EFFECTS OF MARINADES ON THE FORMATION OF HETEROCYCLIC AMINES IN GRILLED BEEF STEAKS

by

FARIBA EMAMGHOLIZADEH

M.D., Azad Tehran University, 1993

A THESIS

submitted in partial fulfilment of the
requirements for the degree

MASTER OF SCIENCE

Food Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas
2008

Approved by;

Major Professor
J. Scott Smith
Animal Sci. & Ind.

ABSTRACT

Heterocyclic amines (HCAs) are a class of toxicological compounds that can be formed during heating of precursors, amino acids, creatinine, creatine, and sugars at high temperature cooking of muscle products. These potent mutagens are suspected to play a role in human cancers. The objective of this study was to investigate a practical method to reduce the amount of HCAs through marinating of beef steaks. We were interested in the potential health benefits of natural extracts containing polyphenols present in commercial marinades. HCAs were compared in marinated and unmarinated steaks. Four common HCAs were investigated: 2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine (PhIP), 1-methyl-9H-pyrido[4,3-b]-indol (harman), and 9H-pyrido[4,3-b]-indol (norharman). Steaks were marinated for one hour and grilled at 400 °F for 5 minutes at each side. Meat samples were extracted by solid phase extraction (SPE) and analysis with HPLC showed the significant decrease ($p < 0.05$) of sum of polar and nonpolar HCAs by 71% compared to untreated steaks. For confirmation of spices potency on reduction of HCA formation, the same experiment was applied to meat with the base of commercial marinade powders excluding the herbs and spices. Lesser reduction of HCAs were shown and in some cases no significant reduction occurred. HPLC analysis showed presence of considerable amount of natural phenolic antioxidants of carnosic acid, carnosol, and rosmarinic acid, which can be related to the reduction effects of HCA formation in commercial marinades. These results revealed that marinating meats before grilling with various spices/herbs containing antioxidants may reduce formation of mutagenic /carcinogenic HCAs markedly.

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ACKNOWLEDGMENTS

I would like to express my gratitude and sincere thankfulness to my major professor J. Scott Smith for giving me this chance to work on this interesting project and complete the study under his great knowledge and guidance. During this study, he showed a great deal of encouragement, huge support, and friendship.

I would like to thank Dr. Donald Kropf for his encouragement and guidance during all my research time. I would like to thank Dr. Curtis Kastner for his kindness in reviewing my thesis, guidance, and his priceless support.

I would also like to thank Dr. K. Kemp for his valuable help in statistical analysis and SAS interpretation.

Finally, I would like to thank all of my co-workers in the lab and friends who helped me with their support and caring through my entire graduate study. I especially would like to thank my precious husband who constantly helped me in all parts of my life and also my parents who always encouraged and supported me. Without the help of these valuable people, it would not be possible for me to accomplish my goal.

PART I. LITERATURE REVIEW

INTRODUCTION

Diet plays an important role in cancer occurrence in humans. Food contains mutagenic and/ or carcinogenic substances such as mycotoxin contaminants, nitrosamines, polycyclic aromatic hydrocarbons (PAH), and plant alkaloids which are considered to be such components that trigger cancer in humans (Doll et al., 1981). However, the human diet is complex and potential carcinogens in the diet can be either exogenous or endogenous substances (Layton et al., 1995).

Between 1975 and 1977, a series of mutagenic-carcinogenic substances were found in heated proteinaceous meat and fish and the presence of heterocyclic amines (HCAs) was shown by Sugimura and coworkers in 1977 for first time. A food safety concern about the formation of carcinogens in foods is based on the fact that meat components are frequently changed to potent mutagens /carcinogens, including HCAs, at normal cooking temperatures (Sugimura et al., 1982; Jägerstad et al., 1984). Currently, more than 20 mutagenic HCAs have been isolated from various cooked meats (Sugimura et al., 2004). Some of these HCAs such as aflatoxin B_1, have much higher mutagenic activity than typical mutagen/carcinogens. Among these amines, Trp-P-1(3-amino-1,4-dimethyl-5-H-pyrido[4,3-b]indol; Trp-P-2 (3-amino-1-methyl-5-H-pyrido[4,3-b]indol; AαC (2-amino-9H-pyrido[2,3-b]indol; MeαA (2-amino-3-methyl-9-H-pyrido[2,3-b]indol; Glu-P-1(2-amino-6-methyldipyrido-[1,2-a:3`,2`-d]imidazole; Glu-P-2 (2-aminodipyrido-[1,2-a:3`,2`-d]imidazole; IQ (2-amino-3methylimidazo[4,5-f]quinoline; MeIQ (2-amino-3,4-imethylimidazo[4,5-f]quinoline; MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline, and

PhIP (2-amino-methyl-6-phenylimidazo[4,5-b]pyridine have been verified as being carcinogenic in rats and mice, and IQ was also found to be carcinogenic in monkeys (Ohgaki et al.,1991; Sugimura et al., 1997). HCAs have been tested for their mutagenic activity in assays in vitro and in vivo with positive results of toxicity for most of them (Schut et al., 1999).

It is known that HCAs are potent bacterial mutagens and have been reported to induce tumors in multiple organs including the colon, prostate, and mammary glands in rodent bioassays (Sugimura et al., 2004). The most significant fact is that HCAs exist in normally cooked foods, but they are not found in raw meats. They are widely distributed in various foods and it seems hard to reduce their human exposure to zero. There are some ways to diminish the formation of HCAs while cooking meat, thus reducing the risk of any human carcinogenesis due to HCAs. Lang (1994), based on some epidemiological studies, reported elevated incidences of colorectal and breast cancers in individuals who frequently consumed heavily browned grilled meats. These findings suggest that exposure to HCAs may contribute to human cancers. Some researchers refer to HCAs as some of the most potent mutagens known to humans (Friedman et al., 1991). Attempts have been made to determine whether HCAs have a consistent carcinogenic effect in humans by conducting epidemiological studies such as trying to correlate the rate of colon cancer with the preference of well-done meat consumption (Sinha et al., 1997; Probst-Hensch et al., 1997). Therefore, it seems important to quantify the amount of these mutagens present in foods in order to estimate intakes and risks to human health. Furthermore, it seems more important to minimize or inhibit formation of these mutagens.

The amount and variety of HCAs formed in cooked meat products depends primarily on processing conditions and cooking time and temperature (Knize et al., 1985). Knize reported that

the major HCAs formed were IQ, MeIQ, and DiMeIQx when beef patties were cooked at 200, 250 and 300 °C for 6 minutes. Skog later demonstrated that with cooking temperatures of 150, 175, 200, and 225 °C and a heating time of 2-11 minutes, the level of HCAs formed in beef and pork increased as time and temperature increased (1993). Despite the trace amount occurrence (parts per billion) of HCAs, some of them are considered to be a human carcinogen based on sufficient evidence in animal models. Unfortunately, humans are continuously exposed to HCAs. However, the presence of HCAs in foods depend largely on cooking method, time, and the presence of relative amounts of precursors and inhibitors.

From the structural view, HCAs are classified and divided into two categories: the amino-carbolines and the amino-imidazo-azaarenes (AIAS). Among the latter, 2-amino-1-methyl-6-phenylimidazol (4,5,b)pyridine (PhIP) accounts for most of the overall HCA exposure (Augustsson et al., 1997). Carbolines are also classified as α, β, and γ-carbolines. Amino-carbolines are so-called "pyrolytic" and they form at temperatures higher than 300 °C. They have an amino group attached to a pyridine ring in their structures. In fact, amino- carbolines are formed when proteinaceous foods are heated to high temperature. For example, harman and norharman are considered amino-carbolines and are called "co-mutagens" since they do not show mutagenicity to *Salmonella serovar typhimurium*. The amino-imidazo-azaarenes are known as "thermic" and form at temperatures below 300 °C. These mutagens have an amino group attached to an imidazole ring and they form via the Maillard reaction through the reactions of creatine or creatinine, amino acids, and hexoses to heating (Busquests et al., 2004). The amount of HCA formation can be decreased in meats by inhibiting the reaction between the Maillard reaction process and creatine.

Because HCAs are formed through free radical reactions, many attempts to inhibit this reaction have been tried. Herbs and spices have been used since prehistoric times to preserve foods. Based on a review of the numerous studies of food products, clove, rosemary, and sage were concluded to be among the most potent antioxidative spices. Therefore, natural antioxidants such as tocopherol (vitamin E) or synthetic antioxidants such as butylated hydroxyanisole (BHA) have been shown to reduce HCAs by scavenging the free radicals (Chen and Chiu, 1998). Pyrazine and pyridine are radicals from the Maillard reaction; thus, antioxidants act to stabilize these intermediates and to reduce the content of HCAs in meat (Chen et al.,1992). For this reason, some researchers attempted to apply natural antioxidants (from herbs, spices, and vegetables) before cooking meat, and they demonstrated the positive effects of these antioxidants. For instance, Murkovic (1998) showed that sprinkling dried spices on the surface of steak decreased the amount of HCAs in meat. Over the past 25 years, extensive studies have been conducted on the chemical and analytic approaches such as isolation and quantification of HCAs in model systems and the possible mechanisms of their formation and mutagenesis. More importantly, the preventive approach aimed at developing strategies to inhibit HCAs formation have been newly studied by investigators. The purpose of this current work is to study and investigate a practical cooking method to reduce HCAs formed during grilling steak. This study has been done on the effort of showing the efficiency of antioxidants and the formation of four important and frequently found HCAs (MeIQx, PhIP, harman, and norharman) on grilled steaks as well as the inhibition of these HCAs by using natural antioxidants present in plants and herbs.

Epidemiology

In 1981, the epidemiologists Richard Doll and Richard Peto estimated that between 10-70% of all cancer deaths in the US are potentially avoidable by dietary changes. Epidemiological studies on the relative risk of cancer from cooked foods have shown that frequent consumers of meat run an increased risk of cancer (Gerhardsson et al., 1990), which might be explained by their intake of mutagenic compounds such as HCAs. Three population-based case control studies in Sweden have found an association between fried foods and pancreatic cancer, urothelial cancer, and colorectal cancer. The study included 347 cases of colon cancer, 212 cases of rectal cancer, and 505 controls, and only one of these studies included questions about the preferred method of frying the meat surface. Results showed that consumption of brown gravy and a preference for a heavily-browned meat surface independently increased the risk for colorectal cancer. These results also indicated that the cooking method is a neglected risk factor for cancer.

In 1986, Norell and colleagues conducted a similar research study on 120 cases with cancer of exocrine pancreas and 162 control subjects (Norell et al.,1986). Diet was assessed with a food frequency questionnaire. They concluded that frequent consumption of meat/pork was associated with an elevated risk of pancreatic cancer compared to consuming meat/pork less than once a week. Further analysis of data showed that the association with pancreatic cancer could be explained by the association with meat that was fried or grilled, while there was no association with pancreatic cancer for meat that was not fried or grilled. Elevated risks were also found to be associated with frequent consumption of bacon or smoked ham and with the consumption of brown gravy, which is commonly made from the pan residue.

Steineck (1990) showed that the weekly consumption of grilled foods and brown gravy

were associated with an increased risk for urothelial cancer. Furthermore, a high intake of fried meat caused a slightly increased risk for urothelial cancer, in contrast to meat cooked in other ways. Several studies were done and verified increased risk of colorectal cancer in case study research. The preference for a heavily-browned meat surface and high frying temperature increased the risk of colorectal cancer. Since laboratory data had shown the high concentration of HCAs in meat crust and in the pan residue, the intake of fried meat and brown gravy was analyzed by degree of browning (Sugimura et al., 1988). The comparison between subjects showed that a high risk for colon cancer was associated with a preference for a heavily browned meat, but the highest risks were found for subjects who also had a frequent intake of fried meat and brown gravy (Table 1).

Table 1 . Adjusted relative risks with 95% confidence intervals for colorectal cancer associated with brown gravy and preferred method of frying the meat surface (De Verdier, 1992).

Exposure	**Colon cancer** (relative risk)	**Rectum cancer** (relative risk)
Brown Gravy		
Once/week	1.6 (1.1-2.3)	1.9 (1.2-3.0)
1-3 times/month	1.0 (0.7-1.5)	1.1 (0.7-1.6)
Less than 1-3 times/month	1.0	1.0
Preferred Meat Surface		
Heavily browned	2.0 (1.2-3.6)	3.4 (1.7-6.7)
Moderately browned	0.5-1.2	1.1 (0.6-1.9)
Lightly browned	1.0	
Temperature when frying		
High	1.8 (1.3-2.4)	1.5 (1.1-2.1)
Medium/Low	1.0	1.0

According to the World Cancer Research Fund and the American Institute for Cancer Research, there is some probable evidence of increased risk of colon and rectal cancers with a high intake of red meat (Verdier et al., 1992). Sinha et al. investigated the association between meat and meat-related mutagens in the prostate, lung, colorectal, and ovarian (PLCO) cancer screening trial (2005). They studied colorectal adenomas because those are precursors of colorectal cancer. In this large study population, they found that increased risks for adenoma of the descending and sigmoid colon were associated with a high intake of bacon, sausage and well-

done red meat. Red meat was associated with increased risk of two meta-analyses of meat intake and colorectal cancer. Meat cooking method and doneness level are used to estimate exposure to mutagens, such as HCAs found in cooked meat. Within a population, exposure to the HCAs will vary depending on the cooking practices and the amount of cooked meat consumption. The genotoxic potential of ingested HCAs depends on the balance between metabolic activity and detoxification. Involvement of enzymes influences susceptibility to the genetic predisposition of individuals. Significance of human exposure to the HCAs is likely to depend upon what combination of these factors pertain to a particular individual (Gooderham et al., 2001). This means that enzymatic metabolically different people may respond differently to HCA exposure in their lives.

History of heterocyclic amine discovery

The discovery that cooked meat can be mutagenic led to efforts to isolate the mutagenic products. It is long known that when we cook food, these mutagenic/carcinogenic compounds form on its surface, although they are in very small amounts. In 1977, it was found that particles of smoke, produced by cooking proteinaceous foodstuffs and trapped on glass-fiber filters, contained significant quantities of mutagens (Hayashi et al., 1977). Because the incidence of human cancers, especially the frequency of development of tumors in various organs, differ widely, racial origin appears to play only a minor role. The effect of diet itself varies depending on the food, the way it is cooked, its nutritional value, and the eating habits as well as hygienic conditions.

Direct mechanical or chemical stimuli by food materials plays an important role in the

development of cancer in humans. In the past, this possibility was not seriously considered. During the 1970s, investigations of various mutagens in foods provided information on the actual carcinogens that might be responsible for human cancers. Cigarette smoking is generally accepted to be a major cause of neoplastic development in humans (Doll et al., 1977). Therefore, it seemed natural to expect that the smoke produced by cooking foods in the kitchen at home or in restaurants would also contain carcinogenic compound. With this idea, Nagao made a simple apparatus to collect smoked-broiled fish particles and he tested for mutagenicity in *Salmonella typhimurium* strains by Ames assay with an S9 mix, that was prepared from rats liver extract. Surprisingly, the mutagenic activity was shown to be much greater than expected and it presented clear differences from polycyclic aromatic hydrocarbons (PAHs). They concluded that there must be other mutagens in existence in addition to PAHs. Therefore, purification and isolation of mutagenic compounds from fried beef was carried out and their structures were identified (Kasai et al., 1984).

Jägerstad et al. (1984) demonstrated that a mixture of creatinine, glycine, and glucose yields MeIQx upon heating it in a solution containing diethylene glycol and water. Later, Negishi confirmed this reaction and isolated two new heterocyclic amines, 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8,-DiMeIQx) and 2-amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline (7,8-DiMeIQx) from a heated mixture of creatinine, threonine, and glucose (1985). Compounds isolated from pyrolyzed amino acids and proteins, such as Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, AαC and MeAαC, are also present in ordinary cooked foods. For instance, Trp-p-1 is present in broiled beef and AαC in broiled chicken (Wakabayashi et al., 1992). Negishi also added three new mutagens to the list of HCAs in cooked foods. These are 2- amino-1-methyl-6-

(4-hyroxyphenyl)imidazo[4,5-b]pyridine (4-OH-PhIP), which had previously been reported as in vivo metabolites of PhIP (Watkins et al.,1991) from broiled beef (Kurosaka et al., 1992). Some HCAs structures that have been definitively determined are shown in Figure 1. Table 2 shows the abbreviations and the years of discovery for different HCAs.

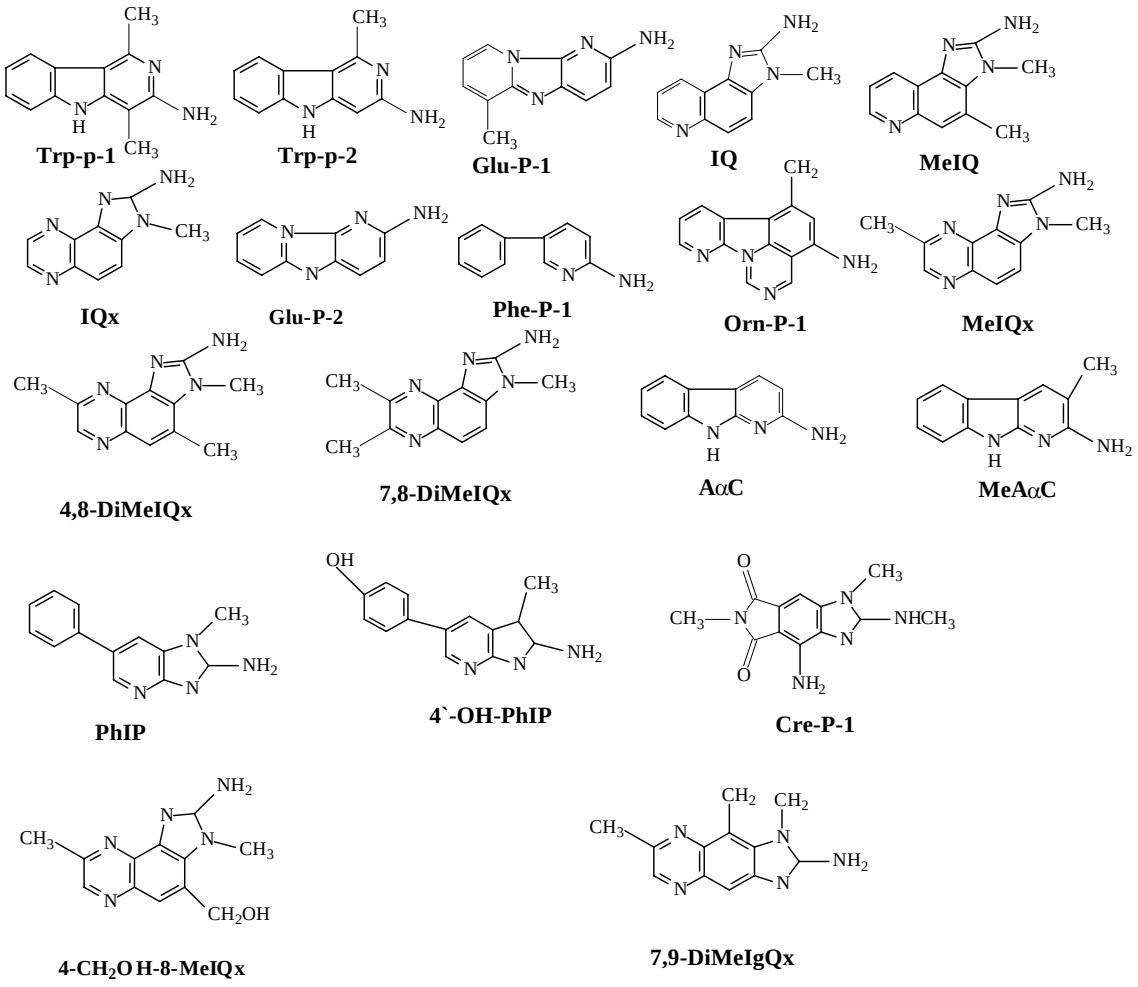


Figure 1. Chemical Structures of Common Heterocyclic Amines.

Table 2. Abbreviations and years of discovery of heterocyclic amines (adopted from Sugimura, 1997).

