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EFFECT OF LIPIDS AND IRON ON HETEROCYCLIC AROMATIC AMINE FORMATION IN AQUEOUS MODEL SYSTEMS

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LISA SCRANTON

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J. S. Gray
Major professor

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EFFECT OF LIPIDS AND IRON ON HETEROCYCLIC AROMATIC AMINE FORMATION IN AQUEOUS MODEL SYSTEMS

By

Lisa Scranton

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ABSTRACT

EFFECT OF LIPIDS AND IRON ON HETEROCYCLIC AROMATIC AMINE FORMATION IN AQUEOUS MODEL SYSTEMS

By

Lisa Scranton

This study was designed to investigate the effect of lipids of varying degrees of unsaturation on heterocyclic aromatic amine (HAA) formation in an aqueous model system. Saturated and unsaturated lipids were added to model systems utilizing creatinine, glucose, glycine or phenylalanine, and water. The model systems were heated at 180°C in closed stainless steel test tubes for 30 minutes. HAAs were isolated by solid phase extraction and separated/quantitated by high performance liquid chromatography. Corn oil, olive oil, and tristearin had no effect on species or yield of HAAs formed. Trilinolein increased MeIQx (2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline) and IQx (2-amino-3-methyl-imidazo[4,5-*f*]quinoxaline) formation in the glycine system. Ferrous sulfate and myoglobin were added to some of the samples. Ferrous sulfate increased MeIQx formation without lipid. Myoglobin decreased IQx formation with or without lipid present. These results indicate that the chemical effect of lipids on HAA formation is probably not significant under household cooking conditions.

To my husband, Alec, and my children Gregg, Audrey, and Elizabeth
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INTRODUCTION

Heterocyclic aromatic amines (HAAs) are a class of compounds containing multiple aromatic rings with at least one exocyclic amino group and often an exocyclic methyl group. These compounds have been found in cooked meat and some other protein-containing foods (Sugimura, 1982), and were first discovered in 1977 by Sugimura and associates after observing the mutagenicity of smoke condensate obtained during the broiling of fish (Sugimura et al., 1977).

The most common HAAs found in food products are IQ (2-amino-3-methylimidazo[4,5f]quinoline), MeIQ (2-amino-3,4dimethylimidazo[4,5f]quinoline), MeIQx (2-amino-3,8 dimethylimidazo[4,5f]quinoxaline), 4,8 DiMeIQx (2-amino-3,4,8 trimethylimidazo[4,5f]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo [4,5b]pyridine) (Skog, 1993). Other HAAs have subsequently been isolated from and identified in foods and model systems, and the current number of HAAs known to occur in foods is now 17 (Johansson et al., 1993).

Mutagenicity of heterocyclic amines is generally measured by the Ames assay (Ames et al., 1975; Sugimura et al., 1988). They are not mutagenic as found, but require activation by cytochrome p450IA2 enzyme to their hydroxyamino forms (McManus et al., 1990). Esters formed with acetic or sulfuric acid probably are the final DNA adducts (Sugimura, 1982). Mutagenicity varies widely between individual heterocyclic amines, but is as high as 661,000 revertants/μg toward *S. typhimurium* TA98. Aflatoxin B1, a documented carcinogen, causes only 6,000 revertants/μg of this strain of *Salmonella.* Not all mutagens are carcinogens, however, and carcinogenicity must be determined with animal or cell culture studies. Rodent and primate assays have shown many HAAs to be multisite carcinogens, including IQ, MeIQ, MeIQx, and PhIP, as well as many of the so-

called nonpolar HAAs (Ohgaki et al., 1991). Some of these neoplastic sites include the liver, small and large intestines, blood vessels, mammary gland, and lungs. While results from these animal studies are highly suggestive of HAAs acting as carcinogens in humans, this point has been more difficult to prove. Davis et al. (1993) found that mutagenic activation of HAAs in the Ames assay was in some cases greater using human microsomes than using those of rodents.

Human consumption of HAAs has been estimated to range from 4-16 μg/day (Wakabayashi et al., 1992), based on analyses of the HAA levels in various meat products by a number of investigators (Felton et al., 1984: Kikugawa and Kato, 1987). However, doses of HAAs used in animal feeding studies have generally been at many thousands of times the level of human lifetime exposure (Stavric, 1994), so it is difficult to extrapolate these studies to human experience. Some epidemiological evidence suggests a link between daily consumption of well-done meat or fish and risk of colorectal cancer (Schiffman and Felton, 1990; Gerhardsson de Verdier et al., 1991). Currently these compounds are considered possible or probable human carcinogens by the International Agency for Research on Cancer, and therefore their occurrence in meats and other foods deserves the close scrutiny of researchers (IARC, 1993).

Given the likelihood that many HAAs are probably human carcinogens, it seems prudent to decrease intake of these compounds, ideally without compromising the nutritional or aesthetic quality of the diet. In order to do so, it is necessary to understand both the mechanism of HAA formation and the effect of possible contributing factors such as cooking methods, time and temperature of cooking, and composition and content of lipid. The exact mechanism of formation of HAAs is unknown, although various postulates have been published for specific compounds (Jagerstad et al., 1983a; Nyhammer, 1986). It is currently accepted that precursors to HAAs are specific amino acids and creatinine, with sugars enhancing formation under some conditions (Skog and Jagerstad, 1990). The reaction

requires heat, and is assumed to proceed utilizing Maillard reaction intermediates (Spingarn and Garvie, 1979; Sugimura, 1982; Jagerstad et al., 1983a; Shibamoto et al., 1981). While contributing factors such as time, temperature, and method of cooking have been investigated by a number of researchers who have published similar findings (Bjeldanes et al., 1983; Knize et al., 1994b; Skog et al., 1992a), there has been no such agreement on the contribution of lipids to HAA formation. Bjeldanes et al. (1983) found that mutagenicity of various beef patties was independent of fat content, while Knize et al. (1985) showed that mutagenicity of beef patties increased with increasing fat content up to 15%, then decreased slightly with higher fat content. Nilsson and colleagues (1986) found that the addition of different types of frying fat increased mutagenicity of meat products. Johansson et al. (1993) utilized an aqueous model system of creatinine, sugar, and an amino acid to show an increase in MeIQx formation upon addition of corn or olive oil to the system.

The studies that have investigated lipid contribution to HAA formation have been hampered by several factors. Most of these studies measured mutagenicity only, not individual HAA formation. Only the experiments done by Johansson et al. (1993) in an aqueous model system separated physical (heat transfer) from chemical (molecular contribution) effects, and this study along with others did not employ adequate statistical analyses. Information which makes the question of lipid contribution to HAA formation more pressing includes the large body of work showing high-fat diets to be epidemiologically linked to cancer (Alavanja et al., 1996; Zhang, et al., 1996) as well as literature demonstrating the ability of fat to act as a cancer promoter (Reddy, 1978, Weisburger et al., 1983).

Based on these observations, the hypothesis for the current studies was as follows: Increasing degrees of lipid unsaturation will lead to increasing amounts of HAA formation in aqueous model systems containing HAA precursors and lipid.

Iron will increase HAA formation in these model systems when lipid is present through accelerated lipid oxidation. Objectives for the studies were:

(1) To determine the effect of specific lipids on the species and quantities of HAAs formed in this model system.

(2) To compare the effects of saturated versus unsaturated lipid on the species and quantities of HAAs formed in this model system.

(3) To study the effect of both heme and nonheme iron on the species and quantities of HAAs formed in the model system, both with and without lipid present.

LITERATURE REVIEW

History of Heterocyclic Aromatic Amines

Discovery

Mutagens and carcinogens have been known to exist in the environment for many decades. Pollutants present in air and water, including pesticides and industrial chemicals, have been studied extensively (Sugimura and Sato, 1983). Mutagenic or carcinogenic fungal toxins such as aflatoxins have been known to contaminate food since the 1950s (Harrison et al., 1993). Several food additives such as AF-2, a nitrofuran derivative used as a preservative in Japan, and *N*-nitroso compounds, formed in cured meats from the interaction of nitrite and secondary amino compounds, have been shown to be mutagenic or carcinogenic (Kada, 1973; Tricker and Preussmann, 1991).

Mutagens/carcinogens which are innately present in foods or formed during cooking of these foods are more difficult to isolate, and their effect on humans is more difficult to determine. HAAs fall under this final category, and have become the subject of extensive research in the last twenty years. The large body of information which now exists regarding HAAs is due in part to the development of the Ames *Salmonella typhimurium* assay for mutagenicity (Ames et al., 1975). The Ames assay allows rapid determination of mutagenic potential which did not exist prior to 1975.

The formation of mutagenic/carcinogenic substances in food as a result of cooking or processing first became the subject of research in the early 1960's, when benzo[α]pyrene, a polycyclic aromatic hydrocarbon, was identified in

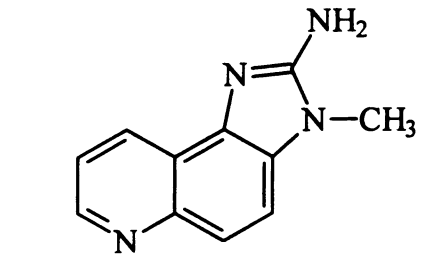
barbecued meats (Lijinsky and Shubik, 1964). About ten years later, Sugimura and associates began to study the mutagenicity of smoke condensates from the cooking of meat and fish, as well as the mutagenicity of the charred meat surfaces (Nagao et al., 1977; Sugimura et al., 1977). The mutagenic levels found were far higher than could be explained by the benzo[α]pyrene content, so a search began for other mutagens in meats. Commoner and associates reported mutagenic compounds in beef and beef broth cooked at temperatures ranging from 190-300°C, and confirmed the absence of mutagens in raw meat. They were the first to attempt to isolate and separate these compounds using chromatography (Commoner et al., 1978; Dolara et al., 1979). Almost twenty years later, at least 17 HAAs have been isolated from meat and other proteinaceous foods, and researchers continue to find new compounds (Johansson et al., 1993).

Categories

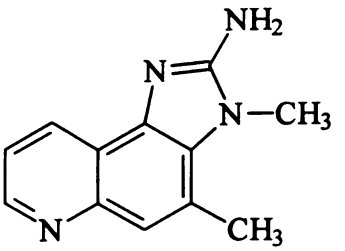
The heterocyclic aromatic amines that have been isolated from food can be divided into two categories based on the temperatures at which they form. "Pyrolytic" mutagens are those which are formed above 300°C and are characterized by a pyridine ring with an amino group attached (Skog, 1993). Pyrolytic mutagens can be deaminated by treatment with nitrite under acidic conditions, after which they are no longer mutagenic (Tsuda et al., 1985). As these mutagens are formed at such high temperatures, they are not generally found in foods cooked under normal household conditions. Exceptions to this rule include the findings of two α-carbolines, 2-amino-9H-pyrido[2,3-b]indole (AαC), and 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeAαC), in grilled fish (Gross and Gruter, 1992).

"Thermic" mutagens are the second category of HAAs based on formation temperature, and includes HAAs formed below 300°C. These compounds, also known as aminoimidazoazaarenes can be further subdivided into quinolines,

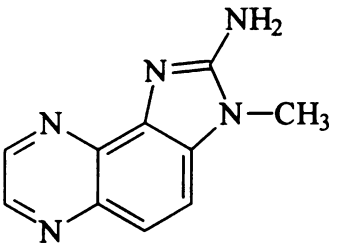
quinoxalines, pyridines, and furopyridines (Skog, 1993). A brief review of the most common HAAs in each category follows. Structures are shown in Figure 1.



