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MECHANISM OF HETEROCYCLIC AMINE FORMATION IN FRIED GROUND BEEF - THE ROLE OF OXIDIZED LIPID AND THE MAILLARD REACTION

By

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ABSTRACT

MECHANISM OF HETEROCYCLIC AMINE FORMATION IN FRIED GROUND BEEF - THE ROLE OF OXIDIZED LIPID AND THE MAILLARD REACTION

By

Jessalin Faulkner

Factors contributing to the formation of heterocyclic amines in meat, and in particular the possible influence of lipid oxidation and its products on their formation, were studied. A procedure involving gas chromatographic - mass spectrometric analysis of the pentafluoropropionyl derivatives of heterocyclic amines was developed to confirm their identity. This procedure proved advantageous over other derivatization methods in that it only took 30 minutes for derivatization. In model systems containing phenylalanine and creatinine or 2-methylpyrazine, fructose and creatinine, the formation of the heterocyclic amine, PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) was confirmed.

When butylated hydroxyanisole and vitamin E were added to ground beef before frying, the concentration of PhIP in the cooked patties was reduced by 56% and 80%, respectively. MeIQx (2-amino-3,4-dimethylimidazo-[4,5-f]quinoline) was not detected in the antioxidant-treated patties, as only trace concentrations (0.3 ng/g) were found in the control patties. This reduction in heterocyclic amines concentration corresponded to a reduction in the overall mutagenicity of the fried ground beef as determined by the Ames assay.

The addition of low concentrations of Maillard reaction products (MRPs) to ground beef reduced the formation of heterocyclic amines in fried patties. However, increasing the concentration of MRPs reduced the inhibitory effect. These studies indicated that a delicate balance exists between suppression and acceleration of heterocyclic amine formation by the addition of MRPs. Thermal lipid oxidation results in the formation of aldehydes and experimental data indicates that these may reduce the formation of heterocyclic amines in meat and model systems by reacting with amino acids, thus preventing their participation in the formation of heterocyclic amines.

Oxidized lard added to ground sirloin tips or slightly oxidized hamburger appeared to reduce the formation of PhIP and MeIQx in the cooked meat. The addition of oxidized lard to water-washed muscle fibers containing phenylalanine and creatinine produced the same effect. These studies infer a relationship between lipid oxidation and heterocyclic amine formation which needs to be developed further.

**To my mother, Janet A. Strong,
for her love, support and caring**

and

**In loving memory of my father,
Lee E. Strong, whose guidance
and love will always be cherished.**

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INTRODUCTION

A class of mutagenic compounds, commonly referred to as heterocyclic amines, are formed when meat is cooked. Many heterocyclic amines have been identified in fried ground beef: IQ (2-amino-3-methylimidazo[4,5-f]quinoline), MeIQ (2-amino-3,4 dimethyl-imidazo[4,5-f]quinoxaline), MeIQx (2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline), DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline) and PhIP (2-amino-1-methyl-6-phenylimidazopyridine) (Hargraves and Pariza, 1983; Felton et al., 1986; Felton and Knize, 1990). These compounds are of concern because they have been shown to be mutagenic both in bacterial tests such as the Ames assay (Sugimura, 1982; Felton et al., 1986; Felton, 1987) and in mammalian cell cultures such as with Chinese hamster ovarian cells (Thompson et al., 1983, 1987; Nakayasu et al., 1983; Alexander et al., 1989; Holme et al., 1989; Tucker et al., 1989). In addition to being mutagenic, heterocyclic amines are known to produce carcinogenic lesions in mice and rats when added to the diet (Takayama, 1984; Ohgaki et al., 1984, 1986, 1987; Esumi et al., 1989). Creatine (or creatinine), amino acids and reducing sugars were proposed by Jagerstad et al. (1983) to be precursors of heterocyclic amines in muscle foods. The formation of heterocyclic amines has been suggested to proceed via a free radical process (Pearson et al., 1992). Reports of phenolic

antioxidants inhibiting the formation of these compounds lends credence to this theory (Chen et al., 1992). In addition to reducing mutagenicity in cooked meat, antioxidants are also known to inhibit lipid oxidation (Gray and Pearson, 1987). This suggests that there might be a relationship between lipid oxidation and mutagen formation.

While sugars and amino acids are precursors of heterocyclic amines, these compounds also participate in the Maillard reaction. Skog and Jagerstad (1990) found that an excess of reducing sugars inhibited the formation of heterocyclic amines in model systems. In addition, Maillard reaction products (MRPs) exhibit antioxidant properties (Lingert et al., 1981; Bailey, 1988). The overall objective of this study was to investigate the factors contributing to the formation of mutagens in meat, and in particular the possible influence of lipid oxidation on the formation of heterocyclic amines in meat systems. Specific objectives of this study were to:

(1) Develop a procedure for confirming the identity of heterocyclic amines extracted from meat and model systems using gas liquid chromatography-mass spectrometry.

(2) Demonstrate the inhibition of specific heterocyclic amines by the addition of antioxidants to ground beef, and characterize the relationship between the reduction of specific compounds and the overall mutagenicity of fried ground beef.

(3) Evaluate the effect of adding a known product of the Maillard reaction on the formation of heterocyclic amines in a model system.

(4) Study the interaction between the Maillard reaction, thermal lipid oxidation and the formation of heterocyclic amines in fried ground beef.

(5) Investigate whether an interaction exists between lipid oxidation and heterocyclic amine formation in meat and model systems.

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LITERATURE REVIEW

Formation of Heterocyclic Amines

Types of mutagenic compounds found in fried ground beef

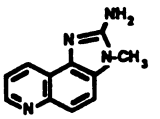
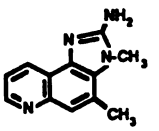
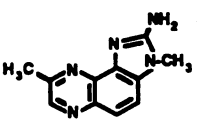
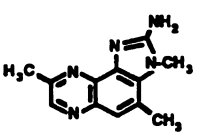
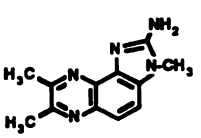
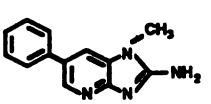
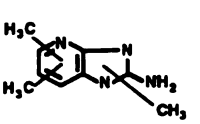
A variety of mutagenic compounds are formed in minute quantities (ng/g concentrations) during the cooking and processing of muscle foods. Examples of mutagenic compounds in cooked meats include cholesterol oxides, polycyclic aromatic hydrocarbons, protein pyrolysate products, and heterocyclic amines (Hotchkiss and Parker, 1990).

Heterocyclic amines are also referred to as amino-imidazoazaarenes and can be broken down into four categories based on the group attached to the aminoimadazo moiety: quinolines, quinoxalines, pyridines, and benzoxazines (Felton et al., 1986; Felton et al., 1988; Overvik and Gustafsson, 1990). The structural formulas are shown in Table 1.

Heterocyclic amines found in fried ground beef

PhIP is the most abundant heterocyclic amine found in cooked meat with concentrations ranging from 0 to 78 ng/g (Hargraves and Pariza, 1983; Felton et al. 1986; Felton and Knize, 1990). Reported concentrations of IQ (<0.2 ng/g), MeIQx (0 to 10.8 ng/g) and DiMeIQx (0 to 3.1 ng/g) are generally smaller (Knize, personal communication).

Table 1. Chemical names, abbreviations and structural formulas of moderate-temperature-induced mutagens (Pearson et al., 1992).

Chemical name	Abbreviation	Structure
2 - amino - 3 - methylimidazo - [4, 5 - f] quinoline	IQ	
2 - amino - 3, 4 - dimethyl - imidazo [4, 5 - f] quinoline	MeIQ	
2 - amino - 3, 8 - dimethyl - imidazo [4, 5 - f] quinoxaline	MeIQx	
2 - amino - 3, 4, 8 - trimethyl - imidazo [4, 5 - f] quinoxaline	4, 8 - DiMeIQx	
2 - amino - 3, 7, 8 - trimethyl - imidazo [4, 5 - f] quinoxaline	7, 8 - DiMeIQx	
2 - amino - 1 - methyl - 6 - phenyl - imidazo - [4, 5 - b] pyridine	PhIP	
2 - amino - N, N, N - trimethyl - imidazopyridine	TMIP	

Mutagenicity of heterocyclic amines

The majority of heterocyclic amines have been shown to be highly mutagenic using bacterial tests such as the Ames assay (Sugimura, 1982; Felton et al., 1986; Felton, 1987) and exhibit moderate mutagenicity in mammalian cell cultures (Thompson et al., 1983, 1987; Nakayasu et al., 1981; Alexander et al., 1989; Holme et al., 1989; Tucker et al., 1989). One exception is PhIP, which was demonstrated to exhibit a high degree of mutagenicity using mammalian cells and much weaker mutagenicity in the Ames assay (Holme et al., 1989; Tucker et al., 1989).

Heterocyclic amines are metabolically activated by cytochrome P450. Metabolic activation of these compounds, in general, involves N-hydroxylation, followed by esterification to an acetyl or sulfate moiety (Okamato et al., 1981; Saito et al., 1985; Paterson and Chipman, 1987; Snyderwine et al., 1987). It is thought that the esterified intermediate hydrolyses to form a nitrenium ion which ultimately reacts with deoxyribonucleic acid (Dirr and Wild, 1988, Asan et al., 1987).

Of the compounds tested so far (IQ, MeIQ, MeIQx and PhIP), all significantly increased the incidence of carcinomas and ademonmas in mice and rats when fed mg/kg quantities in the diet. The majority of the tumors observed for IQ, MeIQ, and MeIQx were found in the liver, lung and forestomach in mice, and in the small and large intestines, zymbal gland and on the skin of rats (Takayama, 1984; Ohgaki et al., 1984, 1986, 1987). PhIP, on the other

hand, has been shown to produce lymphomas (Ohgaki et al., 1987).

Mechanism of heterocyclic amine formation

Reactants

The mechanism by which heterocyclic amines are formed in foods is not completely understood. However, creatinine (the cyclized form of creatine), amino acids and sugars were proposed by Jagerstad et al. (1983) to be the precursors of heterocyclic amines in muscle foods.

Supporting evidence for creatine/creatinine involvement is the low or nonexistent mutagenic activity in foods high in protein but lacking in creatine (Felton and Knize, 1990). Examples of such foods are tofu, beans, cheese, liver and kidney (Felton and Knize, 1990). In muscle foods, creatine is present at concentrations of about 0.5% by weight (Felton and Knize, 1990). When additional creatine is added to meat and meat products before cooking, mutagenicity is enhanced (Nes, 1986; Becher et al., 1988; Knize et al., 1988).

The mutagenicity of bouillon has also been shown to increase with increasing concentrations of creatine. Reutersward et al. (1987) analyzed the creatine/creatinine content of 13 commercial meat extracts and bouillons and compared the mutagenicity of the samples using the Ames Salmonella/microsome test. Samples containing low amounts of creatine (1-10 µmol/g dry matter) had little or no mutagenicity using Salmonella typhimurium TA98. The mutagenicity increased to about 100 revertants/g dry matter at creatine concentrations of 15-40 µmol/g dry matter. At a

creatine content of 150 µmol/g dry matter, the mutagenicity increased to 2500 revertants/g dry matter.

In a second study by Reutersward et al. (1987), pan fried patties of bovine tissue taken from the muscle (Longissimus dorsi), heart, tongue, liver or kidney were compared for overall mutagenicity using Salmonella typhimurium TA98. In kidney and liver, the amount of creatine before cooking was very low (about 2 µmol/g wet tissue) and both showed little to no mutagenicity when fried at 150, 175 or 200 °C. On the other hand, the heart, tongue, and muscle with creatine contents ranging between 19 and 33 µmol/g wet tissue, were highly mutagenic when fried. The mutagenicity at 175 and 200°C ranged between 6000 and 19,6000 revertants/100 g initial raw weight.

In addition to creatine, amino acids play a role in the formation of heterocyclic amines in model systems. The mutagenic effect of amino acids added to meat systems before cooking has not been fully elucidated. Overvik et al. (1989) demonstrated that the individual addition of 15 amino acids to pork before cooking enhanced the mutagenic activity by 1.5 to 43 times. On the other hand, Ashoor et al. (1980) reported that only proline out of 17 amino acids added to ground beef before cooking increased the mutagenic activity. Other parameters, such as cooking methods and fat content may have accounted for the observed differences in the two studies.

In addition to amino acids and creatinine, sugars were proposed by Jagerstad et al. (1983) to be precursors of

heterocyclic amines in meat. However, the role of sugars remains unclear. Although not obligatory when added to a model system containing amino acids and creatinine, the quantities of heterocyclic amines are increased and the ratio of products altered. For example, when phenylalanine (253mg), creatinine (173mg) and glucose (138mg) were heated in an oven to 200°C, the overall mutagenicity of the mixture was 1.7 x 10^6 TA1538 revertants/10^8 bacteria and resulted in the formation of 1.6 µg of IQ and 188 µg of PhIP (Taylor et. al., 1987). Without glucose, the overall mutagenicity was lower at 0.9 x 10^6 revertants/10^8 bacteria, and the ratio of products changed. The amount of PhIP produced increased to 247 µg, whereas only 0.9 µg of IQ was formed (Taylor et al., 1987). This suggests that there may be two pathways for the formation of heterocyclic amines: (1) with sugar, via the interaction of products of the Maillard reaction with creatinine (Jagerstad et al., 1983; Nyhammer, 1986; Pearson et al., 1992) and (2) without sugar, via breakdown of amino acids producing carbon fragments that react with creatinine (Felton et al., 1986).

When sugars are present in excess, mutagen formation is partially inhibited. Skog and Jagerstad (1990) reacted creatine (0.9mmole) and glycine (0.9mmole) with varying concentrations (0-2.4mmole) of glucose, fructose, sucrose or lactose in a diethylene glycol-based model system. When the glucose concentration was about half the molar concentration of creatine, the mutagenicity was the highest. By further increasing the concentration of glucose, the mutagenicity of

the reaction mixture was reduced. A similar trend was obtained for all of the sugars tested.

In an investigation of the recovery of creatine/creatinine, Skog and Jagerstad (1990) demonstrated that excess sugar reacted with creatine/creatinine. They suggested that Maillard reaction products, such as 5-hydroxymethyl-2-furfural (HMF), reacted with creatinine rendering it less available to form heterocyclic amines. When HMF was added to the system at 1, 10, and 25% of the molar concentration of creatine, the mutagenicity was reduced. Interestingly, at 50% of the creatine level, HMF reduced the mutagenicity by 75%.

In addition to creatine, amino acids and sugar, water may also play a role in the formation of heterocyclic amines. The majority of model systems that have been used to study the formation of heterocyclic amines have been heated in a diethylene glycol-based system containing water (Jagerstad et al., 1983; Skog and Jagerstad, 1990). On the other hand, Taylor et al. (1987) was able to produce PhIP from phenylalanine and creatinine without the addition of water. Water may act as a carrier, allowing water-soluble precursors to move to the surface of meat where heterocyclic amine formation occurs. In model systems where there is direct contact between precursors, it is possible that water might not be needed for the reaction to occur.

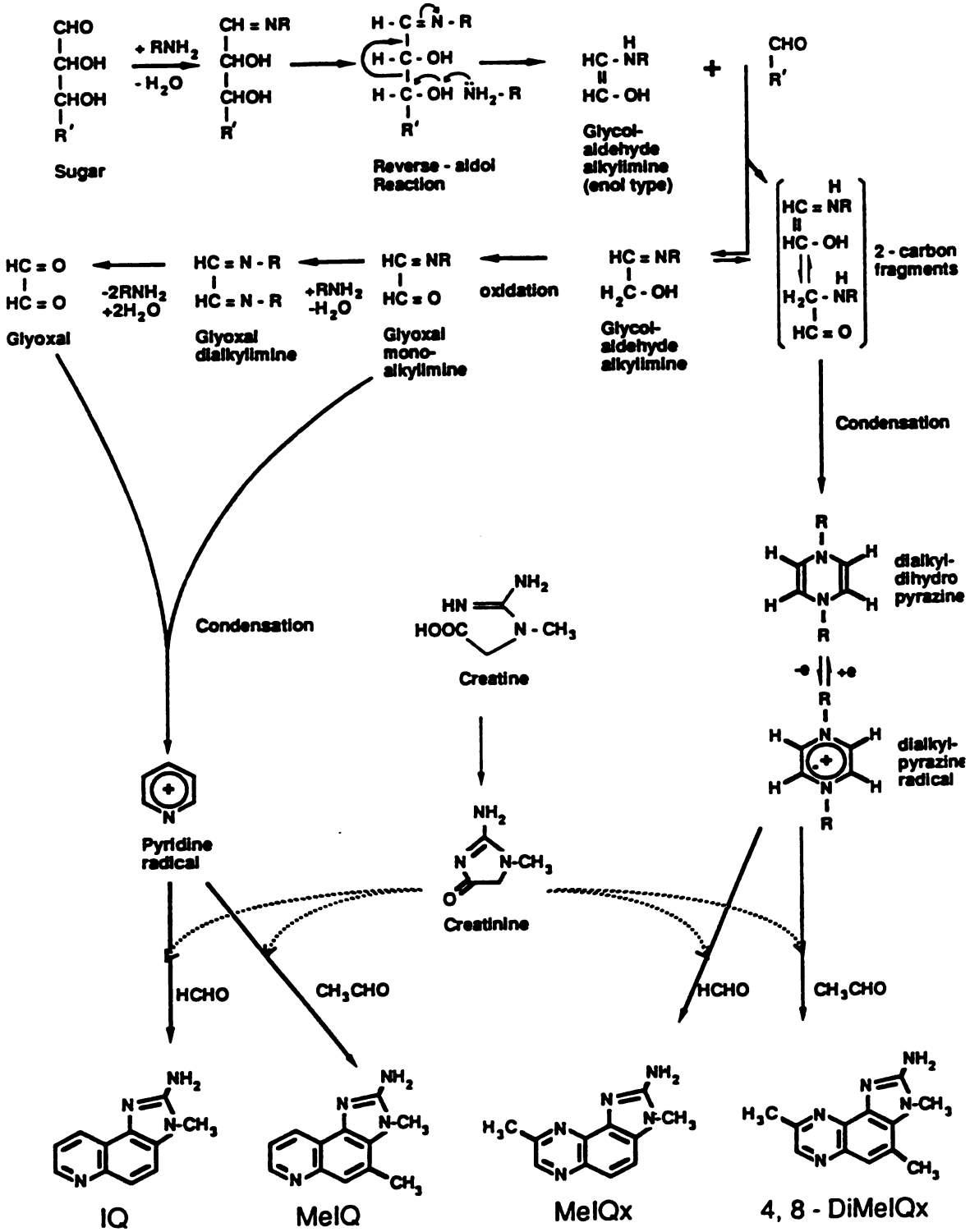
The Maillard reaction and formation of heterocyclic amines

Sugars react with amino acids by way of the Maillard reaction. The Maillard reaction may contribute to the formation of heterocyclic amines by forming pyrazine and pyridine radicals which were proposed by Nyhammer (1986) to be intermediates in the formation of heterocyclic amines (Figure 1).