Abbreviation of HCAs	Year of discovery
Trp-P-1	1977
Trp-P-2	1977
Glu-P-1	1978
Glu-P-2	1978
Phe-P-1	1977
Orn-P-1	1981
AαC	1978
MeAα C	1978
IQ	1980
MeIQ	1980
IQx	1988
MeIQx	1981
4,8-DiMeIQx	1985
7,8-DiMeIQx	1984
PhIP	1986
4-OH-PhIP	1992
Cre-P-1	1991
4-CH2OH-8-MeIQx	1994
7,9-DiMeIgQx	1994

Human exposure

Formation of HCAs have been investigated in model systems and requirements for the presence of sugars, amino acids, and creatine or creatinine have been established (Jägerstad et al., 1991). These naturally occurring substances are found in red meat and they react together during the process of heating in a Maillard reaction. HCA compounds have been detected in all kinds of meats: beef, lamb, pork, chicken, and fish, especially when grilled, fried, or roasted. Although human exposure to these HCAs is low, chronic exposure over a life time is considerable and they can account for the majority of the genotoxic potentials, especially if heavily browned meat is consumed daily. Thus, depending upon dietary preferences, an individual`s daily exposure to HCAs is likely to range from microgram quantities to essentially zero in the case of vegetarians (Gooderham et al., 2001). In studies of the fate of administered HCAs, it was found that irrespective of the route of administration (oral, intraperitoneal, or intravenous) similar amounts of radiolabeled HCAs were extracted in the urine and the feces (Gooderham et al., 1987). This, and the fact that most of the radioactivity was eliminated in the first 24 hours indicated that the HCAs were rapidly and extensively absorbed (Gooderham et al., 1991). Analysis of the excreted material showed that the majority of an administered dose of HCAs was biotransformed except for a small proportion (about 2-5%) that was excreted in the urine as unchanged parent amine. Similar results were obtained in studies of human volunteers fed fried beef meals (Murray et al., 1989). In one study, known amounts of HCAs in fried beef fed to healthy volunteers and urinary excretion of unchanged HCAs was examined. About 2% of ingested MeIQx and PhIP were excreted unchanged in the urine within 24 hours of the test meal

and the majority was eliminated within 8 hours. Daily human exposure levels were estimated at the range of 0.3-3.9 μg/g for MeIQx and 0.005-0.3 μg/g for PhIP (Wakabayashi et al., 1995). The exposure levels might be determined by urine analysis in humans after collecting 24 hours of urine. For each type of meat, the variation in HCAs intake between individuals varies daily and also varies with cooking practices. For a 54 Kg individual, the reported average intake of total PhIP was 899 ng/day, total MeIQx was 141 ng/day, and total DiMeIQx was 44 ng/day (Layton et al., 1995).

In fact, through elimination of carcinogens from the environment or avoidance of human exposure to suspected carcinogens, the incidence of certain types of human cancers dramatically decreased. Turteltaub suggested that MeIQx may form DNA adducts in human tissue even at doses as low as what are considered normal human exposure levels (1990).

Environmental occurrence

Early studies on human cancer revealed the presence of occupation-related cancers. Much effort was expended by investigators to identify many chemicals as carcinogenic substances in the environment. Long term feeding experiments of chemicals using rodents have been performed and some of the chemicals identified have been responsible for the development of some types of human cancer. Through elimination of carcinogens from the environment or avoidance of human exposure, the incidence of particular types of human cancer dramatically decreased. Investigating the exposure of HCAs may vary methodologically. Measuring intake with a food frequency questionnaire is important in identifying the most frequently eaten fried dishes in the population under study.

Classification of Heterocyclic Amines

Amino-imidazo-azaarenes

HCAs, which are mutagens/carcinogens isolated by a *Salmonella* test from foods and protein pyrolysates, can be divided into two types. One is the IQ type, which includes aminoimidazoles such as IQ, MeIQx ,and PhIP. All of these aminoimidazoazarens have a 2-amino-imidazo group and a methyl group attached to a nitrogen in the imidazole ring. The 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) is a HCA from which many others are named according to their structures, such as 2-amino-3,4-dimethylimidazo[4,5-f]-quinoline (MeIQ). The 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP) was the first HCA identified as the most common of all HCAs, but is not the most mutagenic (Verdin et al., 2002). The IQ type was highly mutagenic to *Salmonella typhimurium* TA98.

Amino-carbolines

The other HCA is the non IQ type, which includes pyridoindoles and dipyridoimidazoles such as Trp-1, Glu-p-1, AαC, and these are derivatives of γ-carboline and amino α-carboline. Harman and norharman are considered as amino-carbolines and they are called co-mutagens since they do not show direct mutagenicity to *Salmonella serovar typhimurium*. Co-mutagens don't have exocyclic amino group. It has been shown that L-tryptophan is necessary as a precursor for harman and norharman formation. These are not only formed in heated-protein food, but have been shown to be normal body constituents (Pfau and Skog, 2004). Although the mechanism responsible for co-mutagenic actions has not been elucidated yet non IQ type HCAs were

generated at higher temperatures, which caused pyrolysis of amino acids and proteins (Figure 2). The IQ type was isolated from ordinary foods such as heated sardines and beef and the precursors for this type of HCAs are amino acids, sugars, and creatin(in)e.

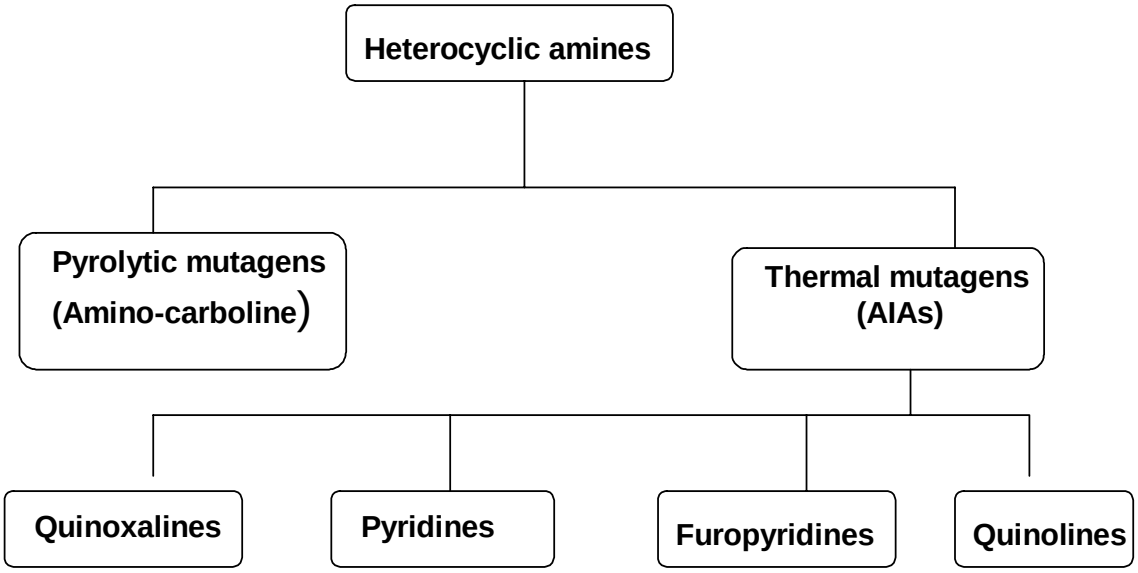


Figure 2. Classification of HCAs: Amino-Carbolines and Amino-imidazo-Azaarenes

Carcinogenesis

Epidemiological studies have shown that life style factors modulate the frequency of human cancer. Genetic predisposition and environmental factors are both implicated in the etiology of cancers. Among the environmental factors that may contribute to the genesis of human cancer, diet is regarded as a major determinant (Doll et al., 1996). HCAs have been shown to be carcinogenic in rodent bioassays. For example, rats treated with PhIP at a concentration of 400 ppm in the diet for 52 weeks developed a high incidence of colon and prostate carcinomas in males, and mammary carcinomas in females (Shirai et al., 1997). Furthermore, development of lymphocytic leukemia was apparently enhanced in males by

administration of HCAs such as PhIP, IQ, MeIQ, Glu-P-1, and Glu-P-2. These were found to induce colon tumors, either adenomas or adenocarcinomas (Ito et al., 1991; Ohgaki et al., 1991). HCAs form DNA adducts in a dose-dependent manner; however, the level of DNA adducts do not simply correlate with target organs. All cancers result from the accumulation of mutations in genes that regulate cellular proliferation. In accordance with the accumulation of genetic alterations relevant to carcinogenic transformation, normal cells move to become fully malignant cells through the stages of less malignant and moderately malignant cells. Most of the genes relevant to carcinogenesis are classified as oncogenes and tumor suppressor genes. Alteration of the genes for transcription regulating proteins result in altered expression of other genes. Interaction with cellular regulatory proteins results in disruption in the ordinary control mechanisms in DNA replication, cell division, cell motility, which may lead to cellular atypism, and metastatic potentials. A common pathway towards cancer occurs when dietary mutagenic agents cause adducts to be formed on genes. Adducts or gene alterations are formed when a carcinogen binds to DNA. When a high enough number of DNA adducts form along gene segments, normal cells can be transformed into cancer cells. If the adducts are not repaired, this can lead to tumor formation. The aminoimidazo-quinoxaline HCAs, such as 2-amino-3,8-dimethylimidazo[4,5-*f*] quinoxaline (4,8-DiMeIQx), and the aminoimidazo-quinoline derivatives, e.g. 2-amino-3-methylimidazo[4,5- *f*]quinoline (IQ), are strongly mutagenic to bacteria (Felton et al., 1991). Pyrido-indole HCAs, 2-amino-9*H*-pyrido[2,3-*b*]indole (A α C), 2-amino-3-methyl-9 *H*- pyrido [2,3-*b*]indole (MeAα C) and 2-amino-1-methyl-6-phenylimidazo[4,5- *b*]pyridine (PhIP), are much less potent (Sugimura et al., 1983). However, Aα C, MeAαC, and PhIP may posses greater genotoxicity in test systems involving mammalian

cells (Thompson et al., 1995). Thompson et al.`s study revealed that PhIP induced more gene mutations in Chinese hamster ovary (CHO) cells than IQ or MeIQx and it was mutagenic in vivo in the mouse in small intestine, whereas MeIQx was not. Among three HCAs, IQ, MeIQx, and PhIP, that were selected for carcinogenesis testing in monkeys, IQ was found to be a potent carcinogen inducing tumors in 70% of the monkeys at a dose of 10 mg/kg five times a week and 100% of the monkeys at a 20 mg/kg dose (Adamson et al., 1995).

There is increasing evidence that hereditary and environmental factors both greatly modify susceptibility to carcinogenesis induced by HCAs. Animals carrying spontaneously induced inherited mutations in genes are significantly more susceptible to HCAs and this should be considered when the carcinogenicity of HCAs is assessed in humans. Most HCAs are hepatocarcinogens, but the small and large intestines, mammary glands, clitoris gland, skin, oral cavity, urinary bladder, forestomach, lung, blood vessels, lymphoid tissue, and hematopoitietic system are among other target organs (Sugimura et al., 1996). Fortunately, the amounts of individual HCAs ingested from the environment might not be sufficient to cause cancer development in man based on a simple calculation of the doses of HCAs required to induce cancer in long term animal experiments at a 50% rate or TD_{50} values (Sugimura et al., 1997). On the other hand, the fact that the number of stem cells among the somatic population in man is 250-times greater than rats suggests the possibility that humans may be 250 times more sensitive to tumor induction than rats (Sugimura et al., 1994).

Heterocyclic Amines and DNA Adduct

Formation of HCA adducts at the C8 position of the guanine base in DNA was first

reported by Hashimoto et al.(1980) working with Glu-P-1 and then Trp-P-2. The structures of adducts of other HCAs, including IQ, MeIQx, and PhIP with guanine bases have been determined (Snyderwine et al., 1988).

All the known HCAs have a planar structure and are efficiently intercalated into guanine-cytosine (GC-pair) rich stretches of DNA. To date, DNA adducts have been characterized for seven different HCAs. Only guanine adducts have been identified. The N-acetoxy derivatives of the HCAs are not highly reactive with other nucleic acid bases (Turesky et al., 1992). All the HCAs examined form an adduct at the C8 position of guanine and MeIQx and IQ each form a minor adduct at the N2 atom of guanine. The first HCA-DNA adducts identified were the C8 guanine adducts of Trp-P-2 and Glu-P-1 (Hashimoto et al., 1979). These adducts were isolated from DNA treated with Trp-P-2 or Glu-P-1 in the presence of hepatic microsomes from polychlorinated biphenyl treated rats and the C8 adduct was the major adduct in the liver of rats (Hashimoto et al., 1982).

The earliest study examining the tissue distribution of HCA-DNA adducts in animals was carried out by Yamashita (1988). There is relatively little data on the binding of HCAs to RNA. Trp-p-2 and Glu-p-1 were reported to form the same adduct in RNA as in DNA, however the binding to RNA was tenfold lower than to DNA (Hashimoto et al., 1982). It is likely that the double strandness of DNA stereochemically facilitates the formation of HCA adducts (Snyderwine et al., 1988). Intercalation of the HCAs or their binding in the groove of double stranded DNA may in part explain the higher binding of these carcinogens to DNA rather than RNA (Hashimoto et al., 1982). Several animal studies have shown that HCA adducts are essentially related to administered doses (Frantz et al., 1995). Since gas chromatography/mass

spectral analysis has been used to explore whether PhIP-DNA adducts can be found in human tissue, spots of six out of 58 surgical samples of colon were detected (Kadulbar et al., 1995).

HCAs form adducts in macromolecules including DNA, RNA, and protein after metabolic activation. The toxicological significance of protein adducts indicates the mutagenicity of HCAs. PhIP induces chromosomal aberrations in freshly obtained human lymphocytes with a minimal concentration of 3.1-12.5 μg/ml (Otsuka et al., 1996). The most direct evidence for genetic damage is through measurement of DNA adducts in cells and their excretion products in urine. In fact, analysis of urinary metabolites can provide information on the ability of humans to metabolically activate or detoxify the procarcinogenic amines.

Mutagenic activity and Ames test

Genotoxic activity of the HCAs has now been studied in various organisms including bacteria, yeast, mammalian cells in vitro, and experimental rodents in vivo. Mutagenicity in bacteria was first used as a marker of the biological effects of HCAs. All HCAs that are mutagenic in *Salmonella* are also mutagenic in Chinese hamster lung cells with a rat liver S9 as a marker (Terada et al., 1986). In this system, harman and norharman are mutagenic without the rat liver S9 mix (Terada et al., 1986). These compounds require phase I and phase II enzymes to react with DNA. If DNA adducts are not repaired by nucleic excision repair, they yield mutations through translation synthesis. The specific mutagenic activity of HCA is determined mostly in *S. typhimurium* TA98. Mutagenicity in *Salmonella* varies vastly (more than 16,000 times) between the strongest and the weakest HCAs. For example, some of the HCAs show very potent mutagenicity. The specific mutagenic activity of IQ is a thousand times higher than that of

benzo[α]pyrene (Sugimura et al., 1997). This vast difference in mutagenic activities may be attributed to variation in reactivity of exocyclic amino groups with cytochrome enzymes, reactivity of metabolic intermediates with DNA, repairability of DNA-adducts, or mutagenic potential of the DNA adducts.

There is an important need to accurately measure the level of these compounds in food products. Initially, modeling was used to identify the mutagenic HCAs and their precursors. During the last decades, several analytical methods for the analysis of HCAs have been described. Such methods should ideally perform accurately and reproducibly and should be low in operator and instrument time usage (Knize et al., 1992). The availability of sophisticated chromatographic and analytical techniques made it possible to isolate, characterize, and analyze several specific compounds formed in early and advanced stages of the Maillard reaction. Gross (1992) has reported a screening method using solid-phase extraction and high performance liquid chromatography (SPE-HPLC) with ultraviolet and fluorescence detection. This method allowed isolation of the HCAs into two groups, a polar extract containing IQ, IQx, MeIQ, MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, PhIP, Glu-P-1 and Glu-P-2, and an apolar extract containing Trp-P-1, Trp-P-2, amino-α carboline, and methyl-amino- α carboline, as well as the non-mutagens norharman and harman. This method was successfully used for the analysis of HCAs such as in pan fried, oven-cooked, and barbecued salmon (Gross and Grüter, 1992). However, for certain industrial products, the isolation was not selective enough and co-eluting interferences spoiled the chromatograms obtained with UV-detection. In such cases, additional purification or mass spectrometric detection after thermospray ionization proved to be successful. The SPE-HPLC method was evaluated in inter-laboratory comparisons. Despite working well, this method has

the drawback of a lengthy sample preparation that requires several hours of bench work. One method for overcoming this drawback is an automated robotic system. Until now, HPLC-MS still appeared to be the only technique able to screen most of the known HCAs simultaneously.

Mutagenicity is determined by the Ames test, which determines whether *Salmonella typhimurium* TA98 were mutated or not by adding the recovered HCAs to their media. This test is performed using the standard plate incorporation assay described by Ames et al. (1976). In fact, the mutagenicity of these compounds has been proven in bacteria by the Ames test (Sugimura et al., 1982 ; Felton et al., 1986) and in mammalian cells like the Chinese hamster ovarian cells (Thompson et al., 1987; Alexander et al., 1989). This is a highly sensitive biological assay to assess the mutagenic potential of a chemical compound, although it does not give information on the different types of mutagens. The bacterium used in the test is a strain of *Salmonella typhimurium* that caries a defective gene that makes it unable to synthesize the amino acid histidine from the ingredients in its culture medium, which means that it requires histidine for growth. In the absence of an external histidine, bacteria cannot grow to form a colony. A colony is resumed if a reversion of a mutation happens.

Salmonella typhimurium TA98 is more suitable for detecting the mutagenic activities of this series of compounds than *Salmonella typhimurium* TA100 (Sugimura et al., 1983).

Formation of Heterocyclic Amines

The formation of mutagenic chemicals in meats has been explained by the condensation of creatine or creatinine with amino acids and sugars or their thermal decomposition products in model systems and meats. In 1998, Kato described two possible pathways for the generation of

HCAs: one is the condensation of dihydropyrazines or pyridines, aldehydes, and creatinine, while the other is the condensation of pyrazine or pyridine cation radicals, carbon centered radicals, and creatinine.

The Maillard reaction, which involves Amadori rearrangement as a key step, results in sugar fragmentation and free radical formation. Participation of the Maillard reaction in the formation of the heterocyclic amines was proposed several years ago. Kikugawa (1999) found that free radical intermediates, pyrazine cation radicals and carbon-centered radicals, generated in the Maillard reaction of sugars/amino acids were involved in the production of mutagenic and carcinogenic imidazoquinoxaline-type heterocyclic amine mutagens (Figure 3). When food is cooked, carbonyl and amino compounds react via the Maillard reaction to produce several hundreds of reaction-intermediate products. Some of these contribute to the color and flavor of the cooked food. The Maillard reaction may also have an impact on the nutritional value of the food. Furthermore, in some cases, the Maillard reaction can lead to the formation of genotoxic compounds of HCAs. These compounds, most of them commercially available, are planar aromatic and stable solids. However the amounts of toxic HCAs formed during cooking are very small. Four factors influence the formation of HCAs: type of meat, cooking time, cooking method, and temperature used. Frying, broiling, and barbecuing produce the largest amounts of HCAs because the meats are cooked at very high temperatures. Oven roasting and baking are done at lower temperatures, so lower levels of HCAs are likely to form; however, gravy made from meat contains a considerable amount of HCAs. For instance, stewing, boiling, or poaching are done at below 100 °C (212 °F).

Using model experiments, Shioya (1987) showed that phenylalanine, creatinine, and

glucose were probable precursors for PhIP. On the other hand, PhIP may also be produced from creatine heated together with leucine, isoleucine, and tyrosine, and glucose does not seem to be a necessary precursor using dry heating conditions (Skog et al., 1998). Aqueous model systems are known to require sugar to produce IQ or IQx compounds (Skog et al., 1993) and the addition of glucose had a significant effect on the formation of these HCAs, resulting in a higher yield and a change in the relative amounts of the HCAs. Creatine, pyrazine or pyridine, and aldehydes are assumed to condense to form IQ compounds (Jägerstad et al., 1983).

