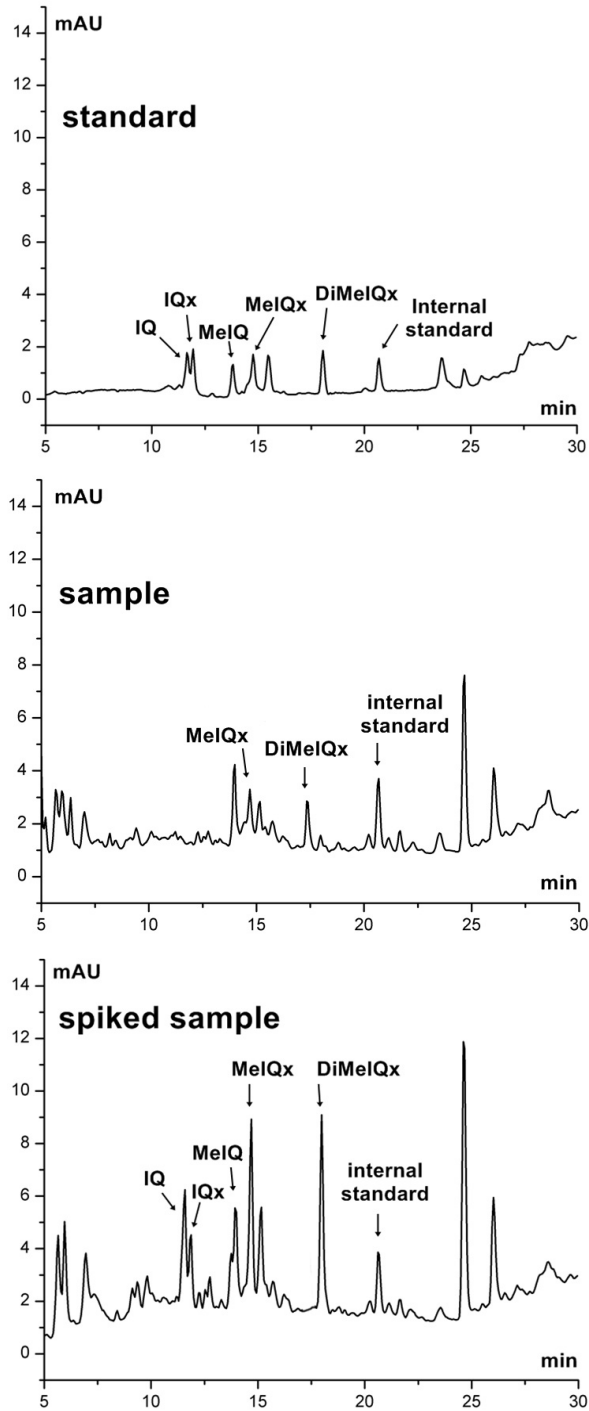


Figure A-6. UV chromatogram of HCA standards (250 ppb each), sample, and spiked sample at concentration 25 ppb of each HCA.