In the Maillard reaction, reducing sugars and amino groups from either amino acids, peptides or proteins combine to form a glycosylamine, which undergoes an Amadori rearrangement to yield a 1-amino-2-keto sugar (Hodge, 1953). This sugar may then be broken down into 2- and 3- carbon fragments by two pathways (3-deoxyhexosone and methyl α-dicarbonyl routes) leading to the formation of a variety of compounds such as aldehydes, ketones and melanoidin pigments. Pyrazines and pyridines can be produced from the interaction of the α-dicarbonyls from the Maillard reaction with amino acids. This reaction is called the Strecker degradation.

Pyrazines and pyridines are also formed prior to the Amadori product, by early carbon fragmentation. Namiki and Hayashi (1983) found that C2 and C3 fragments were produced prior to the Amadori rearrangement by a reverse-aldol reaction of the glycosylamine forming glycolaldehyde

Figure 1: Suggested pathway for formation of IQ-like compounds. See Table 1 for chemical names for IQ, MeIQx and 4,8-DiMeIQx (Pearson et al., 1992).


Sugar
Reverse - aldol Reaction
Glycol-aldehyde alkylimine (enol type)
2 - carbon fragments
Glycol-aldehyde alkylimine
oxidation
Glyoxal
Glyoxal dialkylimine
Glyoxal mono-alkylimine
Condensation
dialkyl-dihydro pyrazine
dialkyl-pyrazine radical
Creatine
Creatinine
Pyridine radical
HCHO
CH3CHO
IQ
MeIQ
MeIQx
4, 8 - DiMeIQx

alkylimines. These compounds could then be oxidized to form glyoxal monoalkylimines. The pyridine radical would result from the interaction of glyoxal monoalklimine with glyoxal (Pearson et al., 1992). Likewise, pyrazines would be produced from the condensation of two glycolaldehyde alkylimines. Namiki and Hayashi (1983) reported that the glycolaldehyde system reacted faster and produced more free radicals than the glyoxal system, therefore; more pyrazine radicals would be produced.

Nyhammer (1986) proposed that heterocyclic amines are formed by an aldol-type condensation between an aldehyde and a pyridine or pyrazine molecule, followed by the cyclic addition of creatinine to yield either an imidazoquinoline or an imidazoquinoxaline. Since more pyrazine radicals are produced, imidazoquinoxalines would predominate. This theory is consistent with the products found in meat, namely MeIQx and 4,8-DiMeIQx predominating over IQ and MeIQ (Felton et al., 1990).

It is possible that the formation of heterocyclic amines involves a free radical process. Free radicals have been shown to occur in the Maillard reaction, both prior to and following the Amadori rearrangement (Namiki and Hayashi, 1975, 1980).

Milic et al. (1993) provided further support for the theory of free radical involvement in the formation of heterocyclic amines. Electron spin resonance was used to detect pyrazine free radicals from heated model systems containing glucose and aminobutyric acids. With the

addition of 2,3-diamino-1,4-naphthohydroquinone to a model system containing glucose and aminobutyric acids, pyridine free radicals were detected using electron spin resonance. The pyrazine and pyridine free radicals were suggested to be precursors of heterocyclic amines.

To further establish the reaction pathway, 2,5-dimethylpyrazine or 2-methylpyridine were heated with creatinine and acetaldehyde. DiMeIQx was detected in the extract from the solution containing 2,5-dimethylpyrazine, while 2-methylpyridine resulted in the formation of MeIQ (Milic et al., 1993). The extracts were analyzed by high performance liquid chromatography, direct probe mass spectrometry and nuclear magnetic resonance.

FACTORS INFLUENCING MUTAGEN FORMATION

The role of fat in mutagen formation

Many studies have investigated the influence of fat content of meat on mutagen formation (Barnes and Weisburger, 1983, 1984; Knize et al., 1985; Chen, 1988). These studies suggest that by increasing fat content to about 15%, heat penetration is increased, thereby increasing mutagen formation. When the fat content is increased above 15%, mutagenicity is slightly decreased by diluting out the precursors.

In addition to fat content, thermal oxidation of lipids during the cooking of meat may have an effect on mutagen formation by interacting with the Maillard reactants. Farmer and Mottram (1990) compared the effect of

triglycerides with three phospholipids (beef phospholipid, egg phosphatidylcholine, and egg phosphatidylethanolamine) on the types and quantities of volatile products obtained from the Maillard reaction between cysteine and ribose. Decomposition products from beef triglycerides produced markedly dissimilar volatile products and odor than the breakdown products from phospholipids. The mixture containing beef triglycerides produced a strongly sulfurous, slightly meaty (ham) odor. In comparison, the mixture containing phospholipids produced a distinctly meaty (chicken, roasted) odor. Phosphatidylethanolamine produced the most characteristic meat odor.

Farmer and Mottram (1990) proposed that lipids could interact in the Maillard reaction by four different pathways:

(1) lipid-derived carbonyl products could react with the amino group of cysteine and with ammonia produced by the Strecker degradation;

(2) The amino group of ethanolamine (in phosphatidylethanolamine) could react with carbonyl compounds produced from sugar fragmentation;

(3) free radicals from peroxidized lipid could interact in the Maillard reaction;

(4) hydroxy and carbonyl lipid oxidation products could react with free hydrogen sulfide from the Strecker degradation.

This study leads to two divergent theories on how the composition of lipid could affect the formation of

heterocyclic amines. Unsaturated lipid such as phospholipids would be more susceptible to thermal oxidation, resulting in the formation of carbonyl products. These products could then react with amino acids reducing the concentration of amino acids available to participate in the Maillard reaction. Since the Maillard reaction produces pyrazines and pyridines that may be intermediates in the formation of heterocyclic amines, the formation of heterocyclic amines would be reduced. On the other hand, thermal oxidation of unsaturated lipids might accelerate the Maillard reaction by contributing free radicals. In this case, the formation of heterocyclic amines would increase. Saturated lipids would be less likely to affect heterocyclic amine formation by this process, since they are less susceptible to thermal oxidation.

Inhibition of mutagen formation

The addition of antioxidants to meat prior to cooking will inhibit mutagen formation. Wang et al. (1982) reported that soy protein concentrates, when added to raw ground beef, reduced the mutagenicity of the fried meat. Overall mutagenicity of the fried ground beef was determined by the Ames assay using Salmonella typhimurium TA98 and the rat liver microsomal fraction for metabolic activation (Ames et al., 1975). Most of the reduction in mutagenicity was attributed to volumetric effects, although some consideration was given to naturally occurring antioxidants in the soy protein concentrates such as chlorogenic acid.

These investigators reported that butylated hydroxyanisole (BHA) also effectively reduced the mutagenicity of fried ground beef when added before cooking.

Defatted glandless cottonseed flour added at a level of 5% (w/w) to ground beef before frying has also been demonstrated to reduce the mutagenicity of the cooked meat (Rhee et al., 1987). Glandless cottonseed contains naturally occurring antioxidants such as quercetin and other flavonoids. However, the investigators did not clearly define whether the reduction in mutagenicity was a result of volumetric effects, i.e. dilution, or through the antioxidant properties of glandless cottonseed components.

The inhibitory effects of antioxidants were more intensively studied by Chen et al. (1992). At 0.1% (w/w) of the fat content, BHA, propyl gallate and tertiary butylhydroquinone reduced the overall concentration of IQ in fried ground beef by 80, 85 and 90%, respectively. At this level of addition, the volumetric effect was minimal. These results clearly established that phenolic antioxidants are effective in reducing the formation of mutagens in meat, and led to the theory of involvement of free radicals in the formation of heterocyclic amines (Pearson et al., 1992).

In addition to antioxidants, sodium bisulfite has been shown to inhibit the formation of mutagens. Sodium bisulfite inhibits the Maillard reaction by forming α-hydroxysulfonates with carbonyl compounds such as glucose.

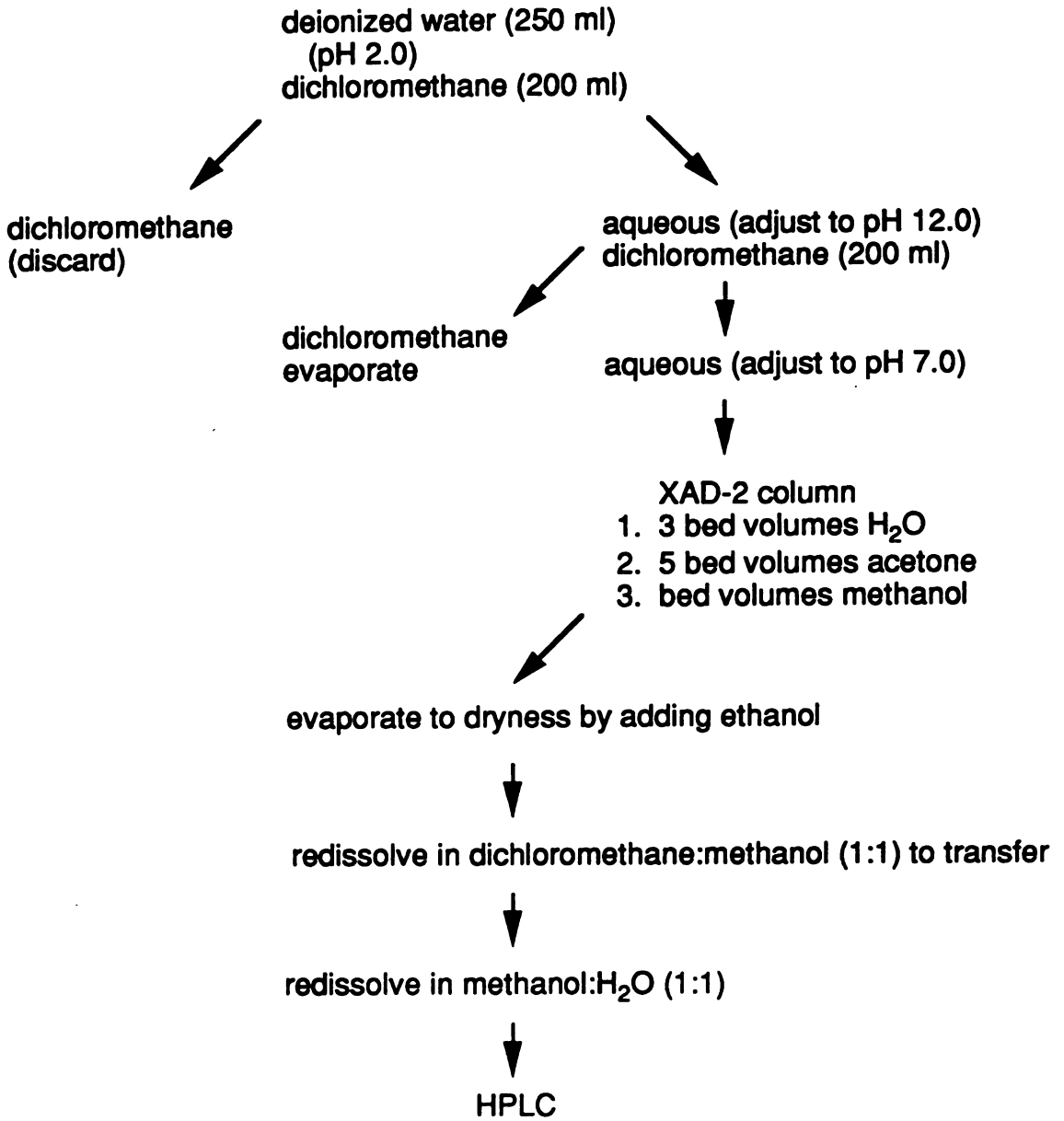
Sodium bisulfite (0.5%) added to canned salmon completely inhibited the mutagenicity as measured by the Ames assay (Krone and Iwaoka, 1987).

Although Maillard reaction products (MRPs) contribute to heterocyclic amine formation, other studies have indicated that MRPs can function as antioxidants in meat systems. Maillard reaction products from a heated model system of histidine and glucose when added to ground beef before frying, reduced the degree of lipid oxidation in the product (Bailey, 1988). Eichner (1980) reported that intermediates in the Maillard reaction, such as Amadori rearrangement products, were strong antioxidants. It would clearly be of interest to evaluate the impact of adding MRPs to meat systems on mutagen formation.

QUANTITATION OF MUTAGENS

The detection of heterocyclic amines has been a challenging undertaking because of the small concentrations (low parts per billion) of these compounds in meats. Until recently, in order to achieve sufficient purity for quantification of these compounds, the following analytical steps were necessary (Figure 2): acid/base partitioning, followed by separation on a column filled with XAD (diatomaceous earth) resin, fractionation on four different high performance liquid chromatography (HPLC) columns, identification of the mutagenic fractions by the Ames assay, and direct probe mass spectrometry (Felton et al., 1986).

Figure 2: Extraction of heterocyclic amines (Felton et al., 1986).


deionized water (250 ml)
(pH 2.0)
dichloromethane (200 ml)
dichloromethane
(discard)
aqueous (adjust to pH 12.0)
dichloromethane (200 ml)
dichloromethane
evaporate
aqueous (adjust to pH 7.0)
XAD-2 column
1. 3 bed volumes H_2O
2. 5 bed volumes acetone
3. bed volumes methanol
evaporate to dryness by adding ethanol
redissolve in dichloromethane:methanol (1:1) to transfer
redissolve in methanol:H_2O (1:1)
HPLC

A faster method of extracting heterocyclic amines from cooked meat was developed by Gross (1990) and involves tandem extraction with diatomaceous earth and an ion exchange resin, followed by clean-up with a C18 column, and subsequent separation and identification of the heterocyclic amines by HPLC (Figure 3). This procedure reduces the sample size needed to detect meat mutagens to approximately 10g and the time of extraction to 4 hours. However, HPLC retention times alone do not provide unequivocal identification of heterocyclic amines, since it is possible that other compounds may co-elute with the specific amine in question. Some researchers have combined HPLC directly with mass spectrometry (MS) to identify heterocyclic amines. Turesky et al. (1988) identified MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline), IQ (2-amino-3-methylimidazo[4,5-f]quinoline) and DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline) in beef extract and fried beef at concentrations ranging from 0.3 to 52 ng/g. Prior to HPLC-MS analysis, the samples were purified by XAD adsorption, acid-base extraction and blue cotton treatment (trisulfo-copper-phthalocyanine). Blue cotton adsorbs compounds with multicyclic planar structures, such as heterocyclic amines. MeIQ and IQ were also identified in broiled salmon using HPLC - thermospray mass spectrometry (Yamaizumi et al., 1986) at concentrations ranging from 0.6-2.8 ng/g and 0.3-1.8 ng/g, respectively. Although the HPLC-

Figure 3: Extraction of heterocyclic amines (Gross, 1990).

Finely grind hamburger in blender, add 10 g ground hamburger to 20 ml 1N NaOH, let partially digest for 20 minutes

Add Extrelut-20 to hamburger mix, fill column with mixture, attach to PRS column containing 2 ml DCM on Visiprep

Rinse columns with 200 ml DCM discard DCM, detach Extrelut column, and dry PRS under vacuum

Add 6ml of 0.1 M HCL to PRS followed by 20 ml methanol:0.1M HCL (4:6), and then 2 ml water

Rinse C-18 column with 1ml methanol followed by 10 ml water

Attach PRS column, rinse with 20 ml of ammonium acetated (pH 8.0), discard PRS, and rinse with 2 ml water

Elute heterocyclic amines with 6ml methanol-conc. ammonia (9:1)

Filter through 0.2 μm disc, evaporate to near dryness under nitrogen

Transfer with 3 X 200 μl aliquots of methanol to 1ml vial, evaporate to dryness, redissolve in 50 μl methanol

MS confirmation has produced promising results, most laboratories are not equipped with the sophisticated instrumentation needed to confirm the identity of these compounds.

Because of the low volatility of heterocyclic amines, derivatization is necessary to facilitate their analysis by gas chromatography - mass spectrometry. Murray et al. (1988) developed a derivatization procedure using 3,5-bistrifluoromethylbenzyl bromide and diisopropylethylamine, as the base, for quantitating MeIQx and DiMeIQx in fried beef by gas chromatography - electron capture negative ion chemical ionization mass spectrometry. Lean minced beef patties were found to contain 1-2.4 ng MeIQx and 0.5-1.2 ng DiMeIQx /g meat.

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CHAPTER ONE

DERIVATIZATION OF HETEROCYCLIC AMINES WITH PENTAFLUOROPROPIONIC ACID ANHYDRIDE AND SUBSEQUENT ANALYSIS BY GAS LIQUID CHROMATOGRAPHY - ELECTRON IONIZATION MASS SPECTROMETRY

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Abstract

A derivatization procedure was developed for analyzing mutagenic heterocyclic amines by gas liquid chromatography-electron ionization mass spectrometry. Pentafluoropropionyl (PFPA) derivatives of heterocyclic amine standards IQ (2-amino-3-methylimidazo[4,5-f]quinoline), MeIQ (2-amino-3,4-dimethylimidazo[4,5-f]quinoline), MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) were synthesized and the mass fragmentation pattern of each analyte was studied. Selected ion monitoring of the base peak for each standard, m/z 225 (IQ), m/z 239 (MeIQ), m/z 240 (MeIQx) and m/z 251 (PhIP), was used to improve the limits of detection to approximately 5 ng. Of the heterocyclic amines examined, only PhIP was identified in model systems containing either phenylalanine and creatinine or 2-methylpyrazine, fructose and creatinine. This procedure is advantageous over other derivatization methods in that it only takes 30 minutes for derivatization, no base is needed to accelerate the reaction, and excess reagent can be readily removed under nitrogen resulting in cleaner chromatograms.

Introduction

Mutagenic heterocyclic amines are formed during the cooking and processing of meat (Chen et al., 1990). Their detection has been a challenging undertaking because of the small concentrations (low parts per billion) of these compounds in meat. Until recently, in order to achieve sufficient purity for quantification of these compounds, the following analytical steps were necessary: acid/base partitioning, followed by separation on a column filled with XAD (diatomaceous earth) resin, fractionation on four different high performance liquid chromatography (HPLC) columns, identification of the mutagenic fractions by the Ames assay, and direct probe mass spectrometry (Felton et al., 1986).

A faster method of extracting heterocyclic amines from cooked meat was developed by Gross (1990) and involves tandem extraction with diatomaceous earth and an ion exchange resin, followed by clean-up with a C18 column, and subsequent separation and identification of the heterocyclic amines by HPLC. This procedure reduces the sample size needed to detect meat mutagens to approximately 10g and the time of extraction to 4 hours. However, HPLC retention times alone do not provide unequivocal identification of heterocyclic amines, since it is possible that other compounds may co-elute with the specific amine in question.