At the beginning of last century, Maillard proposed the browning reaction to account for the brown pigments produced from the reaction of the amino group of an amino acid and the carbonyl group of a sugar. At some stages of the browning reaction, pyrazines are formed that are involved in the formation of HCAs. Experiments using 14 C-labeled glucose in a model system with threonine and creatinine showed that the radioactive C-atoms of the glucose are incorporated into the HCAs. A combination of these results lead to the suggestion that the carbon atoms from the pyridine or pyrazine moiety originate from glucose (Figure 4).

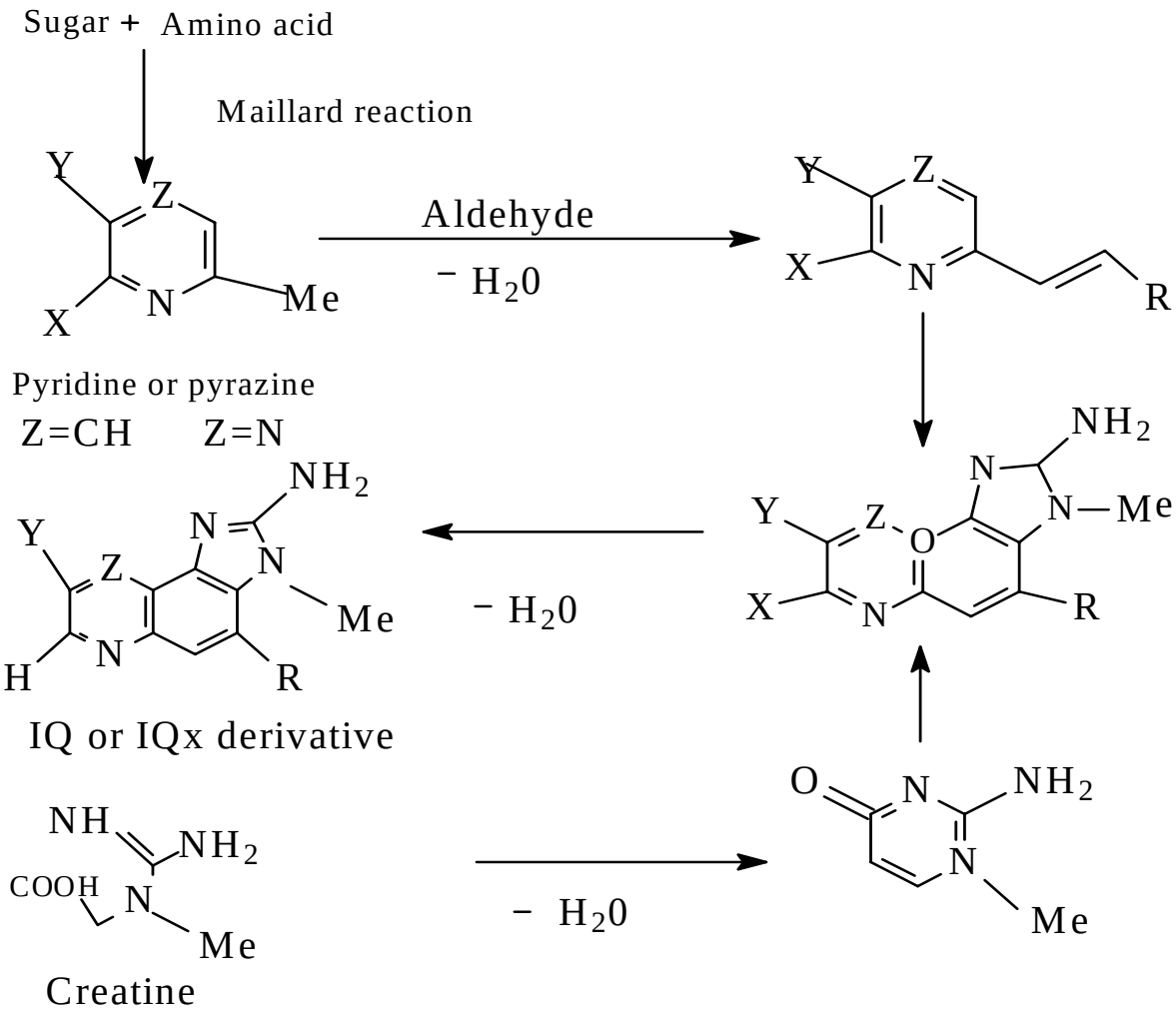


Figure 3. Maillard reaction theory mechanism (adopted from Jägerstad et al., 1998).

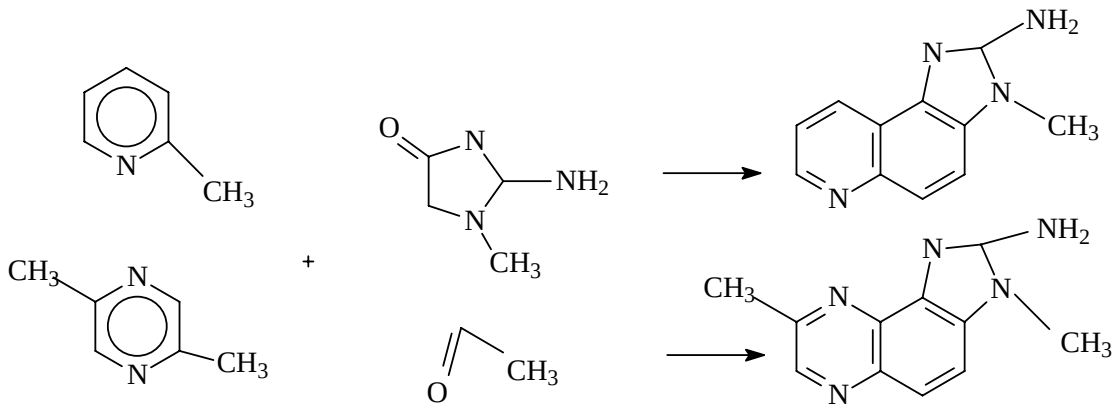


Figure 4. Suggested formation of imidazoquinolines and imidazoquinoxalines from products of the Maillard reaction (2-methyl-pyridine, 2,5-dimethyl-pyrazine) with acetaldehyde and creatinine (Adopted from Murkovic, 2004).

A defined mechanism was described for the formation of norharman, a non-polar HCA. Norharman itself is not mutagenic, but in the presence of aniline, it becomes a co-mutagen. According to the mechanism shown in Figure 5, the tryptophan Amadori rearrangement product in the furanose form undergoes a dehydration reaction, which is followed by ß-elimination and assisted by the lone pair of electrons of the ring oxygen forms with a conjugated oxonium ion (Yaylayan et al., 1990).

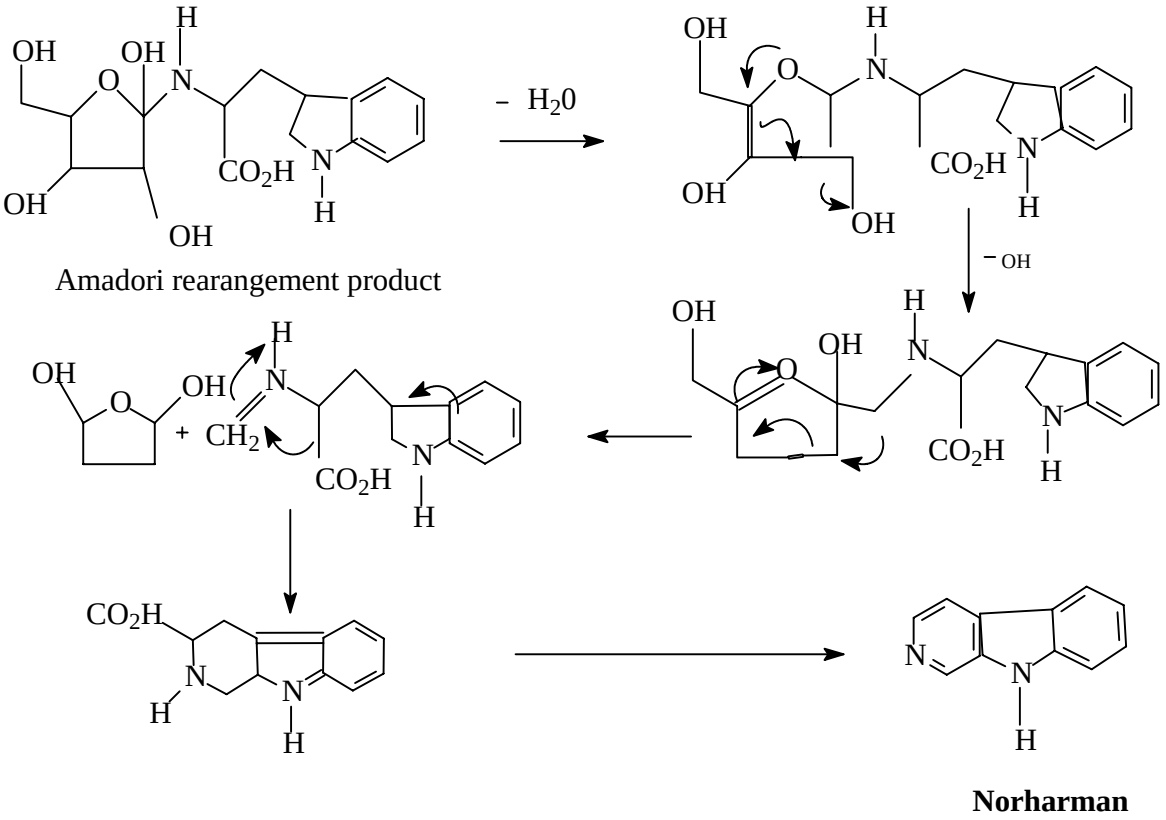


Figure 5. Formation of norharman from tryptophan Amadori rearrangement product (Adopted from Yaylayan, 1990).

The results from model system experiments show that combination of creatine and free amino acids or dipeptides together with mono or disaccharides or protein hydrolysates from vegetable or animal sources are precursors of the HCAs known today.

For all the imidazo containing HCAs (e.g. IQ, IQx derivatives), creatinine is a crucial precursor. Creatinine occurs in all muscle cells where it constitutes an energy-rich metabolite-containing phosphate group. Creatine is converted to creatinine when heated. When meat is

cooked, creatine is transported to the surface and concentrated in the crust, where the temperature is high enough to transform creatine to creatinine. Creatinine forms the amino- imidazo part of the molecule by cyclization and water elimination, whereas the remaining parts of the IQ compounds arise from Strecker degradation products like pyridine and pyrazine (Jägerstad et al., 1991).

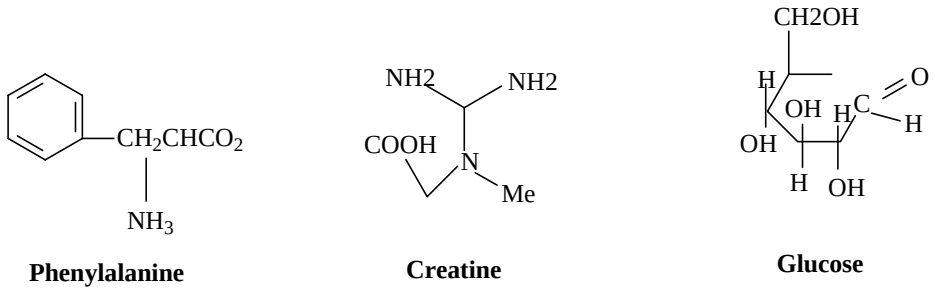
Jägerstad et al. (1983) also described that through the Maillard reaction followed by Strecker degradation pyrazines, pyridines and aldehydes are assumed to condense to form IQ (imidazoquinoline) compounds. Meanwhile, Pearson et al. (1992) suggested two different pathways for the formation of imidazoquinoline and imidazoquinoxaline meat mutagens. They found a contribution of the free radicals from the Maillard reaction and the Amadori rearrangement of sugar fragmentation. Skog and Jägerstad (1991) showed that by adding glucose to phenylalanine and creatine, PhIP was formed. Formation of PhIP was also investigated by Felton`s group (1986), and after heating phenylalanine and creatine, they showed that the phenyl ring from phenylalanine is incorporated intact and the 3-carbon atom and the amino nitrogen from phenylalanine are incorporated into PhIP. Other researchers used labeled creatine and found that 1-nitrogen, the methyl carbon, and the amino-nitrogen from creatine are each incorporated into PhIP (Taylor et al., 1991). From these experiments, it can be assumed that the carbon atoms of phenylalanine form a part of the pyridine moiety and that creatine forms the imidazol part (Zochling et al., 2002).

Factors affecting the formation and yield of Heterocyclic amines

Factors affecting HCA production include cooking time, temperature, pH, precursor

concentration, and amino acid profile. The chemical structures of precursors are shown in Figure 6. Formation of HCAs has been shown to require three classes of precursors: creatine/creatinine, free amino acids or dipeptides, and sugar. Jägerstad et al. (1983) demonstrated that the mutagenic activity in beef increased if a solution of creatine was spread over the surface of meat before frying. According to Jägerstad et al. (1998), unstable free radical intermediates have been shown to be involved in the formation of HCAs.

Mutagenic activity was first reported in protein foods, however, when proteins were added instead of amino acids in a model system, no mutagenic activity was detected (Jägerstad et al., 1983). These results indicate that amino acids and not proteins participate in the mutagenic formation. It is therefore important to distinguish between the content of proteins and the content of free amino acids in meat.



Phenylalanine **Creatine** **Glucose**

Figure 6. Chemical structures of precursors for IQ type HCAs.

Metabolism of Heterocyclic Amines

Heterocyclic amines are likely to be extensively metabolized (Gooderham et al., 1987). In a study using a Cytochrome P450 inhibitor, more than 90% of MeIQx and nearly 70% of PhIP elimination could be inhibited in vivo, demonstrating the extent to which activation occurs in

humans (Gooderham et al., 1997). Several different types of metabolizing enzymes are involved in the biotransformation of chemical carcinogens. Among these enzymes, Cytochrome P450

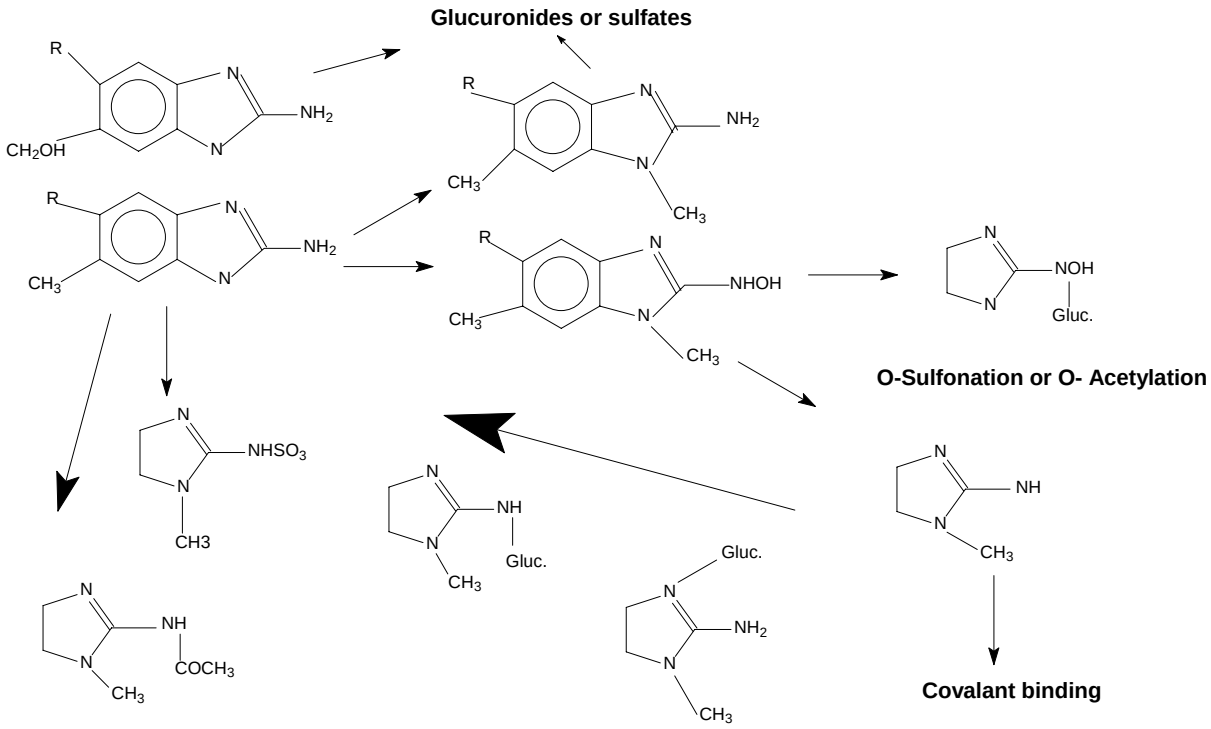


Figure 7. Major metabolic pathways (oxidation and conjunction) of HCAs. (modified from Nagata, 2000).

species are the keys to carcinogen biotransformation. In vitro studies with MeIQx and PhIP using liver microsome preparations from rats, mice, and rabbits showed that at least two oxidative metabolites were formed from each compound, a ring-hydroxylated product and the N-hydroxy derivative (Gooderham et al., 1987). In addition, both the parent amines and primary oxidative metabolites can be further biotransformed to a variety of phase II metabolites including

glucuronides (Kaderlik et al., 1994), sulfate esters (Chou et al., 1995), and acetylated products (Lee et al., 1995). Studies were performed on the metabolic fate of these mutagenic HCAs performed with isolated enzymes, subcellular fractions, and cells in vitro. Investigation with samples from experimental animals have shown that both IQ and non IQ type compounds undergo N-oxidation and subsequent esterification prior to causing DNA damage. Work performed with samples from humans and metabolic-activating pathways of both types of HCAs have been shown to be consistent (Figure 7).

Using liver microsomes and recombinant P450 systems, IQ and MeIQ have been shown to undergo N-hydroxylation as the major pathway of oxidative metabolism (Hammons et al., 1997).

Human P450 including CYP1A1, CYP1A2, CYP1B1, CYP2A6,CYP2E1, and CYP3A4/5 are known to mediate activation of carcinogens. CYP1A1, CYP1A2, CYP1B1 and CYP3A4 are mostly involved in metabolic activation of HCAs through the N-oxidation (Crofts et al., 1997; Hammons et al., 1997). Interestingly, the human tissue content of P450 is known to differ markedly among individuals. The diet is believed to be a major factor modifying the content of hepatic CYP1A2 (nmol/mg microsomal protein), which show more than thirty individual variations in humans (Yamazoe et al., 1988). Therefore, based on the established mechanisms of chemical carcinogenesis, genetic differences in metabolizing enzymes could play a major role in cancer susceptibility.

Marinades

Today, there is increasing evidence that dietary antioxidants reduce the formation of HCAs and interfere with their metabolic activation. It has been suggested by Skog (1993) that

marinating beef steaks before frying can reduce precursors of mutagenic substances. A marinade may act as a "barrier" keeping flames from directly touching the meat. Or the protective powers may lie in the ingredients of a typical marinade. Herbs and spices seem to contribute at least partially to the prevention of HCA formation. According to Gu et al.(2001), seasoning with commercial seasoning consisting of soy sauce, sesame oil, sugar, garlic, and onion powder for four hours at room temperature decreased mutagenicity ($P < 0.01$) of pan-roasted beef except the very well-done sample, which had unchanged HCA contents.

Tikkanen et al. examined the effects of commercial marinades on the mutagenic activity and sensory quality of grilled chicken and reported that processing conditions appear to have a distinctive effect on the mutagenicity of the final products. The amounts of HCAs were determined and results showed the marked reduction of HCAs compared to unseasoned control samples. The ingredients of marinades were a complex mixture of several components, including spices and flavonoids, and therefore it was hard to evaluate which factors exactly inhibited the formation of mutagenicity in the chicken samples (Tikkanen, 1996). Obviously, due to the variation of spice contents of different marinades, reduced levels of HCAs were different. It is suggested that a very simple but efficient method to reduce exposure to HCAs would be coating and marinating meats for even a short time with spices and herbs before grilling.

Inhibition or Reduction of Heterocyclic amines

Since HCAs represent potential risk factors in the etiology of human cancer, it is desirable, although difficult, to control or eliminate levels of mutagens in food. Minimization of HCA formation relates to cooking procedure, temperature, and the role of added food ingredients.