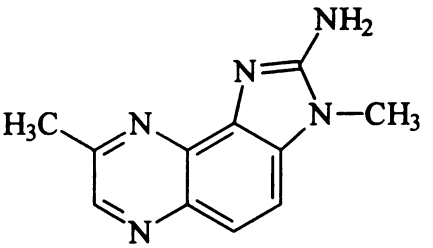
IQ



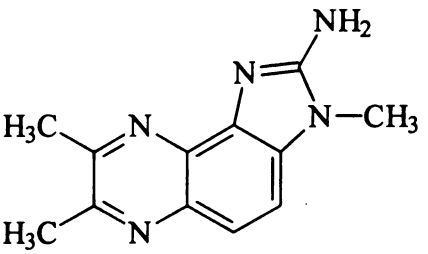
MeIQ



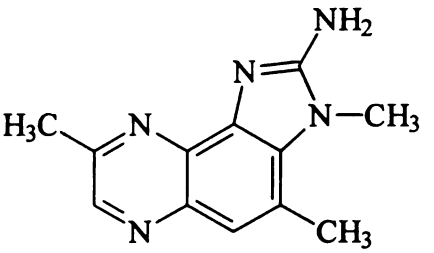
IQx



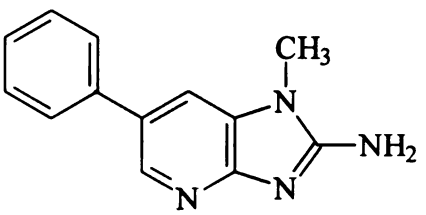
MeIQx



7,8-DiMeIQx



4,8-DiMeIQx



PhIP

Figure 1. Chemical structures of some HAAs in cooked foods (Skog, 1993).

Quinolines

Two distinct imidazoquinoline compounds have been identified in foods. The HAAs, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3,4-methyl-imidazo[4,5-*f*]quinoline (MeIQ), were initially isolated from broiled sardines (Kasai et al., 1980). They have subsequently been identified in fried ground beef (Barnes et al., 1983; Felton et al., 1984; Felton et al., 1986a), fried ground pork (Vahl et al., 1988), and various fishes (Yamaizumi et al., 1986; Zhang et al., 1988). IQ has also been found in beef extract (Taylor et al., 1985; Turesky et al., 1989), and has been formed in model systems containing creatinine, glucose or fructose, and a variety of amino acids (Table 1). MeIQ is generally not found in most model systems.

Quinoxalines

The quinoxaline, 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), was first isolated from fried ground beef by Kasai et al. (1981). MeIQx has subsequently been found in fried ground pork, chicken, mutton, fish, and beef extract (Gry et al., 1986; Wakabayashi et al., 1986; Sugimura et al., 1988; Turesky et al., 1988, Vahl et al., 1988, Gross, 1990; Gross and Gruter, 1992). This HAA has also been produced in a number of model systems containing creatinine, a monosaccharide or disaccharide, and one of five amino acids (Table 1). MeIQx can also be produced in model systems without a sugar, using only creatinine plus threonine, serine, or alanine (Overvik et al., 1989).

Two other quinoxalines which have been isolated from meat are 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) and 2-amino-3,7,8-tri-methylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx). The latter compound has been found in only a few meat products such as fried ground beef (Turesky et al., 1988) and roasted eel (Lee and Tsai, 1991). However, 4,8-DiMeIQx has been isolated from fried ground beef (Grivas et al., 1985; Felton et al., 1986a, 1992; Turesky et

al., 1988; Murray et al., 1988; Sugimura et al., 1988), fried ground pork (Gry et al., 1986; Vahl et al., 1987), chicken (Sugimura et al., 1988), mutton (Kato et al., 1986), fish (Zhang et al., 1988), and beef extract (Turesky et al., 1988; Gross, 1990). Both these mutagens have also been produced in model systems containing creatinine, a monosaccharide or disaccharide, and an amino acid. As with MeIQx, 4,8-DiMeIQx is produced when one of five amino acids (threonine, alanine, glycine, phenylalanine, or lysine) is used, but 7,8-DiMeIQx has only been isolated from a system using glycine as the amino acid (Table 1).

A fourth quinoxaline, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx) has been isolated from model systems (Knize et al., 1988; Skog and Jagerstad, 1993; Johansson et al., 1993), but only found once in a meat system (Becher et al., 1988).

Pyridines

The dominant HAA formed in cooked meat is a pyridine, 2-amino-1-methyl-6-phenylimidazo[4,5-*f*]pyridine, abbreviated PhIP. This HAA was first isolated from the crust of fried ground beef in 1986 (Felton et al., 1986b). Since then, many other researchers have added to the body of knowledge regarding the occurrence, chemistry, and mutagenicity/carcinogenicity of PhIP. According to findings from model system studies, phenylalanine is the major amino acid that contributes to PhIP formation (Shioya et al., 1987; Felton and Knize, 1990; Skog and Jagerstad, 1991; Manabe et al., 1992). Leucine may also be involved in PhIP formation (Overvik et al., 1989). PhIP has been isolated from most of the same meat products as the quinolines and quinoxalines. Fried beef, fried ground pork, broiled chicken, mutton, fish, and beef extract all contain PhIP (Felton et al., 1986a; Gry et al., 1986; Vahl et al., 1987; Hayatsu et al., 1991; Gross and Gruter, 1992). The mass of PhIP in beef has been estimated to be 10 times the weight of other known HAAs combined (Felton et al., 1986b). Therefore, even though PhIP is less mutagenic

than the other HAAs (Table 2), it may play a larger role in the etiology of cancer (Stavric, 1994).

Two other pyridines have been identified in meat, 2-amino-n,n,n-trimethyl-imidazopyridine (TMIP) and 2-amino-1,6-dimethylimidazopyridine (DMIP), but occur in much smaller quantities than PhIP (Becher et al., 1988, 1989; Felton et al., 1984).

New HAAs

Purification and isolation methods for HAAs continue to improve, and as a result of these improvements, new HAAs are being identified. Many of these HAAs contain oxygen. The structure of a furopyridine, first reported by Gry et al. (1986), was elucidated by Knize et al. (1990) to be $C_{10}H_{10}N_4O$. A pyridine similar to PhIP, 4-OH-PhIP, was shown by Kurosaka et al. (1992) to be mutagenic. Knize et al. (1991) identified a mutagen from a model system with a molecular weight of 244 and composition of $C_{12}H_{12}N_4O_2$. In Japan, a new quinoxaline, 4-CH_2OH-8-MeIQx, was isolated from beef extract (Kim et al., 1994). Each new finding adds to the accumulated knowledge of HAA formation in foods, and also illustrates the need for continuing research in this area.

Mutagenicity/Carcinogenicity

The development of the Ames assay for mutagenicity in 1975 made possible the rapid, consistent classification of various compounds as potentially mutagenic or nonmutagenic. Following the lead of Sugimura et al. (1977), many research groups began utilizing the Ames assay to determine mutagenicity in food products. HAAs were discovered to be potent mutagens once activated by liver microsomes (Sugimura et al., 1977; 1979). The initial activation step is thought to be N-hydroxylation, mainly by the cytochrome p450IA2 mediated mixed-function

Table 1. Heterocyclic aromatic amines produced in model systems (Skog, 1993).

Compound	Yield*	Amino Acid	Su-gar	Heating Conditions	Reference
IQ	0.4	pro	—	Dry	Yoshida et al. (1984)
	1.0	gly	fru	DEG-Water	Grivas et al. (1986)
	3.0	phe	—	Dry	Felton and Knize (1990)
	13.5	phe	glu	Dry	Felton and Knize (1990)
	3.7	ser	—	Dry	Knize et al. (1988)
MeIQ	nd	ala	fru	DEG-Water	Grivas et al. (1985)
IQx	2.7	ser	—	Dry	Knize et al. (1988)
	nd	gly	glu	Water	Skog and Johansson (unpublished, 1993)
	nd	thr	glu	Water	Skog and Jagerstad (1993)
MeIQx	4.4	gly	glu	DEG-Water	Jagerstad et al. (1984)
	0.9	ala	glu	DEG-Water	Muramatsu and Matsushima (1985)
	1.8	ala	rib	DEG-Water	Muramatsu and Matsushima (1985)
	4.2	lys	rib	DEG-Water	Muramatsu and Matsushima (1985)
	nd	thr	glu	DEG-Water	Negishi et al. (1985)
	6-7	gly	fru	DEG-Water	Grivas et al. (1986)
	nd	ser	—	Dry	Overvik et al. (1989)
	nd	ala	—	Dry	Overvik et al. (1989)
	nd	tyr	—	Dry	Overvik et al. (1989)
	4	gly	glu	DEG-Water	Skog and Jagerstad (1990)
	nd	phe	glu	DEG-Water	Skog and Jagerstad (1991)
	10	ala, thr	glu	DEG-Water	Skog et al. (1992b)
	8.8-17.9	gly	glu	Water	Johansson et al. (1993)
	7-10	gly	glu	Water	Skog and Jagerstad (1993)
	9	thr	glu	Water	Skog and Jagerstad (1993)
	nd	gly	glu	Water	Johansson and Jagerstad (1993)
4,8-DiMeIQx	nd	thr	glu	DEG-Water	Negishi et al. (1985)
	1.9-2.6	ala	fru	DEG-Water	Grivas et al. (1985)
	4.2	ala	glu	DEG-Water	Muramatsu and Matsushima (1985)
	1.5	ala	rib	DEG-Water	Muramatsu and Matsushima (1985)
	26.1	lys	rib	DEG-Water	Muramatsu and Matsushima (1985)
	nd	gly	glu	DEG-Water	Skog and Jagerstad (1990)
	nd	phe	glu	DEG-Water	Skog and Jagerstad (1991)
	36	ala, thr	glu	DEG-Water	Skog et al. (1992b)
	30	thr	glu	Water	Skog and Jagerstad (1993)
	nd	gly	glu	Water	Johansson et al. (1993)_
7,8-DiMeIQx	1.1	gly	glu	DEG-Water	Negishi et al. (1984)
	nd	gly	glu	DEG-Water	Skog and Jagerstad (1990)
	nd	gly	glu	Water	Johansson and Jagerstad (1993)

Table 1. (cont'd)

4,7,8-TriMeIQx	6	ala, thr	glu	DEG-Water	Skog et al. (1992b)
PhIP	3.6	phe	glu	DEG-Water	Shioya et al. (1987)
	735	phe	—	Dry	Felton and Knize (1990)
	560	phe	glu	Dry	Felton and Knize (1990)
	nd	phe	—	Dry	Overvik et al. (1989)
	nd	leu	—	Dry	Overvik et al. (1989)
	20.9	phe	glyu	DEG-Water	Skog and Jagerstad (1991)
	6.4	phe	—	DEG-Water	Skog and Jagerstad (1991)
	<0.06	phe	glu	DEG-Water	Manabe et al. (1992)

*Yield is in nmol/mmol creatin(in)e
Dry = dry heating at 180 or 200°C for 1 hour
DEG-Water = reflux boiling in diethylene glycol/water (5:1) or 14% water
Water = heated in water in closed metal tubes at 180°C for up to 30 minutes
Amino acids: pro = proline, gly = glycine, phe = phenylalanine, ser = serine, ala = alanine, thr = threonine, lys = lysine, tyr = tyrosine, leu = leucine.
Sugars: fru = fructose, glu = glucose, rib = ribose.
nd = not determined

oxidase system, although the p450IA1 system also may catalyze the reaction (Kato and Yamazoe, 1987; McManus et al., 1990). After hydroxylation occurs, esterification with acetic or sulfuric acid probably forms the final product which can then bind to DNA. These adducts cause mainly frameshift mutations in the Ames assay using *Salmonella typhimurium* strains TA98 and TA 100 (Sugimura, 1982; Sugimura et al., 1988). In *vivo*, these frameshift mutations would then cause abberrant proteins to be formed through the processes of translation and transcription.