Some researchers have combined HPLC directly with mass spectrometry (MS) to identify heterocyclic amines. Turesky et al. (1988) identified MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline), IQ (2-amino-3-methylimidazo[4,5-f]quinoline) and 4,8-DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline) in

beef extract and fried beef at concentrations ranging from 0.3 to 52 ng/g. Prior to HPLC-MS analysis, the samples were purified by XAD adsorption, acid-base extraction and blue cotton treatment (trisulfo-copper-phthalocyanine). MeIQ (2-amino-3,4-dimethylimidazo[4,5-f]quinoline) and IQ were also identified in broiled salmon using HPLC - thermospray mass spectrometry (Yamaizumi et al., 1986) at concentrations ranging from 0.6-2.8 ng/g and 0.3-1.8 ng/g, respectively. Although the HPLC-MS confirmation has produced promising results, most laboratories are not equipped with the sophisticated instrumentation needed to confirm the identity of these compounds.

Because of the low volatility of heterocyclic amines, derivatization is necessary to facilitate their analysis by gas chromatography - mass spectrometry. Murray et al. (1988) developed a derivatization procedure using 3,5-bistrifluoromethylbenzyl bromide and diisopropylethylamine, as the base, for quantitating MeIQx and DiMeIQx in fried beef by gas chromatography - electron capture negative ion chemical ionization mass spectrometry. Lean minced beef patties were found to contain 1-2.4 ng MeIQx/g meat and 0.5-1.2 ng DiMeIQx/g meat.

The majority of the studies involving mass spectrometry have focused on the analysis of aminoimidazoquinolines (IQ and MeIQ) and aminoimidazoquinoxalines (MeIQx and DiMeIQx), although the most abundant heterocyclic amine present in cooked meat is PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) (Felton et al., 1986, Felton and Knize, 1990). The purpose of this study was to develop a derivatization procedure that would allow for the

confirmation of PhIP, and apply it, initially, to the identification of heterocyclic amines in model systems.

Material and Methods

Materials

2-Methylpyrazine and pentafluoropropionic acid anhydride were purchased from Sigma Chemical Company (St. Louis, MO). Extrelut-20 columns were obtained from EM Separations (Gibbstown, NJ). Bond-Elut PRS (500 mg) and C18 (100 mg) cartridges were purchased from Varian, Inc. (Harbor City, CA). All solvents were of HPLC or glass distilled reagent grade. The heterocyclic amine standards (IQ, MeIQ, MeIQx, DiMeIQx, TriMeIQx {2-amino-3,4,7,8-tetramethylimidazo[4,5-f]quinoxaline} and PhIP) were obtained from Toronto Research Chemicals (Toronto, Canada).

Methods

Derivatization of standards - A stock solution containing 5.0 ng/µl of each standard (IQ, MeIQ, MeIQx and PhIP) was prepared. From the stock solution, 10, 100, and 1000 µl aliquots were drawn with a HPLC syringe and placed in 1 ml reaction vials, and evaporated under nitrogen to dryness. Each standard mix was then redissolved in 50 µl of acetonitrile / ethyl acetate (1:1) to which 50 µl of pentafluoropropionic acid anhydride were added for derivatization. The reaction vials were then heated in a heating block at 60°C for 30 minutes. After cooling to room temperature, the derivatized standards were concentrated under nitrogen and redissolved in 20 µl of ethyl acetate.

Mass spectrometric analysis - Two µl of each derivatized mixture were injected into a JEOL 505 gas chromatograph mass spectrometer (Japanese Electric Optical Lab, JEOL-USA, Peabody, MS) operating

in the standard electron ionization mode with an accelerating electron energy of 70 eV. The gas chromatograph was equipped with a 15m DB-1 fused silica capillary column (Supelco, Inc. Bellefonte, PA) which was routed through the separator oven maintained at 260°C and directly into the mass spectrometer ion source. The gas chromatograph oven temperature was held at 180°C for 5 minutes and then increased to 300°C at a rate of 10°C per minute.

Model systems - Model systems containing alanine (5.7 mmoles), creatinine (4.4 mmoles) and fructose (2.8 mmoles) were placed in an oven heated to 200°C for a period of 2 hours. Similarly, 2-methylpyrazine (5.5 mmoles) was added to a system containing fructose (2.8 mmoles) and creatinine (4.4 mmoles) and heated under the same conditions. Because of the volatility of 2-methylpyrazine, all samples were weighed into thick-walled glass ampules specially prepared by the MSU Department of Chemistry glass blowing shop, partially submerged in a mixture of dry ice and acetone, and vacuum sealed. Vacuum sealing was necessary to prevent the glass ampules from exploding during heating.

After heating, the glass ampules were allowed to cool to room temperature before breaking. Heterocyclic amines were extracted from the model systems using essentially the method developed by Gross (1990). Modifications in amounts of solvents used to elute the heterocyclic amines from the various columns were made in order to optimize the recovery of the heterocyclic amines. In addition, a vacuum manifold was used to elute the compounds from all of the columns used in the extraction process. The contents of each glass ampule were dissolved in 60 ml of 1N

sodium hydroxide. A 20 ml aliquot of the extract was mixed, using a spatula, with the packing material (diatomaceous earth) from an Extrelut-20 cartridge (20 ml) which was then packed back into the column. The Extrelut-20 column was connected to a cation exchange resin (500 mg Bond-Elut propylsulfonic acid silica, PRS cartridge) placed on a Visiprep solid phase extraction vacuum manifold (Supelco, Inc., Bellefonte, PA). Dichloromethane (200 ml) was used to elute the heterocyclic amines from the Extrelut-20 column onto the cation exchange resin. Using 20 ml of a methanol/0.1N hydrochloric acid (40:60 v/v) solution, apolar amines were eluted from the PRS column and discarded. Apolar amines that have been found in cooked meat include α- and ß-carbolines, tryptophan pyrolysate products (Trp-P-1 and Trp-P-2), norharman and harman (Gross, 1990).

The heterocyclic amines held on the PRS column were eluted onto 100 mg Bond-Elut C18 cartridges using 20 ml of 0.5 M ammonium acetate adjusted to pH 8.0 ± 0.2 with ammonia hydroxide. The heterocyclic amines were then eluted off the C18 column using 6 ml of methanol/ammonia hydroxide (9:1 v/v). The extract was then filtered through a 0.2-µ Gelman (Ann Arbor, MI) HPLC disc filter evaporated to near dryness under a stream of nitrogen, quantitatively transferred to a 1-ml microvial, dried again under nitrogen and redissolved in 50 µl of acetonitrile / ethyl acetate (1:1). The pentafluoropropionyl derivatives were prepared as previously described.

Results and Discussion

Mutagenic heterocyclic amines, IQ, MeIQ, MeIQx and PhIP, were derivatized using pentafluoropropionic acid anhydride. The derivatization conditions were relatively mild and resulted in a mono-pentafluoropropionic derivative. Watson (1985) indicated that pentafluoropropionic acid anhydride reacts with free amino groups, displacing a hydrogen and forming an amide bond (Figure 1). Therefore, the derivatization of the heterocyclic amines would most likely occur at the site of the amino-imidazo moiety.

The derivatized heterocyclic amines eluted off the column in the following order: IQ, MeIQ, MeIQx and PhIP (Figure 2). Each derivatized heterocyclic amine gave rise to a distinct fragmentation pattern. In all cases, the base peak resulted from the loss of the pentafluoro group (CF_2CF_3) from the pentafluoropropionyl group attached to the amino nitrogen. A tabulation of the molecular ion and base peak for each derivatized heterocyclic amine is provided in Table 1. Using selected ion monitoring, the detection limit was approximately 5 ng of derivatized standard with a signal-to-noise ratio of 4:1. Derivatization with pentafluoropropionic acid anhydride was shown to be an effective procedure in that it only took 30 minutes to derivatize the standards, no base was necessary for the reaction to proceed, and unreacted pentafluoropropionic acid anhydride was easily removed under nitrogen resulting in a cleaner chromatogram.

Model systems containing either phenylalanine and creatinine or 2-methylpyrazine, fructose and creatinine were used to generate heterocyclic amines during heating. The derivatization

procedure was subsequently used to confirm the identity of heterocyclic amines. Model systems were chosen because higher concentrations of heterocyclic amines (μg/g) were generated compared to those present in fried meat, thus facilitating the analysis. In addition, the model systems showed that the derivatization reactions could be carried out in the presence of excess residue such as unreacted precursors, melanoidin pigments and other products.

From literature reports, it was expected that the model system containing phenylalanine and creatinine would produce both PhIP and IQ (Taylor et al., 1987), whereas the model system containing 2-methylpyrazine, fructose and creatinine would produce MeIQx, DiMeIQx, and PhIP (Jagerstad et al., 1984; Grivas et al., 1985). Of the heterocyclic amines examined, only PhIP was identified in model systems containing either phenylalanine and creatinine or 2-methylpyrazine, fructose and creatinine (Figure 3). The concentration of PhIP was high enough that selected ion monitoring was not necessary. It is possible that with selected ion monitoring, small amounts of the other heterocyclic amines could have been identified in the model systems.

In conclusion, pentafluoropropionic acid anhydride derivatization was shown to be a relatively simple and beneficial procedure for confirming the identity of PhIP and possible other heterocyclic amines in model systems. Using selected ion monitoring, a low detection limit of 5ng of derivatized standard was achieved. This procedure will be utilized in subsequent studies involving fried ground beef.

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Table 1: Mass-to-charge values for molecular ions and base peaks of the pentafluoropropionic acid-derivatized heterocyclic amines

Heterocyclic amine	Molecular ion	Base peak
IQ	345	225
MeIQ	358	239
MeIQx	359	240
PhIP	370	251

Figure 1: Derivatization of a free amino group with pentafluoropropionic acid anhydride.

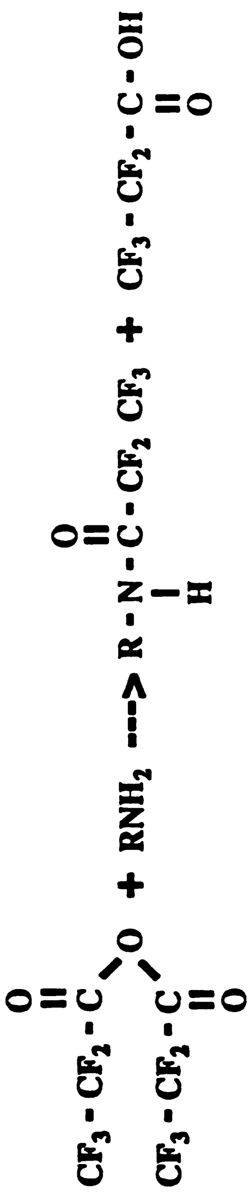

CF3 - CF2 - C
CF3 - CF2 - C
O
+ RNH2 ---> R - N - C - CF2 CF3 + CF3 - CF2 - C - OH
H
O
O

Figure 2: Reconstructed total ion current chromatogram of (A) standard heterocyclic amines derivatized with pentafluoropropionic acid anhydride: IQ (5.0 min), MeIQ (6.3 min.), MeIQx (7.4 min.) and PhIP (7.7 min.) and (B) derivatized PhIP isolated from the heated model system containing phenylalanine and creatinine.

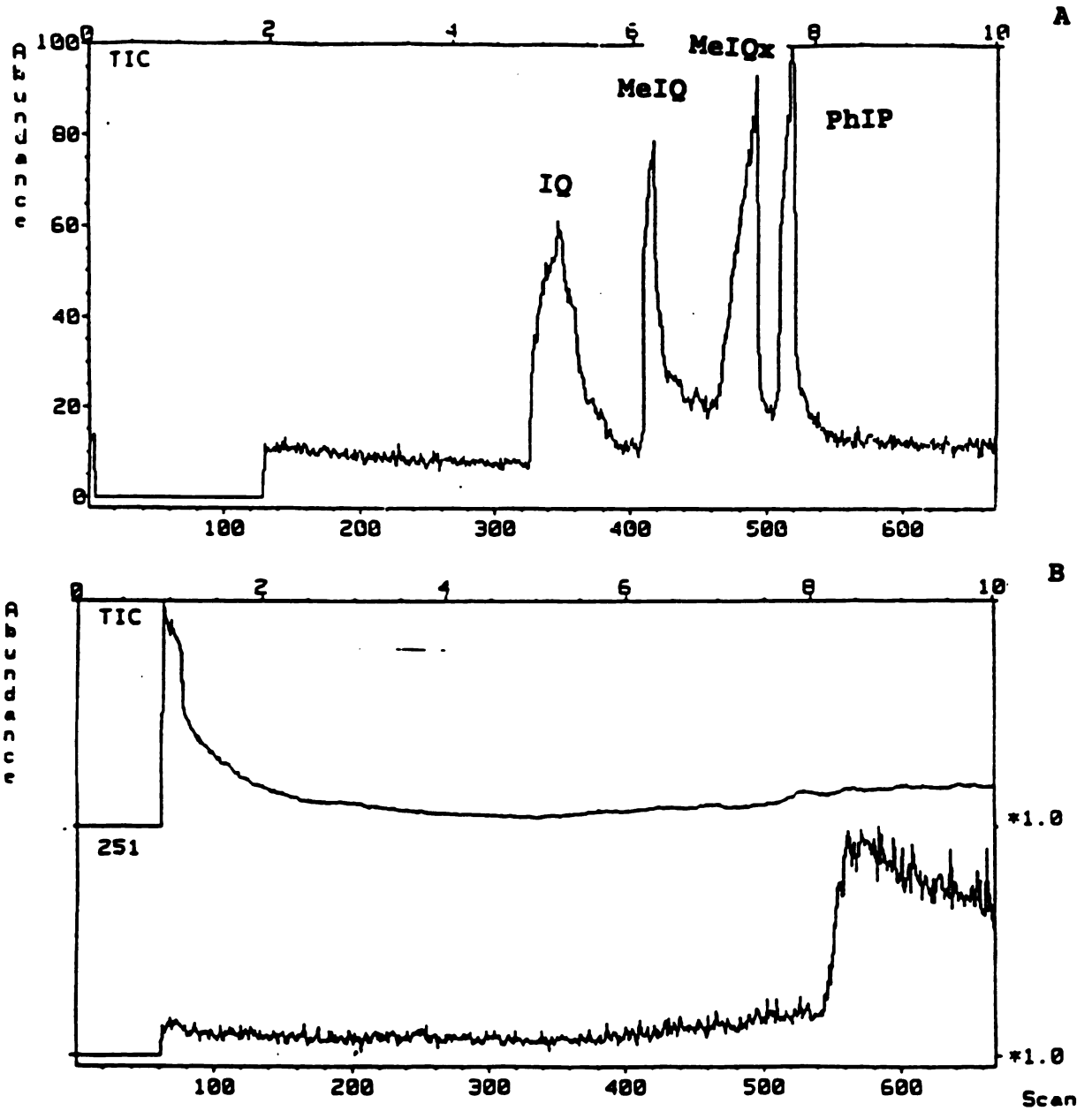
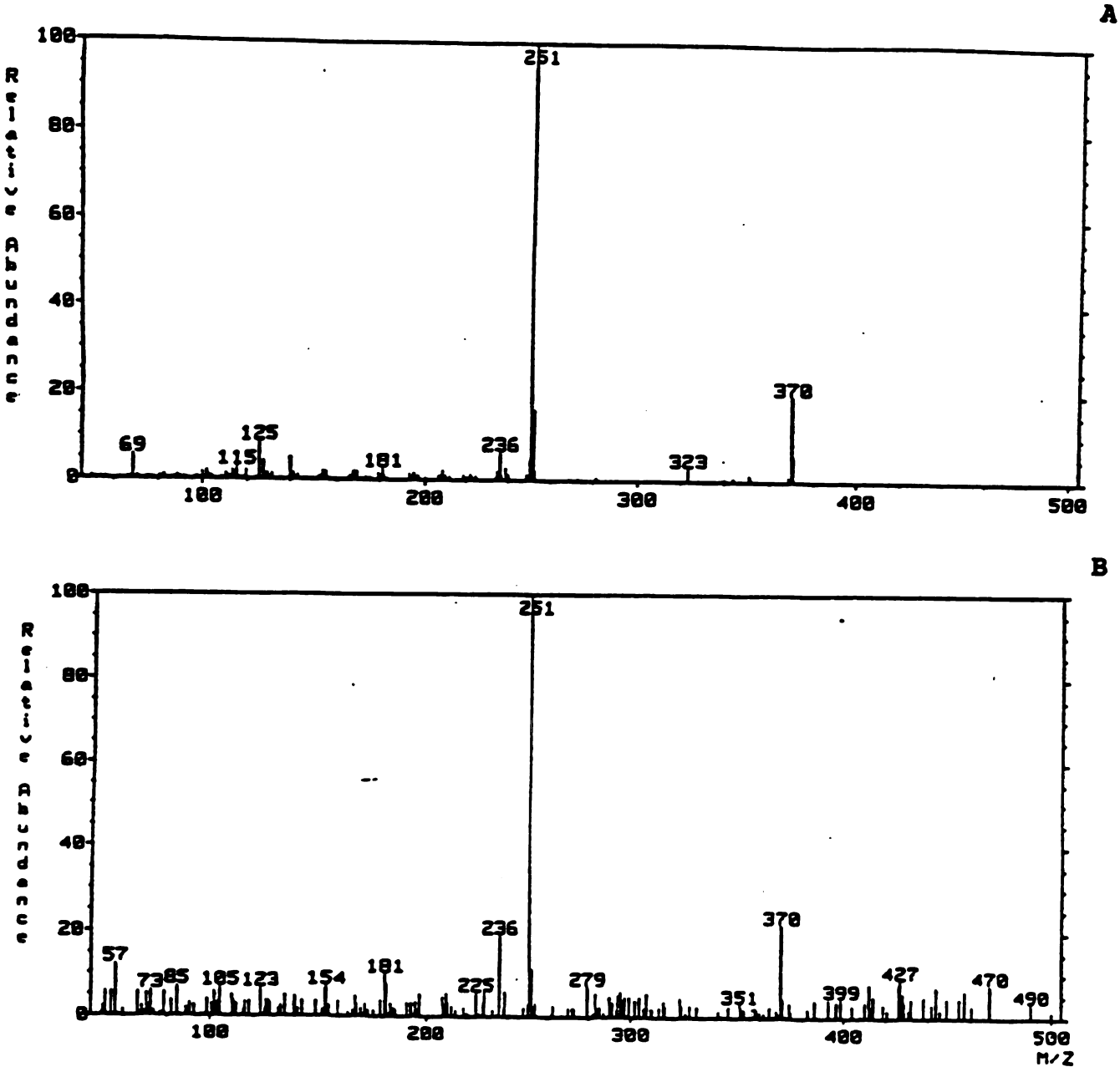

A
Abundance
TIC
IQ
MeIQ
MeIQx
PhIP
B
TIC
251
*1.0
*1.0
Scan

Figure 3: Mass spectra of (A) derivatized PhIP standard and (B) derivatized PhIP isolated from the heated model system containing phenylalanine and creatinine.