Well-done meat contains more than 10 times the concentration of mutagens that rare cooked meat does, so keeping the temperature as low as possible should minimize mutagen formation. Although a certain amount of glucose is needed, excess glucose, lactose, and milk powder inhibit the formation of mutagens in fried beef patties. Microwave cooking and discarding the dripping before frying resulted in the production of a low level of mutagens, presumably because the water soluble precursors for mutagen formation such as amino acids, glucose, and creatine were discarded. Soaking or marinating beef steaks before frying may achieve lower mutagens. Arimoto (1993) and Hayatsu (1993) separately reported that porphyrins such as hemin, chlorophyll, chlorophylin, and phthalocyanine inhibit the mutagenicity of HCAs and some other mutagens. Edenharder (1995) reported that chlorophyll in green beans, broccoli, and spinach reduces considerably the mutagenicity of IQ and MeIQx. Kikugawa (1999) also showed that intermediates of HCAs were eliminated and stabilized in a model system when adding butylated hydroxy anisole (BHA), seasamol, or epigallocatechin gallate (EGCG), as demonstrated by Electron Spin Resonance (ESR).

Antioxidants

In several studies, the addition of antioxidants has proved to inhibit the formation of HCAs due to both mechanisms scavenging the intermediary pyrazine cation radicals as well as protecting lipids against oxidation. Extensive studies have focused on the prevention of the Maillard intermediates from reacting with creatinine. The use of antioxidants as free radical scavengers have demonstrated efficiency in stabilizing these intermediates, thereby reducing the HCA levels (Verdin et al., 2002 ; Nagao et al., 2000). Natural antioxidants found in some spices

have been demonstrated to decrease the levels of HCA in several muscle foods. Rosemary, thyme, sage, and garlic have been shown to decrease the formation of common HCAs in fried meat products (Murkovic et al., 1998). Oregano (*Origanum vulgare*), from the *Labiatae* family, is known to contain naturally occurring antioxidants such as rosmarinic acid, polyhydroxybenzoic acid, cinnamic acid, caffeic acid, and water soluble glycosides (Shahidi, 1997).

In recent years, a great deal of interest has been devoted to preparing antioxidants from natural sources by extraction, purification, and fractionation. The mechanism of protection given by an antioxidant was postulated to occur at the initial stage and more effectively during the propagation stage of oxidation by peroxy radical (ROO·) scavengers such as phenolic compounds (Torel et al., 1986).

Antioxidants tested in model experiments and in meat products included vitamins like tocopherol, ß-carotene and ascorbic acid, carotenoids from tomatoes, phenolic compounds like butylatedhydroxyanisol, propyl gallate, epigallocatechin gallate, sesamol and esculetin, tea and tea polyphenols, cherry tissue as well as single phenolic compounds and extracts from herbs and spices. Surprisingly, and in contrast to the view that a free radical-dependent mechanism lies behind HCA formation, in a study by Johansson and Jägerstad (1994) most antioxidants used increased formation of MeIQx in a model system. However, when ß-carotene and α-tocopherol was added to model mixtures containing the pro-antioxidants hydroquinone and iron sulfate, the formation of MeIQx decreased. The free radical scavengers, ß-carotene and-tocopherol were probably able to scavenge the hydroquinone radicals, thus inhibiting HCA formation (Felton et al., 1999).

Oguri (1998) investigated the effect of 14 food derived phenolic antioxidants on the formation of MeIQx and PhIP in a model system. Green tea catechins, epigallocatechin gallate, luteolin, quercetin, and caffeic acid clearly suppressed the formation of both MeIQx and PhIP. These antioxidants also reduced the mutagenicity of the heated mixtures. However, some antioxidants showed contradictory effects on the yield of HCAs such as nordihydroguaiaretic acid (NDGA), which markedly increased the yield of MeIQx but reduced the yield of PhIP.

Green tea catechin, a plant phenol, showed the tendency to reduce the incidence, multiplicity, and the mean size of mammary adenocarcinomas in rats treated with PhIP (Hirose et al., 1998). Chlorophyllin, a water soluble salt of chlorophyll, creates complexes with HCAs and reduces absorption and increases their excretion via feces (Guo et al., 1995).

Antioxidants can inhibit lipid oxidation and color changes during storage in cooked meat and poultry products. Recently, interest in natural antioxidants has increased because of questions about the long-term safety and negative consumer perception of synthetic antioxidants. Antioxidative effects of rosemary extracts have been observed in ground pork products and several food systems. The main antioxidative effect of rosemary has been reported to relate to the presence of three phenolic compounds: carnosic acid, carnosol, and rosmarinic acid (Cuvelier et al.,1996). The structures of these compounds are shown in Figure 8.

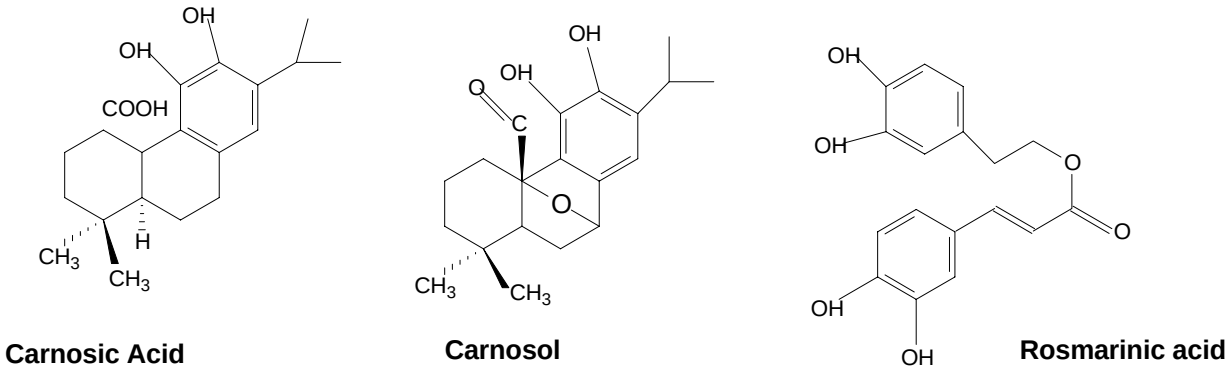


Figure 8. Chemical structures of carnosol, carnosic acid, and rosmarinic acid.

The amount of HCA formation can be decreased in meats by inhibiting the reaction between the Maillard reaction and creatine. Because HCAs are formed by free radical reactions, attempts to inhibit this reaction have been tried. Herbs and spices have been used since prehistoric times to preserve foods. Based on a review of numerous studies of food products, clove, rosemary and sage were concluded to be among the most potent antioxidative spices. Therefore, natural antioxidants such as tocopherol (vitamin E) or synthetic antioxidants such as butylated hydroxyanisole (BHA) have been shown to reduce HCAs by scavenging the free radicals (Chen and Chiu, 1998). Pyrazine and pyridine are radicals from the Maillard reaction, thus antioxidants act to stabilize these intermediates and reduce the content of HCAs in meat (Chen et al., 1992). In 1998, Murkovic showed that sprinkling dried spices on the surface of steak decreased the amount of HCAs in meat. The application of spices (rosemary, thyme, sage, and garlic) successfully reduced the content of the HCAs below 60% of the amount found in the

control (Murkovic et al., 1998).

This current study also has been done to show antioxidants efficiency at preventing the formation of the most abundantly observed HCAs on grilled steaks, as well as the inhibition of these HCAs by using natural antioxidants present in plants.

Carbohydrates

Mutagenic activity decreases as the amounts of carbohydrates added to meat increases, especially the inhibitory effect that is pronounced when glucose and lactose are present. In a study by Skog (1992), minced meat was mixed with starch from golden bread crumbs or potatoes with and without glucose and the patties were fried for three minutes at 150-180° C in a double-sided fryer.

The mutagenic activity was determined using the Ames test. As a result, the addition of glucose or lactose was inhibited by 34-76%. A similar inhibition of the mutagenic activity was observed with powdered milk. Addition of both starch from bread crumbs as well as potatoes with glucose inhibited mutagenic activity up to 54%. Although creatine can be converted to creatinine during the frying, the greater the amount of carbohydrates mixed with meat, the more creatine recovered and the less creatinine formed (Skog et al., 1992).

Lipids

Several studies investigated the effects of fat on the formation of HCAs (fat has the physical effect of improving the heat transfer through the food matrix) as well as chemical effects of interacting in the formation mechanism of HCAs. It has been suggested that lipids may

participate in the formation of Maillard reaction products. Alldrick (1987) has investigated the role of dietary fat as a modifier of metabolic enzyme activity. They concentrated on the effect of fat on the conversion of three HCAs: IQ, MeIQ, and MeIQx. High fat diets increased the conversion of these compounds into bacterial mutagens, and the magnitude of the increase was dependent on the type of fat used (olive oil gave the greatest response and sunflower oil the least). Johansson showed that the addition of oils in a model system was found to significantly increase the formation of MeIQx (1993). The reported effects of lipids on the formation of MeIQx might be explained by an enhanced formation of pyridines, pyrazines, and Strecker aldehydes in the Maillard reaction (Arnoldi et al., 1990). Johansson (1993) also found that the addition of corn oil, olive oil, or linoleic and/or linolenic acid to a model system increased the amount of MeIQx formed compared to the amount observed without fatty acid addition. These finding suggest that the formation of food mutagens can increase when frying is prolonged in the presence of certain fats. This is often the case during deep fat frying of meat and fish with oils heated for several hours. However, fat may be involved chemically in the formation of HCAs by generating free radicals via lipid oxidation or by participating in the Maillard reaction (Arnoldi et al., 1990).

Microwave Treatment

Efforts in many laboratories have been directed at reducing the amounts of mutagenic compounds during the cooking procedure and one of the most efficient way was microwave cooking. In microwave cooking, the heat generates inside the product and the temperature does not increase above the temperature of other parts and no crust is formed. Removing the known precursors of HCAs from beef patties by microwave pretreatment before frying was shown by

Taylor (1986). Felton (1994) examined the effect of varying microwave pretreatment times to remove creatine, creatinine, amino acids, and fat in meat, as well as to determine the mutagenic activity formed during the subsequent frying of the meat at 200 or 250 °C. They used solid-phase extraction and HPLC analysis to measure the level of MeIQx, DiMeIQx, IQ, Trp-p-1, Trp-p-2, AαC, and PhIP formed as a result of frying after varying microwave pretreatment time from 0-3 minutes. Then they compared the mutagenic activity measured in the extracts of the beef patties. The amount of creatine in the meat after microwave treatment is shown in Figure 9.

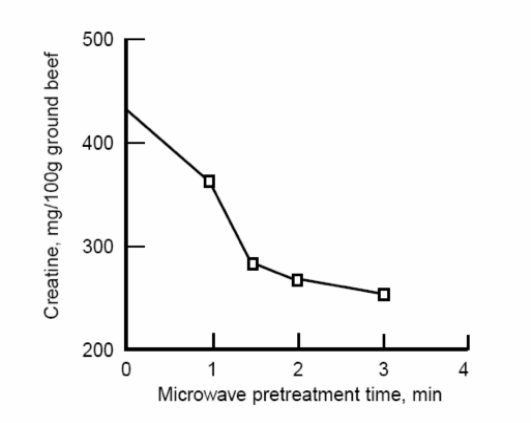


Figure 9. Graph of creatine remaining after varying microwave pretreatment times (Adopted from Felton,1994).

Cooking Procedures

Food preparation methods have a significant influence on the formation of mutagenic activity, and much research has been devoted to the mutagenic activity in fried/broiled food. In fact, the main food mutagens found in cooked meat products are HCAs. The aim of frying meat is to obtain a golden brown crust with flavor, which is induced by the Maillard reaction or non

enzymatic browning. Flavor in meat is derived from water-soluble precursors such as amino acids and sugars, which form during heating from different pyridines, pyrazines, and other volatile products (Ledl, 1990).

Heat can be transferred to the food in different ways, including conduction, convention, or radiation. A high temperature in the outer layer of the meat in combination with decreased moisture content causes chemical and physical changes that form the crust (1-3 mm thick).

Several groups have investigated the most important variables affecting mutagens formation: cooking temperature, cooking time, and cooking method. Mutagenic activity of cooked products increases progressively with the increase of temperature and also with the increase of cooking time (Knize et al., 1985). Of the two variables of temperature and time, temperature seems more important in formation of mutagens. Investigations of several cooking methods have shown that contact frying, deep fat frying, and broiling all cause much higher levels of mutagenic activity than other cooking methods such as roasting, stewing, and microwave cooking (Hargraves and Pariza, 1984).

Table 3. Heterocyclic amines in cooked beef patties fried for 6 min per side following various microwave pretreatment.

Microwave time (min)before Frying at 200 °C	IQ*	MeIQx	DiMeIQx	PhIP
0	ND	3.0 ± 0.2	0.3 ± 0.1	2.7 ± 0.4
1.0	ND	1.3 ± 0.3	0.1 ± 0.0	0.7 ± 0.2
1.5	ND	0.5 ± 0.3	0.2 ± 0.0	1.6 ± 0.2
2.0	ND	0.5 ± 0.1	0.2 ± 0.1	1.6 ± 0.2
3.0	ND	ND	0.1 ± 0.0	2.2 ± 0.3
Microwave time (min)before Frying at 250 °C				
0	1.0 ± 0.2	5.1 ± 0.7	1.2 ± 0.3	13.3 ± 1.8
1.0	ND	1.7 ± 0.1	0.3 ± 0.1	9.4 ± 2.5
1.5	ND	ND	0.7± (0.1)	3.3± (0.5)
2.0	ND	ND	0.5± (0.2)	1.9± (0.1)
3.0	ND	ND	0.1± (0.0)	2.2± (0.3)

* ng/g ± SD

The reduction of HCAs and concomitant mutagenic activity is possible by eliminating the known precursors. Microwave cooking alone has been shown not to form mutagenic activity during cooking in three studies (Dolara et al., 1986).

Therefore, it is suggested that reduction of HCAs and mutagenic activity is possible by eliminating the known precursors of HCAs through microwave treatment. Creatine, sugar, and amino acids were reduced up to 30%, whereas HCAs were reduced up to 90% after two minutes microwave pretreatment (Felton et al., 1994). The reason for this phenomenon is water loss during microwaving and preventing the transport of small molecules of precursors to the meat surface.

[1,2]PART II. EFFECTS OF MARINADES ON THE FORMATION OF HETEROCYCLIC AMINES IN GRILLED BEEF STEAKS

ABSTRACT

Heterocyclic amines (HCAs) are suspected human carcinogens formed in muscle foods during high temperature grilling or cooking. Inhibition of HCAs by commercial marinades were evaluated at 204 °C (400 °F). Levels of four HCAs were investigated: 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP), 1-methyl-9H-pyrido[4,3-*b*]indol (harman), and 9H-pyrido[4,3-*b*]indol (norharman). The marinades were formulated according to the package label instructions in an oil, water, vinegar mixture, and the steaks were treated for one hour prior to grilling. All three marinades, Caribbean, Southwest, and Herb, significantly decreased the imidazo-azaarene HCAs (MeIQx, PhIP) as contrasted to controls and liquid blanks. The marinading liquid mixture had no significant effect on any of the HCAs. The Caribbean mixture showed the highest decrease in the total HCA content (88%), followed by the Herb (72%), and Southwest (57%). With a few exceptions, there were significant decreases in HCAs for treatments with only the marinade bases (ingredients without any spices/herbs). As measured by HPLC, the marinades contained considerable amounts of the polyphenolic antioxidants carnosic acid, carnosol, and rosmarinic acid with the Caribbean being the highest. Commonly available spice-containing marinades can be effective inhibitors of HCA formation and provide reduced exposure to some of the carcinogens formed during grilling.

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[2] Abstract presented in part at IFT Annual Meeting, 2006. http://ift.confex.com/ift/techprogram/paper_003F-4.htm

MATERIALS AND METHODS

Standard HCAs 2-amino-3,8-dimethylidazo[4,5-f] quinoxaline (MeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP), 1-methyl-9H-pyrido[4,3-b]-indol (Harman),and 9H-pyrido[4,3-b]-indol (norharman) were obtained from Toronto Research Chemicals (Toronto, Canada). Rosmarinic acid (97% purity) was obtained from Aldrich Chemicals Co. (Milwaukee, WI.,USA). Carnosic acid was obtained from Essential Oils University (New Albany, In.,USA), and carnosol was purchased from A.G. Scientific, Inc. (San Diego, CA, USA). Solvents and chemicals such as acetonitrile (HPLC grade), methanol (HPLC grade), methylene chloride (HPLC grade), and sodium hydroxide (ACS-grade) were all obtained from Fisher Scientific Co.(Fairlawn, NJ, USA). Ammonium acetate and triethylamine were purchased from Aldrich Chemicals Company, Inc. (Milwaukee, WI, USA). Phosphoric acid was obtained from Sigma Chemicals Company, Inc. (St. Louis, MO, USA). Deionized water was processed by a Sybron/Barnstead PCS unit (Barnstead/Thermolyne, Inc.; Dubuque, IA, USA). Bond Elut® propyl-sulfonic acid (PRS) 500mg cartridges, C-18 (100 and 500 mg) cartridges and the coupling adaptors were purchased from Varian Sample Preparation (Harbor City, CA, USA).

Sample preparation, marinades, and grilling of steaks

Eye round steaks (*Semitendinousus*, average wt 96 g, 0.5 cm thickness) were purchased from a local supermarket in Manhattan, KS. They were frozen until use and then thawed at 4 °C overnight prior to cooking. Before grilling, the intact steaks were immersed in the marinates containing the ingredients listed in Table 4 for one hour at 4 °C. They were turned several times during this period to insure that all surfaces were coated with the marinade. The experiment was

created with a randomized design for control samples (plain steaks), blank samples (steaks marinated with water, vegetable oil, and vinegar), and three different commercial marinated samples. Only the outer layer (about 4 mm thickness) of the steaks was trimmed off with a sharp knife and analyzed (Table 4). All the marinades contained at least several spices from the *Lamiacease* (mint) family. According to the commercial marinade product labeling, the ingredients of the Caribbean marinade included salt, thyme, red pepper, black pepper, allspice, rosemary, sugar, caramel color, and chives. Three separate marinade packets (weight 30 ± 1g) were purchased from a local grocery store. The Southwest marinade contained paprika, red pepper, oregano, thyme, black pepper, garlic, onion, salt, sugar, maltodextrin, and silicon dioxide. The Herb marinade contained oregano, basil, garlic, onion, jalapeno pepper, parsley, red pepper, salt, sugar, maltodextrin, corn starch, soy protein, whey solids, wheat gluten, and calcium silicate.

The marinade powders or base formulations were suspended in a mixture of 60 mL water, 30 mL soybean oil, and 30 mL of vinegar prior to use, thus producing the treatments. In addition, the study included control samples (plain steaks), blank samples (steaks marinated with the liquid only), and bases (marinade ingredients without spices). The ingredients of the different marinade mixtures examined in this study are shown in Table 4.

Table 4. Ingredient and spice composition of the different marinade treatments based on the product labels.

Treatment[1]	Ingredients[2]	Spices
Control (unmarinated and nothing added)	none	none
Marinading liquid blank	water, soybean oil, vinegar	none
Caribbean base + liquid	salt (5.4%), sugar, caramel color	none
Caribbean marinade	salt, sugar, caramel color	thyme, red pepper, black pepper, allspice, rosemary, chives
Southwest base + liquid	salt (3.1%), sugar, maltodextrin, silicon dioxide	none
Southwest marinade	salt, sugar, maltodextrin, silicon dioxide	paprika, red pepper, oregano, thyme, black pepper, garlic, onion
Herb base + liquid	salt (2.2%), sugar, maltodextrin, modified corn starch, whey solids, soy protein, wheat gluten, calcium silicate	none
Herb marinade	salt, sugar, maltodextrin, modified corn starch, whey solids, soy protein, wheat gluten, calcium silicate	oregano, basil, garlic, onion, jalapeno pepper, parsley, red pepper

[1] The liquid used to suspend the bases and marinades contained 60 mL water, 30 mL of soybean oil, and 30 mL of vinegar. The pH value of the liquid blank was 2.70.