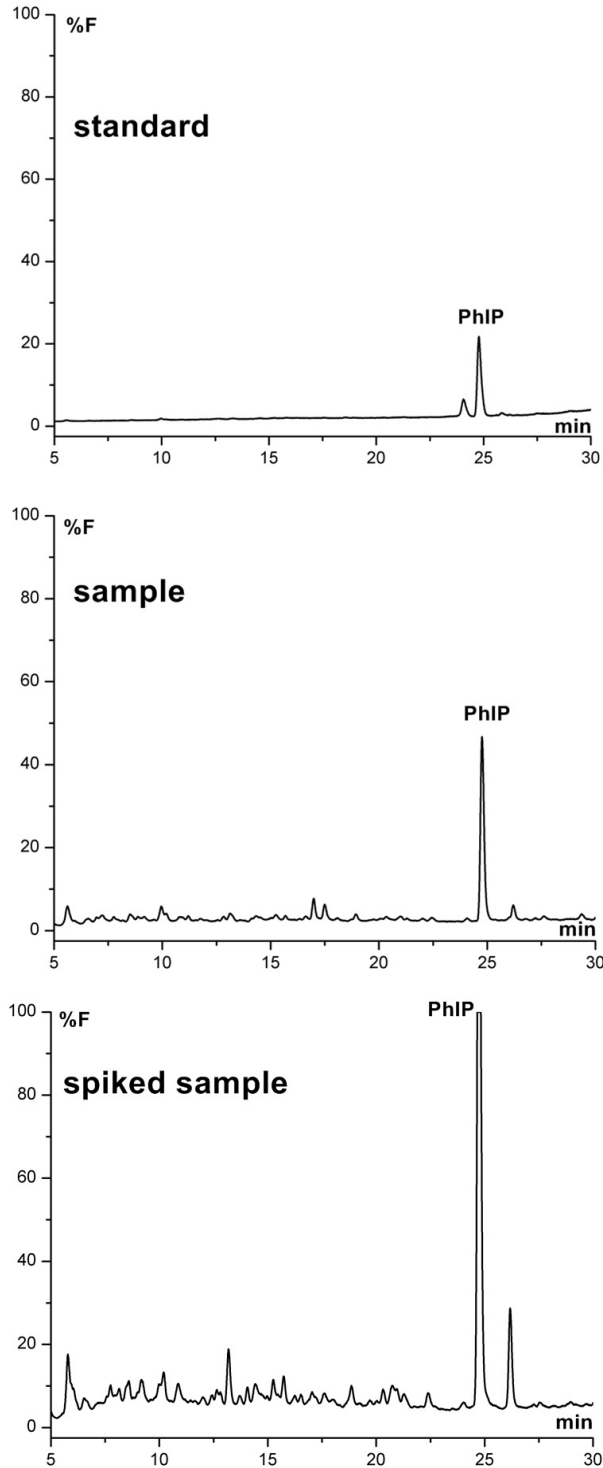


Figure A-7. FLD chromatogram of HCA standards (250 ppb each), sample, and spiked sample at concentration 25 ppb of each HCA.

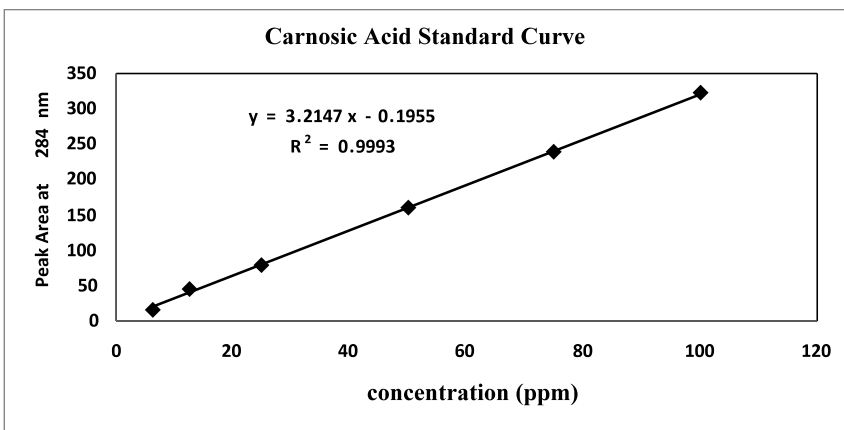
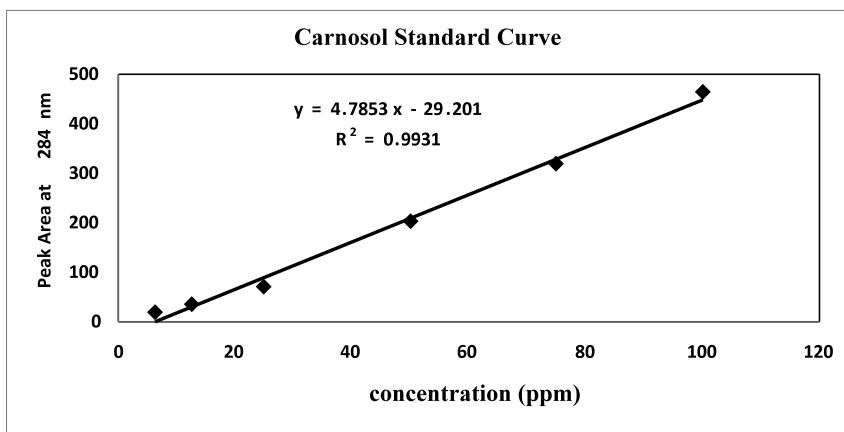
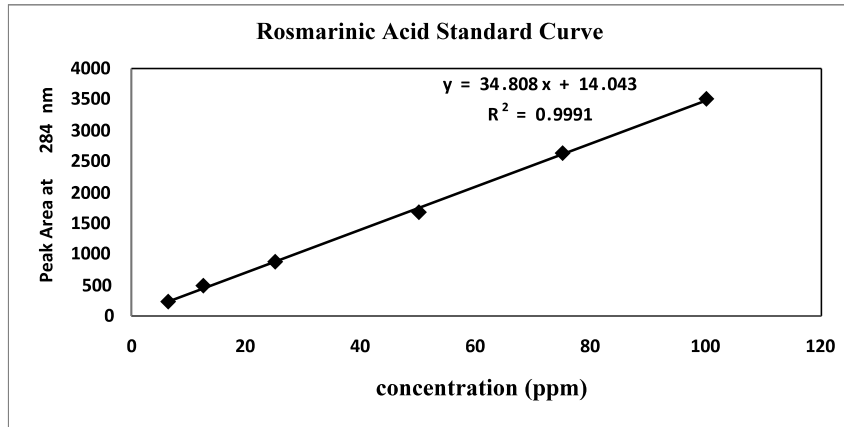