A
Relative Abundance
100
80
60
40
20
0
251
370
69
125
115
181
236
323
100
200
300
400
500
B
Relative Abundance
100
80
60
40
20
0
251
370
236
57
73
85
105
123
154
181
225
279
351
399
427
470
490
100
200
300
400
500
M/Z

CHAPTER TWO

REDUCTION OF MUTAGENS IN GROUND BEEF PATTIES BY THE ADDITION OF PHENOLIC ANTIOXIDANTS

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Abstract

The effect of phenolic antioxidants, butylated hydroxyanisole (BHA) and vitamin E, on the formation of heterocyclic amines and on overall mutagenicity in fried ground beef patties was evaluated. Individual heterocyclic amines were quantified by a high performance liquid chromatographic procedure, while mutagenicity was determined with the Ames assay using Salmonella typhimurium TA 98. Two heterocyclic amines, MeIQx (2-amino-3,4-dimethylimidazo[4,5-f]quinoline) and PhIP (2-amino-1-methyl-6-phenylimidazo-[4,5-f]quinoxaline) were detected in the control patties (no antioxidants) at concentrations of less than 0.3 ng/g and 2.4 ng/g, respectively. No MeIQx was detected in the antioxidant-treated patties, while PhIP concentrations were reduced 56% and 80% by BHA and vitamin E, respectively.

Overall mutagenicity in the fried ground beef patties was also reduced when antioxidants were added to the system. Butylated hydroxyanisole reduced the number of revertants/patty from 7000 to 2800, a reduction of 60%. Likewise, when vitamin E was added to the ground beef patties, the overall mutagenicity was reduced by 70%.

Introduction

Over the past decade, much research has been directed toward the isolation and identification of a number of mutagenic compounds in cooked meat (Hotchkiss and Parker, 1990). Of these compounds, heterocyclic amines, also referred to as aminoimidazoaarenes, are of major concern because their precursors are endogenous to muscle tissue and they arise as a result of cooking.

Heterocyclic amines that have been identified in fried ground beef include IQ (2-amino-3-methylimidazo[4,5-f]quinoline), MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline), DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline) and PhIP (2-amino-1-methyl-6-phenylimidazo-[4,5-f]pyridine). The most thorough studies on fried ground beef have been performed by Hargraves and Pariza (1983) and in particular, by Felton et al. (1986) and Felton and Knize (1990). PhIP concentrations range from 0 to 78 ng/g, while those for IQ (<0.2 ng/g), MeIQx (0 to 10.8 ng/g) and DiMeIQx (0 to 3.1 ng/g) are generally smaller (Knize, personal communication).

Factors influencing the formation of heterocyclic amines in ground beef have been intensively investigated. Studies have focused on the method of cooking (broiling, frying and microwaving) and the time/temperature relationship of mutagen formation (Dolara et al., 1979; Pariza et al., 1979; Spingarn and Weisburger, 1979; Hatch et al., 1982). It is well established that frying produces greater quantities of these mutagens than other cooking procedures (Overvik et al., 1989). Thus, alternative cooking procedures have been investigated to reduce the concentrations of heterocyclic amines present in fried ground beef. One such method involves partially cooking the ground beef in a microwave

oven before frying, and draining off the cooked-out fluid containing the water-soluble precursors (Taylor et al., 1986).

Inhibition of mutagen formation by the addition of antioxidants to the meat prior to cooking has received less attention. Wang et al. (1982) reported that soy protein concentrates, when added to raw ground beef, reduced the mutagenicity of the fried meat. Overall mutagenicity of the fried ground beef was determined by the Ames assay using Salmonella typhimurium TA98 and the rat liver microsomal fraction for metabolic activation (Ames et al., 1975). Most of the reduction in mutagenicity was attributed to volumetric effects, although some consideration was given to naturally occurring antioxidants in the soy protein concentrates such as chlorogenic acid. These investigators reported that butylated hydroxyanisole (BHA) also effectively reduced the mutagenicity of fried ground beef when added before cooking.

Defatted glandless cottonseed flour added at a level of 5% (w/w) to ground beef before frying has also been demonstrated to reduce the mutagenicity of the cooked meat (Rhee et al., 1987). Glandless cottonseed contains naturally occurring antioxidants such as quercetin and other flavonoids. However, the investigators did not clearly define whether the reduction in mutagenicity was a result of volumetric effects, i.e. dilution, or through the antioxidant properties of glandless cottonseed components.

The inhibitory effects of antioxidants were more intensively studied by Chen et al. (1992). At 0.1% (w/w) of the fat content, BHA, propyl gallate and tertiary butylhydroquinone reduced the overall concentration of IQ in fried ground beef by 80, 85 and 90%, respectively. At this level of addition, the volumetric effect was minimal. These results clearly established that phenolic antioxidants

are effective in reducing the formation of mutagens in meat, and led to the theory of involvement of free radicals in the formation of heterocyclic amines (Pearson et al., 1992).

The inhibitory effects of antioxidants on mutagen formation in meats have been determined by the Ames mutagenic assay. However, there is a scarcity of data on the inhibition of mutagens in meats by antioxidants as determined by specific analytical techniques such as high performance liquid chromatography (HPLC). The major objective of this study, therefore, was to demonstrate the inhibition of specific heterocyclic amines, namely MeIQx and PhIP, by the addition of antioxidants to ground beef, and to characterize the relationship between the reduction of specific compounds and the overall mutagenicity of fried ground beef.

Material and methods

Materials

Freshly ground beef (hamburger) was obtained from a local supermarket and used within one hour of purchase. Vitamin E and BHA were purchased from Sigma Chemical Company (St. Louis, MO). Extrelut-20 columns were obtained from EM Separations (Gibbstown, NJ). Bond-Elut PRS (500 mg) and C18 (100 mg) cartridges were purchased from Varian, Inc. (Harbor City, CA). All solvents were HPLC or glass-distilled reagent grade. The heterocyclic amine standards (IQ, MeIQ, MeIQx, DiMeIQx, TriMeIQx {2-amino-3,4,7,8-tetramethyl-imidazo-[4,5f]quinoxaline} and PhIP) were obtained from Toronto Research Chemicals (Toronto, Canada).

Methods

Treatments and frying conditions - Based on the fat content of the ground beef, as determined by the Marmer and Maxwell procedure (1981), BHA (0.1%) and α-tocopherol (1%) were dissolved in 1 ml of corn oil and added as separate treatments to ground beef before frying. The concentrations of antioxidants used were based on those described earlier by Chen et al. (1992). Ground beef, to which was added 1 ml of corn oil, served as the control. Patties weighing 100 g were formed in a petri dish (9 cm dia. x 1.5 cm thickness), and fried for six minutes on each side at 204°C in an aluminum, teflon-coated electric frying pan without a lid.

Extraction - The heterocyclic amine content of the fried ground beef was determined using essentially the method developed by Gross (1990). Modifications in amounts of solvents used to elute the heterocyclic amines from the various columns were made in order to improve the recovery of the heterocyclic amines (Figure 1). In addition, a vacuum manifold was used to elute the compounds from all of the columns utilized in the extraction process.

In order to ensure sample uniformity, each fried patty was finely blended in a Waring blender for 1 minute at speed 6. From each ground patty, a 10 g sample was removed for the analysis of heterocyclic amines. Three patties from each treatment were individually analyzed.

The ground meat samples were soaked in 20 ml of 1N sodium hydroxide for 30 minutes, allowing the samples to be partially digested. The packing material from a 20 ml Extrelut-20 cartridge (diatomaceous earth) was mixed, using a spatula, with the meat sample and then packed back into the column. The Extrelut-20 column was connected to a cation exchange resin (500 mg Bond-Elut propylsulfonic acid silica, PRS

cartridge) placed on a Visiprep solid phase extraction vacuum manifold (Supelco, Inc., Bellefonte, PA). Dichloromethane (200 ml) was used to elute the heterocyclic amines from the Extrelut-20 column onto the cation exchange resin. The apolar amines were eluted from the PRS column with 20 ml of methanol/0.1N hydrochloric acid (40:60 v/v) solution and discarded. Apolar amines that have been found in cooked meat include α- and ß-carbolines, tryptophan pyrolysate products (Trp-P-1 and Trp-P-2), norharman and harman (Gross, 1990).

The heterocyclic amines held on the PRS column were eluted onto 100 mg Bond-Elut C18 cartridges using 20 ml of 0.5M ammonium acetate adjusted to pH 8.0 ± 0.2 with ammonia hydroxide. The heterocyclic amines were then eluted off the C-18 column with 6 ml of methanol/ammonia hydroxide (9:1 v/v). The extract was then filtered through a 0.2-µ Gelman HPLC disc filter (Ann Arbor, MI), evaporated to near dryness under a stream of nitrogen, transferred to a 1 ml microvial, dried again under nitrogen, and redissolved in 50 µl of methanol.

High Performance Liquid Chromatography A 20-µl aliquot of the extract was injected onto a Waters 501 HPLC (Milford, MA) equipped with a Model 450 variable wavelength detector and a Waters 420 AC fluorescence detector (excitation 254 nm and emission 375 nm). In order to improve peak shape, triethylamine (1.4 ml/liter) was added to HPLC grade water. The mobile phase was then vacuum filtered and adjusted to pH 3.3 with dilute phosphoric acid. Acetonitrile was used as the second mobile phase. A reversed phase silica HPLC column (TSK-Gel ODS-80TM column; 4.6 mm ID X 25 cm; Tosoh Haas; Montgomeryville, PA) was used to separate the heterocyclic amines. The particle size of the column packing material was 5 µ in diameter. The mobile phase flow rate was set at 1

ml/minute. The acetonitrile concentration in the mobile phase was increased from 5% to 25% during the first 20 minutes of analysis. Percent acetonitrile was then increased to 55% in 10 minutes and held at this concentration for 5 minutes to elute the heterocyclic amines from the column. Over a 5-minute period, the percent of acetonitrile was decreased to its original concentration (5%) and the column was allowed to equilibrate for 20 minutes before the next injection.

Ames Test - The remainder of each fried ground beef patty was used to determine its overall mutagenicity by the Ames mutagenic assay. The samples were prepared for the Ames test by first separating the mutagens by acid/base partitioning with dichloromethane (Chen et al., 1992). The extract was then evaporated to dryness, and the residue was redissolved in 0.8 ml chloroform : methanol (80:20 v/v). The solution was transferred to a 1 dram vial, evaporated again to dryness and the residue redissolved in 0.2 ml dimethylsulfoxide. Two 50-µl aliquots of the extract were then mixed in a test tube containing 2 ml of top agar, 100 µl of Salmonella typhimurium TA98 broth and 500 µl of filtered rat liver S-9 fraction, and plated on petri dishes containing bottom agar (Ames et al., 1975). The S-9 fraction was necessary to metabolically activate the heterocyclic amines to mutagenic compounds. After incubation for 48 hours at 37°C, the number of revertant colonies were counted on each plate.

Statistical Analysis - Statistical analysis of the heterocyclic amine content of fried ground beef was based on two replicates for the addition of BHA and vitamin E, and on four replicates for the control. All treatments were from the same source of meat. Likewise, the statistical analysis of the mutagenicity of fried ground beef, as

measured by the Ames assay, was based on three replicates for each treatment. Each replicate was plated twice. The results were analyzed by a statistical computer program MSTAT developed at Michigan State University (Department of Crop and Soil Sciences) for a one-way analysis of variance (ANOVA). The F-test was used to determine significant differences between mean squares of the samples and the t-test was used to determine the significance of the treatment compared to the control (Steel and Torrie, 1980).

Results and Discussion

Two heterocyclic amines (MeIQx and PhIP) were identified in the cooked beef patties based on a comparison of their retention times with those of standard heterocyclic amines that were either co-injected with the sample or injected alone. With the variable wavelength detector set at 264 nm, the peaks corresponding to MeIQx and PhIP on the HPLC chromatogram were small. The amount of MeIQx detected in the samples was determined to be less than 0.3 ng/g of sample. Since PhIP has fluorescent properties (Gross, 1990), the compound was also monitored using a fluorescent detector. This improved the sensitivity of the chromatographic analysis by approximately 1000 fold. The mean average beef patties contained 2.4 ± 0.15 ng PhIP/g raw meat correcting for recovery using an external standard of PhIP.

The percent recoveries of the heterocyclic amines in the samples were determined by adding 100 ng each of PhIP and TriMeIQx to 20 ml of 1 N sodium hydroxide that was then added to a blank Extrelut-20 cartridge, and extracted as per the samples. Percent recoveries for PhIP and TriMeIQx were 62% and 54%, respectively. It was assumed that the percentage recovery of MeIQx would be similar to that for TriMeIQx

since the compounds are structurally similar. To serve as an internal standard, 200 ng of TriMeIQx were added to each sample during extraction, following the addition of sodium hydroxide. Samples in which the recoveries for TriMeIQx were outside the range of 40-60% were not included in the analysis, and the analysis of these samples were repeated.

The concentrations of PhIP and MeIQx detected in the fried ground patties fall within the range of concentrations of heterocyclic amines reported by other investigators (Hargraves and Pariza, 1983; Felton et al., 1986; Felton and Knize, 1990). PhIP is the most abundant heterocyclic amine found in cooked meat with reported concentrations ranging from 0 to 78 ng/g. Reported concentrations for IQ (<0.2 ng/g), MeIQx (0 to 10.8 ng/g) and DiMeIQx (0 to 3.1 ng/g) are generally smaller (Knize, personal communication).

When BHA or vitamin E was added to the ground beef before frying, the concentrations of PhIP formed in the fried patties were significantly ($P<0.005$) reduced (Table 1). PhIP concentrations were reduced by 60% to 1.2 ± 0.16 ng/g on adding BHA, while an 80% reduction in PhIP concentrations was achieved by the addition of vitamin E.

Chen et al. (1992) also reported a reduction in mutagen formation in fried ground beef on adding BHA and vitamin E. When BHA (0.1% based on fat content) was added to the ground beef, the concentration of IQ-like compounds was reduced from 7316 ng/g meat to 3244 ng/g meat, a reduction of 56%. Likewise, the addition of vitamin E (1% based on fat content) reduced total mutagen formation (IQ, MeIQx and DiMeIQx) by 50% (Chen, 1988).

When antioxidants were added to ground beef before frying, the overall mutagenicity (Ames assay) of the cooked meat was reduced. The

addition of BHA significantly ($P < 0.005$) reduced mutagenicity by 60% from 7000 revertants to 2800 revertants/100g raw meat. A 70% reduction ($P < 0.005$) in mutagenicity was achieved with vitamin E (Table 2). These results are comparable to those reported by Wang et al. (1982) for BHA inhibition of mutagen formation.

A comparison between PhIP formation and overall mutagenicity of the fried ground beef patties is illustrated in Figure 2. These results indicate that antioxidants are effective in blocking the formation of heterocyclic amines, thus diminishing the mutagenic potential of fried ground beef. Although PhIP contributes less than 18% of the total mutagenic activity of meat, it is the most abundant heterocyclic amine formed in cooked beef (Felton et al., 1986; Felton and Knize, 1990). Therefore, it would be expected that the reduction of mutagenicity in this study would also correspond to a reduction in the formation of other heterocyclic amines, such as MeIQx and DiMeIQx, that contribute significantly to the overall mutagenicity as determined by the Ames assay. The formation of MeIQx was inhibited in the patties containing vitamin E or BHA. It follows then that the reduction in overall mutagenicity of the fried ground beef patties was partially attributable to the elimination of MeIQx in the cooked beef.

The mechanism(s) by which phenolic antioxidants function to inhibit mutagen formation has not been fully elucidated. One possibility is that antioxidants act as free radical scavengers. Nyhammer (1986) proposed that pyrazine and pyridine radicals are intermediates in the formation of heterocyclic amines, thus antioxidants might act to stabilize these intermediates (Pearson et al., 1992).

Phenolic antioxidants inhibit lipid oxidation by interrupting the free radical chain mechanism of the oxidation process. It also remains

to be determined if there is a relationship between lipid oxidation and the formation of heterocyclic amines.

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Table 1. Reduction of PhIP concentrations in fried ground beef by the addition of antioxidants. [1,2,3,4]

	Control	BHA	Vitamin E
ng PhIP/ g raw beef	a 2.4 ± 0.15	b 1.1 ± 0.16	c 0.51 ± 0.04

[1]Values are expressed on a raw ground beef basis.
[2]Concentrations of BHA (0.1%) and vitamin E (1.0%) were based on the work of Chen (1988).
[3]Each value represents the mean of at least two samples ± standard deviation.
[4]Means with different superscripts are significantly different ($P < 0.005$).

Table 2. Reduction in mutagenicity of fried ground beef by the addition of antioxidants.[1,2,3]

	Control	BHA (0.1%)	Vitamin E (1%)
Revertants/ 100g raw beef	7078 ± 991 a	2794 ± 880 b	1905 ± 180 b

[1] Salmonella typhimurium TA 98 with the S-9 microsomal rat liver fraction for metabolic activation was used as a measure of the number of revertant colony-forming units.

[2] Each value represents the mean of at least three samples in which duplicate analysis has been averaged ± standard deviation.

[3] Means with different superscripts are significantly different ($P < 0.005$).

Figure 1: Extraction of heterocyclic amines from fried ground beef following the Gross method (Gross, 1990).

Finely grind hamburger in blender, add 10 g ground hamburger to 20 ml 1N NaOH, let partially digest for 20 minutes

Add Extrelut-20 to hamburger mix, fill column with mixture, attach to PRS column containing 2 ml DCM on Visiprep

Rinse columns with 200 ml DCM discard DCM, detach Extrelut column, and dry PRS under vacuum

Add 6ml of 0.1 M HCL to PRS followed by 20 ml methanol:0.1M HCL (4:6), and then 2 ml water

Rinse C-18 column with 1ml methanol followed by 10 ml water

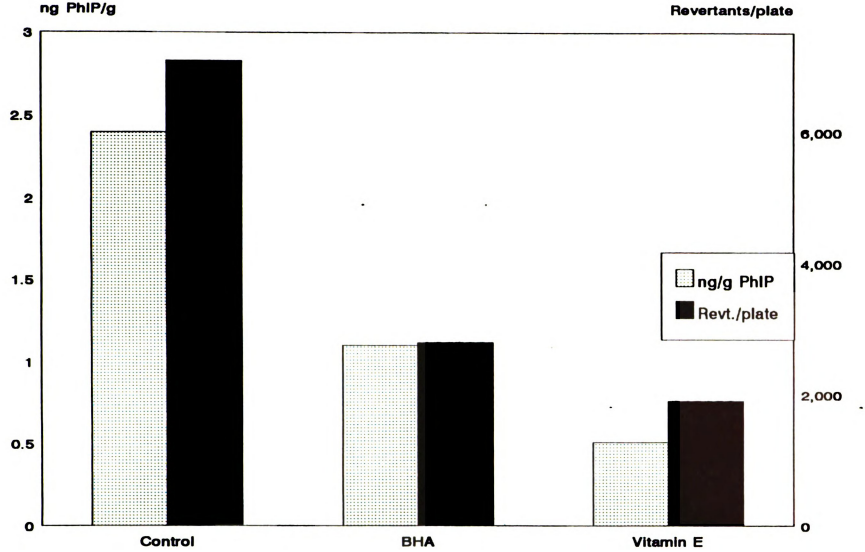
Attach PRS column, rinse with 20 ml of ammonium acetated (pH 8.0), discard PRS, and rinse with 2 ml water

Elute heterocyclic amines with 6ml methanol-conc. ammonia (9:1)

Filter through 0.2 µm disc, evaporate to near dryness under nitrogen

Transfer with 3 X 200 µl aliquots of methanol to 1ml vial, evaporate to dryness, redissolve in 50 µl methanol

Figure 2: A comparison between the overall mutagenicity, as measured with the Ames assay, and PhIP concentration in fried ground beef by the addition of antioxidants.


ng PhIP/g
Revertants/plate
3
2.5
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0.5
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ng/g PhIP
Revt./plate
Control
BHA
Vitamin E

CHAPTER THREE

CONTRIBUTION OF THE MAILLARD REACTION TO THE FORMATION OF HETEROCYCLIC AMINES IN MODEL SYSTEMS

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Abstract

The contribution of the Maillard reaction to the formation of heterocyclic amines in model systems was investigated. Three model systems (alanine, creatinine; alanine, creatinine and fructose; and 2-methylpyrazine, creatinine and fructose) were heated for 2 hours in an oven at 200°C. In all three model systems, a mutagenic fraction isolated between 80-90 minutes and corresponding to the HPLC retention time of PhIP (2-amino--1-methyl-6-phenylimidazo-[4,5-b]pyridine) was identified. The heatining of model systems containing either alanine and creatine or 2-methylpyrazine, fructose and creatinine also resulted in the formation of a mutagenic fraction appears between 10-30 minutes. Unequivocal identification of this fraction was not possible based on retention times since more than one standard heterocyclic amine eluted during this time interval. The formation of PhIP in the model systems was confirmed by mass spectrometry using pentafluoropropionic acid anhydride for derivatization. This study suggests that dual pathways for the formation of PhIP are possible since PhIP was formed without an amino acid.