[2] Salt values are from the product nutritional labels

Instrumentation

A Teflon-covered electric grill with a temperature controller (Toastmaster, Denver, CO, USA) was used to grill the steaks. The temperature profile of the grill is shown in Table 5. Steaks were grilled at 400 °F for five minutes on each side. A temperature profile of the grill surface was measured using a surface probe thermometer (Barnant Company, Barrington, IL, USA). A Supelco Visiprep SPE (Bellefonte, PA, USA) vacuum manifold was used for manipulation with solid-phase extraction cartridges. High performance liquid chromatography (HPLC), HP Series II, 1090A (Palo Alto, CA, USA) fitted with a photodiode array ultraviolet(UV)-visible detector (HP 1040) and a fluorescent detector was used for separation and identification of HCAs. Detection of HCAs was achieved by both UV and fluorescence detectors. The UV detector was set at 263 nm wavelength for monitoring MeIQx. The fluorescence detector was programed according to the maximum excitation and emission wavelengths each of Harman, norharman, and PhIP. For Harman, the excitation wavelength was 243 nm and the emission wavelength was 437 nm. For norharman, the excitation wavelength was 245 nm and the emission wavelength was 448 nm. For PhIP, the maximum excitation was 229 nm and the emission was 437 nm.

Table 5. Grill surface temperature profile with center area set at 400 °F. The bold numbers represent the areas used for grilling.

305.1	319.5	321.6	328.9	330.3	335.7	336.9	339.1	338.1	339.2	329.2	315.3
299.1	331.3	339.5	345.3	339.8	346.8	349.9	359.6	365.9	366.6	364.9	389.4
281.1	338.1	**387.0**	**398.9**	**403.2**	**395.9**	**398.3**	**399.2**	**401.0**	**399.9**	**405.3**	410.8
279.3	340.0	**390.2**	**389.0**	**375.1**	**372.2**	**370.0**	**373.2**	**383.5**	**391.0**	**407.8**	406.7
281.2	349.8	**389.3**	**381.5**	**380.0**	**378.3**	**393.2**	**402.3**	**395.4**	**394.6**	**409.9**	387.8
281.1	338.1	**392.3**	**400.0**	**401.2**	**397.9**	**399.1**	**398.1**	**398.0**	**400.2**	**406.7**	399.4
309.2	349.8	342.3	350.9	371.3	382.9	379.2	381.5	380.5	378.8	391.0	386.2
301	303.3	315.7	337.1	342.9	362.2	337.0	339.3	330.1	332.3	319.3	318

Extraction of Marinades

The antioxidants were extracted following the procedure described by Wellwood and Cole (2004). Ethanol (95.27% purity) was the solvent used to dissolve the powdered spice, which was then placed in a glass test tube. The test tube was placed in a 35 °C water bath for about three hours, shaken well, evaporated to dryness by an evaporator (Rotavapor, Model RE-121 B, Büchi/Brinkmann), and then the residue was suspended in 2 mL of ethanol and stored in amber vials at 2 °C prior to HPLC analysis. All the solutions were passed through a 0.45 µm nylon filter (Whatman, Clifton, NJ, USA) before injection into the HPLC.

Solid- Phase Extraction of HCAs

HCAs were extracted and purified from meat using a solid-phase extraction method described by Gross and Grüter (1992). Three grams of the external surface of meat were

homogenized by 12 mL of NaOH (1M) in a commercial Waring blender for three minutes at medium speed and mixed with 24 grams of Extrelut refill material and then poured into the empty Extrelut columns. For determination of recovery, selected homogenate samples were spiked with 50 ng of each standard. The recovery of each HCAs was obtained based on ratio of the calculated concentration to added concentration. The Bond-Elut PRS (polysulfonic acid, 500 mg) cartridges were adopted onto the vacuum manifold and conditioned with 5 mL of ethyl acetate, and another 2 mL applied under positive pressure. The Extrelut® columns fitted onto the PRS cartridges for weak cation exchange. Polar HCAs (MeIQ, and MeIQx) were eluted at 1 mL/min with 60 mL of ethyl acetate into the PRS cartridges. The PRS cartridges were then dried under vacuum pressure at 15 "Hg for four minutes and then the PRS was rinsed at 1 mL/min with 6 mL of 0.1 N HCL and this was discarded. The methanol-HCL and water elutes containing the nonpolar HCAs (Harman, norharman, and PhIp) were neutralized with an ammonia solution (NH_4OH) and diluted with 20 mL of deionized water to decrease the methanol concentration. The nonpolar HCAs were transferred to a C-18 cartridge (500 mg) that had been conditioned with 1 mL of methanol and 10 mL of water at the 4-5 mL/min. Then the cartridges were dried under nitrogen pressure and eluted with 1.4 mL methanol/ concentrated ammonium solution (9:1). For elution of polar HCAs (MeIQ, MeIQx), the 100 mg C-18 cartridges, which already pre- conditioned were adopted to PRS cartridges and 20 mL of 0.5 M NH_4OAc (ammonium acetate) was used to elute the polar HCAs into the C-18 cartridges. The polar HCAs were eluted with 0.8 mL of methanol/NH_4OH (9:1) and were concentrated under nitrogen gas before suspension in 25 μL of methanol.

HPLC Analysis of HCAs

The chromatographic separation of HCAs was achieved using a reversed phase TSK-Gel ODS-80 TM column (25 X 4.6 mm, 5 µm, 80 Å) (Toso Haas, Montgomeryville, PA, USA). Optimal separation was achieved with a binary mobile phase at a flow rate of 1 ml/min. The two mobile phases used for the HPLC separation were described by Knize (1995): solvent A, 0.01 M triethylamine (PH 3.6 adjusted with 1 M H_3PO_4) and solvent B, acetonitrile. The elution program used consisted of 95% A: 5% B to 75% A:25% B from 0 to 30 min. At the end of 30 minutes, a post-run rinse of mobile phase 55% A: 45% B was done for 10 minutes followed by 12 minutes of equilibration with the starting mobile phase. The column temperature was set at 40 °C.

HPLC Analysis of Antioxidants

For the extraction analysis of antioxidants, a method using ethanol was adapted from Wellwood and Cole (2004). For HPLC a method was adapted from Cuvelier and others (1996) on a reversed phase C-18 Hypersil-ODS column (25 cm X 4.6 mm, 5 µm pore size) (Altima, Altech Assoc., Deerfield, IL) using a C-18 guard column. The UV diode-array detector monitored separation at 284 nm. The mobile phase was a linear gradient from 90% A (840 mL of deionized water with 8.5 mL of acetic acid) and 10% B (methanol), and changed to 30 % A, 55% B, and 15% C (acetonitrile) in 20 minutes. At 40 minutes, the solvent was 100% B. A 25 minute post run was set to equilibrate the column. The flow rate was 1 mL/min and the temperature of the oven was set at 40 °C. Each sample were analyzed twice. The compounds were identified by comparison with the relative retention time of standards and from an on line saved library. Rosmarinic acid (1mg/mL) was used as external standard and to verify the performance of HPLC.

Head Space Analysis of Antioxidants (SPME-GC-MS)

The GC-MS was performed with a HP 5890 (Agilent Technologies, Palo Alto, CA) fitted with a HP-5 MS column (crosslinked 5% Ph Me siloxane, 30 m x 0.22 mm x 0.25 μm film thickness), and a HP MSD 5070 detector. Marinade samples in 0.1 gram increments 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 75 μm carbon-polymethysiloxane (CAR-PDMS) obtained from Supelco (Bellefonte, PA, USA). The fiber was immersed in head space for 30 min at the same temperature. After each extraction, the fiber was introduced into the injection port of the GC-MS. Oven temperature was programmed to an initial temperature of 70 °C for 3 min, followed by an increase at a rate of 7 °C/min to 180 °C and maintained for 5 min. Injector and detector temperature were 260 and 300 °C, respectively. The carrier gas was helium at a flow rate of 1 mL/min. Head space-SPME injections were carried out in splitless mode. Volatile compounds were identified by comparison of the spectra with those in a saved general library.

Standard Curves and Recoveries

Heterocyclic amines used as standards were handled according to Environment Protection Agency (EPA) guidelines. The contents of the vials (10 mg) were transferred to 10 mL volumetric flasks and were increased volume with methanol to make a solution of 1000 μg/mL (1ppm), then these solutions were used for making serial dilution of each HCA. The dilutions made with methanol were 500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.2 ng/mL, 15.6 ng/mL,7.8 ng/mL, and 3.9 ng/mL for polar HCAs and lower concentrations (under 50 ng/mL) were used for PhIP, Harman, and norharman because of their high sensitivity to a fluorescence

detector. Dilution factor and peak area were used to establish standard curves. The coefficient of determination (r^2) for all HCAs was above 0.99, which was used to evaluate the linearity of the standard curves. The limits of detection (LOD) for the HCAs analysis were 0.16 ng/mL for MeIQx and 0.08 ng/mL for Harman, norharman, and PhIP. The range of concentrations used for standard curves of antioxidants was between 1.5 μg/mL-100 μg/mL. The average extraction recoveries for the various HCAs were 58% for MeIQx, 56% for PhIP, 41% for harman, and 52% for norharman. The recoveries of 64% for MeIQx, 57% for PhIP, 36% for harman, and 58% for norharman reported by Tsen et al. (2006) are comparable to current study. Balogh also reported recoveries of 67-92% for MeIQx and 32-62% for PhIP (2000). Knize and collogues (1995) reported a 68% recovery of MeIQx and a 30% recovery for PhIP.

Statistical Analysis

The Statistical Analysis System (SAS) version 9.1 (SAS Institute Inc., Cary, NC, USA, 2002) was used for the analysis of the data. The experiment was a randomized complete block design. One way analysis of variance (ANOVA) was performed to determine the significant differences among treatments at $p < 0.05$. Tukey method was selected and pair wise differences were done for all observations. The data was then checked for normality between populations and matched to the pattern drawn from normal distribution. Because of the funnel shape of residual plot, and in order to have the basic assumption of equal variance, we decided to do log transformation.

RESULTS AND DISCUSSIONS

Effects of commercial marinades were evaluated for their influence on HCAs formation. For this research, three different commercial marinades were tested individually. All the marinades contained two or more spices from the *Lamiaceae* family, which are rich in the polyphenolic antioxidants such as rosmarinic acid, carnosol, and carnosic acid (Shan and others 2005). Since HCAs are mainly formed on the surface, a better inhibition may be observed with the surface application of the spices. Therefore, the exterior surface of steaks (2-4 mm thickness) was trimmed off and analyzed. To confirm that HCAs are formed mostly in the external surface of the steaks, the amount of HCAs were measured both in internal part and external part of meat and the comparison (as shown in Figure 10) revealed the higher amount of HCAs present in the external surface of steaks. The internal part of the meat contained 2.27 ng/g MeIQ and 2.14 ng/g MeIQx, while the external part in the control steaks contained 12.78 ng/g MeIQ and 24.23 ng/g MeIQx (it is about 5.6 fold higher for MeIQ and 11.2 fold higher for MeIQx). Table 7. shows the amount of HCAs (ppb) in the exterior part of crust and compare it to the whole part of the steak.

Marinated and unmarinated beef steaks had differences in appearance and texture. Marinated steaks were less browned in appearance and were juicer and more tender in taste. Internal temperature was monitored continuously in all treatments and the range was between 68-74 °C.

The results of comparing marinated and unmarinated steaks are shown in Table 6. The quantity of the four HCAs was figured using the solid-phase extraction and HPLC analysis method, which was calculated per gram of meat after cooking. In this study, the dominant

observed HCA in beef steaks was MeIQx, followed by PhIP. Our results indicated that marinated steaks had significantly lower HCAs compared to unmarinated samples (Figure 11). An average decrease greater than 71% in sum of HCAs observed. Obviously, due to the variation of the spice contents of different marinades, reduction levels of HCAs were dissimilar in different marinades. The control steak (unmarinated) had 30.2 ± 4.1 ng/g of MeIQx, and 17.4 ± 4.7 ng/g of PhIP. These amounts decreased to 87% of MeIQx and 87.3% of PhIP in the Caribbean marinated steak. With the exception of the Caribbean marinade, all marinades contained maltodextrin and /or modified starch ingredients that might be expected to inhibit HCA formation due to water retention. The cooking loss of steaks was evaluated as several reports and indicated that weight loss of meat may result in increased HCA formation. This has been explained due to an increased transport of water-soluble precursors to the surface where the reactions occur (Jägerstad and others, 1998). In our studies, there was no pattern of cooking loss or significant differences between the controls, marinades, or their bases (Table 6). The control steaks had one of the lower cooking losses as contrasted to most treatments. All marinades contained significant amounts of salt (2.2-5.4%), which could be a factor in reducing HCA production due to high water-holding capacity (Persson and others 2003). As expected, the dominant HCA observed in most treatments was MeIQx, followed by PhIP and lesser amounts of harman and norharman. There was a significant reduction (75%) in both harman and norharman amounts in steak marinated with the Caribbean marinade and a 65% reduction in harman with the Southwest marinade. The Herb base reduced harman by over 50%. All three marinades significantly decreased the imidazo-azaarene HCA (MeIQx, PhIP) as contrasted to control and liquid blanks. The marinading liquid mixture has no significant effect on any of the HCAs. The Caribbean

mixture was responsible for the highest decrease in the total HCA content (88%), followed by the Herb (72%), and Southwest (57%). These decreases were significant for all the imidazo-azaarene HCAs as contrasted to the controls and blanks. The Caribbean base reduced total HCAs by 54%, the Southwest by 23%, and the Herb by 48%.

There were slight changes in the beta carbolines, harman, and norharman with the various treatments. The Caribbean significantly decreased norharmn, while the Southwest and Herb did not show any inhibition effect. Murkovic (2007) has summarized some of the literature where values range accordingly: MeIQx 0-10 ppb, PhIP 0-35 ppb, harman 0-17 ppb, and norharman 0-800 ppb.

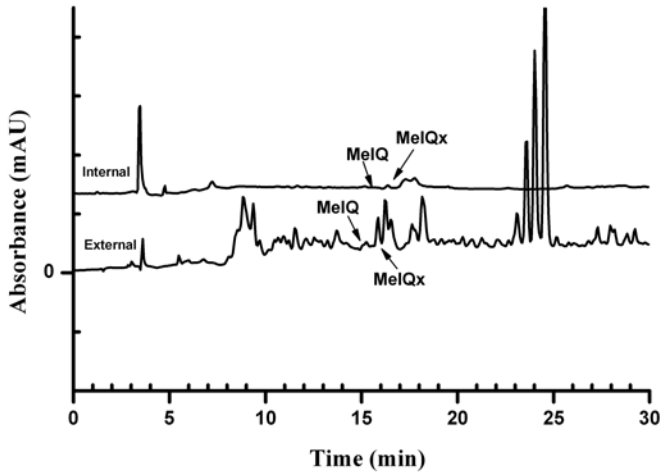

Figure 10. UV chromatogram comparison of internal MeIQ and MeIQx with the external portion of the steaks.

Steaks marinated for one hour using the Caribbean marinade showed the highest reduction in HCAs. We also analyzed all of the marinades for the three major non volatile polyphenolics: rosmarinic acid, carnosol, and carnosic acid. Phenolic antioxidants such as rosemary and thyme were already shown to effectively inhibit the formation of IQ type HCAs because they contain free radical scavengers (Murkovic et al., 1998; Kikuguwa et al., 2004). Free radical reactions have previously been suggested to be involved in the formation of HCAs and antioxidants have inhibitory effects (Yoshida et al., 1985). Involvement of free radicals in the formation of HCAs and prevention by phenolic antioxidants were also examined by Kikugawa (1999) and it was indicated that the effectiveness of the phenolic antioxidants in minimizing the development of the imidazoquinoxaline type of HCAs in cooking and all the phenolic antioxidants examined in their study reduced the mutagen development in the heated model system. The Caribbean marinade contained the highest levels of polyphenolics and was the strongest inhibitor of HCA formation. The degree of inhibition of the Southwest and Herb marinades did not exactly match the measured polyphenolic levels. Some of these differences may be due to the volatile phenolic compounds that are present. The Herb marinade contained basil and a significant amount of eugenol was found in it. The Caribbean marinade contained allspice, which is rich in eugenol and methyl eugenol, thus the polyphenolic antioxidants most likely do not account for all of the observed HCA inhibition.

Table 6. HCA concentrations (ng/g) in beef steaks pretreated with retail marinades or bases prior to grilling at 400 °F (204 °C) for five minutes per side. The liquid blank is water, oil, and vinegar that was used to constitute the marinades and bases (no added spices).

Treatment	**Cooking Loss (%)**	**MeIQx**	**PhIP**	**Harman**	**Norharman**
Control	26.4 ± 6.4^{a}	30.2 ± 4.17^{a}	17.4 ± 4.73^{a}	2.26 ± 0.35^{ab}	2.20 ± 0.07^{ab}
Liquid Blank	29.8 ± 1.0^{a}	30.3 ± 6.01^{a}	24.8 ± 10.1^{a}	2.86 ± 0.55^{a}	2.21 ± 0.69^{ab}
Caribbean Base	25.4 ± 3.9^{a}	14.4 ± 0.61^{c}	4.10 ± 0.26^{ed}	1.24 ± 0.05^{bcd}	1.41 ± 0.10^{bc}
Southwest Base	26.9 ± 3.6^{a}	22.6 ± 2.50^{ab}	9.26 ± 1.75^{b}	$1.2\ 0 \pm 0.10^{cd}$	3.46 ± 0.35^{a}
Herb Base	28.5 ± 8.8^{a}	12.8 ± 0.41^{c}	8.20 ± 1.35^{bc}	1.10 ± 0.17^{cd}	2.60 ± 0.17^{ab}
Caribbean	27.0 ± 4.7^{a}	3.10 ± 0.62^{e}	2.33 ± 0.15^{f}	0.54 ± 0.05^{e}	0.52 ± 0.18^{c}
Southwest	28.1 ± 3.3^{a}	15.8 ± 2.20^{bc}	5.03 ± 0.50^{cd}	0.80 ± 0.40^{ed}	1.63 ± 0.85^{bc}
Herb	22.0 ± 8.2^{a}	7.16 ± 1.04^{d}	3.00 ± 0.32^{ef}	1.70 ± 0.05^{bac}	1.96 ± 1.10^{abc}

Means with the same superscript letter within the same column are not significantly different at $P < 0.05$. Values are means of three replicates ± SD.

As shown in Table 6, in the case of MeIQx, the amount of MeIQx in control and blank did not have any significant difference. The base of the Caribbean marinade and the base of Herb marinade decreased the amount of MeIQx. Caribbean marinade inhibited MeIQx at a higher rate than the Herb marinade and Southwest marinade did. All three marinades inhibited the amount of MeIQx significantly, and this was higher than their correspondent bases. In the case of PhIP, all three marinades inhibited PhIP significantly and were stronger inhibitors than their corresponding

bases. In the case of harman, all three marinades decreased the amount of harman significantly and the Caribbean marinade was stronger than its relevant base, because it had the greatest decrease although the bases were not different from each other. In the case of norharman the amount of norharman in the control and the blank were not significantly different from each other. The Caribbean marinade significantly decreased norharman, while the Southwest marinade and the Herb marinade did not show any inhibiting effect. Our results are similar to other studies that investigated the effects of marinades on HCA formation. Tikanen and others (1996) evaluated the effects of commercial marinades on the mutagenic activity of grilled chicken. Their results showed a 50% reduction of PhIP with increase in MeIQx and DiMeIQx. The ingredients of the marinade were a complex mixture of spices and flavonoids. Similar studies by Salmon (1996) showed that marinating chicken with a mixture of sugar, olive oil, vinegar, garlic, mustard, lemon juice, and salt reduced the total HCAs by 92%.