The mutagenicity of HAAs is well documented and is, in several cases, greater than that of compounds which are known to be potent carcinogens, such as aflatoxin B_1 (Table 2). While many mutagens are also carcinogens, this is not necessarily the case, and cell culture or whole-animal assays must be performed to determine the carcinogenic potential of a compound. Long-term feeding experiments with rodents and primates have demonstrated the carcinogenicity of a

number of the HAAs, including IQ, MeIQ, MeIQx, 4,8-DiMeIQx, and PhIP. The primary sites of DNA-adducts caused by the IQ and IQx-type compounds appear to be the liver, followed by kidney, heart, and bladder (Snyderwine et al., 1992). Tumors have been observed in the liver, colon, small intestine, stomach, liver, bladder, mammary gland, and pancreas of rodents fed IQ-type compounds. (Takayama et al., 1984; Tanaka et al., 1985; Weisburger et al., 1986). PhIP, in contrast to its weak mutagenic activity in the Ames assay, shows strong mutagenicity in mammalian cell cultures (Thompson et al., 1987). Colon and mammary gland carcinomas have been induced by PhIP in rodents, as well as lymphomas (Esumi et al., 1989; Ito et al., 1991). In attempting to extrapolate animal studies to humans, it should be noted that human microsomes show a two-fold greater ability than rat microsomes to activate PhIP (Davis et al., 1993).

Incidence and Consumption of HAAs

There remains little doubt as to the potent carcinogenic potential of HAAs in many animal species. Questions do exist regarding the interpretation of the results of these studies (Stavric, 1994). Many of the studies used doses equivalent to thousands of times the human lifetime exposure. Two studies using graduated doses of MeIQx found a linear dose-response relationship between ingestion of MeIQx and hepatic DNA adducts, even at doses too small to produce tumors in the test animals (Yamashita et al., 1990; Ohgaki et al., 1991). Thus it is possible that there exits a threshold level for HAA consumption below which cancer does not form. It is more likely that the many HAAs consumed daily, along with exposure to other carcinogens and promoters, exert some type of additive effect on human cancer risk. In order to examine human risk more closely, it is necessary to turn to epidemiological evidence and our knowledge of the HAA content of various foods.

Table 2. Mutagenicity of heterocyclic aromatic amines and typical carcinogens in *Salmonella typhimurium* (Sugimura and Sato, 1982; Sugimura et al., 1988)

	Revertants/μg	
Compound	**TA98**	**TA100**
IQ	433,000	7,000
MeIQ	661,000	30,000
IQx	75,000	1,500
MeIQx	145,000	14,000
4,8-DiMeIQx	183,000	8,000
7,8-DiMeIQx	163,000	9,900
PhIP	1,800	120
Trp-P-1	39,000	1,700
Trp-P-2	104,200	1,800
Glu-P-1	49,000	3,200
Glu-P-2	1,900	1,200
Orn-P-1	56,800	–
AαC	300	20
MeAαC	200	120
Aflatoxin B_1	6,000	28,000
AF-2	6,500	42,000
4-Nitroquinolin 1-oxide	970	9,900
Benzo[a]pyrene	320	660
N-Methyl-N'nitro-N-nitrosoguanidine	0.00	870
N-nitrosodiethylamine	0.02	0.15
N-Nitrosodimethylamine	0.00	0.23

The average daily consumption of HAAs from food in the Western hemisphere has been estimated to range from 0.1 μg/day to 16 μg/day (Turesky et al., 1993; Wakabayashi et al., 1992). The actual intake is quite variable, based on amount and type of meat eaten as well as cooking technique and degree of doneness. Lifetime cancer risk for humans based on intake of 80 ng/kg/day is significant (Felton et al., 1986a; Adamson, 1990). Actual epidemiological studies considering HAA intake and cancer risk are sparse and contradictory. Lyon and Mahoney (1988) reported no association between ingestion of fried or broiled meat and the risk of colon cancer. However, the data collection procedure which they utilized has been criticized as being inaccurate (Schiffman and Felton, 1990). A population study in Sweden found that total meat intake, consumption of brown gravy, and heavily browned meat each increased risk of colon cancer independently (Gerhardsson-de-Verdier et al., 1991). These same authors concluded in a later review article that the epidemiological literature is insufficient to justify any recommendations at this time (Steineck, et al., 1993).

Heterocyclic aromatic amines appear to be possible or probable human carcinogens on the basis of animal studies and bacterial assays (IARC, 1993). Clearly, more long term, in-depth human studies are needed to definitively classify HAAs as human carcinogens. In order to elucidate an accurate relationship between HAA consumption and carcinogenesis, it is also necessary to have precise knowledge of the HAA content of foods and the factors which affect their formation. Accurate values are not easy to obtain. The methods used to isolate and quantitate HAAs in foods vary widely, as do the results (Felton et al., 1992). The most accurate method currently in wide use is the solid-phase extraction procedure developed by Gross (1990) and refined by Gross and Gruter (1992). Briefly, this procedure utilizes tandem extraction with diatomaceous earth and an ion exchange resin followed by further clean-up with a C18 column. Separation and quantification of the HAAs is achieved with high performance liquid

chromatography (HPLC) using a photodiode array detector. Another accurate approach which is not in general use involves spiking with heavy-isotope-labeled standards, a clean-up procedure, then analysis by gas chromatography-electron-capture negative ion chemical ionization mass spectrometry (Murray et al., 1988). Table 3 lists average values of HAAs in meat products (Skog, 1993).

Table 3. Average values of HAAs formed in selected food products (Skog, 1993)

	Heterocyclic Aromatic Amines*				
Food	**IQ**	**MeIQ**	**MeIQx**	**4,8-DiMeIQx**	**PhIP**
Fried ground beef	3.69	not determined	1.58	1.13	13.19
Salmon	0.85	1.55	0.93	—	—
Chicken	—	—	2.22	0.81	38.1
Fried ground pork	0.04	0.02	1.4	0.6	4.5
Beef Extract	4.70	—	19.99	3.45	3.62

*Values are expressed in ng/g cooked or uncooked food, and are an average of those quoted by Skog (1993).

Mechanism of Formation of HAAs

The next step beyond establishing mutagenic/carcinogenic potential of HAAs and ascertaining levels formed in the food supply is to elucidate the mechanism of HAA formation. Without this understanding, it is difficult to determine contributing factors or formulate methods to control HAA formation.

Much is not yet known about the mechanism by which HAAs are formed. It is generally accepted that one route of HAA formation is through intermediates of the Maillard or nonenzymatic browning reaction. This concept was first proposed

not long after HAAs were initially isolated (Spingarn and Garvie, 1979; Powrie et al., 1981; Shibamoto et al., 1981; Wei et al., 1981). The precursors which have been found to be essential for HAA formation are creatine or creatinine (Yoshida and Okamoto, 1980a,b,c; Bjeldanes et al., 1982a,b) and amino acids (Taylor et al., 1984, 1985). Sugars and water appear to be involved in the formation of some HAAs (Jagerstad et al., 1983b; Skog and Jagerstad, 1990). The contributions of the various reactants are detailed later in the text.

Chemical Formation

The Maillard reaction is actually a series of reactions between reducing sugars and proteins or amino acids, catalyzed by heat, that lead to the formation of hundreds of compounds. Melanoidins are some of the color compounds formed during the Maillard reaction that lead to the characteristic brown color of baked breads and fried meats. Aldehydes, furanones, sulfur-containing heterocycles, pyridines, pyrazines, and pyrroles all are volatile Maillard reaction products which contribute to flavor and aroma (Rizzi, 1994).

The Maillard reaction begins when a reducing sugar reacts with the amino group of an amino acid to form a glycosylamine. This compound rearranges to give an N-substituted amino ketose, or Amadori compound. Amadori compounds are thermodynamically unstable, and under alkaline conditions, these rings fragment to 2- and 3-carbon compounds, which give rise to melanoidins (Hayashi and Namiki, 1986). Slightly acidic conditions will cause the Amadori compounds to lose their amine and give rise to 1-deoxyosones (Pischetsrieder and Severin, 1994). Strongly acidic conditions will generate a 3-deoxyosone. Deoxyosones undergo cyclization and dehydration, then fragment to low molecular weight 1,2-dicarbonyls. These dicarbonyls react with amino acids (the Strecker degradation) to form aldehydes, pyrazines, pyrroles, pyridines, and many other volatile compounds (Rizzi, 1994).

The first hypothesis for the mechanism of HAA formation through Maillard reaction pathways was proposed by Jagerstad et al. (1983a). This hypothesis assumes the formation of pyridines and pyrazines through the Maillard reaction pathway outlined above. One molecule of a pyridine or pyrazine then reacts with an aldehyde (which has also been formed through the Maillard reaction) to form the quinoline or quinoxaline portion of the HAA. Creatine, when heated, dehydrates and cyclizes to form creatinine, and one molecule of creatinine adds to the aldehyde to form an IQ-or IQx-type heterocyclic aromatic amine (Figure 2).

Confirmation of the reactants involved in HAA formation was offered by Jagerstad et al. (1983b) in the way of a model system study that included refluxing creatinine, amino acids, and glucose in a water/diethylene glycol mixture at 128°C. This mixture exhibited high mutagenic potential in the Ames assay. Excluding any one of the three reactants reduced the level of mutagenicity significantly (Jagerstad et al., 1983b). Addition of synthetic pyridines or pyrazines increased the mutagenic potential by 50%. Grivas et al (1985) utilized a similar model system, employing fructose as the sugar, and glycine as the amino acid. From this system, they isolated MeIQx and IQ, offering further confirmation of the precursors needed to form HAAs.

The theory of Jagerstaad et al. (1983a) assumes that condensation first occurs between pyridines or pyrazines and aldehydes, followed by ring closure with creatinine. A variation of this theory was offered by Nyhammar (1986), who hypothesized that condensation first occurs between creatinine and an aldehyde, with the addition of a pyrazine or pyridine as the last step. Support for the condensation of aldehydes with creatinine was offered by Jones and Weisburger (1988), who proposed a "concerted condensation" model of IQ-type HAA formation after finding "IQ-like" mutagens resulting from the reaction of aldehydes and creatinine. This model involves the condensation of two molecules of acetaldehyde with creatinine in one step to form IQx-type compounds. They

found no mutagenic activity resulting from the reflux of 2-vinylpyrazine with creatinine, which may indicate that aldehydes are necessary for HAA formation. It should be emphasized that very few mechanistic studies have actually been performed to verify reaction sequences for HAA formation.

An alternative explanation of early Maillard reaction chemistry raises intriguing questions about HAA formation. A series of experiments performed by Namiki and Hayashi (1981, 1983) challenges the long-held belief that Amadori rearrangement follows aldol condensation in the Maillard reaction sequence (Hodge, 1953). Using electron spin resonance (ESR) to look for free radicals, these investigators followed the Maillard reaction of various sugars and amino acids. It was found that N,N'-disubstituted pyrazine cation radicals were formed prior to Amadori rearrangement. The reaction sequence was determined to first involve the formation of glucosylamine, then fragmentation to a 2-carbon glycolaldehyde alkylimine and other products. The glycolaldehyde alkylimines can then proceed through a series of further reactions to form glyoxal, or condense to form a dialkylpyrazine radical. According to Namiki and Hayashi (1981), the Amadori rearrangement occurs after the formation of this radical.

Milic et al. (1993) performed ESR studies of HAA formation which confirmed the pyrazine/pyridine free radical cation findings. They were unable to determine if these free radicals participate in HAA formation. Therefore the possibility of a free radical mechanism for HAA formation does exist.