Introduction

In the Maillard reaction, reducing sugars and amino groups from either amino acids, peptides or proteins react to produce a variety of compounds such as aldehydes, ketones, and melanoidin pigments which contribute flavor and color to food (Hodge, 1953). The interaction between α-dicarbonyls, produced during the Maillard reaction, and amino acids, i.e., the Strecker degradation, leads to the formation of various heterocyclic compounds including pyrazines and pyridines. The Maillard reaction and Strecker degradation contribute to mutagen formation since pyrazine and pyridine compounds that are formed have been postulated to be intermediates in the formation of mutagenic heterocyclic amines (Nyhammer, 1986; Pearson et al., 1992). These compounds, also known as amino-imidazoazaarenes, have been isolated from heat-processed muscle foods (Overvik et al., 1989; Gross, 1990).

The mechanism by which heterocyclic amines are formed in meat is not fully understood. Two theories have been suggested: (1) heterocyclic amines are formed via the interaction of products of the Maillard reaction between amino acids and reducing sugars with creatinine (Jagerstad et al., 1983; Nyhammer, 1986; Pearson et al., 1992); (2) heterocyclic amines are formed from the reaction of amino acids and creatinine (Felton et al., 1986). In both theories, temperatures ranging between 128° and 200°C are needed for the formation of these compounds.

Supporting evidence for creatinine (the cyclized form of creatine) involvement is the low or nonexistent mutagenic activity in foods high in protein but lacking in creatine, such as tofu, liver and kidney. In muscle, creatine is present at concentrations of approximately 0.5% by weight (Knize et al., 1988). When additional creatine is added to meat

and meat products before cooking, mutagenicity is enhanced (Ness, 1986; Becher et al., 1988; Knize et al., 1988; Overvik et al., 1989). Creatinine is thought to contribute to the imidazo-moiety of the heterocyclic amine structure (Chen et al., 1990).

The quinoline, quinoxaline or pyridine moieties of the mutagens are believed to arise from the interaction of sugars and amines. Many studies support the theory of Maillard reaction involvement in the formation of heterocyclic amines. For example, Grivas et al. (1985) identified the mutagen 4,8-DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline) when creatinine, fructose and alanine (1:0.5:1 molar ratio) were heated in diethylene glycol containing 14% water for 2 hours at 128°C. MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline) was identified when creatinine, glucose and glycine were heated under similar conditions (Jagerstad et al., 1984). When creatinine, glucose and phenylalanine were heated in a like manner, PhIP (2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine) was formed (Overvik et al., 1989.)

On the other hand, Taylor et al. (1987) demonstrated that the Maillard reaction was not necessary for the formation of heterocyclic amines. When phenylalanine and creatinine (2:1 molar ratio) were placed in a beaker and heated in an oven at 200°C, two heterocyclic amines, PhIP and IQ (2-amino-3-methyl-imidazo[4,5-f]quinoline), were identified. When glucose was added to the system, at twice the molar concentration of creatinine, both PhIP and IQ were formed but the ratio of their concentrations was altered. The amount of PhIP formed decreased, whereas the yield of IQ increased.

The objective of this study was to evaluate further how the Maillard reaction might affect the formation of heterocyclic amines in model systems. If pyrazines and pyridines from the Maillard reaction

are involved in the formation of heterocyclic amines, then the addition of a known pyrazine to a system containing creatinine and fructose should enhance the formation of heterocyclic amines.

Materials and Methods

Materials

2-Methylpyrazine and XAD-2 resin were purchased from Sigma Chemical Company (St. Louis, MO). Extrelut-20 columns were obtained from EM Separations (Gibbstown, NJ). Bond-Elut PRS (500 mg) and C18 (100 mg) cartridges were procured from Varian, Inc. (Harbor City, CA). All solvents were of high performance liquid chromatographic- or glass distilled-reagent grade. The heterocyclic amine standards (IQ, MeIQ {2-amino-3,4-dimethyl-imidazo[4,5-f]quinoline}, MeIQx, DiMeIQx, TriMeIQx {2-amino-3,4,7,8-tetramethylimidazo[4,5-f]quinoxaline} and PhIP) were obtained from Toronto Research Chemicals (Toronto, Canada).

Methods

Model systems - Model systems containing alanine (5.7 mmoles) and creatinine (4.4 mmoles), with or without fructose (2.8 mmoles), were placed in an oven heated to 200°C for a period of 2 hours. A third system of 2-methylpyrazine (5.46 mmoles), fructose (2.8 mmoles) and creatinine (4.4 mmoles) was heated under similar conditions. Because of the volatility of 2-methylpyrazine, all samples were weighed into thick walled glass ampules, partially submerged in dry ice, and vacuum-sealed. Vacuum sealing was necessary to prevent the glass ampules from exploding during heating. The Ames assay was used to assess the formation of mutagens in the heated model systems (Ames et al., 1975) following extraction and HPLC fractionation (Knize et al., 1988 - Procedure 1). Only one sample was analyzed for each treatment. In a complementary

study, the recent method of Gross (1990) was used to confirm the formation of PhIP in heated model systems (Procedure 2).[1] Unequivocal confirmation of the identity of PhIP was achieved by the pentafluoropropionic acid anhydride derivatization procedure described in Chapter One.

Procedure 1:

Extraction and clean-up - After heating, the glass ampules were allowed to cool to room temperature before breaking. Based on the extraction procedure outlined by Knize et al. (1988), the samples were dissolved in 250 ml of deionized water adjusted to pH 2.0 ± 0.2 with dilute hydrochloric acid. Using a separatory funnel, any nonpolar compounds were extracted with 200 ml of dichloromethane and discarded. The aqueous phase was adjusted to pH 12.0 ± 0.2 with sodium hydroxide and then washed twice with 200 ml aliquots of dichloromethane. The combined dichloromethane extracts were reduced in volume to approximately 25 ml using a rotary evaporator. The pH of the aqueous phase was then adjusted to pH 7.0 with dilute hydrochloric acid and passed through an XAD-2 column. Three bed volumes of deionized water were used to rinse the column, and the heterocyclic amines were eluted with 5 bed volumes of acetone, followed by 3 bed volumes of methanol. The acetone and methanol fractions were reduced in volume to approximately 25 ml by

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1. When this study was initiated, the Gross method (1990) had not been published.

rotary evaporation, combined with the dichloromethane extract, and evaporated to dryness using ethanol to form an azeotropic mixture. The amount of ethanol used for each sample varied between 50 and 200 ml, depending on the amount of water carried over from the XAD-2 column extraction for each sample. The concentrate was redissolved in dichloromethane:methanol (1:1), transferred to a 2 dram vial and evaporated to dryness under nitrogen. The final mixture was redissolved in 2 ml of 50% methanol in water (v/v) for HPLC analysis.

High performance liquid chromatography - A 500-µl aliquot of the sample extract was injected onto a Waters 501 HPLC (Milford, MA) equipped with a Model 450 variable wavelength detector and a Waters 420 AC fluorescence detector (excitation 254 nm and emission 375 nm). A reversed phase preparative column, PRP-1, (styrene-divinyl-benzene, 10 X 350 mm, Hamilton Co., Reno, NV) was used to separate the heterocyclic amines. The mobile phase containing 14% acetonitrile in water, with 1% diethylamine added to improve peak shape, was run isocratically for 70 minutes, and then the concentration of acetonitrile was increased to 100 % over a period of 40 minutes. Fractions of the eluant were collected every two minutes using a Model 1850 Isco fraction collector (Omaha, NE) and analyzed for mutagenicity using the Ames test with Salmonella typhimurium TA 98 and rat liver microsomal S-9 fraction.

Ames Assay - From each fraction, a 100-µl aliquot was added to a test tube containing 2 ml of top agar, 100 µl of Salmonella typhimurium TA 98 broth, and 500 µl of filtered rat liver S-9 fraction (Ames et al., 1975). The S-9 fraction was necessary to metabolically activate the heterocyclic amines to mutagenic compounds. After incubation for 48 hours at 37°C, the number of revertant colonies were counted on each plate.

Procedure 2:

Extraction and clean up: The extraction of heterocyclic amines from the model systems was determined using essentially the method developed by Gross (1990). Modifications in amounts of solvents used to elute the heterocyclic amines from the various columns were made in order to improve the recovery of the heterocyclic amines. In addition, a vacuum manifold was used to elute the compounds from all of the columns used in the extraction process. HPLC conditions were also modified and adapted for a biphasic mobile phase.

The contents of each glass ampule was dissolved in 60 ml of 1N sodium hydroxide. A 20-ml aliquot of the extract was mixed, using a spatula, with the packing material (diatomaceous earth) from an Extrelut-20 cartridge and then packed back into the column. The Extrelut-20 column was connected to a cation exchange resin (500 mg Bond-Elut propylsulfonic acid silica, PRS cartridge) placed on a Visiprep solid phase extraction vacuum manifold (Supelco, Inc., Bellefonte, PA). Dichloromethane (200 ml) was used to elute the heterocyclic amines from the Extrelut-20 column onto the cation exchange resin. Using 20 ml of a methanol/0.1N hydrochloric acid (40:60 v/v) solution, apolar amines were eluted from the PRS column and discarded. Apolar amines that have been found in cooked meat include α- and ß-carbolines, tryptophan pyrolysate products (Trp-P-1 and Trp-P-2), norharman and harman (Gross, 1990).

The heterocyclic amines held on the PRS column were eluted onto 100 mg Bond-Elut C18 cartridges using 20 ml of 0.5 M ammonium acetate adjusted to pH 8.0 ± 0.2 with ammonia hydroxide. The heterocyclic amines were then eluted off the C-18 column using 6 ml of methanol/ammonia hydroxide (9:1 v/v). The extract was then filtered

through a 0.2-μ Gelman HPLC disc filter (Ann Arbor, MI) evaporated to near dryness under a stream of nitrogen, transferred to a 1 ml microvial, dried again under nitrogen and redissolved in 50 μl of methanol.

High performance liquid chromatography A 20-μl aliquot of the extract was injected onto a Waters 501 HPLC (Milford, MA) equipped with a Model 450 variable wavelength detector and a Waters 420 AC fluorescence detector (excitation 254 nm and emission 375 nm). In order to improve peak shape, triethylamine (1.4 ml/liter of water) was added to HPLC grade water. The mobile phase was then vacuum filtered and adjusted to pH 3.3 ± 0.05 with dilute phosphoric acid. Acetonitrile was used as the second mobile phase. A reversed phase silica HPLC column (TSK-Gel ODS-80TM column; 4.6 mm ID X 25 cm; Tosoh Haas; Montgomeryville, PA) was used to separate the heterocyclic amines. The particle size of the column packing material was 5 μm in diameter. The mobile phase flow rate was set at 1 ml/minute. The acetonitrile concentration in the mobile phase was increased from 5% to 25% during the first twenty minutes of the analysis. Percent acetonitrile was then increased to 55% in 10 minutes and held at this concentration for 5 minutes to elute the heterocyclic amines from the column. Over a 5-minute period, the percent of acetonitrile was decreased to its original concentration (5%) and allowed to equilibrate for 20 minutes.

Mass spectrometry - To confirm the identity of the formed heterocyclic amines, each model system extract was evaporated under nitrogen and redissolved in 50 μl of acetonitrile / ethyl acetate (1:1) to which 50 μl of pentafluoropropionic acid anhydride were added for derivatization (Chapter 1). The mixture was then heated on a heating block at 60°C for 30 minutes. After cooling, the samples were evaporated under nitrogen

to dryness and redissolved in 20 µl of ethyl acetate. Each sample was then injected onto a JEOL 505 gas chromatograph-mass spectrometer (Japanese Electro Optical Lab, JEOL-USA, Peabody, MS) in the standard electron ionization mode with an accelerating electron energy of 70 eV. The gas chromatograph was equipped with a 15-m DB-1 fused silica capillary column (Supelco, Inc.) which was routed through the separator oven maintained at 260°C and directly into the mass spectrometer ion source. The temperature of the gas chromatograph was set initially at 180°C for 5 minutes, and then increased to 300°C at a rate of 10°C per minute.

Results and Discussion

The purpose of this study was to examine, using model systems, the contribution of the Maillard reaction to the formation of heterocyclic amines. Heterocyclic amines were identified from model systems based on the mutagenic profile of fractions collected from the HPLC and from comparing the mutagenic profile with the retention times of standard compounds. When alanine and creatinine were reacted at 200°C, mutagenic compounds were produced corresponding to HPLC fractions ranging from 10-30 minutes and 80-90 minutes (Figure 1). In the range between 10-30 minutes, the first fraction (10) was highly mutagenic with a plate count of approximately 1525 revertants/100 µl. The remainder of the first range of fractions (11-30) resulted in an overall mutagenicity of approximately 400 revertants/100 µl. Three standard heterocyclic amines eluted during this time interval (IQ, MeIQx and DiMeIQx) and the mutagenicity of this range (10-30 minutes) was not distinctly resolved into three separate peaks corresponding to the retention time of the standards, thus, no further identification was possible by this

procedure. A HPLC chromatogram of the heterocyclic amine standards is illustrated in Figure 2.

The second mutagenic range of fractions (80-90 minutes) for the reaction between creatinine and alanine produced a mutagenicity of approximately 700 revertants/100 µl. The retention time of this range corresponded to that of PhIP which was well resolved from the other heterocyclic amines (Figure 2).

Reducing sugars such as fructose and amino acids react via the Maillard reaction giving rise to a variety of products that contribute flavor and color to food (Hodge, 1953). The contribution of the Maillard reaction to the formation of heterocyclic amines was studied by the model system containing fructose, alanine and creatinine. Based on the work of Grivas et al. (1985) and Skog and Jagerstad (1990), fructose was added to the system at half the molar ratio of alanine and creatinine to obtain the greatest mutagenic response. At molar concentrations higher than creatinine, an inhibition in mutagenic response, as determined by the Ames assay using Salmonella typhimurium TA 98) was reported by Skog and Jagerstad (1990). They suggested that excess reducing sugar enhances the formation of Maillard reaction products such as 5-hydroxymethyl-2-furfural that would block the formation of heterocyclic amines by reacting with creatine. However, in this study, the mutagenic response from this model system was considerably lower than the response from the model system containing only alanine and creatinine. Only the fractions collected between 80-90 minutes indicated a mutagenic response of approximately 260 revertants/100 µl, and based on the retention times of standards, the active mutagen was tentatively identified as PhIP (Figure 3). The reduction in mutagenicity by the addition of fructose in the model

system containing alanine and creatine might be attributable to differences in the viability of the Salmonella typhimurium TA 98 strain, since the Ames assay was performed on separate days (Maron and Ames, 1983).

Mutagen formation in model systems has also been investigated by Grivas et al. (1985) who reported that DiMeIQx and MeIQx were produced when creatinine, alanine and fructose (1:1:0.5 molar ratio) were refluxed at 128°C for 1 hour. Skog and Jagerstad (1990), using a model system containing creatine, glucose and glycine heated at 180 °C for 10 minutes, identified three major mutagenic peaks based on HPLC fractionation and the Ames assay corresponding to MeIQx, DiMeIQx and possibly PhIP. Unequivocal identification of PhIP was not possible because the mutagenic fraction was too low in amount for mass spectral analysis.

When 2-methylpyrazine, fructose and creatinine were combined in a model system, mutagenicity was detected in HPLC fraction haveing retention times of: 18-22 minutes, 58-64 minutes and 86-96 minutes. The mutagenicity of these fractions ranged from 300 revertants/100 µl to 500 revertants/100 µl. Based on the retention times of standard heterocyclic amines, the fraction between 80-90 minutes appeared to correspond to PhIP. The fraction between 58-64 minutes did not correspond to any of the heterocyclic amine standards listed previously and the fractions between 18-22 minutes embraced the retention times of IQ, MeIQx and DiMeIQx (Figure 4).

In the Maillard reaction, a reducing sugar and an amino acid react to form a glycosylamine, which undergoes an Amadori rearrangement to yield a 1-amino-2-keto sugar. This sugar may then be broken down into 2- and 3- carbon fragments by two pathways (3-deoxyhexosone and methyl

α-dicarbonyl routes) leading to the formation of a variety of compounds such as aldehydes, ketones and melanoidin pigments (Whitfield, 1992).

Pyrazines and pyridines can be produced from the interaction of the α-dicarbonyls from the Maillard reaction with amino acids via the Strecker degradation. Nyhammer (1986) proposed that heterocyclic amines are formed by an aldol-type condensation between an aldehyde and pyridine or pyrazine molecule, followed by the cyclic addition of creatinine to yield either an imidazoquinoline or an imidazoquinoxaline.

With 2-methylpyrazine as a reactant, a quinoxaline-type structure would have been expected to be formed in the model system. The addition of 2,5-dimethylpyrazine or 2-methylpyridine to a system containing creatinine and acetaldehyde resulted in the formation of DiMeIQx and MeIQ, respectively (Milic et al., 1993). Based on the HPLC retention times of heterocyclic amine standards, the mutagenic peak corresponding to the fraction collected between 18-24 minutes for the 2-methylpyrazine system suggests the formation of quinoxaline structures. In all three model systems, a mutagenic peak corresponding to the HPLC retention time of PhIP was observed. In order to provide further evidence of the formation of PhIP in these systems, the experiment was repeated using two model systems: (i) alanine and creatinine and (ii) 2-methylpyrazine, creatinine and fructose. A more recently developed extraction procedure was used for these systems that was fast, easy and provided a better clean-up of the sample (Gross, 1990). The model system containing alanine, creatinine and fructose was not used because the high degree of browning interfered with the extraction procedure. Based on the HPLC retention times of standards using both ultraviolet (264nm) and fluorescence detection (excitation 254nm, emission 345 nm), PhIP was detected in both model systems. Its identity was confirmed by mass

spectral confirmation of its pentafluoropropionyl derivative (Chapter 1). The mass spectrum of derivatized PhIP with its fragmentation pattern of m/z 370, m/z 251 (base peak) and m/z 236 is shown in Figure 5.