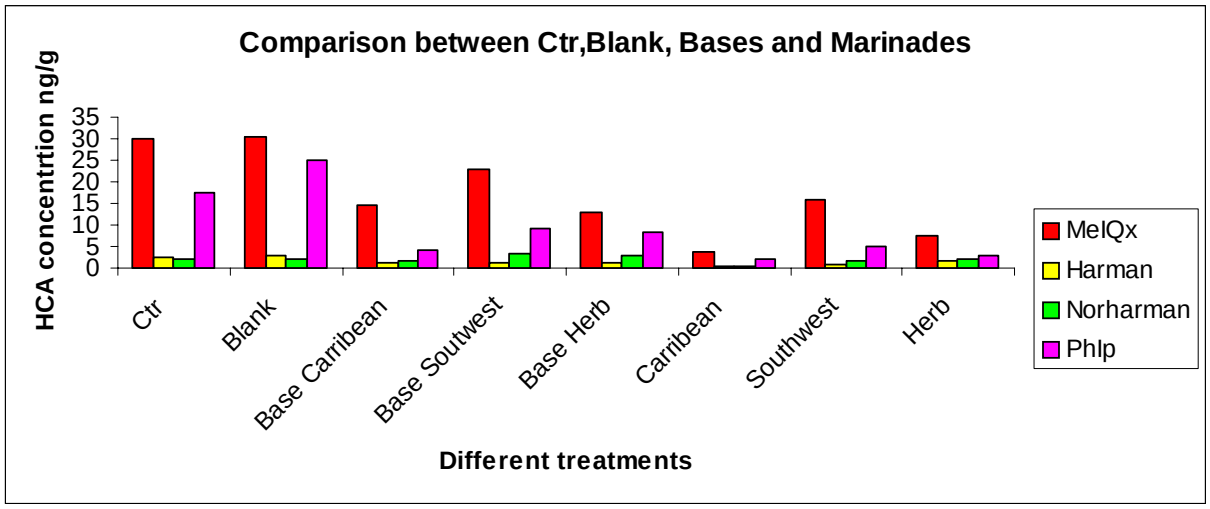

Figure 11. Comparison of HCA levels among the groups of control, blank, marinades, and their relevant bases.

Interestingly, the commonly used spice and flavoring agent rosemary, which is derived from the leaves of the plant *Rosmarinus officinalis,* displays antioxidant properties in foods and in biological systems. Moreover, in animal models, rosemary components were found to inhibit the initiation and tumor promotion phases of carcinogenesis (Offord et al., 1995). Rosemary is known as one of the spices with the best antioxidant activity (Nakataki et al., 1997). The antioxidant activity of rosemary is due to the presence of flavonoids as well as diterpenes. The phenolic structure is in fact free radical acceptor. Phenolic diterpenes like carnosol and carnosic acid are present in this spice. Carnosol can also be extracted from sage (Nakataki et al., 1997). Murkovic and co-workers found that the addition of spices, including rosemary, thyme, and sage, to ground beef reduced the concentration of HCAs in fried beef (1998). Balogh and co-workers also showed that rosemary oleoresin added directly to the ground beef patties or to the surface of the patties before frying, produced a reduction of 44% of PhIp after cooking (2000). In our study, extraction of each marinade by ethanol and water bath method were done and three major antioxidants detected by HPLC in all three marinades (Figure 12). These three antioxidants including rosmarinic acid, carnosol, and carnosic acid, were detected and the level of each measured and compared. Figure 13 shows the UV chromatographs of the three marinades containing the three potent antioxidants of carnosol, carnosic acid, and rosmarinic acid. Figure 14 shows the standard mix of these three antioxidants together.

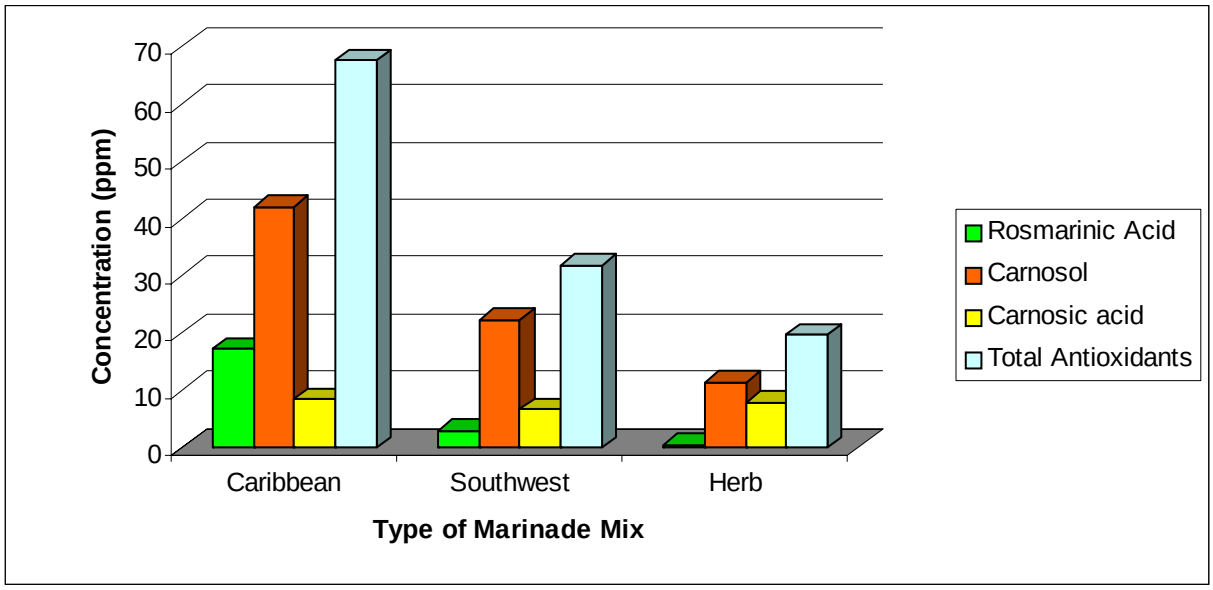

Figure 12. Antioxidant levels (carnosic acid, rosmarinic acid, carnosol) and total antioxidants detected by HPLC in each marinade.

Table 7. The amount of HCAs(ppb) detected in the crust (exterior) portion of the steak and comparison with the amount in the whole steak. Abbreviation used are: Carb-Caribbean, SW-Southwest, Herb, and Mar-marinade.

Concentration of HCAs (ppb) in crust					**Concentration of HCAs (ppb) in whole steak**			
	MeIQx	**PhIP**	**Harman**	**Norharman**	**MeIQx**	**PhIP**	**Harman**	**Norharman**
Control	25.5	14.5	2.6	2.25	8.13	4.62	0.83	0.72
Control	32	14.9	2.3	2.2	9.44	4.40	0.68	0.65
Control	33.3	22.9	1.9	2.1	10.30	7.08	0.59	0.65
Blank	27.5	17.3	2.5	1.45	7.58	4.77	0.69	0.40
Blank	37.2	36.3	3.5	2.8	9.12	8.90	0.86	0.69
Blank	26.2	20.8	2.6	2.4	10.18	8.08	1.01	0.93
Carb Base	14.6	4.2	1.22	1.45	5.14	1.48	0.43	0.51
Carb Base	15	4.3	1.2	1.3	5.28	1.51	0.42	0.46
Carb Base	13.8	3.8	1.3	1.5	4.86	1.34	0.46	0.53
SW Base	22.8	9.3	1.2	3.5	6.55	2.67	0.34	1.01
SW Base	25	11	1.15	3.8	7.18	3.16	0.33	1.09
SW Base	20	7.5	1	3.1	5.74	2.15	0.29	0.89
Herb Base	12.8	8.3	1.3	2.8	3.71	2.40	0.38	0.81
Herb Base	13	9.5	1	2.5	3.76	2.75	0.29	0.72
Herb Base	12.2	6.8	1	2.5	3.53	1.97	0.29	0.72
Carb Mar	3.8	2.2	0.53	0.54	1.11	0.64	0.15	0.16
Carb Mar	2.9	2.5	0.6	0.7	0.91	0.79	0.19	0.22
Carb Mar	2.6	2.3	0.5	0.34	0.80	0.71	0.15	0.10
SW Mar	15.8	5.1	0.8	1.6	3.10	1.00	0.16	0.31
SW Mar	17.9	5.5	1.2	2.5	4.74	1.46	0.32	0.66
SW Mar	13.5	4.5	0.4	0.8	2.64	0.88	0.08	0.16
Herb Mar	7.5	3	1.6	2.1	2.36	0.95	0.50	0.66
Herb Mar	8	3.1	1.7	0.8	1.98	0.77	0.42	0.20
Herb Mar	6	2.5	1.7	3	1.92	0.80	0.55	0.96

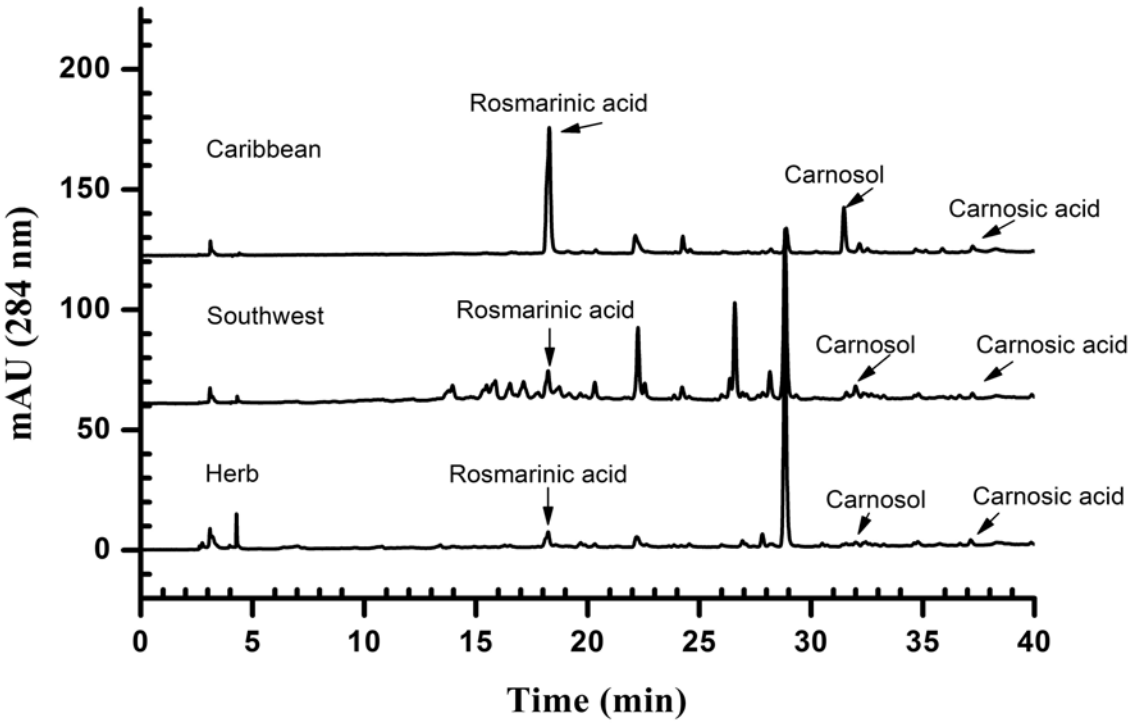

Figure 13. HPLC chromatograph of the antioxidants present in the three marinades.

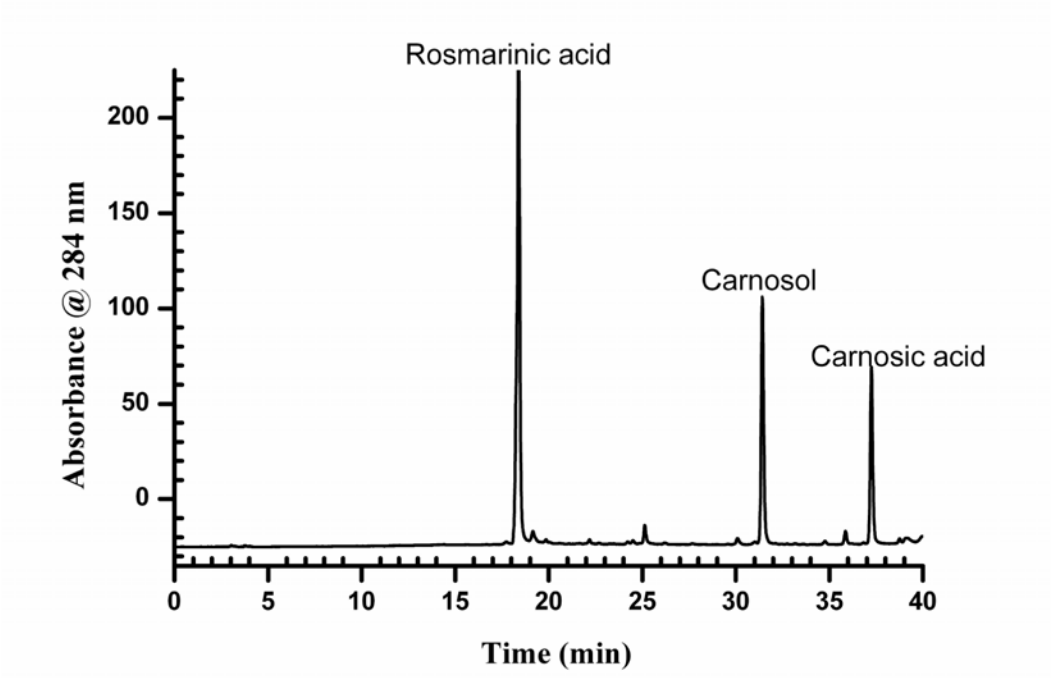

Figure 14. HPLC chromatograph of the standard mix of the antioxidants rosmarinic acid (100 ppm), carnosol (1000 ppm), and carnosic acid (100 ppm).

Both carnosol and carnosic acid can effectively scavenge HO˚ radicals. In fact, antioxidant polyphenols have positive effects and the ability to terminate radical chains, scavenge active oxygen species, and trap electrophiles (Offord et al., 1997). Radicals play an important role during the formation of HCA compounds in the Maillard reaction. The development of the Maillard reaction occurs through a free radical mechanism that has been shown to play an important role in the formation of imidazoquinoxalines and imidazoquinolines (Jägerstad et al., 1998). The reactive intermediates such as pyrazinum and pyridinum cation radicals can be inactivated by the antioxidative effect of spices. The antioxidative activity of rosemary accounts

for over 90% of all antioxidant activity of the combination of rosemary, carnosol, and carnosic acid (Offord et al., 1997). In our study, an average of 62.3 % carnosol and 12.3 % carnosic acid, and 25.3% rosmarinic acid were shown in the Caribbean marinade. Amounts for the Southwest marinade were 70% carnosol, 20.8% for carnosic acid, and 9% rosmarinic acid. The Herb marinade had 58% carnosol, 39.7% carnosic acid, and 2.1% rosmarinic acid (Figure 11).

In a study by Monti (2001), frying meat in virgin olive oil (VOO) with a high content of phenols has been suggested as a way of reduce the content of HCAs in beefburgers since the amounts of PhIP, norharman, and harman were considerably lower in the beefburgers fried in fresh VOO. They concluded that the presence of pholyphenols was important in reducing the negative effect of HCAs.

Harman and norharman are B-carbolines that have been termed co-mutagens and they don't have an exocyclic amino group; therefore, they don’t provide mutagenic activity in the Ames test. It is proved that L-tryptophan is necessary as a precursor for their formation. Harman and norharman are not only formed in heated proteinaceous food, but have been shown to be normal body constituents (Pfau and Skog, 2004). Although the mechanisms responsible for co-mutagenic action have not been elucidated as yet, in a study by de Meester (1995), formation of norharman was reduced by 64%, 60%, and 100% (not detectable) by 1.0% grape seed extract and 1.0% pine bark extract.

In fact, antioxidants can act as inhibitors along the different pathways of reaction, preventing the mutagens formation through radical quenchers and free radical scavengers activity or preventing the biotransformation of premutagens into reactive metabolites by inhibiting metabolic activation (Dashwood et al., 2002).

Carnosol and carnosic acid had stronger antioxidant activities than butylated hydroxy toluene (BHT). As a result, the high antioxidant capacity of carnosol and carnosic acid may be beneficial not only in combating degeneration of foods through oxidation, but also in scavenging free radicals that are implicated in human disease (Offord et al., 1995).

Thyme has been shown to decrease MeIQx by 70% when added to the surface of beef steaks (Murcovic et al., 1998). Oregano was also reported to reduce PhIp (50-78%), while rosemary and thyme decreased HCAs by 75% when applied to the surface of beef (Murkovic et al., 1998). Antioxidants probably react as inhibitors, and they can change the concentration of precursors as well as scavenging free radicals such as pyrazine and thus showing a reduction effect on HCAs. It is suggested that free radical scavenger-type antioxidants may stabilize the sugar fragments or else react with the alkylpyridine free radicals or dialkylpyrazine free radicals (Pearson et al., 1992). According to Salmon (1997), marinating chicken before grilling greatly decreased the amount of MeIQx and PhIp (87%). In his study, HCA formation and the effects of seven component marinade in the surface treatment of chicken breast meat was investigated. The marinade was a mixture of brown sugar, olive oil, cider vinegar, garlic, mustard, lemon juice and salt applied four hours prior to grilling. Similar to our results, Murkovic reported that the application of dried rosemary, thyme, sage, and garlic to the surface of meat prior to heating resulted in significant reduction in total HCA contents (1998). This reduction is explained by the antioxidative properties of the spices. The reactive intermediates such as pyridinium and pyrazinum cation radicals can be inactivated by antioxidants, and therefore reduce the formation of HCA. Verdin (2002) also studied several spices as antioxidants and possible HCA reducers. The spices used were basil, garlic, ginger, onion, oregano, rosemary, sage, thyme, and turmeric.

These spices were in powder form, as they usually are used in a household. The antioxidants in rosemary and sage were expected to lower HCAs activity the most, which is in agreement with our results.

Head-space GC/MS analysis of each marinade for the volatile polyphenolics was done separately by HP 5890 fitted with a HP-5 MS column and a HP MSD 5070 detector. Caribbean marinade contained a considerable amount of eugenol and methyl eugenol which are potent antioxidants (Figure 15). Five volatile polyphenolics were identified in the Caribbean mixture. Considering that the synergistic effects of both volatile and nonvolatile phenolics exist in the Caribbean marinade, the high potential for the reduction of HCAs can be explained. The Southwest marinade contained thymol and 4-hydroxy-3-methyl acetophenone (Figure 16). The Herb marinade also showed a similar amount of thymol and 4-hydroxy-3-methyl actophenone as part of the volatile phenolics (Figure 17).

A marinade may act as a "barrier" keeping flames from directly touching the meat. The protective powers may lie in the ingredients of a typical marinade. Herbs and spices seem to contribute at least partially to the prevention of HCA formation. On the other hand, marinades are complex mixtures of several chemical compounds and it is difficult to evaluate the exact factors that inhibited the formation of HCAs. In our study, marinading steaks with base powders of each marinade showed that MeIQx and PhIP was significantly reduced in the Caribbean marinade (Table 6). Reduction of MeIQx and PhIP in the base (without any herbs and spices) was less than the reduction from the marinade, which indicates that the base materials might slightly reduce the formation of HCAs, but the major reduction occurs after marination with spices and herbs.