Figure A-8. Standard curves obtained by UV detection (284 nm) for rosmarinic acid, carnosol, and carnosic acid at concentration 5 to 100 ppm.

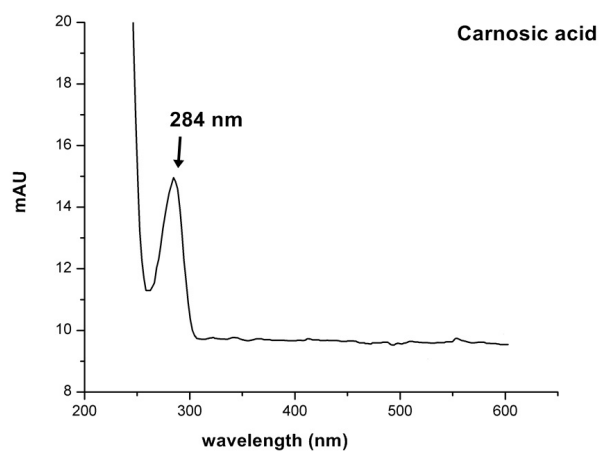
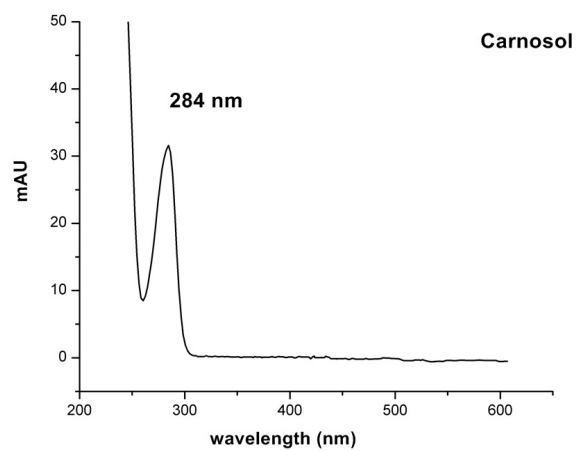
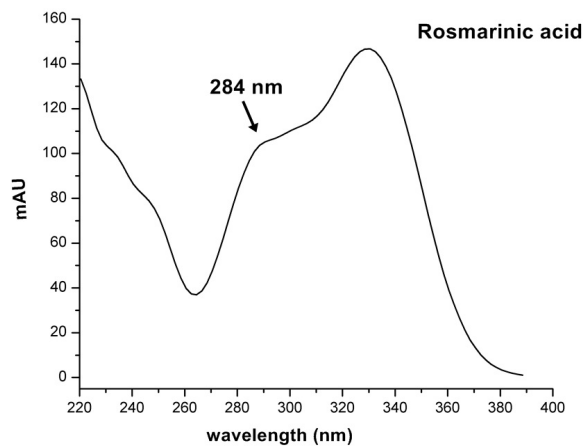