Maillard reaction products have been reported to form other heterocyclic amines when heated with creatinine and acetaldehyde. Milic et al. (1993) synthesized quinoxaline and quinoline heterocyclic amines by reacting creatinine, acetaldehyde with 2,5-dimethylpyrazine or 2-methylpyridine, respectively. The mixture was heated in a 11% diethylene glycol solution for 3 hours at pH 6.0 and at a temperature of 130°C. These compounds were confirmed by nuclear magnetic resonance and mass spectrometry. It is possible that the lower temperature favored the formation of quinoline and quinoxaline compounds over PhIP. It is also possible that PhIP was formed but not identified in this system.

In summary, the Maillard reaction intermediate, 2-methylpyrazine, when heated with creatinine and fructose, resulted in the formation of PhIP, and possibly minute amounts of quinoline and quinoxaline heterocyclic amines. In the absence of fructose, alanine and creatinine reacted to produce PhIP. This suggests the possibility of dual pathways for the formation of heterocyclic amines. Further validation by isotopic labelling would be beneficial in substantiating these pathways.

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Figure 1: The mutagenic profile of HPLC fractions of a heated alanine/creatinine model system [as measured by the Ames assay using Salmonella typhimurium TA98].

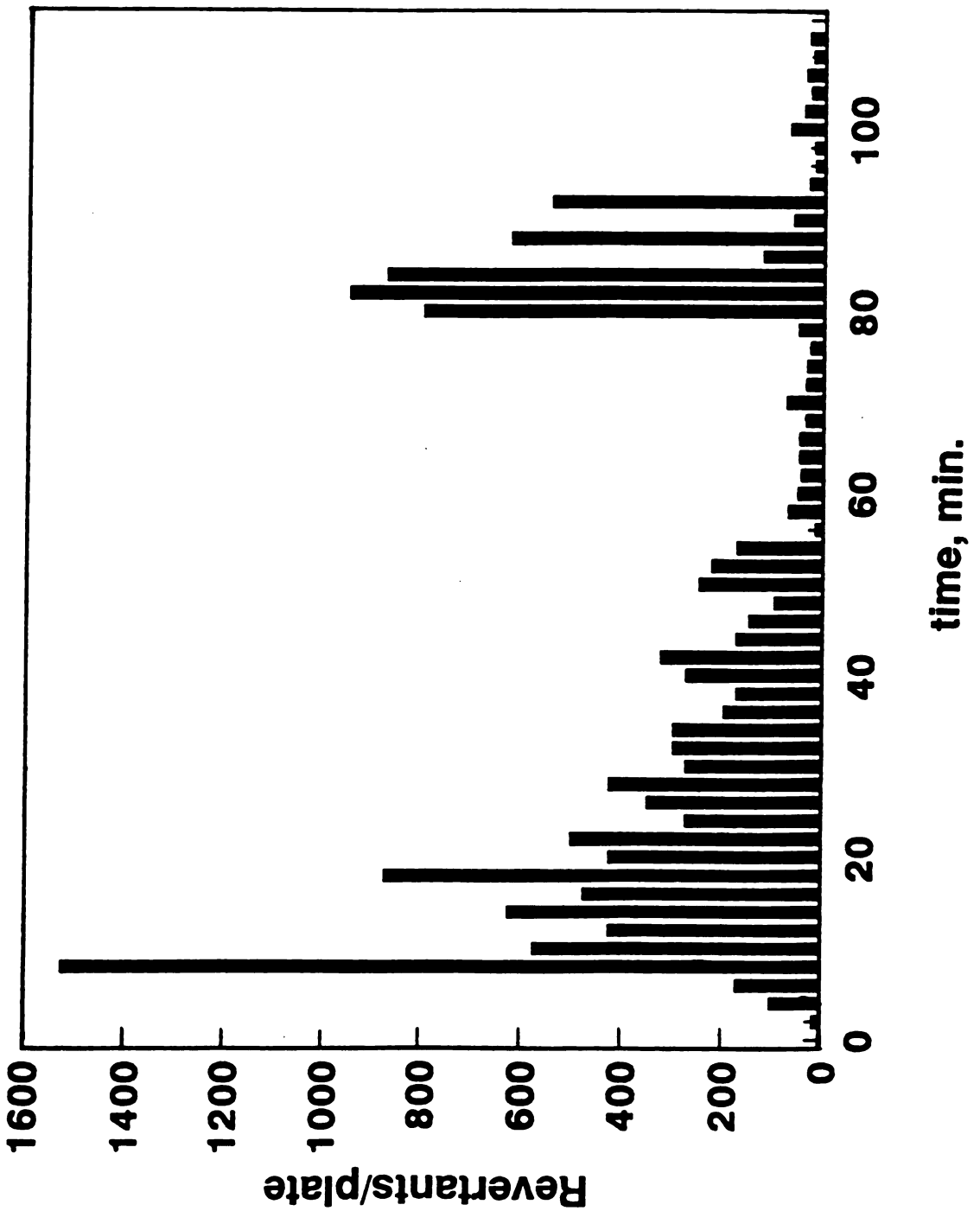

Revertants/plate
1600
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
100
time, min.

Figure 2: A high performance liquid chromatogram of heterocyclic amine standards: IQ (14 min.), MeIQ (26 min.), DiMeIQx (28 min.) and PhIP (83 min.). [HPLC conditions as outlined in Procedure 1 with uv detection at 264nm.]

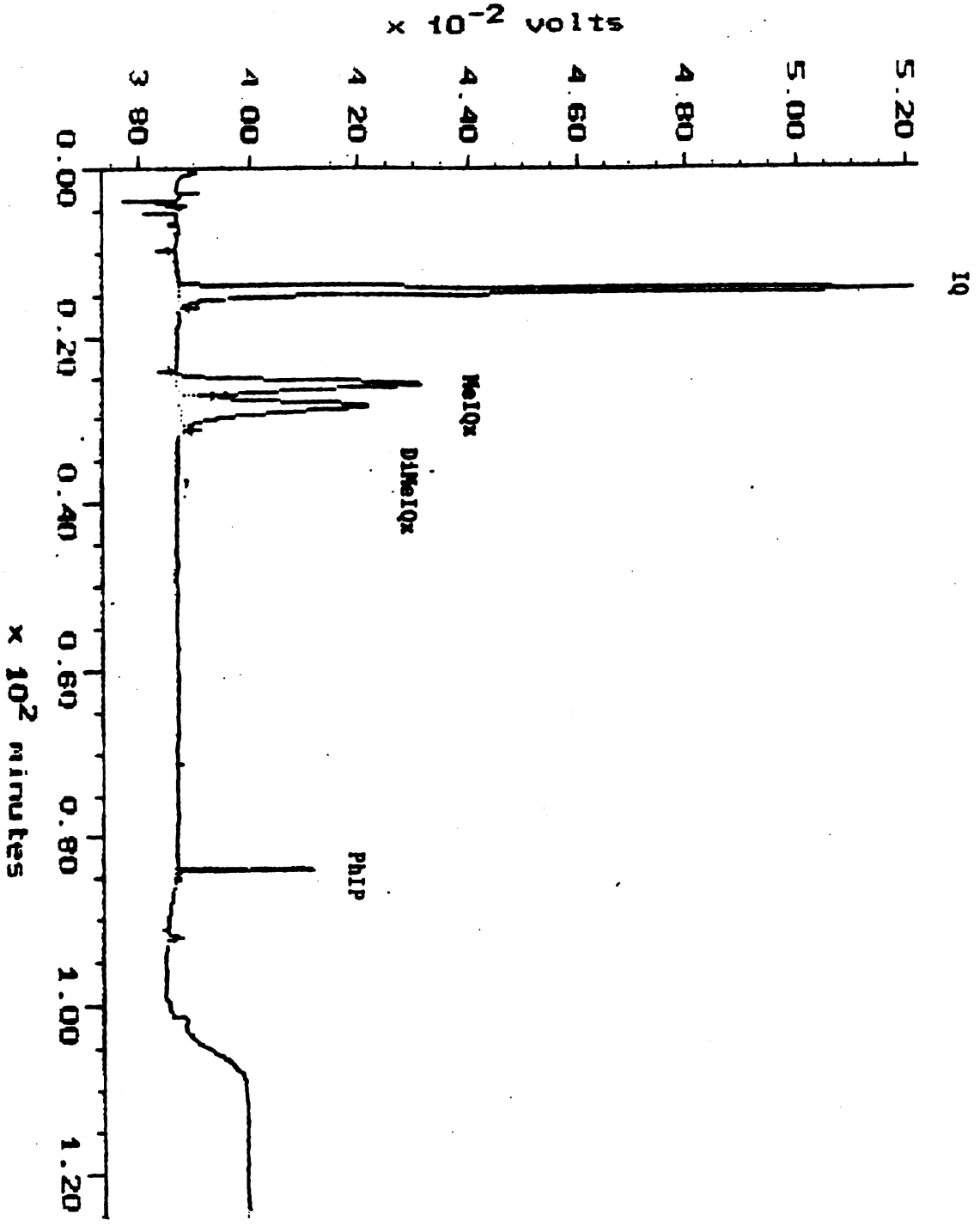

x 10⁻² volts
3.80
4.00
4.20
4.40
4.60
4.80
5.00
5.20
0.00
0.20
0.40
0.60
0.80
1.00
1.20
x 10² minutes
IQ
MeIQx
DiMeIQx
PhIP

Figure 3: The mutagenic profile of HPLC fractions of a heated alanine/fructose/creatinine model system [as measured by the Ames assay using Salmonella typhimurium TA98].

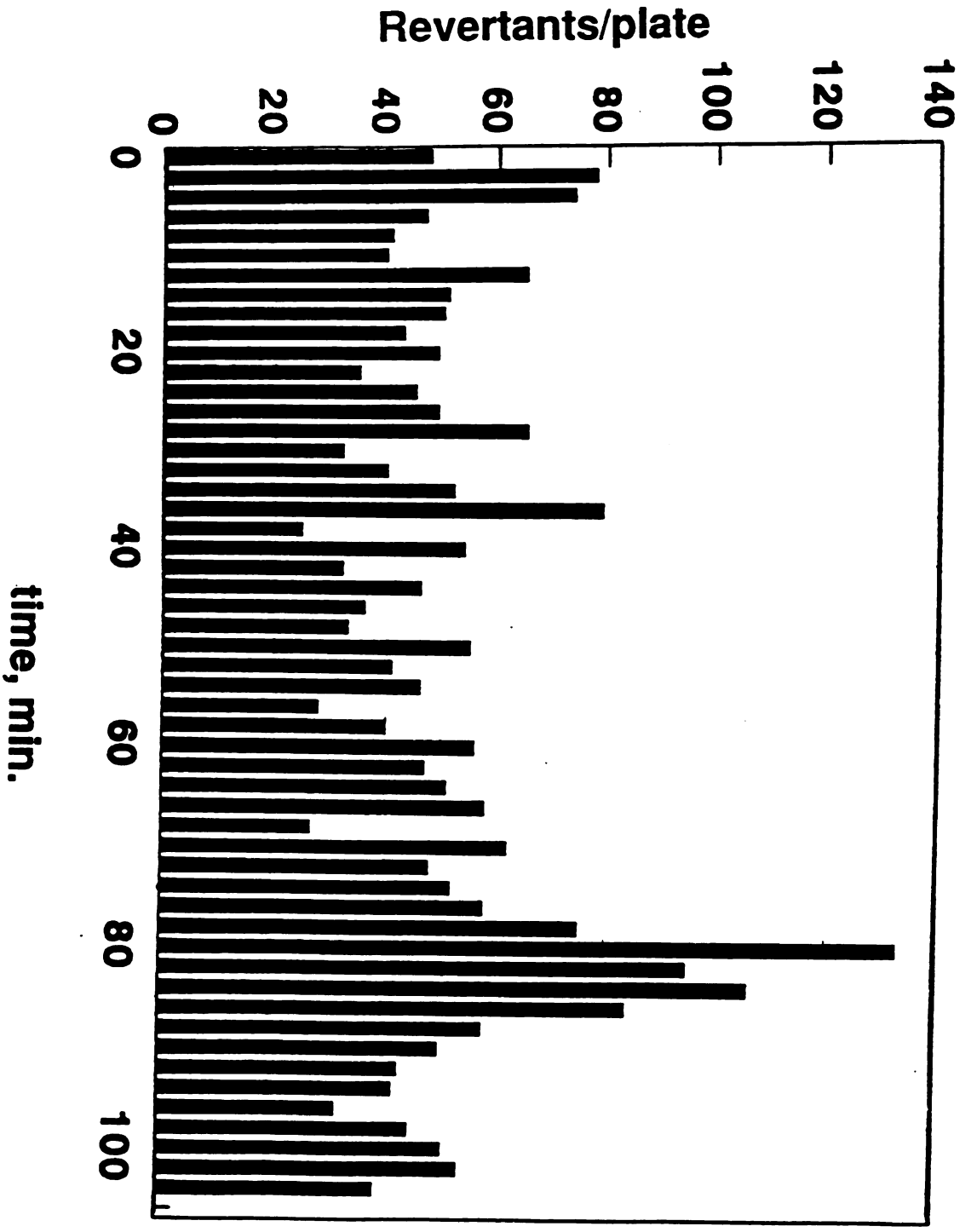

Revertants/plate
0
20
40
60
80
100
120
140
time, min.
0
20
40
60
80
100

Figure 4: The mutagenic profile of HPLC fractions of a heated 2-methylpyrazine/fructose/creatinine model system [as measured by the Ames assay using Salmonella typhimurium TA98].

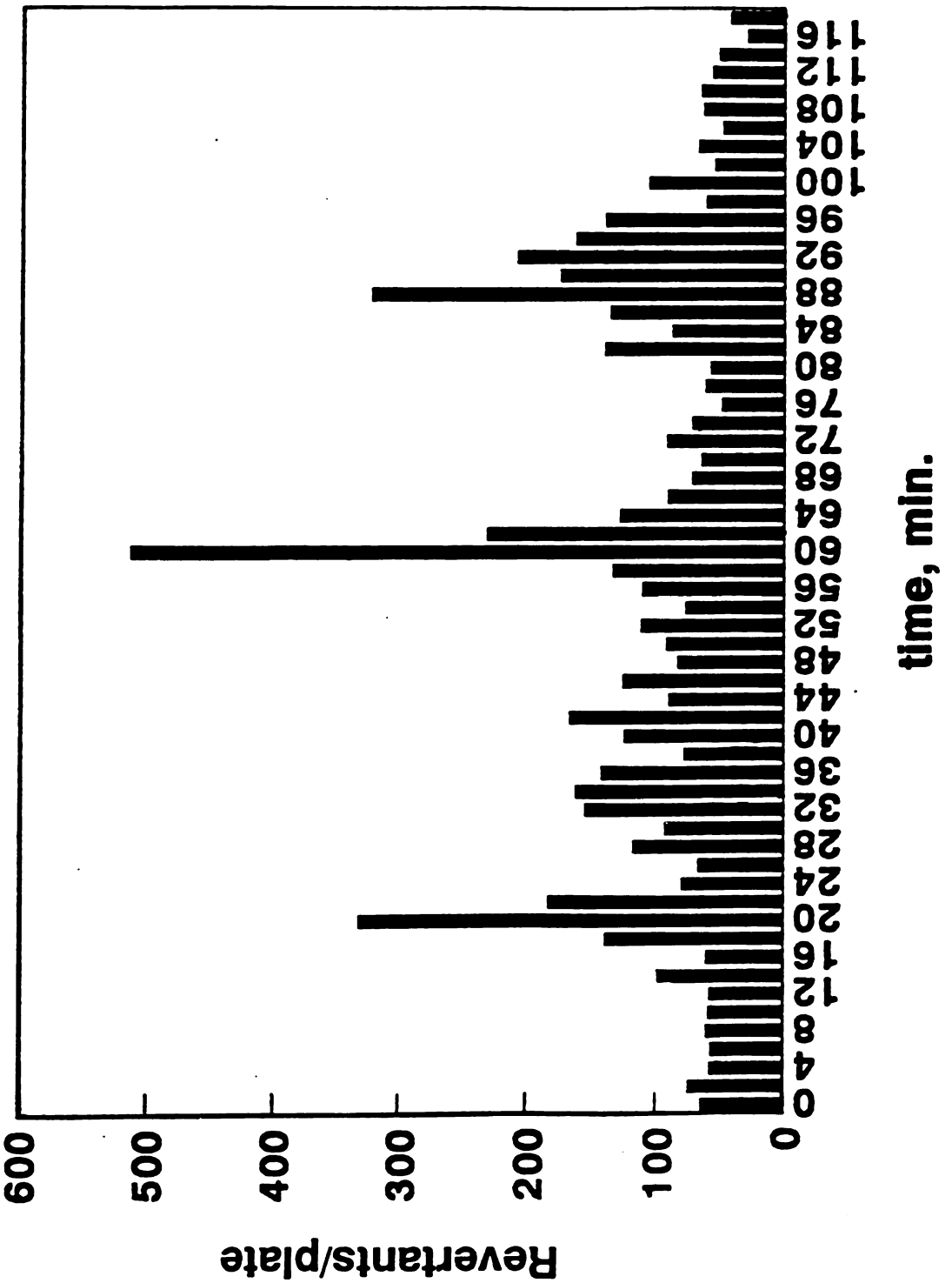
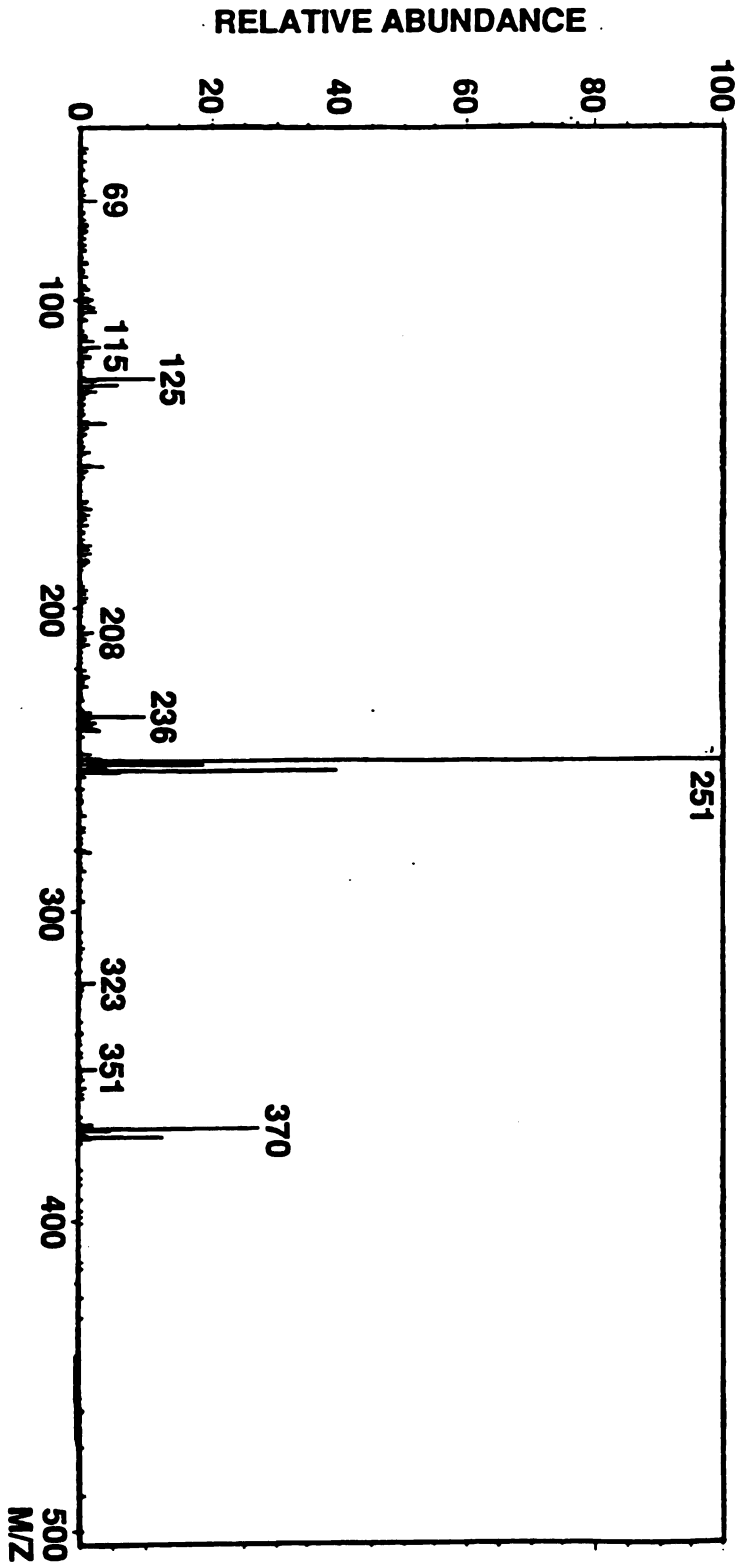

Revertants/plate
600
500
400
300
200
100
0
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
64
68
72
76
80
84
88
92
96
100
104
108
112
116
time, min.