Factors Contributing to HAA Formation

Creatinine

Creatine exists in vertebrate animals as creatine phosphate, which acts as an energy reserve for muscles. Creatine can be cyclized to form creatinine through dehydration at elevated temperatures (Laser-Reutersward et al., 1987a). Creatinine has been clearly implicated, through both model system and meat

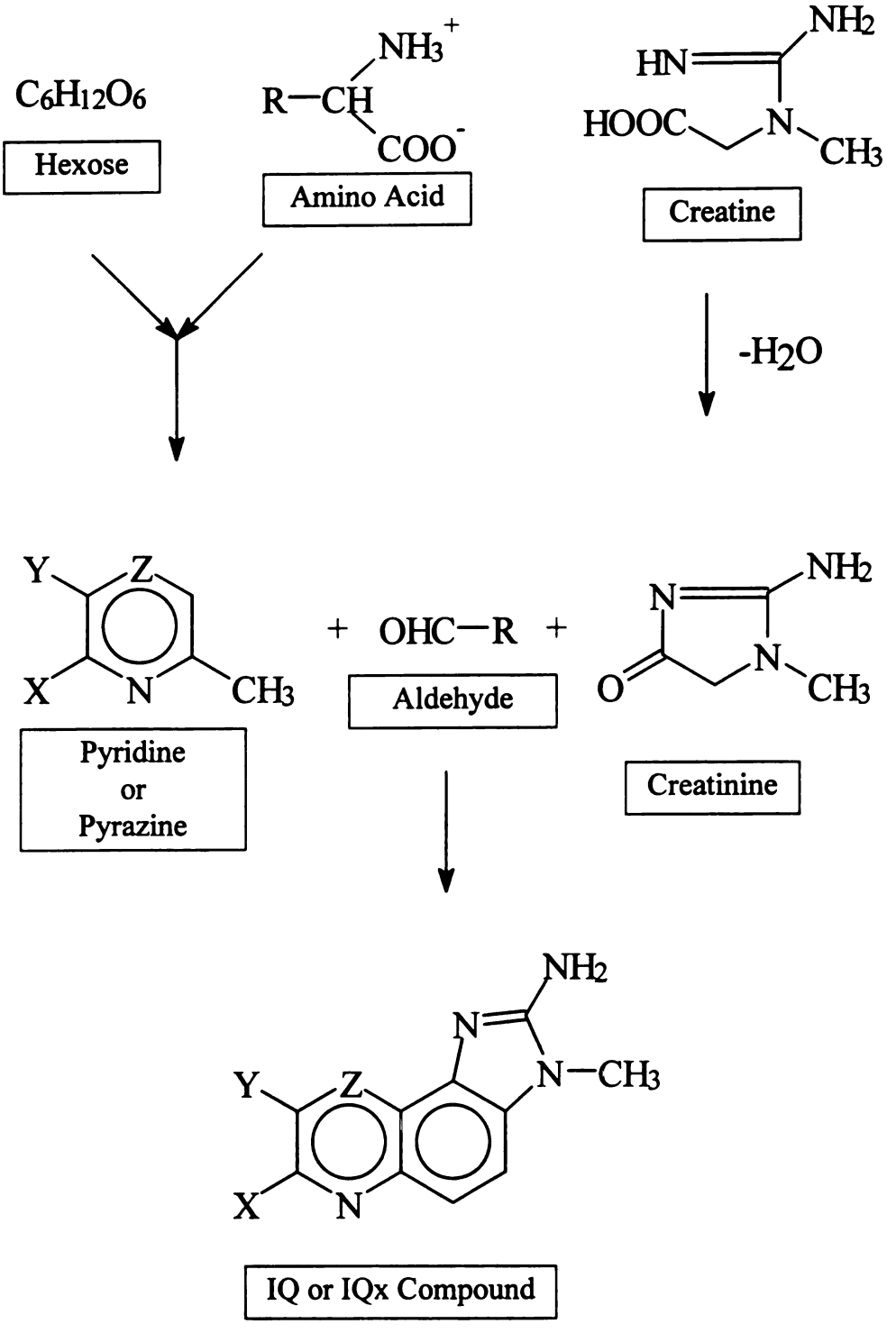


Figure 2. Theoretical reaction pathway for formation of IQ and IQx compounds. R, X, and Y may be H or CH_3: Z may be CH or N (Jagerstad et al., 1983a).

studies, as a precursor of HAAs. The first indication that muscle meats contain a compound which causes mutagen formation was published by Bjeldanes et al. (1982a,b). These researchers found low mutagenicity in cooked dairy products, organ meats, and shrimp, compared to the high mutagenicity reported for cooked muscle meats. Jagerstad et al. (1983a) proposed the involvement of creatinine in HAA formation and demonstrated a 50% increase in mutagenicity of beef when creatine was added before cooking. Other researchers subsequently verified the increased mutagenicity of meat systems containing added creatine (Taylor et al., 1985; Becher et al., 1988; Knize et al., 1988; Overvik et al., 1989). Laser-Reutersward et al. (1987a) confirmed earlier findings of low mutagenicity in non-muscle, high protein meat. They evaluated the levels of mutagenicity developed during the cooking of a variety of bovine tissues which exhibited a wide range of creatine concentrations. Kidney and liver were found to have very low levels of creatine, and also low mutagenicity, while muscle portions, heart, and tongue exhibited much higher mutagenic potential.

Model system studies provide the most definitive proof of creatinine as a HAA precursor, as they exclude other compounds contained in meat. Many model system studies have involved the heating of an amino acid with creatine/creatinine and a sugar to produce IQ-, IQx-, and pyridine-type HAAs (Table 1). Yoshido and Okamoto (1980a,b) were the first to use creatine and amino acids in a model system to produce mutagenic activity in the Ames assay. Knize et al. (1988) and Felton and Knize (1990) were able to isolate IQ and PhIP from dry-heating amino acids with creatinine with no sugar present. Many other investigators have utilized diethylene glycol as a reflux medium for model systems containing creatinine, amino acids, and sometimes sugars (Skog and Jagerstad, 1990; Muramatsu and Matsushima, 1985; Grivas et al., 1985). An aqueous system was employed by Johansson et al. (1993). Despite the many differences in the model systems employed, all these systems have in common amino acids and creatine/creatinine

as reactants. These are the only model systems from which HAAs have been isolated, a fact that provides significant evidence for creatinine as a necessary precursor of HAAs. Felton and Knize (1990) have been able to show incorporation of labeled isotopes of creatine into the PhIP molecule, giving definitive evidence of creatine's role in the formation of PhIP.

Amino Acids

As previously discussed, all model systems which have produced isolatable HAAs have contained amino acids (see Table 1). Ever since Commoner et al. (1978) demonstrated mutagenicity in cooked beef, researchers have shown repeatedly that HAA formation is limited to specific protein-rich foods (Nagao et al., 1977; Bjeldanes et al., 1982a,b). Mutagenicity is present at low levels in some grain-based cooked foods, but none of the known heterocyclic aromatic amines have been isolated from these foods (Knize et al., 1994a). Some form of protein, therefore, appears to be required for HAA formation. Several researchers have attempted to determine the form of protein involved in this process. Jagerstad et al. (1983b) found that substituting proteins for amino acids in a model system produced no mutagenic activity. Ashoor et al. (1980) reported that out of 19 amino acids tested, only proline and hydroxyproline increased mutagenicity of fried beef patties in the Ames assay. Taylor et al. (1985) demonstrated that HAA production markedly increased in boiled beef extract when the amino acid tryptophan was added to the reaction mixture. Overvik et al. (1989) recorded increases in the mutagenicity of broiled pork up to 43-fold with the addition of 1% by weight of various amino acids. The dipeptide, carnosine (alanine plus histidine), has also been found to contribute to mutagenic activity at approximately the same level as single amino acids when utilized in a model system with creatinine and glucose (Laser-Reutersward et al., 1987b).

The research to date clearly points to the free amino acid content of meats rather than their protein content as a determining factor in HAA formation (Skog, 1993). An interesting point that is made in several papers is that not all amino acids contribute equally to mutagenicity (Ashoor et al., 1980; Jagerstad et al., 1983b; Overvik et al., 1989). Threonine appears to have the greatest mutagenic potential. Most HAAs can be formed from more than one amino acid (Table 1). Often, however, one amino acid causes greater amounts of a specific HAA to be formed than does another amino acid. As more research is conducted in this area, findings may indicate that only certain amino acids are truly significant contributors to HAA formation.

Sugars

The role of sugars in the formation of HAAs is less clear than that of amino acids and creatinine. Many HAAs have been isolated from model systems using only creatinine and amino acids as reactants (see Table 1). However, sugars have been shown to increase HAA yield and change the relative ratio of HAAs formed in these model systems (Muramatsu and Matsushima, 1985; Skog and Jagerstad, 1990; Knize et al., 1991). Skog and Jagerstad (1991) further proved this point in a model system experiment where phenylalanine was heated with creatine at 180° to yield only PhIP. When glucose was added to the reaction mixture under the same conditions, the yield of PhIP increased three-fold, and MeIQx and 4,8 DiMeIQx were also detected. Concentration of sugars appears to play a role in mutagen formation as well. High concentrations of monosaccharides or disaccharides (greater than half the molar amount of the creatinine) acted as inhibitors to the formation of HAAs in a model system study employing increasing amounts of these compounds. (Skog and Jagerstad, 1990).

The actual mechanism by which sugar enhances HAA formation was unknown until Skog and Jagerstad (1993), using ^{14}C-labeled glucose, produced

definitive evidence that monosaccharides actually contribute carbon atoms to the HAAs IQx, MeIQx, and 4,8-DiMeIQx. Labeled carbon atoms from the glucose were incorporated into all three HAAs. Jagerstad et al. (1983a) suggested that the pathway involving sugars is the preferred route of HAA formation in actual meat systems. They tested mutagenicity in low-glucose beef, which was produced through animal exhaustion and thus glycogen breakdown prior to slaughter. These low-glucose beef patties produced very little mutagenic activity in the Ames assay, but mutagenic potential increased two-to three-fold when glucose was added prior to frying.

Other Factors

Several factors other than the immediate precursors have been found to exert significant influence over HAA formation. Fat is one such element, and will be considered separately in the next section. The factors with the best-defined relationship to HAA production are time, temperature and method of cooking.

Methods of cooking that result in lower mutagen formation include microwave cooking, roasting, and stewing (Dolara et al., 1979; Bjeldanes et al., 1983; Miller and Buchanan, 1983). Microwave cooking of meat is of extremely short duration, does not cause formation of a brown meat surface, and the pan residue has been shown to contain the water-soluble precursors of HAAs. These facts may explain the absence of mutagen formation in microwaved meats (Felton et al., 1994). Contact frying and deep-fat frying are cooking methods which give higher levels of mutagen production in meats (Nader et al., 1981).

Temperature has been demonstrated by many researchers to be an important factor in HAA formation. In meat systems, mutagen formation has been demonstrated to increase with increasing cooking temperature (Weisburger and Spingarn, 1979; Bjeldanes et al., 1983; Knize et al., 1994b; Balogh, 1995). Mutagenic activity has been reported at frying temperatures as low as 130°C

(Laser-Reutersward et al., 1989). The exact mathematical relationship between cooking temperature and mutagen or HAA formation is unknown, although some studies have suggested a roughly linear relationship within normal cooking temperatures (Bjeldanes et al., 1983; Knize et al., 1994b).

Cooking time also affects HAA formation. Generally, longer cooking times produce greater mutagenic potential and larger quantities of HAAs (Weisburger and Spingarn, 1979; Miller and Buchanan, 1983; Knize et al., 1994b). A lag period of 2 to 4 minutes during which the surface temperature of the meat is less than 100°C has been demonstrated. During this lag time, little mutagen formation is found (Spingarn and Weisburger, 1979; Knize et al., 1985). More information on time and temperature relationships to HAA formation in ground beef has been detailed by Balogh (1995). Time and temperature relationships to HAA formation are less predictable in model systems (Skog, 1993).

Due to the proven mutagenic and carcinogenic potential of HAAs, a number of compounds have been tested to determine if they inhibit HAA formation. The mechanisms of inhibition of these compounds vary, and much remains unknown as to their modes of action. High concentrations of sugars were discovered accidentally to inhibit mutagenic activity in beef stock (Taylor et al., 1986), and this effect has since been demonstrated consistently by other researchers (Skog and Jagerstad, 1990; Skog et al., 1992a). Soy protein concentrate and defatted glandless cottonseed flour, both of which contain naturally occurring antioxidants, were shown to decrease mutagenicity in cooked beef (Wang et al., 1982; Rhee et al., 1987). Polyphenolic compounds present in tea have been shown to be effective antioxidants (Sorata et al., 1984; Chen et al., 1990) and inhibitors of HAA formation in model systems (Weisburger et al., 1994; Yen and Chen, 1995). Vitamin E is another effective antioxidant that has been shown to inhibit HAA formation by as much as 80% (Faulkner, 1994; Balogh, 1995).