Figure A-9. UV spectra recorded for rosmarinic acid, carnosol, and carnosic acid standards.

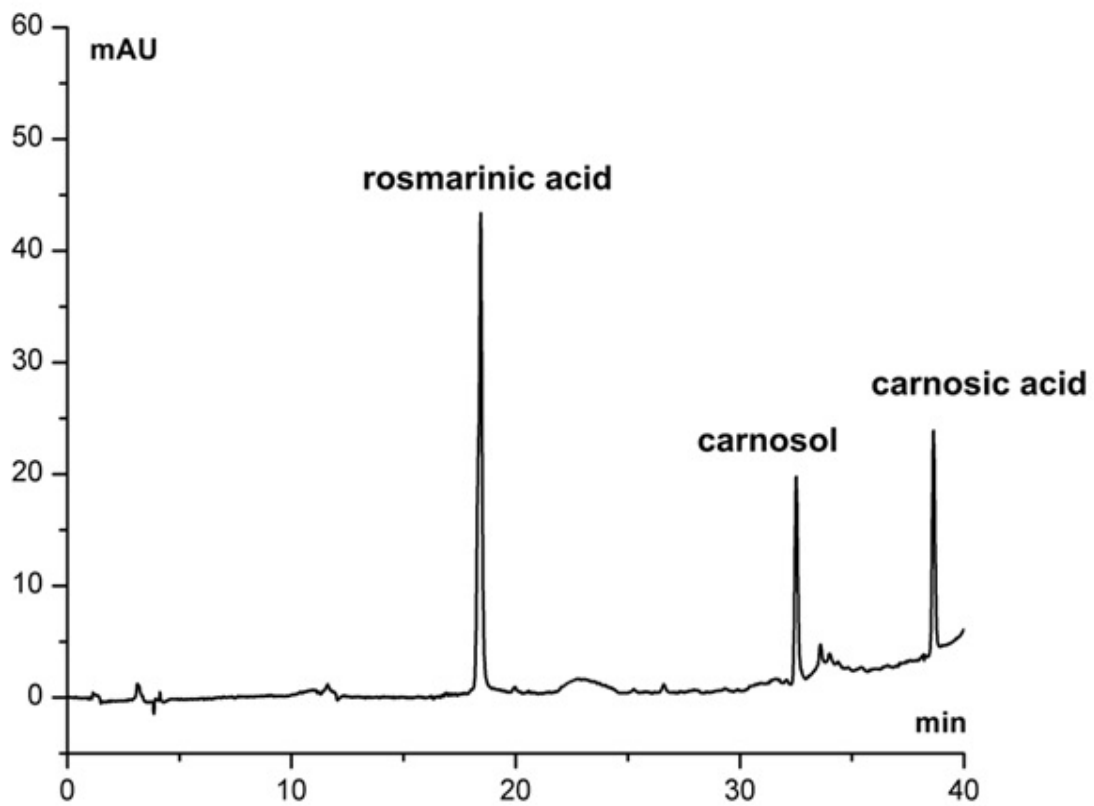


Figure A-10. Chromatogram of rosmarinic acid (25 ppm), carnosol (100 ppm), and carnosic acid (100 ppm) standards (20  $\mu$ L injection) using UV detection at 284 nm.