Figure 5: Mass spectrum of PFPA-derivatized PhIP isolated from a heated model system containing 2-methylpyrazine, creatinine and fructose.


RELATIVE ABUNDANCE
0
20
40
60
80
100
69
100
115
125
200
208
236
251
300
323
351
370
400
500
M/Z

CHAPTER FOUR

INTERACTIONS BETWEEN THE MAILLARD REACTION, THERMAL LIPID OXIDATION AND HETEROCYCLIC AMINE FORMATION IN FRIED GROUND BEEF

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Abstract

Interactions involving products from the Maillard reaction, thermal lipid oxidation and heterocyclic amine formation in fried ground beef were investigated. A reduction in heterocyclic amine concentration in fried ground beef was observed on adding a solution (10% v/w) of Maillard reaction products (MRPs) formed by heating a mixture of glucose and histidine (15 mM, 50 mM and 200 mM per reactant) before frying. The lower the concentration of MRPs added to the ground beef, the greater the inhibitory effect on the formation of PhIP and MeIQx.

The addition of lipid oxidation products also reduced heterocyclic amine formation in cooked meat. Inhibition of PhIP and MeIQx was achieved by the addition of dodecanal to the ground beef patties before frying. While dodecanal is not a product of lipid oxidation, it was chosen as a representative aldehyde from the breakdown of fats. The addition of glycolaldehyde only slightly reduced the heterocyclic amine concentration in the cooked meat.

Furthermore, thermal oxidation of sunflower oil reduced the formation of PhIP in a heated model system containing phenylalanine and creatine.

Introduction

Interactions between products of thermal lipid oxidation and Maillard reactants/reaction products occur when meat is cooked. Mottram and Edwards (1983) demonstrated that aliphatic lipid breakdown products altered the flavor of cooked beef by suppressing the formation of pyrazine and other heterocyclic compounds characteristic of the Maillard reaction. One proposed mechanism by which the formation of pyrazine compounds could be altered was by rendering fewer amino acids available to react with reducing sugars to form pyrazine compounds. End products of lipid oxidation (carbonyls) have been shown to react with free amino groups of amino acids such as cysteine, thus reducing the available pool of amino acids in muscle tissue (Farmer and Mottram, 1990).

Maillard reaction products (MRPs) arise as the result of a series of reactions between reducing sugars and the primary amino groups of amino acids, peptides or proteins (Nawar, 1985). This reaction results in a complex mixture of products such as furfurals, furanones, dicarbonyls, pyrazines, pyridines, and aliphatic aldehydes (Whitfield, 1992). These compounds contribute many positive characteristics to meat such as overall flavor and the browning pigments characteristic of cooked meat (Whitfield, 1992). Maillard reaction products (MRPs) have been shown to

possess antioxidant potential. For example, reaction products of histidine and glucose when added to ground beef before frying, reduced the degree of lipid oxidation (Bailey, 1988).

On the other hand, certain MRPs such as pyrazines and pyridines have been proposed to be intermediates in the formation of mutagenic heterocyclic amines (Nyhammer, 1986). These compounds are thought to arise from the reaction of amino acids, creatine and reducing sugars. Heterocyclic amines that have been identified in ground beef include: IQ (2-amino-3-methylimidazo[4,5-f]quinoline), MeIQ (2-amino-3,4-dimethylimidazo[4,5-f]quinoline), MeIQx (2-amino-3,4-dimethylimidazo[4,5-f]quinoxaline), DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline) and PhIP (2-amino-1-methylimidazo[4,5-b]pyridine (Felton and Knize, 1990).

The objectives of this study were to: (1) to ascertain how the addition of selected MRPs would influence heterocyclic amine formation in fried ground beef as these compounds have been suggested as potential antioxidants in meat (Bailey, 1988); (2) to study the effect of glucose and selected aldehydes (dodecanal and glycolaldehyde) on heterocyclic amine formation in fried ground beef; and (3) to investigate the effect of saturated and unsaturated fats on the formation of heterocyclic amines in heated model systems containing creatinine and phenylalanine.

Material and Methods

Materials - Freshly ground beef containing 27% fat, as determined by a Hobart F101 Fat tester (Liu, 1991), was obtained from a local supermarket and used within one hour of purchase. Extrelut-20 columns were obtained from EM Separations (Gibbstown, NJ). Bond-Elut PRS (500 mg) and C18 (100 mg) cartridges were purchased from Varian, Inc. (Harbor City, CA). All solvents were high performance liquid chromatographic (HPLC)- or glass-distilled reagent grade. The heterocyclic amine standards (IQ, MeIQ, MeIQx, DiMeIQx, TriMeIQx {2-amino-3,4,7,8-tetramethyl-imidazo-[4,5f]quinoxaline} and PhIP) were obtained from Toronto Research Chemicals (Toronto, Canada).

Methods

The influence of MRPs on heterocyclic amine formation in ground beef - In order to form MRPs, several equimolar concentrations (15, 50 and 200 mM) of glucose and histidine in phosphate buffer (pH 5.7) were heated in a round bottom flask at 100°C for 48 hours (Bailey, 1988). An aliquot of the reaction mixture was added to ground beef to give a concentration of 10% (v/w). The ground beef was then shaped into 100g patties using a standard size petri dish (9-cm dia. X 1.5-cm thickness). The patties were fried at 204°C for a total of 12 minutes in an aluminum teflon coated - electric frying pan without a lid. After 6 minutes, the patties were flipped so that browning occurred on each side. Four patties from each treatment were analyzed for heterocyclic amine content.

Effect of glucose and aldehyde addition on the formation of heterocyclic amines in ground beef patties - The effect of the addition of glucose and aldehydes on the formation of heterocyclic amines in fried ground beef was investigated. Glucose, glycolaldehyde, dodecanal and a mixture of glucose and dodecanal (0.16 mmoles) were added to ground beef before frying. The amount of each compound added was based on the work of Skog and Jagerstad (1990) who reported that sugars added at this concentration (twice the molar concentration of creatine in ground beef) produced the greatest inhibition of mutagenicity in ground beef during cooking.

Patties weighing 30 g were then formed using a small petri dish (5.5-cm dia. X 1.5-cm thickness) and then fried at 204°C for 6 minutes per side in a teflon - coated electric frying pan without a lid. Three patties from each treatment were analyzed for heterocyclic amine content.

Effect of saturated and unsaturated fats on the formation of heterocyclic amines in heated model systems - Tristearin (2.5 ± 0.1 g) or sunflower oil (2.5 ± 0.1 g) was weighed into a 250-ml beaker to which was added 1.0 g phenylalanine and 4.0 g creatine. The beakers were covered with aluminum foil and placed in a 200°C oven, and heated for 30 minutes. After allowing the beakers to cool to ambient temperature, 60 ml of 1N sodium hydroxide was added to each beaker to dissolve the contents. Three replicates for each treatment were then analyzed for heterocyclic amines.

Extraction of heterocyclic amines from fried patties and model systems - Heterocyclic amines were extracted following the procedure developed by Gross (1990), as outlined in Chapter One.

High Performance Liquid Chromatography - A 20-µl aliquot of the extract was injected onto a Waters 501 HPLC (Milford, MA) equipped with a Model 450 variable wavelength detector and a Waters 420 AC fluorescence detector (excitation 254 nm and emission 375 nm). In order to improve peak shape, triethylamine (1.4 ml/liter) was added to HPLC grade water. The mobile phase was then vacuum filtered and adjusted to pH 3.3 with dilute phosphoric acid. Acetonitrile was used as the second mobile phase. A reversed phase silica HPLC column (TSK-Gel ODS-80TM column; 4.6 mm ID X 25 cm; Tosoh Haas; Montgomeryville, PA) was used to separate the heterocyclic amines. The particle size of the column packing material was 5µ in diameter. The mobile phase flow rate was set at 1 ml/minute. The acetonitrile concentration in the mobile phase was increased from 5% to 25% during the first 20 minutes of analysis. Percent acetonitrile was then increased to 55% in 10 minutes and held at this concentration for 5 minutes to elute the heterocyclic amines from the column. Over a 5-minute period, the percent of acetonitrile was decreased to its original concentration (5%) and the column was allowed to equilibrate for 20 minutes before the next injection.

Statistical Analysis - Statistical analysis of the heterocyclic amine data for fried ground beef was based on three replicates for each treatment. All treatments were from the same source of meat. The results were analyzed by a statistical computer program (MSTAT) developed at Michigan State University (Department of Crop and Soil Sciences) for a one way analysis of variance (ANOVA). The F-test was used to determine significant differences between mean squares of the samples and the t-test was used to determine the significance of the treatment compared to the control (Steel and Torrie, 1980).

Results and Discussion

Effect of MRPs on heterocyclic amine formation in fried ground beef - Two heterocyclic amines, MeIQx and PhIP, were identified in the fried ground beef patties through comparison of retention times with those of standard compounds. The concentrations of heterocyclic amines were greatest in the control beef patties, i.e., no MRPs added. The fried control patties contained, on average 3.6 ± 0.6 ng MeIQx/g and 12.6 ± 9.5 ng PhIP/g, (Table 1) corrected for recoveries of 54% and 30%,respectively, as determined in Chapter Two .

The smallest concentration of MRPs had an inhibitory effect on the formation of heterocyclic amines in the fried ground beef patties. A signficant ($P < 0.005$) reduction (60%) in MeIQx (1.4 ± 0.4 ng/g) was observed when the products of the heated 15 mM histidine and glucose solution

were added to the ground beef. Likewise, the amount of PhIP present in the fried ground beef was significantly ($P < 0.1$) reduced by 87% to 1.1 ± 1.6 ng/g.

The 50 mM and 200 mM solutions of glucose and histidine reduced mutagen formation, but were not as effective as the 15 mM solution. Both solutions appeared to reduce the formation of PhIP by approximately 70%. A significant ($P < 0.005$) reduction (50%) in MeIQx concentration in the cooked patties occurred when the 50 mM solution was added before frying. On the other hand, the 200 mM solution did not reduce the formation of MeIQx.

One explanation as to why higher concentrations of MRP's are less effective in suppressing mutagen formation in ground beef may be attributed to the dual functionality of MRPs. At low concentrations, MRPs inhibit heterocyclic amine formation by reacting with creatine (Skog and Jagerstad, 1990) or through their antioxidant properties (Lingert et al., 1981; Bailey, 1988). Some MRPs with antioxidant properties are reductones and maltol, and could function like phenolic antioxidants which have been shown to suppress heterocyclic amine formation (Chen et al., 1992). At high concentrations, the system is flooded with heterocyclic amine intermediates such as pyridines and pyrazines, and the inhibitory efffect of MRPs at the lower concentrations is neutralized by the greater potential to supply heterocyclic amine precursors at the higher concentrations.

Evidence of the inhibitory effects of MRPs was demonstrated by Skog and Jagerstad (1990) through the addition of a number of sugars (glucose, sucrose, fructose and lactose) to a model system containing glycine and creatine. When sugars were added in molar amounts less than that of creatine, the yield of heterocyclic amines, MeIQx and DiMeIQx, was increased until an optimum was reached. For glucose, the optimum mutagenicity (Ames assay) was reached when the molar ratio of glucose was half that of glycine and creatine. By further increasing the molar amount of glucose beyond the optimum level, the mutagenicity was reduced.

Skog and Jagerstad (1990) proposed that the inhibition of heterocyclic amines from sugars was an effect of the interaction of the Maillard reaction products with creatine. This was supported by data showing a lower recovery of unreacted creatine with increasing concentration of glucose. In addition, when 5-hydroxymethyl-2-furfural, a Maillard reaction product, was added to the model systems at 1, 10 and 25% of the molar amount of creatine, the mutagenicity was reduced with increasing concentration. The amount of recoverable unreacted creatine was lower when the highest amount of 5-hydroxymethyl-2-furfural was added.

Efect of glucose, dodecanal and glycolaldehyde on mutagen formation in fried ground beef - Thermal oxidation of lipids and the Maillard reaction occur simultaneously when beef is cooked. Whitfield (1992) extensively reviewed the formation of volatile compounds from the interaction of the

Maillard reaction and products of lipid oxidation. This study focused on the effect of adding products of lipid oxidation and the Maillard reaction on the formation of heterocyclic amines in fried ground beef. Glucose was added in order to increase the extent of browning via the Maillard reaction. Dodecanal, although not a common product of lipid oxidation, and glycolaldehyde, served as representative aldehydes from lipid oxidation and the Maillard reaction, respectively. Both glucose and dodecanal, and the equimolar mixture of the two, were effective in reducing the formation of PhIP and MeIQx. The amount of PhIP present in the fried ground beef was reduced from 8.1 ± 6.1 ng/g to 2.4 ± 1.0 ng/g (cooked weight) when glucose was added to the meat. Smaller amounts (1.3 ± 1.5) of PhIP were detected when dodecanal was added to the ground beef, while no PhIP was detected when a mixture of glucose and dodecanal was added. However, the reductions were not significant at $P < 0.10$ because of the large standard deviation associated with the PhIP concentrations in the control samples (Table 2).

MeIQx concentrations in the fried patties appeared to be reduced by the addition of glucose and dodecanal. Only the dodecanal treatment produced significant ($P < 0.10$) reductions when the data were statistically analyzed. The combination of dodecanal and glucose virtually eliminated MeIQx and PhIP formation. However, no statistical treatment of these data was possible because standard deviations were not derived (Table 2).

Glycolaldehyde, on the other hand, appeared to only

slightly reduce the formation of PhIP and MeIQx in the fried ground beef. The concentrations of PhIP and MeIQx formed in the fried ground beef containing glycolaldehyde were 6.2 ± 0.8 ng/g and 2.7 ± 0.4 ng/g cooked beef, respectively (Table 2). Glycolaldehyde might have been ineffective in inhibiting the formation of heterocyclic amines because it is formed as an early carbon breakdown product in the Maillard reaction which contributes to the formation of pyrazine and pyridine molecules (Namiki and Hayashi, 1975; Hayashi and Namiki, 1980). Pyrazine and pyridine molecules could then participate in the formation of heterocyclic amines (Nyhammer, 1986).

Results of these studies indicate that lipid oxidation may suppress heterocyclic amine formation. Breakdown products from lipid oxidation such as carbonyls, may react with amino acids, thereby reducing the extent of browning. This would result in the formation of smaller concentrations of pyrazines and pyridines (Farmer and Mottram, 1990), compounds that have been suggested to be intermediates in the formation of heterocyclic amines (Nyhammer, 1986) It would then follow that heterocyclic amine formation would also be reduced.

Alternatively, the interaction of carbonyls with amino acids would reduce the availability of the latter compounds to react with creatine in the muscle tissue, again reducing the formation of heterocyclic amines.

Effect of tristearin and sunflower oil on heterocyclic amine formation in heated model systems containing phenylalanine and creatine - To investigate the possible role of thermal lipid oxidation on heterocyclic amine formation in fried beef, saturated and unsaturated lipids were heated in model systems containing phenylalanine and creatine.

Previous studies have shown that PhIP is formed on heating phenylalanine and creatine (Chapter 3). In this study, when phenylalanine and creatine were heated, 655 ± 66 ng of PhIP/g were formed. The addition of tristearin to the model system only slightly reduced the concentration of PhIP to 581 ± 58 ng/g (Table 3). Tristearin is composed of three molecules of stearic acid (18:0) attached to a glycerol moiety. On the other hand, when an unsaturated fat (sunflower oil) was added to the model system containing phenylalanine and creatine, the formation of PhIP was significantly ($P < 0.005$) reduced to approximately 92 ± 15 ng/g (Table 3). Sunflower oil is composed of 52% linoleic acid and 29% oleic acid (Mattil et al., 1964). The unsaturated fatty acids in sunflower oil may have broken down during heating to produce carbonyls that reacted with phenylalanine, thereby reducing the formation of heterocyclic amines. Mottram (1987) has described thermal oxidation of lipids and the broad spectrum of compounds, including carbonyls, that are formed.

Overall, these studies indicate that formation of heterocyclic amines is not an independent process in meat. Other reactions that occur during the cooking of meat such

as thermal lipid oxidation influence their formation. A delicate balance appears to exist between optimal conditions to suppress their formation. Further studies are needed to comphehensively evaluate the relationship between lipid oxidation and heterocyclic amine formation and the inhibitory role of MRPs at concentrations likely to prevent lipid oxidation in cooked meats.