Role of Fat in HAA Formation

The role of lipids in HAA formation is an especially interesting topic due to the epidemiological and laboratory evidence that high-fat diets in general, and perhaps specific lipids as well, may play a role in cancer promotion (Reddy et al., 1978; Brennan-Craddock et al., 1990; Alldrick et al., 1987; Alavanja et al., 1996; Ritskes et al., 1996). Oxidation products of lipids have been shown to produce mutagenic activity in the Ames assay (Levin et al., 1982). A logical extension of these data is to examine the possibility that fat increases the production in meats of the probable human carcinogens, HAAs. Many researchers have examined the effect of lipid source and content on HAA formation, mostly in meats but also in model systems. Results have been variable.

Bjeldanes et al. (1983) examined the effect of meat composition on mutagen formation in ground beef. Fat content was found to have minimal effect on the mutagenicity of the samples, with defatted products actually displaying slightly higher mutagenic potential. Spingarn et al. (1981) obtained opposite results when examining the mutagenicity of fried ground beef patties. Mutagenic potential increased with the fat content of the beef up to a maximum at 16.4% fat. Results similar to those of Spingarn et al. (1981) were obtained by Knize et al. (1985), who found maximum mutagenicity in 15% fat ground beef. Lower fat content produced lower mutagenic potential, and 30% fat patties had slightly decreased mutagenicity compared to 15% fat patties. The authors attributed the decrease at 30% fat to dilution of the reactants.

Two studies have examined the effects of different types of frying fat on mutagen formation in pork. Nilsson et al. (1986) investigated the mutagenicity of pork fried in butter, lard, three types of margarine, and three types of vegetable oil at two different temperatures. Mean mutagenicity was higher in samples fried in fat versus samples fried without added fat at 250°C, but the investigators were unable to correlate mutagenic potential and degree of unsaturation of the frying fat.

Overvik et al. (1987) performed a similar study, frying pork in the same margarines and oils, and examining mutagenicity of the pan residues as well as the meat. Again, mean mutagenicity was greater in both pan residues and meat fried with fat at 250°C versus samples fried without fat. No significant difference in mutagenic potential between samples fried with fat and those fried without fat at 200°C was observed in either study. These investigators attributed the increased mutagenicity formed with fat present to the enhancement of heat transfer caused by cooking with fat. Barrington et al. (1990) examined the effect of cooking methods and type of frying fat on mutagen formation in lamb and beef. They demonstrated that the class of frying fat influenced mutagen formation, with butter and margarine producing higher mutagenic potential then oil, despite oil-fried meat achieving the highest maximum cooking temperature. However, no statistical treatment of the data was apparently performed for this part of the study.

One of the first studies to quantitate the formation of a specific HAA in response to two different levels of fat in meat was performed by Barnes et al. (1983), who determined the amount of IQ formed in beef patties containing either 10.6% or 27.5% fat. Forty-fold higher quantities of IQ were formed in the high-fat beef than in the low-fat beef. It was hypothesized that this effect was not due to increased heat transfer, but could be due to a number of factors, including the formation of free radicals during lipid peroxidation. These free radicals might then enhance some Maillard reactions leading to HAA formation. Barnes et al. (1983) also postulated that lipid oxidation will lead to increased formation of certain Maillard reaction intermediates that are possibly HAA precursors such as aldehydes and N-heterocyclic compounds.

In a more comprehensive study, Johansson and Jagerstad (1994) examined HAA formation in cooked meats and their pan residues, with specific emphasis on the role of fat content. Results indicated a significant positive correlation between fat content and IQ formation. No relationship to fat content was reported for any

other HAA. Mutagenicity was not determined, so it is not known if total mutagenicity increased with increasing IQ formation. It was noted that the total HAAs formed were ten times greater in pan residue from the beefburgers fried in butter versus those fried in vegetable oil. The authors postulated that this difference may be due to naturally occurring antioxidants in the vegetable oils.

The first researchers to investigate the effect of fat on mutagenicity in model systems were Barnes and Weisburger (1983, 1984). Their results indicated that several different types of fats increased mutagenic potential in the model system, including beef suet, corn oil, and lard. Larger amounts of fat (20%) increased mutagen formation even further. However, this study did not control for the physical effect of fat, which is greater efficiency of heat transfer. A model system was designed by Johansson et al. (1993) that permitted the use of water as a solvent, and basically eliminated the heat transfer effect of added fat. Using this model system, these investigators tested the formation of HAAs in a glucose/glycine/creatinine mixture with addition of various fatty acids and edible oils at approximately 20% by weight. In this system, MeIQx formation was found to nearly double when corn or olive oil was added to the system. Fatty acids had no predictable effect. However, due to small sample size and large standard deviations, no statistical analysis was performed.

Johansson and Jagerstad (1993) utilized this same model system to study the effects of oxidized fat, tocopherol content, and iron on HAA formation. The oxidation status of fat seemed to make little difference in HAA formation. Naturally occurring tocopherols did not affect the quantities of HAAs formed. Addition of iron as ferrous sulfate significantly increased HAA formation, both in the presence of fat and without fat. In samples containing lipid, HAA formation was only significantly greater than in the control (no lipid) when iron was present. MeIQx formation was greater in samples containing 40% fat than in samples containing 20% fat. These results differ somewhat from the study by Johansson et

al. (1993), where addition of oil caused MeIQx formation to increase relative to the control which contained no oil. Johansson and Jagerstad (1993) explained the influence of iron as catalyzing free radical reactions which lead to greater formation of pyrazines and pyridines. They hypothesized that these pyrazine compounds are immediate precursors of HAAs, and their increase leads to a higher yield of HAAs.

Most of the aforementioned studies found some increase in mutagenicity or HAA formation in samples with higher levels of intrinsic or added fat. Several theories have been proposed to explain these observations. First, it is well known that fat is an efficient conductor of heat. Large amounts of fat in or surrounding the meat could effectively increase the cooking temperature at the meat surface, or increase heat transfer into the meat (Knize et al., 1985; Nilsson et al., 1986). This phenomenon is known as the physical effect of fat on HAA formation. It is unlikely that this is the only explanation for the increase of HAAs formed in the presence of fat. Barrington et al. (1990) observed higher mutagenicity in meat products fried in butter or margarine than in those fried in oil, despite higher surface temperatures in the oil-cooked products. Nilsson et al. (1986) and Overvik et al. (1987) reported no greater mutagenicity in pork products fried in fat at 200°C than in those fried without fat. Barnes et al. (1983) observed no differences in surface temperature of meats cooked in fat versus those cooked without fat, despite higher levels of IQ formation in the former. Johansson and Jagerstad (1993) and Johansson et al. (1993) confirmed the possibility that a chemical effect of fat may exist through their model system studies which eliminated the heat transfer effect.

The nature of the chemical effect of fat on HAA formation is still quite speculative. Pyrazine and pyridine formation, which occurs during the Maillard reaction, is known to be enhanced by the presence of lipids (Watanabe and Sato, 1971; Buttery et al., 1977; Parihar et al., 1981; Arnoldi, 1990). If pyrazines and pyridines are immediate precursors of HAAs, as proposed in the theory of

Jagerstad et al. (1983a), lipids could have a chemical role in increasing HAA formation. Lipid oxidation, which is catalyzed by heat, can produce aldehydes and carbonyl compounds (Farmer and Mottram, 1990), which may also be precursors of HAAs (Barnes et al., 1983; Jagerstad et al., 1983a; Jones and Weisburger, 1988). Peroxidized lipids can also cause formation of free radicals (Pokorny, 1981), and based on the effectiveness of phenolic antioxidants in controlling HAA formation (Faulkner, 1994; Balogh, 1995), free radicals may be involved in HAA formation.

MATERIALS AND METHODS

Materials

Creatinine, L-phenylalanine, L-glycine, trilinolein (*cis-cis* 9,12), tristearin, D-glucose, 1-monostearoyl-*rac*-glycerol, ferrous sulfate heptahydrate, caffeine, triethylamine, and horse-heart myoglobin were purchased from Sigma Chemical Company (St. Louis, MO). All solvents, including water, were HPLC grade. The heterocyclic amine standards (IQx, MeIQx, 4,8-DiMeIQx, TriMeIQx, and PhIP) were obtained from Toronto Research Chemicals (Toronto, Canada). The heterocyclic aromatic amine standard FEMA (Flavour and Extracts Manufacturers' Association) was a kind gift from Dr. Mark Knize, Lawrence Livermore National Laboratory, University of California, CA. The FEMA standard contained IQ, MeIQ, MeIqx, 4,8-DiMeIQx, and PhIP, each at 5.0 ng/μl.

Extrelut-20 columns and Extrelut refill diatomaceous earth were obtained from EM Separations (Gibbstown, NJ). Propyl-sulfonic acid (PRS) Bond-Elut columns (500 mg), C18 cartridges (100 mg), and Hydromatrix diatomaceous earth were purchased from Varian, Inc. (Harbor City, CA). The heating module was a Reacti-Therm III, model 18835, made by Pierce Co. (Rockford, IL). Stainless steel test tubes, 2.3 ml. capacity, with threaded, self-sealing stainless steel caps were manufactured by the Engineering Research Complex Machine Shop at Michigan State University. A separate set of stainless steel test tubes was used for each amino acid to avoid carryover of HAAs. A Waters 510 high performance liquid chromatography machine (HPLC), equipped with a 991M photodiode array UV detector and 420 Waters fluorescence detector (Millipore, Milford, MA) and a

Rheodyne 7125 injector with a 50 µl loop (Rheodyne, Inc., Cotati, CA) were used for separation and quantitation of the HAAs.

Methods

Three model system studies were performed as described below. All procedures mentioned apply to each study unless otherwise stated. Briefly, the three studies were designed as follows:

Study #1: Effect of Trilinolein versus Tristearin on HAA Formation in Aqueous Phenylalanine and Glycine Model Systems

Study #1 examined the effects of a completely saturated lipid (tristearin) and a highly unsaturated lipid (trilinolein) on HAA formation in a phenylalanine and a glycine model system. Each amino acid system consisted of a control (no lipid), a treatment with tristearin, and a treatment with trilinolein. The control and treatments were replicated five times each. The control for the glycine system consisted of 0.6 mmol creatinine, 0.3 mmol glucose, 0.6 mmol glycine, and 0.02 g monostearin in 1.5 ml water. The phenylalanine control was composed of 0.6 mmol creatinine, 0.3 mmol glucose, 0.6 mmol phenylalanine, and 0.02g monostearin in 1.5 ml water. Treatment #1 for each model system was the addition of 0.3 g tristearin to the given reactants. Treatment #2 for each system was the addition of 0.3 g trilinolein to the aforementioned reactants.

Study #2: Effect of Corn Oil and Olive Oil on HAA Formation in an Aqueous Phenylalanine Model System

Study #2 examined the effect of two vegetable oils with different degrees of unsaturation on HAA formation in the phenylalanine model system. This study consisted of a control (tristearin), and two treatments (olive oil and corn oil).

Creatinine (0.6 mmol), glucose (0.3 mmol), phenylalanine (0.6 mmol), monostearin (0.02 g), and tristearin (0.3 g) in 1.5 ml water comprised the control. Treatment #1 substituted corn oil (0.3 g) for the tristearin. Treatment #2 substituted olive oil (0.3 g) for the tristearin. Each treatment and the control were replicated seven times. Three extra treatment #1 replications were performed and extracted with Hydromatrix to compare extraction efficiency of Extrelut and Hydromatrix diatomaceous earth.