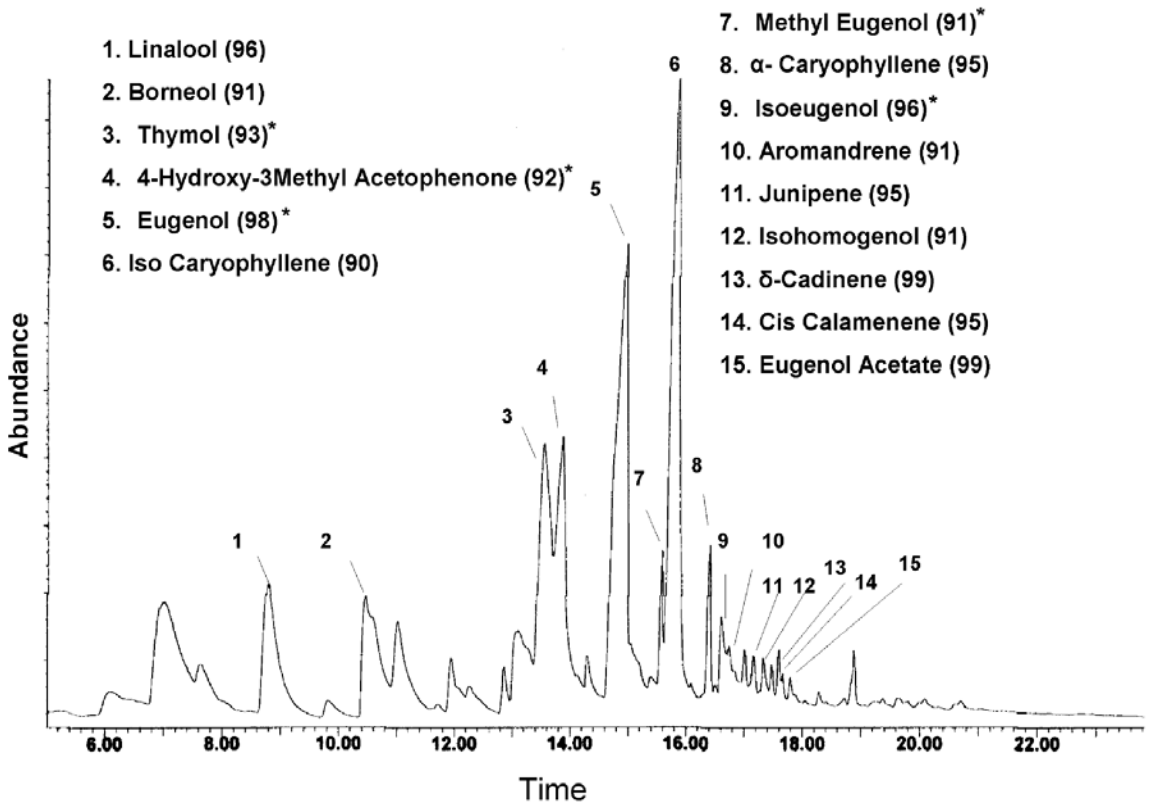

Figure 15. GC/MS chromatogram of SPME headspace analysis of the volatile compounds in the Caribbean marinade powder. Compounds marked with an asterisk contain a phenolic group and would be expected to exhibit antioxidant activity. The number in parenthesis is the probability match factor relative to the Wiley/NTST MS spectral library. Values higher than 90 (on a scale of 100) are considered a positive identification.

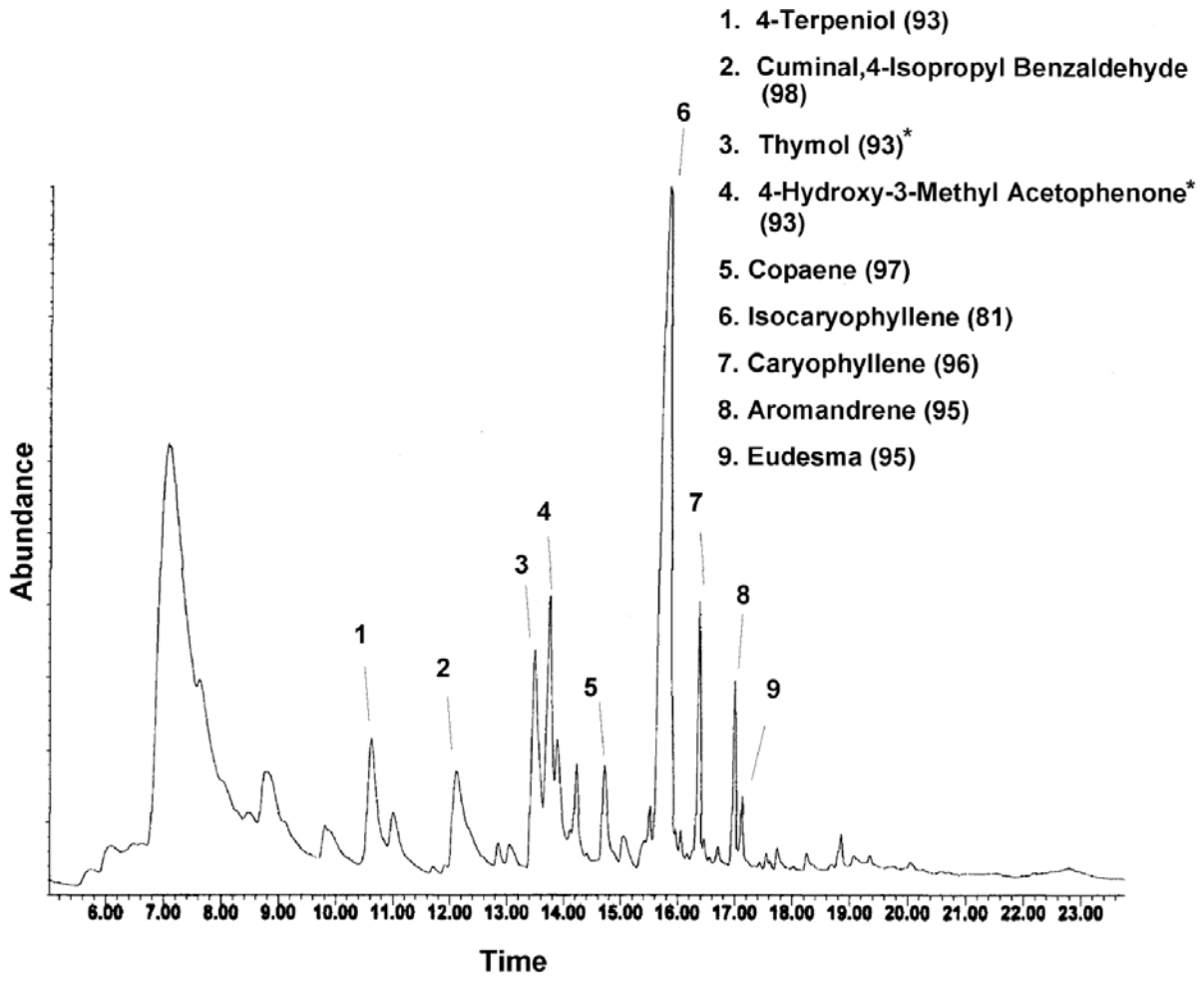

Figure 16. GC/MS chromatogram of SPME headspace analysis of the volatile compounds in the Southwest marinade powder. Compounds marked with an asterisk contain a phenolic group and would be expected to exhibit antioxidant activity. The number in parenthesis is the probability match factor relative to the Wiley/NTST MS spectral library. Values higher than 90 (on a scale of 100) are considered a positive identification.

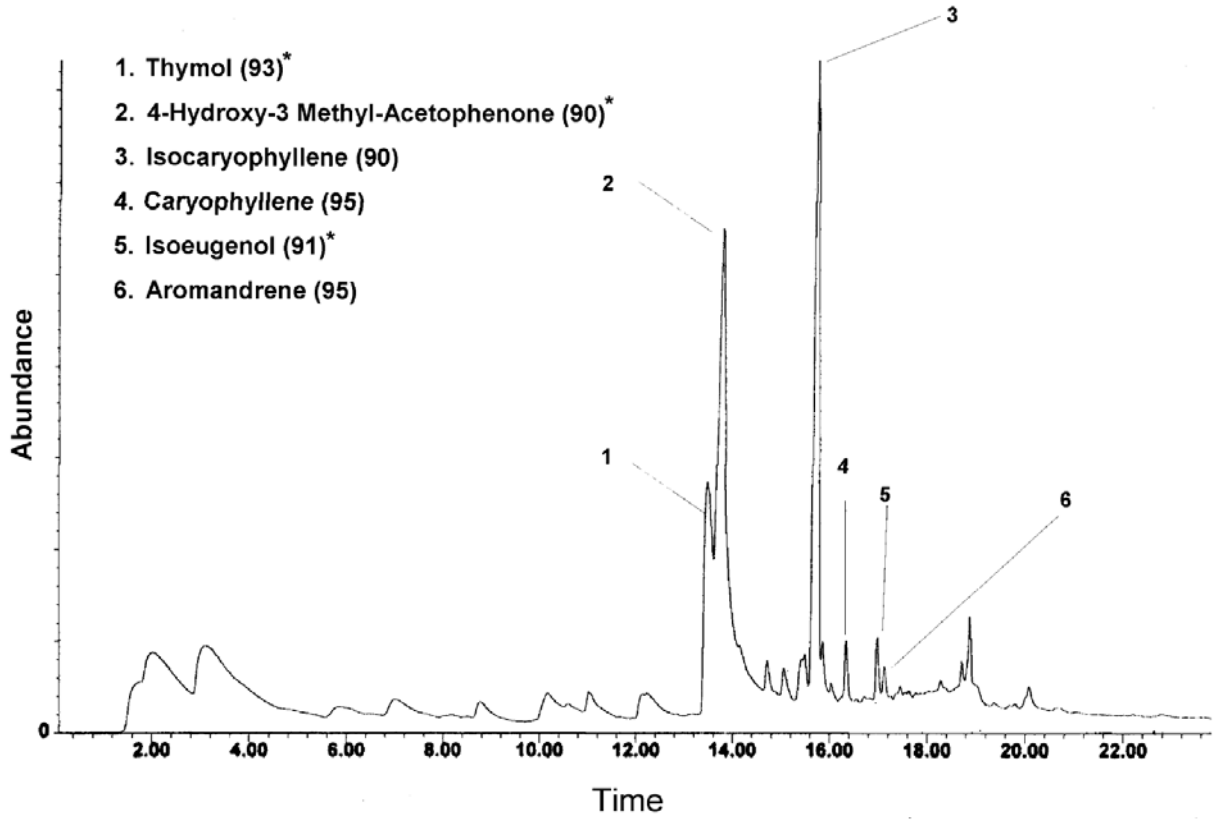

Figure 17. GC/MS chromatogram of SPME headspace analysis of the volatile compounds in the Herb marinade powder. Compounds marked with an asterisk contain a phenolic group and would be expected to exhibit antioxidant activity. The number in parenthesis is the probability match factor relative to the Wiley/NTST MS spectral library. Values higher than 90 (on a scale of 100) are considered a positive identification.

However, different marinades might be one reason for the variation between similarly prepared grilled foods. It is suggested that a very simple but efficient method to reduce exposure to HCAs would be covering and marinating meats for even a short time with spices and herbs before grilling. Another point can be the dilution of precursors of HCA formation, which can happen in the presence of the marinade`s contents. Further investigations are needed to determine the possible factors in commercial marinades that reduce the amounts of HCAs. The goal of understanding and reducing carcinogenic HCA incidence is worthwhile. In our study, we evaluated the positive capability of spices that are normally used during household preparation of meat on the formation of carcinogenic HCA. As shown in the present study, the formation of HCAs can be affected by the addition of antioxidants as well as the concentration level of those antioxidants in the marinade. A well-designed, functional marinade can alter mutagen/carcinogens and possibly decrease human health problems. We demonstrated that marinating meats in solutions rich in antioxidants before grilling, reduced potential hazards to human health.

SUMMARY

Heterocyclic amines (HCAs), a group of toxic chemicals formed during high temperature cooking of meat and fish, are potent mutagens and are suspected to play a role in colorectal cancer. This study suggests that marinating steaks with different commercial marinades can offer a practical way to reduce the formation of HCAs. The objective of the present study was to investigate a practical home-style cooking method to reduce the amount of HCAs formed during

cooking. The antioxidant level of each of three marinades was detected separately and compared with each other. We examined the effect of varying pretreatment of different spice marinades added to steak one hour before grilling at 400 °F. A decrease in HCA amounts after marinating steaks was observed (71%). The powder base of each marinade (excluding herbs and spices) was obtained from the manufacturer and the experiment was repeated with these bases. A decrease in HCA formation showed that marinades containing antioxidants were able to inhibit the formation of HCAs more than their bases. The amount of phenolic antioxidants present in each marinade was detected and quantitated by HPLC method and confirmed. Similarly, antioxidants in herbs and spices may play an important role in inhibiting the formation of HCAs.

The inhibition is not due to differences in cooking loss, nor does the application of a marinading liquid alone appear to be beneficial. In fact, recommendations to marinate muscle foods in oil and vinegar prior to grilling do not appear to reduce HCAs.

Thus, commonly available marinades can be effective inhibitors of HCA formation and provide a reduction in the exposure to some of the carcinogens formed during grilling.

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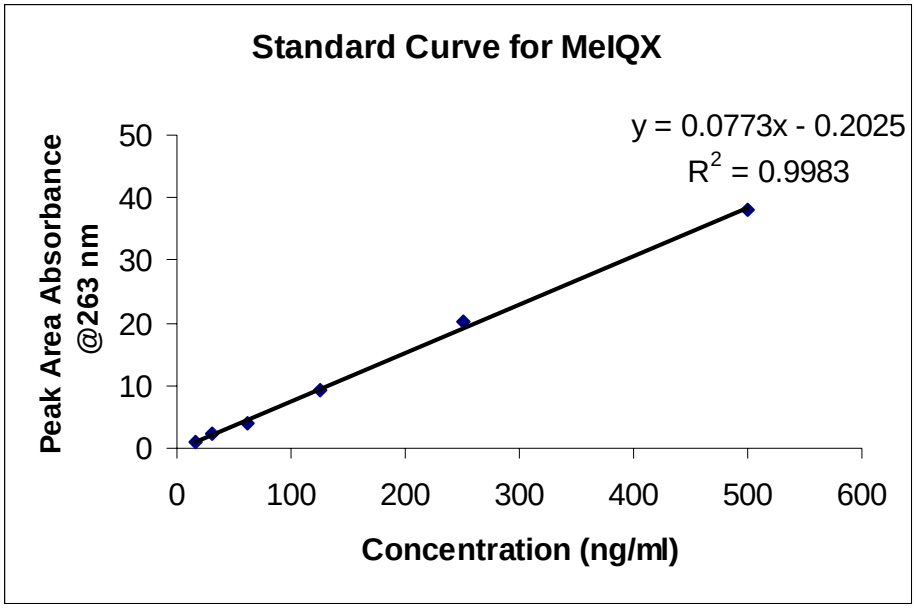


Figure A-1. Standard curve for MeIQx obtained by UV detection at 263 nm.

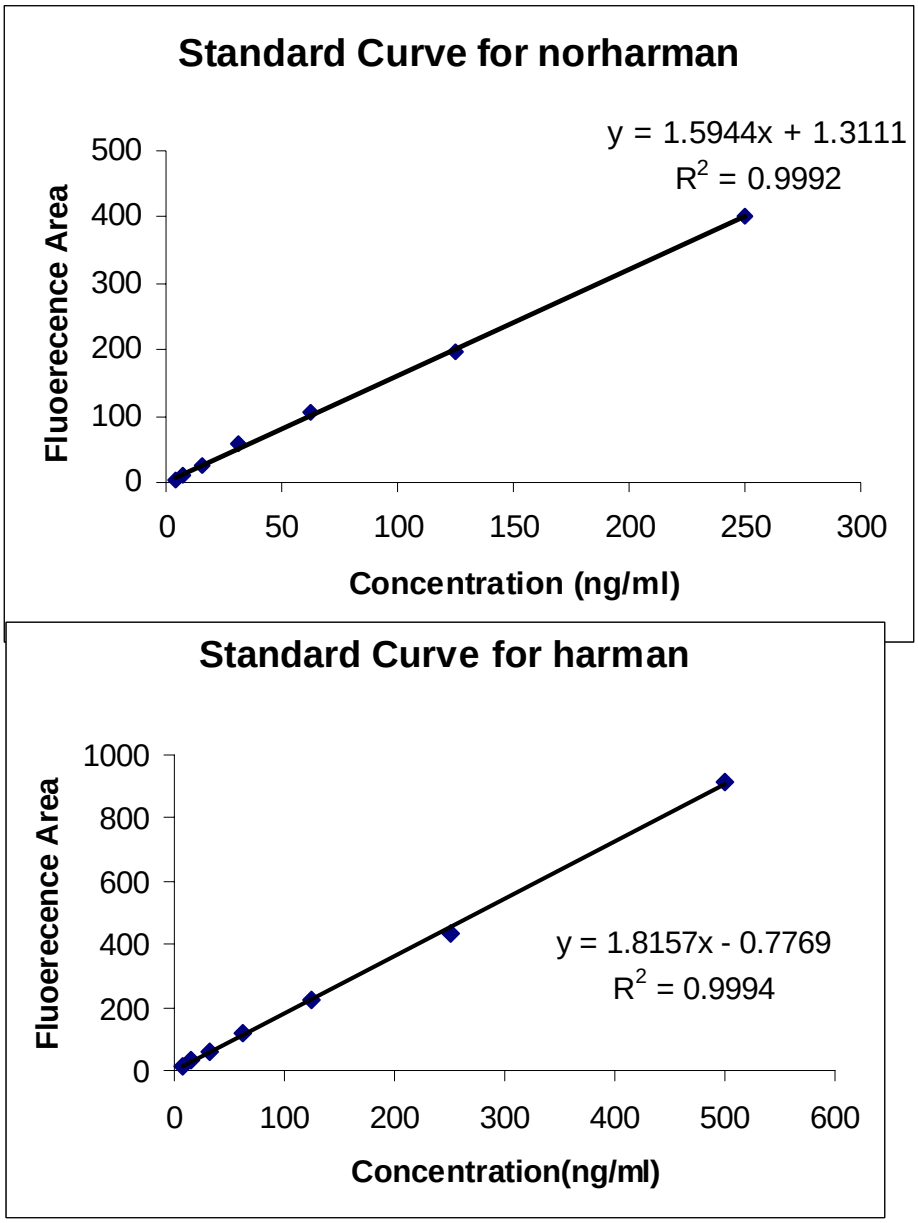


Figure A-2. Standard curves for norharman and harman obtained by FLD detection.

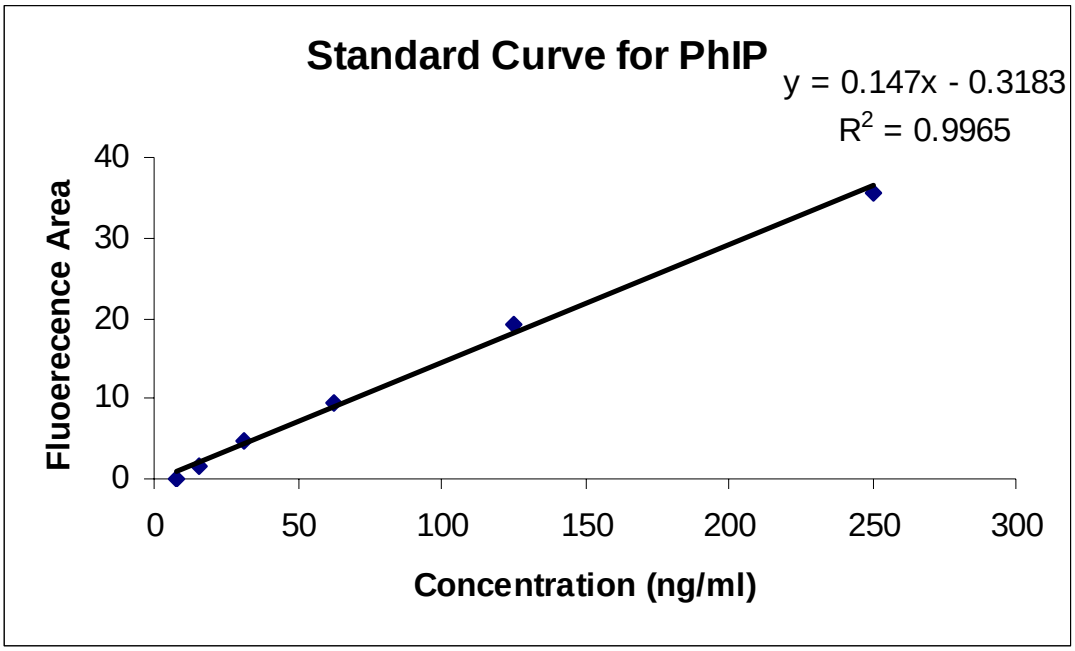
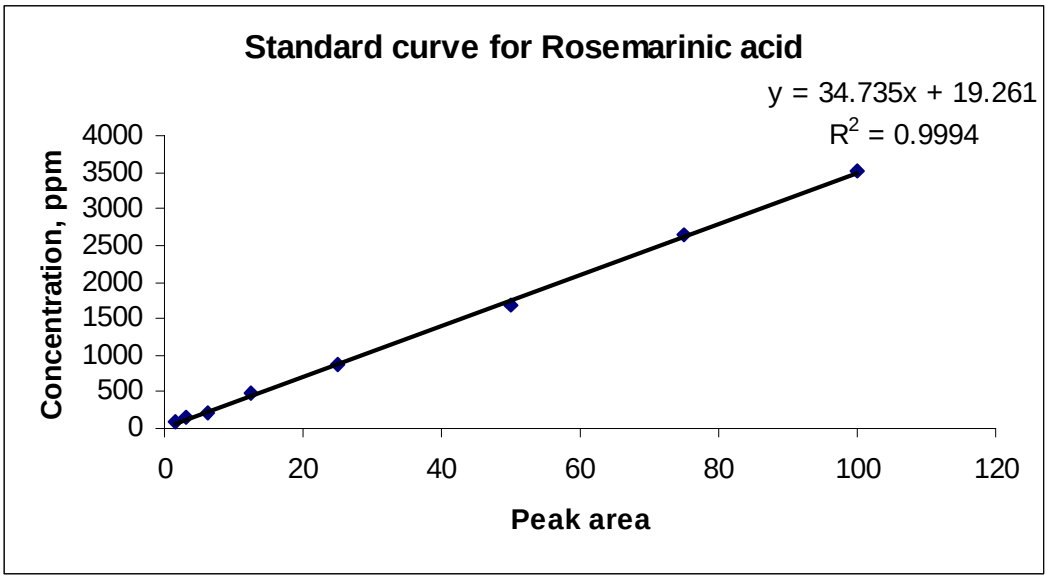


Figure A-3. Standard curve for PhIP obtained by FLD detection.