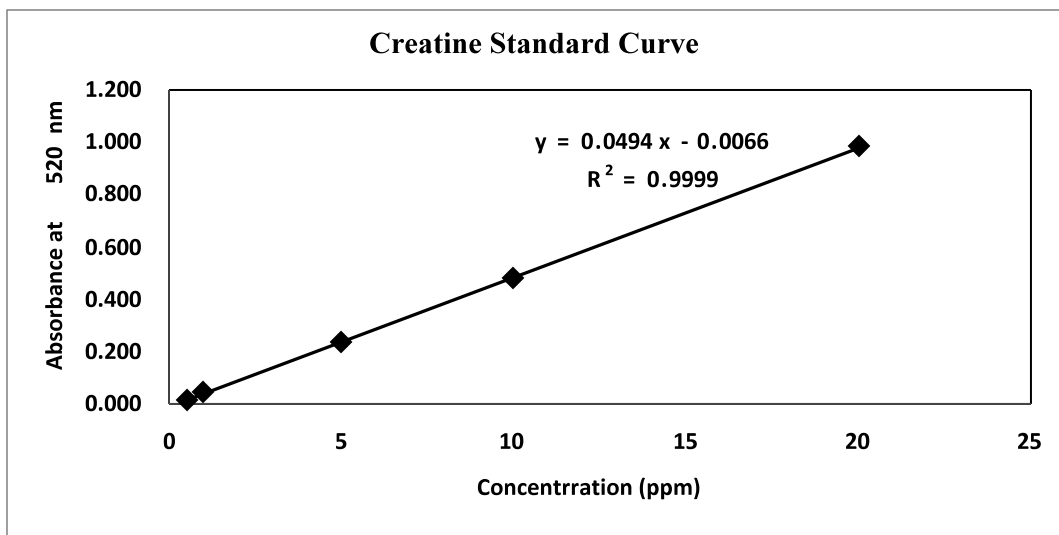


Figure A-11. Standard curves obtained by UV detection (520 nm) for creatine standards at concentration 0.5 to 20 ppm for creatine determination.

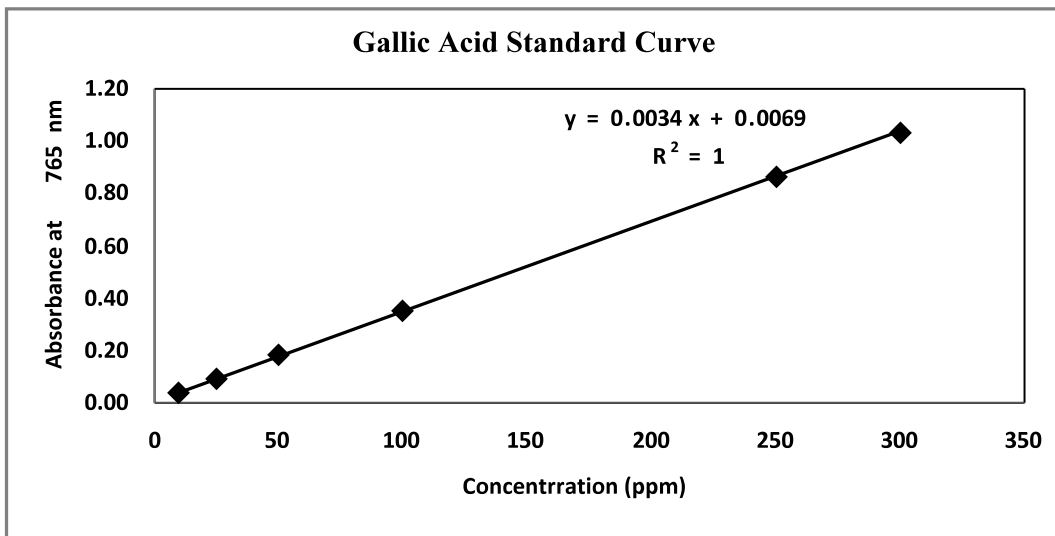


Figure A-12. Standard curves obtained by UV detection (765 nm) for gallic acid standards at concentration 2.5 to 300 ppm for total phenolic determination.