Study #3: Effect of Ferrous Sulfate and Myoglobin on HAA Formation in a Glycine Model System with and without Lipid

Study #3 examined the effect of two sources of iron present in meat (ferrous sulfate and myoglobin) on HAA formation in the glycine system, both in the presence and absence of an unsaturated lipid. The five treatments entailed addition to a glycine system of one of the following: ferrous sulfate, myoglobin, trilinolein, ferrous sulfate plus trilinolein, or myoglobin plus trilinolein. The control contained creatinine (0.6 mmol), glucose (0.3 mmol), and glycine (0.6 mmol) in 1.5 ml water. A control for the lipid-containing samples consisted of the above reactants plus 0.3 g trilinolein and 0.02 g monostearin. Treatment #1 added ferrous sulfate to both the model system with trilinolein and the system without trilinolein in the amount of 0.01 mmol/test tube. Treatment #2 added myoglobin to both model systems also in the amount of 0.01 mmol. Each treatment plus the control were replicated five times. Three extra replications of the glycine system control and three replications of the glycine plus lipid control had nitrogen bubbled through the reactants and the headspace filled with nitrogen immediately prior to capping the test tubes. These extra replications were not included in the statistical analysis.

Safety of Designed System

Safety of the investigator and other laboratory workers was a major consideration when designing the model system. Small, stainless steel test tubes with tight-fitting caps were chosen rather than glass to minimize the hazard of explosion during heating. The reaction was carried out in a closed hood separated from the rest of the laboratory. Test tubes were not opened until cooled below 20°C. Asbestos gloves were used to handle the hot test tubes and heating block. All standard laboratory safety precautions such as goggles, lab coat, and latex gloves for handling chemicals were followed as well.

Weighing/Mixing of Ingredients

Creatinine (0.6 mmol), glucose (0.3 mmol), and either phenylalanine or glycine (0.6 mmol) were weighed accurately (± 0.5 mg) and poured into clean glass test tubes. Monostearin (an emulsifier) was added at a level of 0.02 g to each tube except those that were the control or control plus iron in study #3. Next, 0.3 g lipid (tristearin, trilinolein, corn oil, or olive oil) was placed in each test tube. HPLC-grade water (1.5 ml) was added to each tube. The tubes were then capped and placed, one at a time, in a beaker of boiling water for 15 seconds to dissolve the emulsifier and tristearin (both of which have melting points above 70°C). The glass test tubes were shaken vigorously for 10 seconds upon removal from the water bath, and the contents immediately poured into the stainless steel tubes and sealed with the threaded caps wrapped with Teflon tape. The only deviations from this procedure were for the controls which did not contain lipid or emulsifier. These samples were mixed directly in the stainless steel test tubes rather than being heated in a glass test tube first. Any samples scheduled to receive iron or myoglobin had the ferrous sulfate or myoglobin dissolved in the water prior to adding the water to the sample, at a concentration which delivered 0.01 mmol iron to each test tube.

Heating

The Reacti-Therm heating module was allowed to preheat for a minimum of 1.5 hours before heating of samples. It was discovered in preliminary experiments that the temperature of the heating block dropped approximately 30°C when loaded with 10 stainless steel test tubes. The heating temperature was 180° ± 5°C, so the heating block temperature reached prior to loading was set at 210°C. Silicon oil (0.5 ml) was placed in each cavity in the heating block to facilitate heat transfer from the block to the test tubes. Samples were heated for exactly 30 minutes. Upon completion of heating, the test tubes were plunged immediately into a beaker of ice, where they were allowed to cool for 30 minutes before transfer of the product to microvials for temporary storage at 5°C.

Extraction of HAAs

Sample preparation for HAA extraction from the model system products began with dissolution of the contents of two identical 1.5 ml microvials in sodium hydroxide (NaOH). Samples containing tristearin were dissolved in 57 ml hot (100°C), 10N NaOH. All others were dissolved in 57 ml 5N NaOH. The NaOH and samples were well-mixed with a spatula. Aliquots (10 ml) were taken from the mixture and placed in four 250 ml beakers. The remainder of the mixture was saved and used if any further extractions were required. Approximately 20 g of Extrelut refill (experiments # 1 and 2) or Hydromatrix (experiment #3) was added to each beaker. Two of the four beakers were spiked with a known amount of a standard mixture of HAAs (Appendix I). This spiking procedure facilitated peak identification during HPLC analysis, and allowed calculation of percent recovery for each HAA. The rest of the extraction procedure followed the method developed by Gross and Gruter (1992) and modified by Balogh (1995). Details of the procedure can be obtained from Balogh (1995).

Briefly, the extraction procedure was performed as follows. The diatomaceous earth was mixed with the sample, and the mixture was packed into the Extrelut 20 columns (20 g diatomaceous earth plus sample in each column). Hydromatrix brand diatomaceous earth required the operator to apply pressure in order to pack 20 g into a column; Extrelut brand did not. The columns were placed under a ventilation hood, and the HAAs were eluted with dichloromethane/toluene (95:5). PRS cartridges, which had been preconditioned with the same solvent mixture and had needles attached, were coupled to the Extrelut columns. Solvent was added periodically to keep the columns full until 45 ml had flowed through the assembly. The PRS cartridges, which retained the HAAs, were removed and dried under vacuum for 10-15 minutes. The PRS cartridges were connected to a peristaltic pump and rinsed consecutively at 2 ml/minute with 6 ml of 0.1 M hydrochloric acid, 15 ml methanol:0.1 M hydrochloric acid (4:6), and 2-3 ml water. C18 cartridges, which had been conditioned with 2 ml methanol and 2 ml water, were connected to the PRS cartridges, and 20 ml 0.5 M ammonium acetate buffer (pH=8.0) was pumped through the assembly to transfer the HAAs to the C18 columns. The C18 cartridges were then rinsed and dried under vacuum for 30 minutes. The HAAs were eluted into microvials using 0.8 ml methanol:ammonia (9:1), and evaporated under nitrogen for temporary storage at –20°C.

High Performance Liquid Chromatography

Separation of HAAs

The dry sample extracts were redissolved in 50 µl of methanol containing 50 ng/µl caffeine (experiment #1) or TriMeIQx (experiments #2 & 3). Samples were injected into the HPLC in aliquots of 20 µl. The first mobile phase was filtered, degassed 0.1 M triethylamine, adjusted to pH 3.2 with either phosphoric acid or hydrochloric acid. The second mobile phase was filtered, degassed acetonitrile. One of two reversed phase silica columns was used to separate the HAAs. The first

experiment utilized a column from Toso Haas (TSK-Gel ODS-80TM column with 5 μm particle size, 4.6 mm ID x 25 cm; Toso Haas, Montgomeryville, PA), and the second and third experiments utilized a Waters Symmetry C8 column (3.9 mm ID x 15 cm, Waters/Millipore, Milford, MA). A precolumn (Supelguard LC-8-DB, Supelco, Bellefonte, PA) was attached between the injector port and column to filter out unwanted compounds, and its cartridge was replaced approximately every 60 injections.

The flow rate of the mobile phase was 1 ml/min. For the first and second experiments, the initial ratio of acetonitrile:buffer in the mobile phase was 8:92, which increased to 17:83 during the first 10 minutes. Acetonitrile concentration continued to increase until the ratio was 25:75 in another next 10 minutes, then 55:45 in the next 10 minutes. This allowed elution of all heterocyclic aromatic amines from the column. Over the next five minutes, the acetonitrile:buffer ratio increased to 80:20 to facilitate elution of other compounds. After 35 minutes, the ratio returned to its original 8:92 for 10 minutes to allow the column to re-equilibrate before the next injection. The third experiment utilized a slightly different gradient based on observations of the Waters column during experiment #2. The original ratio of acetonitrile:buffer was again 8:92, but changed to 20:80 over the first 15 minutes, then to 80:20 over the next three minutes. Cleaning of the column was accomplished with an 80:20 ratio for 8 minutes, then the gradient returned to its original 8:92 ratio for the last 9 minutes. This gradient allowed adequate separation of HAAs while decreasing each run time to 35 minutes instead of 45 minutes.

Detection of HAAs

Detection of the HAAs was performed by a photodiode array UV detector. The IQ standards were detected at 254 nm, IQx compounds at 262 nm, and PhIP at 316 nm PhIP was also detected by a fluorescence detector with excitation set at

330 nm and emission at 375 nm. The software package used for peak observation and integration was the Millennium 2000 Chromatography Manager (Millipore, Milford, MA).

Mass Spectrometry

Fractions from the glycine system were collected from the HPLC for analysis by mass spectrometry at the Michigan State University-NIH Mass Spectrometry Facility. The samples were found to be contaminated by phosphate and potassium, which blocked the spectrophotometric signal. Multiple attempts were made to clean the HPLC and collect fractions for analysis, but the contamination was still present. An extracted sample that had not been fractionated on the HPLC was analyzed at the Mass Spectrometry facility by direct probe fast atom bombardment (FAB). This sample was shown to contain a compound of molecular weight 199, corresponding to IQx, a compound of molecular weight 214, corresponding to MeIQx , and a compound of molecular weight 228, corresponding to DiMeIQx. As further analysis was not possible due to the impurity of the sample, it is not known if this was 4,8-DiMeIQx or 7,8-DiMeIQx. A compound with molecular weight 242 was also found, corresponding to TriMeIQx. It is not known if this last compound was actually TriMeIQx or another substance with the same molecular weight, as TriMeIQx was not identified by retention time during HPLC of samples.

Quantitation of HAAs

Before HPLC separation of samples for each experiment, four aliquots of the standard mixture of HAAs, four aliquots of internal standard, and four aliquots of FEMA (10, 15, 20, and 25 μl) were injected. Linear regression (ng compound versus peak area) was performed for each HAA in each mixture. A correlation coefficient of 0.99 or greater was considered acceptable for FEMA or internal

standard, and 0.97 or greater was acceptable for the laboratory standard mixture of HAAs.

Each peak area corresponding to an HAA was first corrected for expected area of the internal standard based on the internal standard regression line. The standard addition method of Gross and Gruter (1992) was then used for determining extraction efficiency and for quantitation of HAAs from the samples. Each data point consisted of four subsamples; two spiked and two unspiked. The average area of the spiked samples minus the average of the unspiked samples allowed comparison with the regression line for the standard mixture. Each data point as determined below was then corrected for its individual extraction efficiency, or percent yield.

Nanograms of each HAA formed were determined using the average of the two unspiked subsamples. The linear regression slope for FEMA was used to determine the exact amount of each HAA formed in each sample. FEMA was employed for this calculation rather than the standard mixture, as the concentration of each HAA in FEMA was known to 1/100 of a nanogram. The exception to this rule was for the compound IQx. This HAA was not present in FEMA, so the standard mixture regression line was used to calculate formation of IQx.

Statistical Analysis

Study #1: Effect of Trilinolein versus Tristearin on HAA Formation in Aqueous Phenylalanine and Glycine Model Systems

Statistical analysis was accomplished using the SPSS statistical software package (student version 6.1 for Windows, 1994, Prentice Hall, New Jersey). One-way analysis of variance (ANOVA) was performed for each heterocyclic amine. Prior to this analysis, a log transformation was performed on the data. This transformation was necessary due to a positive correlation between ng HAAs

formed and standard deviations. Student-Newman-Keuls post-hoc tests were performed.

Study #2: Effect of Corn Oil and Olive Oil on HAA Formation in an Aqueous Phenylalanine Model System

Statistical analysis was again performed with SPSS software. One way ANOVAs were used to analyze differences in HAA formation between treatments for each HAA. No post-hoc tests were necessary. Hydromatrix replications were not included in any statistical treatment. Extraction efficiencies were virtually identical in Hydromatrix and Extrelut samples.

Study #3: Effect of Ferrous Sulfate and Myoglobin on HAA Formation in a Glycine Model System with and without Lipid

Statistical analysis was again accomplished with one way ANOVAs using the SPSS statistical software. Student-Newman-Keuls post-hoc tests were performed with the same software.