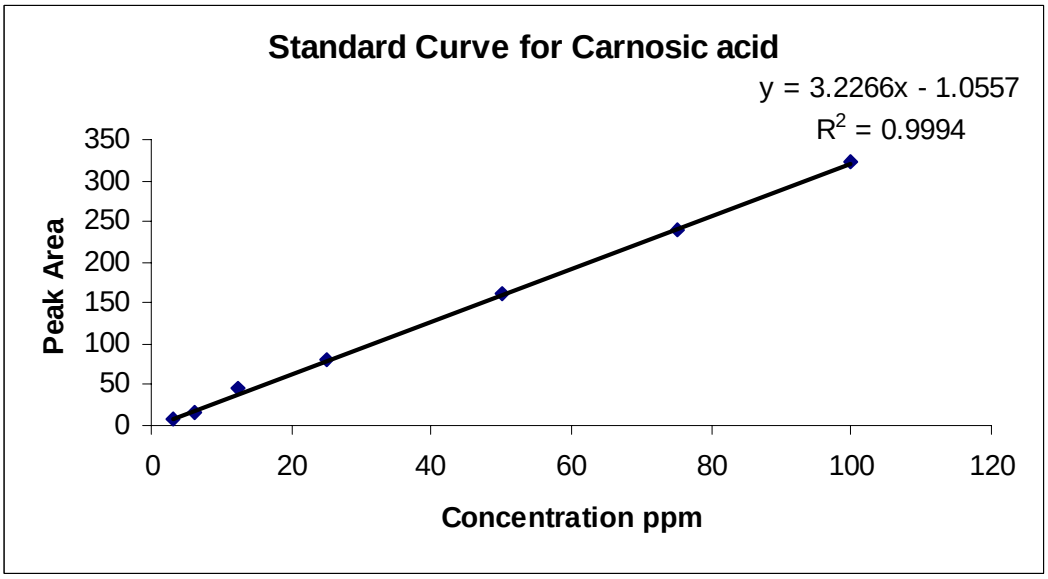


Figure A-4. Standard curves for rosmarinic acid and carnosic acid obained by UV detection at 284 nm.

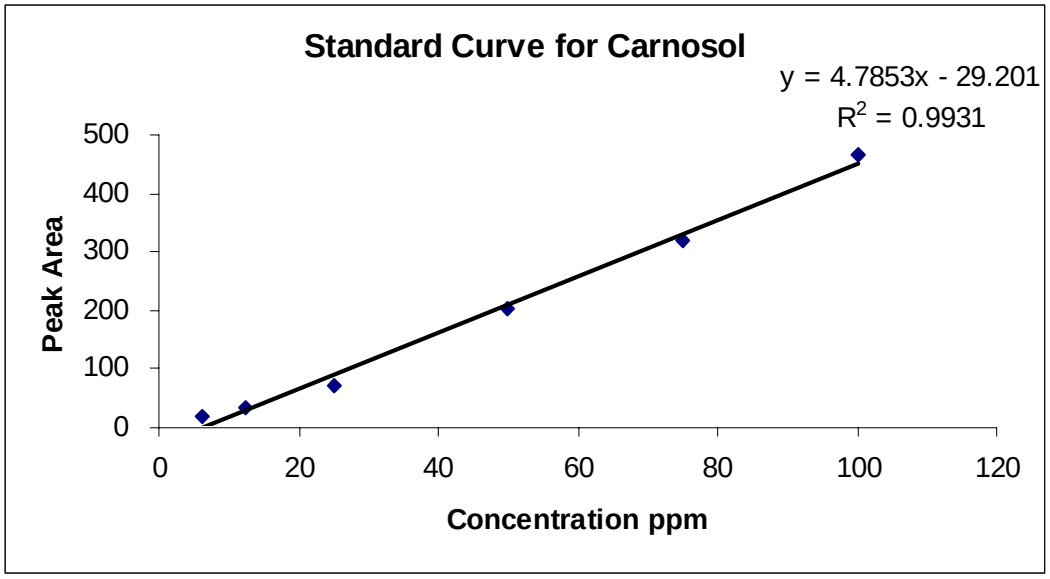


Figure A-5. Standard curve for carnosol obtained by UV at 284 nm.

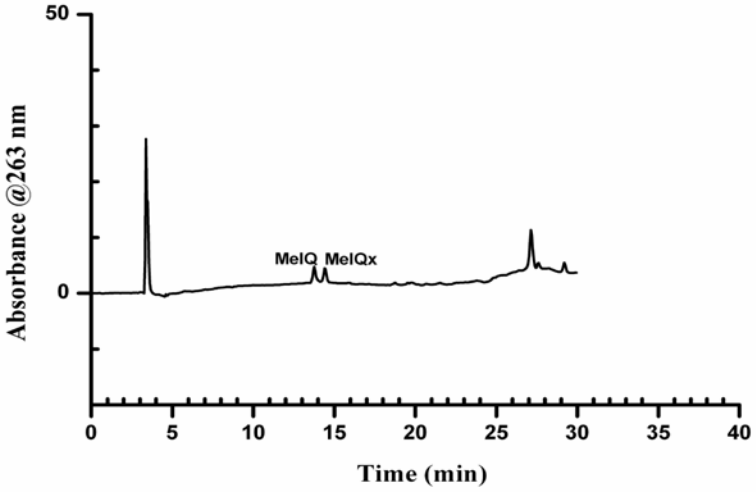


Figure A-6. Representative UV chromatogram of standard mixed HCAs, MeIQ, and MeIQx (250 ppb).

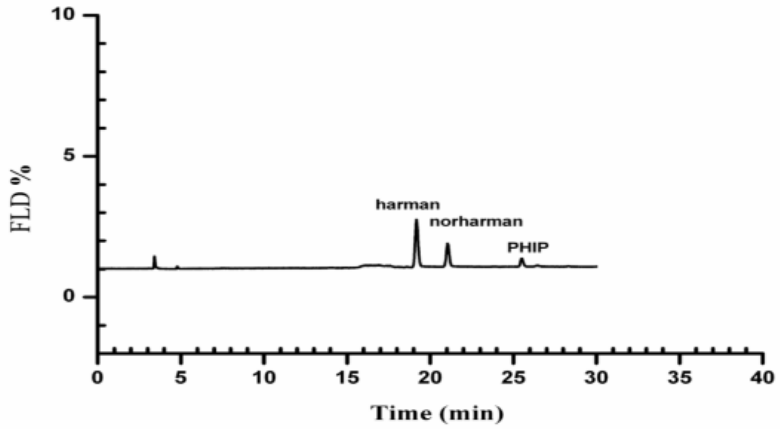


Figure A-7. Representative FLD chromatogram of standard mixed harman, norharman, and PhIP (50ppb)

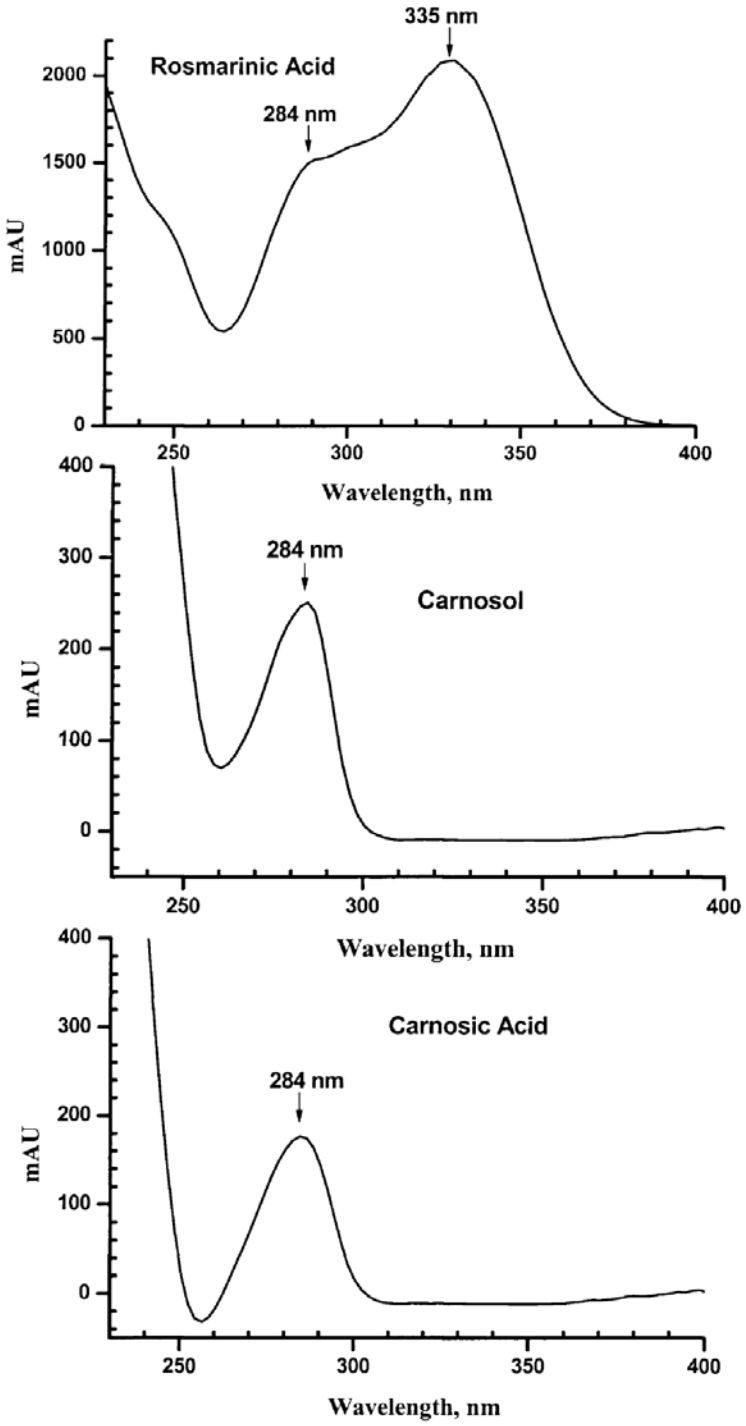


Figure A-8. UV Spectrum of standard of rosmarinic acid, carnosol, and carnosic acid.

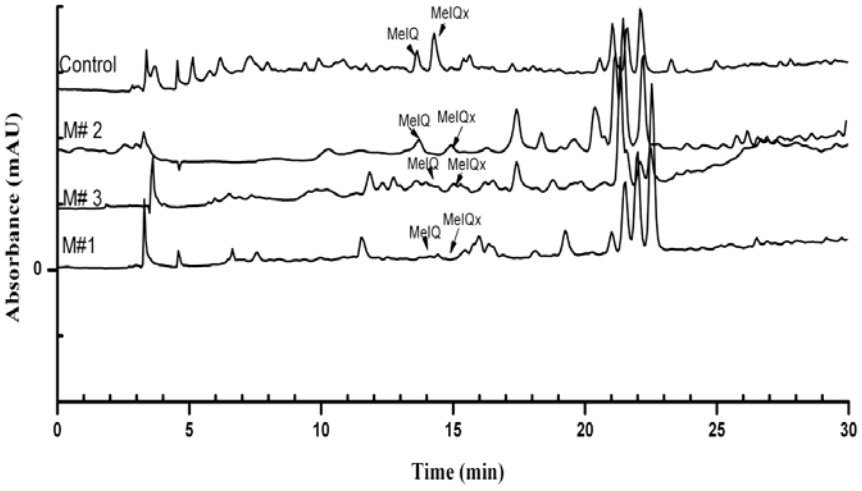


Figure A-9. Comparison of chromatograms showing MeIQ, and MeIQx peaks in control, and different marinades detected by UV detector of HPLC at 263 nm.

Table A-1. HCA levels (ng/g) in different treatments before and after marinating beef steaks.

	MeIQx	PhIP	Harman	Norharman
Control	25.5	14.5	2.6	2.25
Control	32	14.9	2.3	2.2
Control	33.3	22.9	1.9	2.1
Liquid (Blank)	27.5	17.3	2.5	1.45
Liquid (Blank)	37.2	36.3	3.5	2.8
Liquid (Blank)	26.2	20.8	2.6	2.4
Caribbean Base	14.6	4.2	1.2	1.45
Caribbean Base	15	4.3	1.2	1.3
Caribbean Base	13.8	3.8	1.3	1.5
Southwest Base	22.8	9.3	1.2	3.5
Southwest Base	25	11	1.15	3.8
Southwest Base	20	7.5	1	3.1
Herb Base	12.8	8.3	1.3	2.8
Herb Base	13	9.5	1	2.5
Herb Base	12.2	6.8	1	2.5
Caribbean	3.8	2.2	0.53	0.54
Caribbean	2.9	2.5	0.6	0.7
Caribbean	2.6	2.3	0.5	0.34
Southwest	15.8	5.1	0.8	1.6
Southwest	17.9	5.5	1.2	2.5
Southwest	13.5	4.5	0.4	0.8
Herb	7.5	3	1.6	2.1
Herb	8	3.1	1.7	0.8
Herb	6	2.5	1.7	3

Table A-2. Concentration of HCAs (ng/g) in control samples grilled at 400 °F

Treatment #	**MeIQX**	**Norharman**	**Harman**	**PhIp**
1	24.23	0.80	4.06	11.60
2	40.80	0.82	4.79	12.02
Average	32.51	0.81	4.41	11.85
1	30.11	0.31	2.87	12.14
2	35.29	0.44	4.02	12.66
Average	32.62	0.38	3.44	10.50
1	24.97	0.67	2.69	10.29
2	21.51	0.81	2.84	10.95
Average	23.26	0.74	2.77	10.63
1	26.17	1.09	2.51	14.20
2	35.56	1.00	1.82	20.70
Average	28.32	1.06	2.16	17.52
1	25.56	0.96	2.57	10.02
2	15.64	1.16	2.00	9.00
Average	20.62	1.06	2.28	9.51
1	25.51	0.93	2.20	10.19
2	18.13	1.24	1.77	13.00
Average	21.83	1.08	1.98	12.21

Table A-3. Concentration of HCA (ng/g) in blank samples grilled at 400 °F

Treatment #	**MeIQX**	**Norharman**	**Harman**	**PhIp**
1	15.70	1.18	1.47	9.41
2	44.40	1.30	3.05	19.63
Average	30.19	1.24	2.25	14.55
1	55.36	2.40	3.52	22.74
2	44.72	0.96	1.55	19.01
Average	49.51	1.04	2.54	15.36
1	35.91	1.58	3.34	20.15
2	17.59	0.95	1.29	16.14
Average	26.75	1.27	2.32	15.57
1	27.79	0.68	2.32	12.79
2	41.28	0.78	2.37	12.38
Average	34.52	0.73	2.35	13.22

Table A-4. Steak weights before and after grilling, and cooking loss.

	Weight of steaks before grilling (g)	**Weight of steaks after grilling (g)**	**Weight of trimmed (g)**	**Fraction of steaks used for analysis (%)**	**cooking loss %**
Control 1	101.3	78.7	25.1	24.78	22.6
Control 2	100.9	77.6	22.9	22.7	23.3
Control 3	102.6	67.9	21	20.47	34.7
Blank 1	89.4	70.7	19.5	21.81	18.7
Blank 2	93.1	75.9	18.6	19.98	17.2
Blank 3	99.3	67.7	26.3	26.49	31.6
Caribbean 1	89.2	68.7	20	22.42	20.5
Caribbean 2	91.6	76.5	24.1	26.31	15.1
Caribbean 3	99.7	77.3	23.8	23.87	22.4
Southwest 1	100.5	76.4	15	14.93	24.1
Southwest 2	99.2	74.8	19.8	19.96	24.4
Southwest 3	98.5	66.6	13	13.20	31.9
Herb 1	86.6	73.9	23.3	26.91	12.7
Herb 2	96	66.4	16.4	17.08	29.6
Herb 3	86.31	68.6	22	25.49	17.71
Caribbean Base	98.09	77.91	24.9	25.38	20.18
Southwest Base	92.83	66.23	21.8	23.48	26.6
Herb Base	95	64.36	19.6	20.63	30.64

Statistical Analysis - SAS program

1-way ANOVA of MeIQ, MeIQx, PhIP, Harman, Norharman by Treatment

14:58 Wednesday, September 26, 2007

The GLM Procedure

Class Level Information

Class	Levels	Values
trt	4	BAS# BLK CTR M#

Number of Observations Read 24

Number of Observations Used 24

1-way ANOVA of MeIQ by Treatment

The GLM Procedure

Dependent Variable: MeIQ

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	3	1868.631667	622.877222	40.10	<.0001
Error	20	310.646667	15.532333		
Corrected Total	23	2179.278333			

R-Square	Coeff Var	Root MSE	MeIQ Mean

The GLM Procedure

Least Squares Mean

Adjustment for Multiple Comparisons: Tukey-Kramer

LSMEAN

trt	MeIQ LSMEAN	Number

Least Squares Means for effect trt

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: MeIQ

1-way ANOVA of MeIQ by Treatment

The GLM Proc

Tukey's Studentized Range (HSD) Test for MeIQ

NOTE: This test controls the Type I experiment wise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha 0.05

Error Degrees of Freedom 20

Error Mean Square 15.53233

The SAS System 16:18 Thursday, October 25, 2007 29

The GLM Procedure

Dependent Variable: Ros

Sum of Source	DF	Squares	Mean Square	F Value	Pr > F
Model	2	323.3132333	161.6566167	17046.4	<.0001
Error	3	0.0284500	0.0094833		
Corrected Total	5	323.3416833			

R-Square	Coeff Var	Root MSE	Ros Mean
0.999912	1.438794	0.097382	6.768333

Source	DF	Type I SS	Mean Square	F Value	Pr > F

Critical Value of Studentized Range 3.95829

Minimum Significant Difference 7.3539

Harmonic Mean of Cell Sizes 4.5

NOTE: Cell sizes are not equal.

Means with the same letter are not significantly different.

Tukey Grouping Mean N trt

Least Squares Means for effect trt

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: lnMeIQ

The GLM Procedure

Tukey's Studentized Range (HSD) Test for lnMeIQ

NOTE: This test controls the Type I experimentwise error rate, but it ggenerally has a higher Type II error rate than REGWQ.

AUTHORIZATION STATEMENT

From: Ameri [mailto:ameriba@gmail.com]

Sent: 03 June 2008 01:33

To: Journals Rights

Subject: Permission Request for a Manuscript in Journal of Food Science

Dear Sir/Mam, With greetings,

I am a Food Science graduate student in Kansas State University. I need to get permission to include the materials of a manuscript in my thesis; I am the second author of this article. The manuscript was approved for publication in the Journal of Food Science in May 2008 and the title of this paper is: *Effect of Marinades on the Formation of Heterocyclic Amines in Grilled Beef Steaks*. The authors are: J. Scott Smith, Fariba Ameri, and Priyadarshini Gadgil. I would appreciate it if you please kindly provide me with a permission letter. My contact information is: Phone # 518-324-4607 and e-mail address: ameriba@gmail.com

Sincerely,

Fariba Ameri

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From: Haine, Julia - Oxford <jhaine@wiley.com>

Date: Jun 3, 2008 4:11 AM

Subject: Permission Request for a Manuscript in Journal of Food Science

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