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Table 2: The effect of the addition of glucose, dodecanal, and glycolaldehyde on the formation of MeIQx and PhIP in fried ground beef.[1,2,3]

	MeIQx ng/g beef	PhIP ng/g beef
Control	3.6 ± 2.8[a]	8.1 ± 6.1[c]
Glucose	1.0 ± 0.5[a]	2.4 ± 1.0[c]
Glycoaldehyde	2.7 ± 0.4[a]	6.2 ± 0.8[c]
Dodecanal	0.5 ± 0.7[b]	1.3 ± 1.5[c]
Glucose & Dodecanal	n.d.	n.d.

[1] Each value represents the mean of three samples ± standard deviation.
[2] Mean values in columns with different superscripts are significantly different at $P < 0.10$ for MeIQx and $P < 0.10$ for PhIP.
[3] n.d.: not detectable; limit of detection of 0.10 ng/g for MeIQx and 0.02ng/g for PhIP.

Table 3: The effect of adding tristearin and sunflower oil to model systems containing phenylalanine and creatine on the formation of PhIP.[1,2,3]

Control	Tristrearin	Sunflower oil
655 ± 66[a]	581 ± 58[a]	92 ± 15[b]

[1] Each value represents the mean of at least two samples ± standard deviation.
[2] Means with different superscripts are significantly different at $P < 0.005$.
[3] Values are expressed as ng PhIP/model system.

CHAPTER FIVE

THE EFFECT OF LIPID OXIDATION ON HETEROCYCLIC AMINE FORMATION IN GROUND BEEF

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Abstract

A variety of chemical reactions occur when ground beef is cooked. The possible interaction between lipid oxidation and heterocyclic amine formation was the focus of this study. Oxidized and fresh ground beef patties containing 27% fat were fried and analyzed for heterocyclic amine content. Two heterocyclic amines, MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) were detected in the cooked meat. The fried oxidized ground beef contained 21% less MeIQx and 30% less PhIP than the freshly cooked meat. For further comparison, oxidized and unoxidized lard were added to ground sirloin tips to give a total fat content of 30%. The fried ground beef containing oxidized lard contained 40% less MeIQx and 56% less PhIP than the ground beef containing the unoxidized lard. In addition, oxidized lard, when added to water-washed muscle fibers containing phenylalanine and creatine, also reduced the formation of PhIP by 60%.

Introduction

Mutagenic heterocyclic amines such as IQ (2-amino-3-methylimidazo[4,5-f]quinoline), MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline), DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-f]pyridine) have been identified in fried ground beef patties (Hargraves and Pariza, 1983; Felton et al., 1986; Felton and Knize, 1990). The formation of these compounds is thought to involve a free radical process (Pearson et al., 1992).

The formation of these mutagens is influenced by a number of factors including the fat content of the beef patties (Chen et al., 1990). Using the Ames mutagenic assay with Salmonella typhimurium TA98, Spingarn and Weisburger (1979) determined that increasing the fat content of beef patties from 7% to 15% enhanced the mutagenicity of the cooked meat. When additional beef fat (up to 30%) was added to the patties, there was a decrease in mutagenicity which was attributed to dilution effects because of the increase in volume. Similarly, Knize et al. (1985) demonstrated that the overall mutagenicity (Salmonella typhimurium TA 1538) of fried ground beef was reduced from 230,000 to 150,000 revertants/kg raw meat when the fat content was increased from 15 to 30%. The mutagenicity in the cooked beef was attributed to three mutagens: IQ, MeIQx, and 4,8-DiMeiQx.

Although fat content is important in heterocyclic amine formation in cooked meat, fat oxidation may also play an

important role since the process is a free radical one and is inhibited by phenolic antioxidants (Nawar, 1985; Gray and Pearson, 1987). Antioxidants have been shown also to reduce the formation of mutagens in fried meat. Wang et al. (1982) reported that butylated hydroxyanisole effectively reduced the mutagenicity of fried ground beef when added before cooking. The inhibitory effects of antioxidants were more extensively studied by Chen et al. (1992). At 0.1% (w/w) of the fat content, butylated hydroxyanisole, propyl gallate and tertiary butylhydroquinone reduced the concentration of IQ in fried ground beef by 80, 85 and 90%, respectively.

It is well established that thermal oxidation of fat during the cooking of meat affects the generation of specific Maillard reaction products (Mottram and Edwards, 1983). It has also been shown that thermal oxidation also influences the formation of heterocyclic amines (Chapter Four). It remains to be determined whether oxidized fat in meat systems has an effect on the these mutagens.

The objective of the present study was to investigate whether an interaction exists between lipid oxidation and heterocyclic amine formation in meat and model systems.

Material and Methods

Materials

Freshly ground beef (hamburger and sirloin tips) was obtained from a local supermarket and used within one hour of purchase. Lard was also purchased at a local supermarket. Extrelut-20 columns were obtained from EM

Separations (Gibbstown, NJ). Bond-Elut PRS (500 mg) and C18 (100 mg) cartridges were purchased from Varian Inc. (Harbor City, CA). Creatine and phenylalanine were purchased from Sigma Chemical Company (St. Louis, MO). All solvents were high performance liquid chromatographic (HPLC) or glass-distilled reagent grade. The heterocyclic amine standards (IQ, MeIQ, MeIQx, DiMeIQx, TriMeIQx {2-amino-3,4,7,8-tetramethyl-imidazo- [4,5f]quinoxaline} and PhIP) were obtained from Toronto Research Chemicals (Toronto, Canada).

Methods

Initiation of lipid oxidation in raw ground beef - A standard size petri dish (9 cm dia. x 1.5 cm thickness) was used to form 75g ground beef patties (hamburger) containing 27% fat, as determined using a Hobart F101 fat tester (Liu, 1991). In order to promote lipid oxidation, the patties were left uncovered and placed under white fluorescent light in a walk-in cooler at 4°C for 4 days. To serve as a control, additional ground beef patties were vacuum-packaged in polyethylene laminated nylon pouches, 3mm thick with an oxygen transmission rate of 9 ml/m^2 for 24 hours at 4 °C (Koch, Kansas City, MO), placed in a freezer at -20°C, and then thawed overnight on the 4^{th} day in the cooler. The extent of lipid oxidation of the patties was measured using the TBA test (Tarladgis et al., 1960), as modified by Crackel et al. (1988). Results were determined as TBARS (thiobarbituric acid reactive substances) values, with the values being expressed as mg malonaldehyde equivalents/kg of

meat. Three replicates for each treatment were analyzed for heterocyclic amine content.

Oxidation of lard and addition to ground beef sirloin tips - Lipid oxidation was initiated by adding 50 mg of ferrous sulfate to 500 g of lard held at 40°C, bubbling air into the melted lard and stirring, with a stir bar, for 4 days. The lard was then allowed to solidify at room temperature and the extent of oxidation was measured using the TBA test. The lard was subsequently added to the ground beef containing 4% fat so that the total fat content of the mixture was 30%. Ground sirloin patties (75 g) were then formed in a standard size petri dish (9 cm dia. x 1.5 cm thickness). Three replicates for each treatment were analyzed for heterocyclic amine content.

Water-washed muscle fibers - A water-washed muscle systme, used frequently to simplify lipid oxidation studies in meat (Monahan et al., 1993) was chosen as the substrate to study the role of lipid oxidation in heterocyclic amine formation. Water-soluble substances were extracted by blending 250 g of the raw meat with 250 ml of deionized water in a Waring blender at speed 6 for 1 minute. The water was previously chilled to 4°C before use. After blending, the mixture was strained through a cheesecloth in order to separate the residue from the water extract. The residue was extracted four more times with 250 ml aliquots of water. All work, except the blending, was performed in a walk-in cooler at 4°C.

Phenylalanine (1% w/w) and creatine (5% w/w) were added to the muscle fibers to serve as precursors for the formation of PhIP. Fresh or oxidized lard was also added to the residue so that the total fat content of the water-washed fibers was 30%. Fifty gram patties were prepared using a standard size petri dish (9-cm dia. x 1.5-cm thickness).

Frying conditions and extraction - All patties were fried for six minutes on each side at 204°C in an aluminum, teflon-coated electric frying pan without a lid. The heterocyclic amine content of the fried patties was determined using essentially the method developed by Gross (1990). Modifications in amounts of solvents used to elute the heterocyclic amines from the various columns were made in order to improve the recovery of the heterocyclic amines. In addition, a vacuum manifold was used to elute the compounds from all of the columns utilized in the extraction process. Details of this procedure have been presented in Chapter 2.

Statistical Analysis: Statistical analysis of the heterocyclic amine content of fried patties was based on three replicates for each treatment for the ground beef patties and three replicates for each treatment form the water-washed muscle fiber systems. The results were analyzed by a statistical computer program MSTAT developed at Michigan State University (Department of Crop and Soil Sciences) for a one-way analysis of variance (ANOVA). The F-test was used to determine significant differences between

the mean squares of the samples and the t-test was used to determine the significance of the treatment compared to the control (Steele and Torrie, 1980).

Results and Discussion

Heterocyclic amine content of fried ground beef (hamburger) - Two heterocyclic amines, MeIQx and PhIP, were identified in the extract of the fried ground beef patties. On frying the ground beef that was not exposed to fluorescent light (TBARS value of 0.21 ± 0.03), concentrations of 7.0 ± 0.4 ng/g and 4.0 ± 0.8 ng/g cooked meat were obtained for MeIQx and PhIP,corrected for recoveries of 54% and 30% as determined in Chapter Two, respectively (Table 1). These concentrations fall within the range cited by other investigators (Hargraves and Pariza, 1983; Murray et al., 1988; Felton et al., 1986; Felton and Knize, 1990). PhIP is the most abundant heterocyclic amine found in cooked meat with reported concentrations ranging from 0 to 78 ng/g. Concentrations reported for MeIQx fall in the range, 0 to 10 ng/g (Knize, personal communication).

Influence of lipid oxidation on heterocyclic amine formation - Exposure to fluorescent light increased the TBARS value of ground beef from 0.21 to 0.79 ± 0.02, thus indicating that some oxidation had taken place. This small increase in lipid oxidation appeared to reduce the formation of heterocyclic amines in the fried ground beef. Ground beef patties subjected to fluorescent light contained 21% less

MeIQx and 30% less PhIP than in the control patties. The MeIQx reductions were significantly different at $P < 0.1$ level. The concentration of MeIQx and PhIP averaged 5.5 ng/g ± 1.3 ng and 2.8 ± 1.6 ng/g (cooked weight), respectively (Table 1).

When oxidized lard (TBARS value of 2.62 ± 0.24) was added to ground sirloin tips to increase the fat content of the patty to 30%, the amount of PhIP and MeIQx formed on cooking the meat was reduced. A 56% reduction in PhIP and a 40% reduction in MeIQx occurred (Table 2). PhIP was reduced from 16.0 ± 6.9 ng/g to 6.9 ± 2.6 ng/g, while, MeIQx was reduced from 5.2 ± 0.2 ng/g to 3.1 ± 0.8 ng/g. These reductions were significant at the $p < 0.1$ and $p < 0.01$ levels, respectively.

Results of these studies indicate that lipid oxidation appears to suppress heterocyclic amine formation. Breakdown products from lipid oxidation such as carbonyls, including hexanal, could react with Maillard reactants (e.g. amino acids) to reduce the extent of browning, therefore producing less pyrazines and pyridines (Farmer and Mottram, 1990). These latter compounds have been suggested to be intermediates in the formation of heterocyclic amines (Nyhammer, 1986) It would then follow that heterocyclic amine formation would also be reduced. Alternatively, the interaction of carbonyls with amino acids would reduce the availability of the latter compounds to react with creatine in the muscle tissue, again reducing the formation of heterocyclic amines.

The effect of adding lard (oxidized and unoxidized) to water-washed muscle fibers - When unoxidized lard (TBARS value of 0.8 ± 0.02) was added to the water-washed residue of muscle fibers containing added creatine and phenylalanine, 484 ± 117 ng PhIP/g muscle fiber was formed on frying. When the more oxidized lard sample (TBARS value of 3.0 ± .42) was added to the muscle fibers, the amount of PhIP formed in the fried fibers was 75% smaller (Table 3). The PhIP reductions were significant at the $P < 0.06$ level.

These studies indicate that lipid oxidation products appear to suppress the formation of heterocyclic amines in fried ground beef patties. Previous studies (Chapter Four) have shown that the addition of aldehydes to ground beef before frying, reduces the formation of MeIQx and PhIP in the cooked meat. Further studies are needed to validate these preliminary data and to investigate further the possible relationship between lipid oxidation and heterocyclic amine formation. Particular attention should be paid to the role of iron, both heme and non-heme, in this regard as iron plays a pivotal role in lipid oxidation and has also been reported to increase the formation of mutagenic compounds in model systems (Taylor et al.,1986).

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Table 1: The effect of lipid oxidation on the formation of heterocyclic amines, MeIQx and PhIP, in fried ground beef patties.[1,2,3,4]

	MeIQx ng/g beef	PhIP ng/g beef
Control	7.0 ± 0.4	4.0 ± 0.8
Oxidized	5.5 ± 1.3	2.8 ± 1.6

[1] Each value represents the mean of three samples ± standard deviation.
[2] Control and oxidized hamburger differ significantly in MeIQx at $P < 0.1$.
[3] Control and oxidized hamburger do not differ significantly in PhIP at $P > 0.1$.
[4] Average TBA value for control hamburger was 0.21 ± 0.03 and for oxidized hamburger was 0.79 ± 0.02.

Table 2: The effect of adding oxidized lard on the formation of heterocyclic amines, MeIQx and PhIP, in fried ground sirloin patties.[1,2,3,4]

	MeIQx ng/g beef	PhIP ng/g beef
Control	5.1 ± 0.2	16.0 ± 6.9
Oxidized	3.1 ± 0.8	6.9 ± 2.6

[1] Each value represents the mean of three samples ± standard deviation.
[2] Control (unoxidized lard) and oxidized lard, fried ground sirloin patties differ significantly in MeIQx at $P < 0.01$.
[3] Control (unoxidized lard) and oxidized lard, fried ground sirloin patties differ significantly in PhIP at $P < 0.1$.
[4] Average TBA value for control lard was 0.14 ± 0.04 and for oxidized lard was 2.62 ± 0.24.

Table 3: The effect of oxidized lard on the formation of heterocyclic amine, PhIP, in fried water-washed muscle fiber model systems[1,2,3]

	PhIP ng/g muscle fiber
Control	484 ± 117
Oxidized	146 ± 34

[1] Each value represents the mean of two samples ± standard deviation.
[2] Control and oxidized lard model systems differ significantly in PhIP concentration at $P < 0.06$.
[3] Average TBARS value for control lard was 0.8 ± 0.02 and for oxidized lard was 3.0 ± 0.42.

SUMMARY AND CONCLUSIONS

A series of studies was designed to investigate the influence of the Maillard reaction and lipid oxidation on the formation of heterocyclic amines in meat and models systems.

A procedure was developed for confirming the identity of heterocyclic amines extracted from meat and model systems by using gas liquid chromatography-mass spectrometry. Pentafluoropropionic acid anhydride (PFPPA) was used to derivatize the compounds of interest. Using conventional EI mass spectrometry, the identities of the derivatized heterocyclic amines were ascertained based on fragmentation patterns. This procedure proved to be advantageous over other derivatization methods in that it only took 30 minutes for derivatization, no base was needed to accelerate the reaction, and excess PFPPA was readily removed under nitrogen resulting in a cleaner chromatogram.

Also studied was the reduction in mutagens in ground beef by the addition of phenolic antioxidants. Specific heterocyclic amines, MeIQx and PhIP, were reduced in concentration along with a corresponding reduction in overall mutagenicity of the beef patties when BHA or Vitamin E was added to the meat before frying. No MeIQx was detected in the antioxidant-treated patties, while PhIP concentrations were reduced 56% and 80% by BHA and vitamin E, respectively.

A third study was designed to evaluate the effect of adding a specific Maillard reaction product on the formation of heterocyclic amines in model systems. When 2-methylpyrazine was heated with creatinine and fructose, PhIP was formed. Alanine and creatinine also produced PhIP when heated as a dry mixture. This suggests the possibility of dual pathways for the formation of heterocyclic amines: (1) with sugar, via the Maillard reaction and (2) without sugar, breakdown of the amino acid to form carbon fragments.

The interaction between the Maillard reaction, thermal lipid oxidation and the formation of heterocyclic amines in fried ground beef was investigated. It was concluded that breakdown products of lipid oxidation such as aldehydes suppress the formation of PhIP in meat and model systems by reacting with amino acids, rendering them unavailable to form heterocyclic amines. Glucose also reduced PhIP formation when added to similar model systems.

Finally, the effect of lipid oxidation on mutagen formation in ground beef was also studied. Smaller quantities of PhIP and MeIQx were produced in the ground beef patties having a higher degree of lipid oxidation. In addition, oxidized lard when added to water-washed muscle fibers containing phenylalanine and creatine, appeared to suppress the formation of PhIP. In conclusion, these studies indicate that products of thermal lipid oxidation suppress the formation of heterocyclic amines in meat and model systems. In addition, lipid oxidation in ground beef may also reduce the formation of such mutagens in the fried

patties. Furthermore, the addition of Maillard reaction products may either suppress or have no influence on heterocyclic amine formation. Since thermal oxidation and the Maillard reaction occur simultaneously when meat is cooked, a delicate balance exists between heterocyclic amine formation and inhibition. Any change in this balance would, in theory, influence the formation of heterocyclic amines.

FUTURE RESEARCH

This study focused on the influence of the Maillard reaction and oxidized lipid on the formation of heterocyclic amines in meat and model systems. A procedure was developed for confirming the identity of heterocyclic amines by gas liquid chromatography-mass spectrometry by derivatization with pentafluoropropionic acid anhydride. This procedure could be further developed to quantify heterocyclic amines using selected ion monitoring. Lower detection limits might be achieved by using electron capture negative chemical ionization mass spectrometry.

Antioxidants were shown to inhibit the formation of heterocyclic amines. This study supports the theory that a free radical mechanism is involved in the formation of these compounds. Using electron spin resonance, direct evidence for the free radical nature of heterocyclic amine formation could be obtained. For example, the formation of free radicals from a heated model system containing phenylalanine and creatine could be measured using electron spin resonance. Since these compounds have been shown to form PhIP, the detection of free radicals would suggest that heterocyclic amines are formed via a free radical process.

Using a model system containing 2-methylpyrazine (a MRP), creatinine and fructose, PhIP was formed. It was also shown that an excess of glucose suppressed the formation of heterocyclic amines in ground beef. A balance between

suppression and acceleration exists. Further studies involving the addition of known MRPs to meat and model systems at varying concentrations to study the effect on heterocyclic amine formation would shed more light on this balance.

Breakdown products from thermal lipid oxidation such as aldehydes have been postulated to suppress heterocyclic amine formation. Future studies are needed to validate preliminary data and to investigate further the possible relationship between lipid oxidation and heterocyclic amine formation. Particular attention should be paid to the role of iron, both heme and non-heme, in this regard as iron plays a pivotal role in lipid oxidation and has also been reported to increase the formation of mutagenic compounds in model systems (Taylor et al., 1986).

Finally, systems need to be developed to optimize cooking conditions, yet minimize heterocyclic amine formation.

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