RESULTS AND DISCUSSION

Study #1: Effect of Trilinolein on HAA Formation in Aqueous Phenylalanine and Glycine Model Systems

Johansson et al. (1993) reported a large increase in MeIQx formation in an aqueous model system of creatinine, glucose, and glycine with the addition of 20% corn oil to the system. This observation is significant in light of the results of many meat studies indicating increasing mutagenicity of meat with increasing fat content up to approximately the same level of fat. The difficulty with investigations into the effect of fat on HAA formation has been the inability to decouple the physical effect of fat from possible chemical effects. An aqueous model system heated in very small test tubes made of material with excellent heat conductivity essentially eliminates the physical effect of fat (increased heat transfer). The model system of Johansson et al. (1993) appears to effectively decouple these two factors and provide evidence of a large increase in HAA formation due to the chemical interaction of fat within the Maillard reaction.

Many questions are raised by these results. It is not known whether HAAs other than MeIQx increase in the presence of fat. There is no information on how different types of fats of varying degrees of unsaturation affect HAA formation. Perhaps the most important note of caution is that a rigorous statistical evaluation of the data was lacking in the study of Johansson et al. (1993). As has been shown by several investigators, data on HAA formation in meat and in model systems have inherently large variability. This variability can be problematic if adequate replications are not performed (Balogh, 1995).

Based in part on some of the questions discussed above, this study was designed utilizing a model system similar to that developed by Johansson et al. (1993). The objective of this study was to elucidate the chemical effect of a completely saturated fat (tristearin) and an unsaturated fat (trilinolein) on the formation of several HAAs in the model system.

The ratios of reactants for this study and the time and temperature of heating were selected to resemble those used by Johansson et al. (1993) while taking into account the small size of the test tubes. Glycine was chosen as the amino acid for system #1 in order to compare results with those of Johansson et al. (1993). Phenylalanine was selected for system #2 to maximize formation of PhIP, the dominant HAA in meat systems. Lipid was added at 15% by weight rather than 20% because of literature evidence indicating that the greatest concentrations of HAAs are formed at this fat level in ground beef patties, and because of the size limitations of the test tubes. The amount of standard used to spike half the samples (1000 ng of each HAA) was based on the expected amount of formation of HAAs. The average amounts of each HAA formed for specific treatments with the standard deviations, as well as the mean recovery are shown in Table 4.

The dominant HAAs formed in the glycine system were IQx and MeIQx. PhIP, IQx, and MeIQx were the major HAAs formed in the phenylalanine system. Small amounts of DiMeIQx were formed in both systems, but DiMeIQx was not present in every sample, and therefore was not quantitated. Recoveries ranged from a low of 33% to 100% (Table 4). Larger amounts of HAAs were formed in these systems than anticipated, which may have caused mean recoveries to be slightly lower than actually calculated. This effect is due to difficulty in accurately determining recovery when the amount of HAAs added to the spiked samples is not greater than the amount of HAAs formed in these samples.

Statistical analysis of the data from this study revealed a significant increase ($p<0.05$) in MeIQx formation when trilinolein was added to the glycine system.

The amount of MeIQx formed when trilinolein was present was also significantly greater than the amount formed with tristearin present. A similar profile was seen with IQx formation in the glycine system. The addition of trilinolein significantly increased formation of IQx above that in the control and in the samples containing tristearin. PhIP was not expected to be formed from glycine in this system and was not observed.

Table 4. Effect of trilinolein on HAA formation in aqueous phenylalanine and glycine model systems[1,2,3,]

System	IQx	MeIQx	PhIP
Gly/Control	5.09±0.16[a]	10.40±2.12[a]	not formed
Gly/Tristearin	4.33±0.52[a]	10.63±2.21[a]	not formed
Gly/Trilinolein	6.30±0.32[b]	15.90±4.42[b]	not formed
Phe/Control	2.59±0.55	4.67±1.55[a]	44.90±4.76
Phe/Tristearin	2.72±0.40	6.72±1.45[b]	39.4±17.57
Phe/Trilinolein	2.80±0.13	6.07±0.82[a,b]	24.6±11.90
Mean Recovery	87.8%*	87.8%	71.3%

[1]Values for each HAA are expressed in nmol/mmol creatinine used.

[2]Each value is the mean of five analyses ± standard deviation.

[3]Means in columns with different superscripts are significantly different from each other within each amino acid system at $p<0.05$.

*Recovery for IQx was calculated based on MeIQx recovery, as the standard did not include IQx.

In contrast to the glycine system, the presence of lipids and their composition did not change the amount of IQx formed in the phenylalanine system. MeIQx was formed in somewhat greater quantity when tristearin was present compared to the lipid-free control. Trilinolein seemed to have no effect on MeIQx in this system. PhIP formation appears to decrease dramatically in the presence of trilinolein versus tristearin or no fat. However, this effect was statistically significant only at the $p<0.26$ level, due to the large standard deviations for PhIP. Figures 3 and 4 depict the changes in HAA yields in both amino acid systems graphically.

The increase in formation of quinoxaline compounds in the glycine system upon addition of an unsaturated lipid agrees with the data of Johansson et al. (1993). These results indicate a chemical role of fat in HAA formation under specific conditions. The fact that tristearin did not increase HAA formation in this system suggests that a certain degree of unsaturation is necessary for fat to participate in reactions leading to HAAs. The role of unsaturated lipids may be to increase the amount of intermediate pyrazine and pyridine compounds formed via the Maillard reaction that lead to HAA formation. This theory was proposed by Barnes et al. (1983) and Jagerstad et al. (1983a). Free radical reactions cause acceleration of lipid oxidation. Thus, greater concentrations of lipid oxidation products such as aldehydes may occur in the system, leading to increased HAA formation (Parihar et al., 1981; Namiki and Hayashi, 1983).

The apparent decrease in PhIP formation in the phenylalanine system due to the presence of trilinolein is difficult to explain. This decrease in PhIP formation, while large, was not statistically significant and likely occurred simply by chance. However, a similar result was achieved by Faulkner (1994) in a phenylalanine model system when an unsaturated lipid was added. Assuming for the sake of discussion that this trend was significant, explanations for this result are purely speculative. There was no significant increase in any other HAA in this system when trilinolein was added, so it is not the case that reactants were diverted to

production of other HAAs. It is possible that reactants in the presence of an unsaturated lipid preferentially produced other compounds, such as melanoidins. Another possibility is that, as mentioned above, lipid oxidation leads to increased amounts of aldehydes and other compounds, but these compounds are not precursors for PhIP. The effect of unsaturated lipids on PhIP formation should be a productive area for further research.

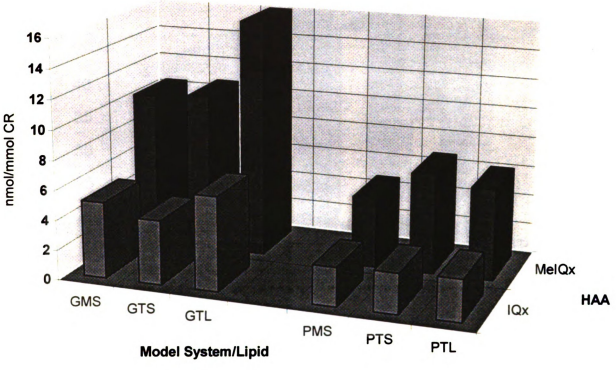


Figure 3. Effect of tristearin versus trilinolein on HAA formation in glycine and phenylalanine model systems.

GMS = glycine control plus monostearin, GTS = glycine system plus tristearin, GTL = glycine system plus trilinolein, PMS = phenylalanine system plus monostearin, PTS = phenylalanine system plus tristearin, PTL = phenylalanine system plus trilinolein.

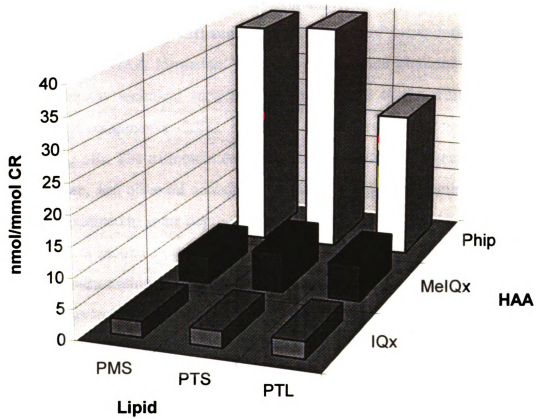


Figure 4. Effect of tristearin versus trilinolein on HAA formation in a phenylalanine model system.

PMS = phenylalanine system plus monostearin, PTS = phenylalanine system plus tristearin, PTL = phenylalanine system plus trilinolein.

Study #2: Effect of Corn Oil and Olive Oil on HAA Formation in an Aqueous Phenylalanine Model System

The results of the first study confirmed a chemical role for unsaturated fat in the formation of HAAs in an aqueous Maillard reaction system. The unsaturated fat in that study was trilinolein, which contains six double bonds per triglyceride molecule. This is a greater degree of unsaturation than any cooking oil would contain. It is unknown how differences in the degree of unsaturation of actual cooking oils affect HAA formation. Based on information obtained from Sigma Chemical Company technical services, and the storage of the trilinolein under argon gas in its container at -20°C, oxidation should have been less than that of typical cooking oils. The trilinolein contained no antioxidants. Vegetable oils such as corn, soybean, and olive oil contain some naturally occurring antioxidants, and generally are oxidized to some degree before use.

Therefore, a second study was designed to address practical aspects of how the degree of unsaturation in vegetable oils affects HAA formation. The objective of this study was to investigate the effect of corn oil and olive oil on the formation of HAAs in the phenylalanine model system.

The major HAAs detected in the phenylalanine model system were IQx, MeIQx, sometimes DiMeIQx, and PhIP. This observation is the same as for study #1. Extraction efficiencies were similar to those of the previous study as well, except for PhIP in the presence of tristearin. This atypically low average recovery of PhIP was due to extraction efficiencies ranging from 16-36% in one treatment. It was proposed that the tristearin complexed with the large quantity of PhIP present in the standard for this study (2500ng/sample) and made it unavailable for extraction. Therefore the average recoveries from the first study were used to calculate this treatment (Table 4).

Table 5. Effect of corn oil and olive oil on HAA formation in a phenylalanine model system.[1,2,3]

System	IQx	MeIQx	PhIP
Phe/Control	3.26±1.04	7.54±2.74	36.75±16.80
Phe/Olive Oil	2.80±0.42	6.06±1.99	40.93±12.01
Phe/Corn Oil	2.94±0.49	7.34±1.13	39.41±13.89
Mean Recovery	81.77%	93.10%	43.87%

[1]Values are expressed in nmol HAA formed/mmol creatinine used.

[2]Each value is the mean of seven replications ± standard deviation.

[3]No significant differences were found at $p<0.05$ level.

Statistical analysis revealed no significant differences between any of the treatments in this study. Absolute values and standard deviations were very similar to those obtained from the phe/control and phe/tristearin treatments in study #1.

Although the results of this study did not corroborate the results obtained with pure unsaturated lipids in study #1, the lack of differences between treatments gives some important information. First, the difference in the degree of unsaturation of olive oil and corn oil is not adequate to influence the formation of HAAs that require phenylalanine as a precursor. Second, it also appears that, at least for PhIP, the degree of unsaturation in the range of zero double bonds/triglyceride (tristearin) to 3.77 double bonds/triglyceride (corn oil) makes no difference in the amount of HAA formation. In comparing data between studies #1 and 2, it also appears that the presence of 15% fat does not affect PhIP formation as long as the degree of unsaturation is ≤ that of corn oil. Here study #2 confirms the statistical analysis of study #1, showing unsaturated oils to have no significant effect on PhIP formation.