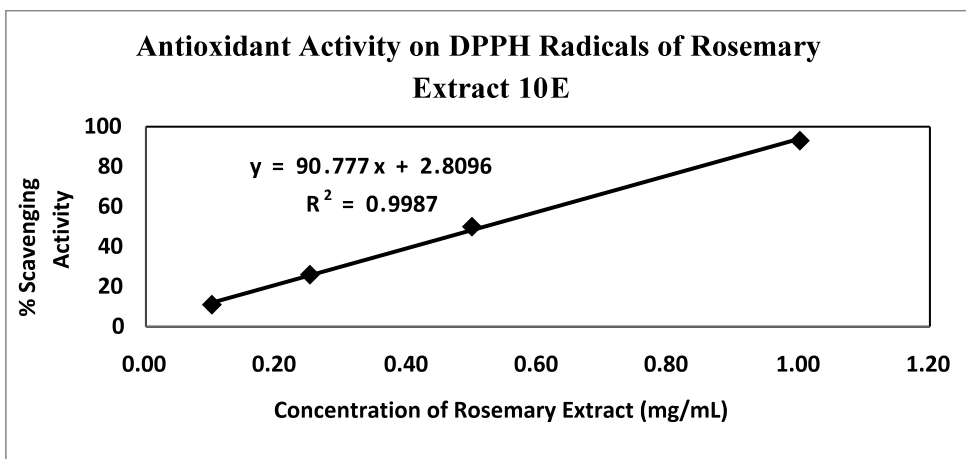
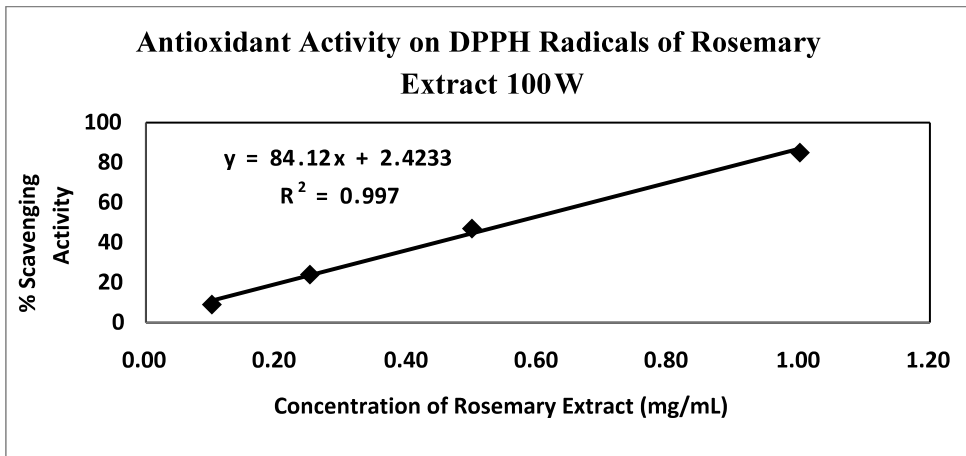


Figure A-13. Scavenging activity on DPPH radicals on rosemary extract 100W and 10E at concentration 0.1 to 1.0 mg/mL for  $IC_{50}$  of antioxidant activity determination.