Several investigators have speculated that naturally occurring antioxidants in vegetable oils might inhibit HAA formation (Johansson et al., 1993). The corn oil used in this experiment contained approximately 0.012% mixed tocopherols per the manufacturer's analysis. No information was available for the olive oil. The presence of naturally occurring antioxidants did not appear to have any effect on HAA formation in this model system. Because antioxidant concentration is so crucial to its effectiveness (Balogh, 1995), it is difficult to say if these vegetable oils would have any effect in a meat system.

Study #3: Effect of Ferrous Sulfate and Myoglobin on HAA Formation in a Glycine Model System.

The third study was designed to investigate the effect of iron on HAA formation in the glycine model system. HAA yields in this system are known to be affected by the addition of highly unsaturated lipids (Table 3; Johansson et al., 1993). If oxidation of lipids plays a role in increasing intermediate compounds necessary for HAA formation, addition of a prooxidant should increase the rate of this oxidation and possibly further increase HAA formation. Iron, in the form of heme compounds as well as free iron, can facilitate the decomposition of lipid hydroperoxides and speed the formation of hydroxyl radicals (Tappel, 1962; Halliwell and Gutteridge, 1986). Johansson and Jagerstad (1993) found that the addition of ferrous sulfate to an aqueous model system significantly increased MeIQx formation without the presence of fat and also with the addition of 40% fat.

The objectives for this phase of the study were to determine the effect of free iron (ferrous sulfate) and myoglobin on HAA formation both in the absence and presence of an unsaturated lipid. Ferrous sulfate was chosen to allow comparison of data with the study of Johansson and Jagerstad (1993). Myoglobin was selected as a representative heme compound in meat systems. The latter group of compounds are effective catalysts of lipid oxidation (Silberstein and Lillard, 1978).

Table 6. Effect of ferrous sulfate and myoglobin on HAA formation in a glycine model system with and without trilinolein.[1,2,3,4]

System	IQx	MeIQx
Gly/Control	7.39±1.23[a,b]	15.48±1.34[a]
Gly/Fe	8.49±2.08[a]	26.29±2.90[b]
Gly/Trilinolein	5.94±1.85[b]	15.79±0.89[a]
Gly/Trilinolein/Fe	5.71±1.69[b]	19.98±6.83[a]
Gly/Mb	2.30±0.74[b,c]	15.18±2.45[a]
Gly/Trilinolein/Mb	1.83±0.40[b,c]	16.27±4.49[a]
Gly/N_2	4.67±1.55	14.37±5.18
Gly/Trilinolein/N_2	5.04±1.17	17.07±2.93
Mean Recovery	63.50%	64.85%

[1]Values are expressed in nmol HAA formed/mmol creatinine used.

[2]All treatments above the dotted line were replicated five times. Treatments below the dotted line were replicated three times and were not subjected to statistical analysis.

[3]Means in the same column with different superscripts are significantly different from each other at $p<0.05$.

[4]Fe denotes ferrous sulfate, Mb denotes myoglobin, N2 denotes nitrogen gas.

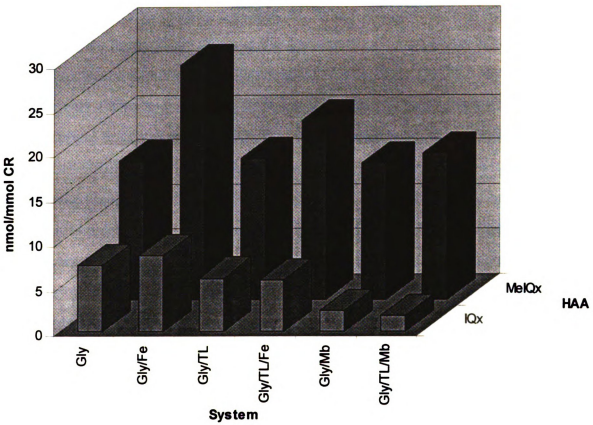


Figure 5. Effect of ferrous sulfate (Fe) or myoglobin (Mb) on the formation of HAAs in a glycine model system with and without trilinolein (TL).

Gly = glycine system control, Gly/Fe = glycine system plus ferrous sulfate, Gly/TL = glycine system plus trilinolein, Gly/TL/Fe = glycine plus trilinolein plus ferrous sulfate, Gly/Mb = glycine system plus myoglobin, Gly/TL/Mb = glycine system plus trinlinolein, plus myoglobin.

A one-way ANOVA with Student-Newman-Keuls post-hoc testing was performed for each HAA, except DiMeIQx, whose presence was not detected in all samples. This analysis revealed that the glycine system with ferrous sulfate added (no lipid) produced significantly greater ($p<0.05$) amounts of IQx and MeIQx than did almost any other treatment (Table 6). When myoglobin was added to the system, MeIQx production remained the same as the control, while IQx formation actually decreased significantly. The presence of trilinolein at 15% by weight made no significant differences in any treatments. The system containing trilinolein plus ferrous sulfate did form more MeIQx than the system containing trilinolein and no iron, but the difference did not attain statistical significance. A notable difference between the results of this study and the first study is that the control of this study formed 50% more MeIQx than the control in study #1. The cause of this difference is unknown, but a similar variance was noted between the model system results obtained by Johansson and Jagerstad (1993) and Johansson et al. (1993). Data from Table 6 are depicted in graph format in Figure 5.

The results of this study are consistent with those of Johansson and Jagerstad (1993). They reported increases in MeIQx formation after 30 minutes of heating when iron was added to the system without lipid, and when iron was added to a system containing 40% lipid (not 20%). Several possible explanations exist for these data. First, it appears that iron may have a role in the Maillard reaction as an initiator of free radical reactions that do not involve lipids. Results of this study, along with the data from antioxidant treatments of meat which suppress HAA formation (Balogh, 1995), support the theory of a free radical component of HAA formation. Second, decomposition of low and moderate levels of lipids (15-20%) in the presence of iron may not produce a sufficient increase in HAA precursors such as pyrazines or pyridines to make a difference in HAA yield.

The apparent lack of involvement of lipid oxidation in this study has several implications. First, it may be that rate of HAA formation is crucial. Iron may be

hastening lipid breakdown and thus formation of HAA precursors, but the reaction may reach steady state before the heating is finished, so the final yield is no different than without iron. Second, it may be that the role of lipid is not due to oxidation products at all, but rather that lipid is involved in the Maillard reaction in another, as yet undetermined, mode.

The decrease in IQx in the myoglobin treatments is likely due to high viscosity and the inability of the reactants to reach each other. Myoglobin denatures at relatively low temperatures, and creates a network of thick strands. When these test tubes were opened after heating, the contents were solidified from top to bottom, due to the high reaction temperature. MeIQx formation was unaffected by the increase in viscosity. It is likely that MeIQx formation occurs prior to or at a faster rate than that of IQx during heating, and therefore MeIQx was produced before being affected by the increased viscosity. It is unknown if the same effect would function in meat systems to keep the reactants physically separated.

Although the data from the test tubes heated in a nitrogen atmosphere were not subjected to a statistical analysis, it appears that the lack of available oxygen did not affect the HAA formation in these samples. The same species and approximately the same yield of HAAs were produced as in many of the other samples. Either enough oxygen was still present for any reactions requiring this compound, or the reactions leading to HAA formation are not oxygen dependent.

Summary and Conclusions

Results of these three studies taken together show an increase in formation of specific quinoxaline-type HAAs formed from one amino acid in the presence of either trilinolein or ferrous sulfate. Through rigorous statistical treatment of the data, several lipids were shown to have no effect on HAA formation in the two model systems utilized (tristearin, corn oil, olive oil).

The three studies discussed here indicate that highly unsaturated lipids may have a small chemical effect on HAA formation, but it is likely that the major result of fat on HAA production is the physical effect of increased heat transfer. It appears that the chemical effect of lipids may differ depending upon the precursors present, as HAA formation increased in the glycine system and apparently decreased in the phenylalanine system. Iron in the form of ferrous sulfate (Fe^{2+}) increased HAA formation in the model system containing no lipid, but not in the system containing lipid. This result points away from lipid oxidation products playing a major role in HAA formation during the Maillard reaction, and toward a free radical mechanism. Results indicating inhibition of the majority of HAA formation by phenolic antioxidants in fried ground beef support the involvement of a free radical mechanism in their formation (Balogh, 1995).

Future Research

The research discussed here indicates that trilinolein has a chemical effect on HAA formation when glycine or phenylalanine is the amino acid precursor. Other model system studies need to be performed to determine whether actual cooking lipids have an effect on HAA formation. If a chemical effect does exist for any of these fats, it should be determined for as many amino acids as possible, as the effect may vary greatly among the amino acid precursors.

Even more fundamental than the issue of a lipid effect on HAA formation is the actual mechanism of formation itself. Each study examining the result of a specific component on HAA formation adds more information regarding the possible mechanism. However, a series of several studies could be designed to give more definitive information. Continuous or intermittant monitoring of a model system reaction containing lipid through electron spin resonance (ESR) could indicate at what point free radicals are present. Thermal gravidimetric analysis of the lipids could pinpoint the exact time/temperature of lipid oxidation, and combined with ESR data, determine whether lipid oxidation products are involved in HAA formation. Another study which could provide valuable information regarding reaction intermediates would be detection and separation of aldehydes, pyrazines, and pyridines by gas chromatography. Aliquots of the reaction mixture could be analyzed at intervals to follow the reaction sequence as it proceeded.

Other areas of research which can provide information about the reaction mechanism of HAA formation are labeled-carbon experiments as performed by Skog and Jagerstad (1993) and Felton et al. (1990). Labeling of probable reaction

intermediates such as aldehydes, pyrazines, and pyridines would contribute the most knowledge to the field, as it is already known that creatinine, amino acids, and in some instances, sugars are HAA precursors. The ongoing studies of inhibition of HAA formation also help our understanding of the mechanism of formation. If we know what substances inhibit or stop HAA formation, we can make inferences regarding the pathways by which they are produced. Such inhibition studies are best performed on actual meats rather than in model systems. Most of the compounds which inhibit HAA formation are also antioxidants, such as vitamin E, tea phenolics, and other plant extracts, and their efficacy is highly concentration dependent. The concentrations of reactants in most model systems is necessarily quite different from those in meat due to lack of the meat matrix and absence of the many other substances which do not contribute to HAA formation. Inhibition data obtained using model systems would not be applicable to meat products. A useful tool would be the availability of a probe, perhaps similar to the enzyme-linked-immunosorbent assays (ELISA) now used for toxin detection, which would allow rapid determination of the presence of HAAs. Such a tool could quickly assess the need for or effectiveness of an inhibitory compound.

Finally, the continuing studies to quantitate the known HAAs in various meat products and isolate new HAAs are very important. As stated earlier, much of the work on HAAs formed in foods was performed before an accurate quantitative method was available (Gross and Gruter, 1992). Values reported in the literature vary widely, and it is important to know where the HAAs are found in the food supply if attempts are to be made to avoid them or decrease their production.

APPENDIX

APPENDIX

Study 1

HAA standard used for study #1 consisted of:

IQ (20 ng/μl)

MeIQ (20 ng/μl)

MeIQx (20 ng/μl)

4,8-DiMeIQx (20 ng/μl)

PhIP (20 ng/μl)

Samples (two of every four) were spiked with 50 μl of this standard, which delivered 1000 ng of each HAA to the sample.

Study 2

HAA standard used for study #2 consisted of:

IQx (20 ng/μl)

MeIQx (30 ng/μl)

4,8-DiMeIQx (20 ng/μl)

PhIP (50 ng/μl)

Two of every four samples were spiked with 50 μl of this standard, which delivered 1000 ng IQx, 1000 ng DiMeIQx, 1500 ng MeIQx, and 2500 ng PhIP to each sample.

Study 3

HAA standard used for study #3 consisted of:

IQx (20 ng/μl)

MeIQx (30 ng/μl)

4,8-DiMeIQx (20 ng/μl)

Two of every four samples were spiked with 50 μl of this standard, which delivered 1000 ng IQx, 1000 ng DiMeIQx, and 1500 ng MeIQx to each sample.

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