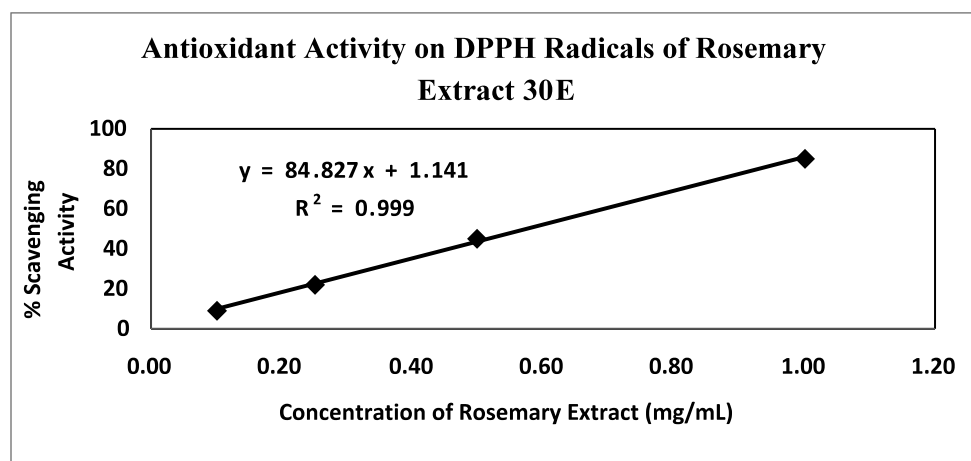
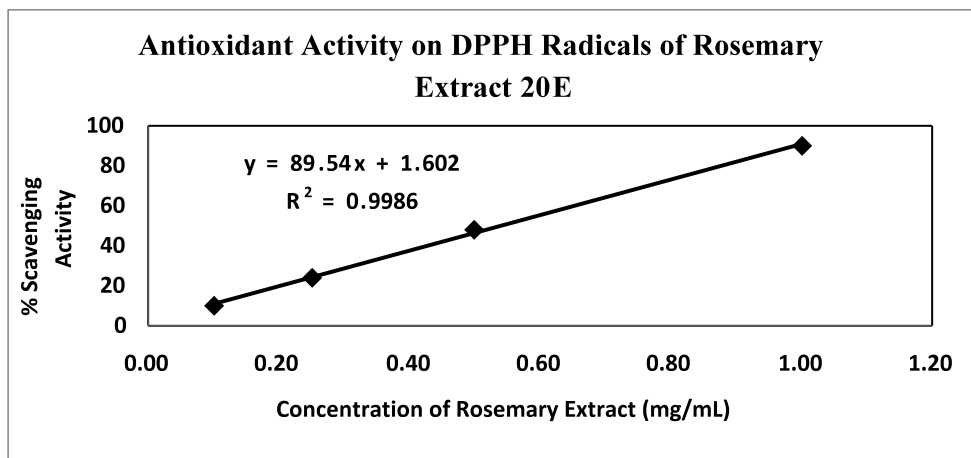


Figure A-14. Scavenging activity on DPPH radicals on rosemary extract 20E and 30E at concentration 0.1 to 1.0 mg/mL for IC<sub>50</sub> of antioxidant activity determination.

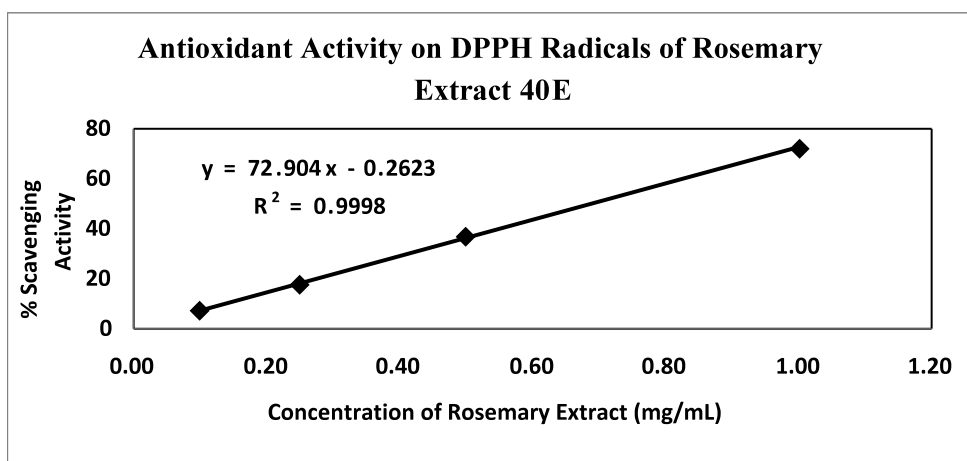


Figure A-15. Scavenging activity on DPPH radicals on rosemary extract 40E at concentration 0.1 to 1.0 mg/mL for  $IC_{50}$  of antioxidant activity determination.