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THE INHIBITION OF HETEROCYCLIC AROMATIC AMINE FORMATION IN GROUND BEEF PATTIES USING TART CHERRIES AND FRACTIONS FROM TART CHERRIES *(PRUNUS CERASUS)*

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By

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ABSTRACT

THE INHIBITION OF HETEROCYCLIC AROMATIC AMINE FORMATION IN GROUND BEEF PATTIES USING TART CHERRIES AND FRACTIONS FROM TART CHERRIES (*PRUNUS CERASUS*)

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The effects of temperature and tart cherry tissue concentration on heterocyclic aromatic amine (HAA) formation in fried ground beef patties and overall mutagenicity were investigated. Tart cherry tissue was added to ground beef patties at three levels (4.0%, 7.5% and 11.5% by weight); the patties were fried at two temperatures (190 °C & 225 °C) for 10min/side. HAAs were isolated by solid phase extraction and quantified by HPLC. Ground beef patties fried at 190 °C with the addition of 11.5% tart cherries showed a reduction in 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) of 53.0% and a reduction in 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine (PhIP) of 87%. The addition of 4.0 and 7.5% cherries showed significant reductions in PhIP. Overall, mutagenicity decreased by 69%. Patties fried at 225 °C with the addition of 4.0, 7.5, and 11.5% tart cherries showed reductions in PhIP formation of 51, 72, and 85%, respectively.

The reductions in HAA formation led us to speculate that anthocyanins, present in tart cherries, may be responsible for inhibiting HAA formation due to their high antioxidant potential. Therefore, the effects of anthocyanins on HAA formation were investigated in a model system and in fried ground beef patties. The model system contained a mixture of three reactants (0.2 mmol phenylalanine, 0.2 mmol creatine, and 0.1

mmol glucose). The three cherry anthocyanins (15 mg), **1** [3-cyanidin 2"-O-β-D-glucopyranosyl-6"-O-α-L-rhamnopyranosyl-β-D-glucopyranoside] (65%), **2** [3-cyanidin 6"-O-α-L-rhamnopyranosyl-β-D- glucopyranoside] (30%), and **3** [3-cyanidin O-β-D-glucopyranoside] (5%), were added to the model system and heated at 180 °C for 30 minutes. Anthocyanins were added to ground beef patties at a dose equivalent to 11.5% (w/w) cherry tissue, and the patties were fried at 225 °C for twenty minutes. No significant inhibition of HAA formation was observed in the model system or ground beef patties with the addition of anthocyanins.

In a third set of experiments, lyophilized Montmorency tart cherries were sequentially extracted with solvents to determine which compound or group of compounds in cherry tissue was responsible for inhibiting the formation of heterocyclic aromatic amines (HAAs). Individual extracts from cherries were added at a dose equivalent to 11.5% (w/w) cherry tissue to ground beef patties and fried at 225 °C. The methanol extract inhibited the formation of PhIP by 62%. Further purification of the methanol extract yielded a fraction containing three sugars (glucose, fructose, and maltose), which inhibited PhIP formation by 49%. Further studies showed no significant difference between using a combination of three sugars or using glucose alone at the same concentration (0.134% w/w). We conclude that reducing sugars are primarily responsible for the HAA inhibitory effect of cherries.

Analysis of several meat samples for concentration of precursor compounds glucose and creatine indicated considerable variation. Instead of promoting HAA formation in these samples as predicted by various models, glucose was inhibitory in meat at all levels tested. Several possible mechanisms for inhibition by glucose are discussed.

To My Wife

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INTRODUCTION

Over the past twenty years heterocyclic aromatic amines (HAAs) have been extensively studied, since their discovery by Sugimura et al. (1977). HAAs are commonly found on the charred surfaces of meat and fish products cooked at temperatures above 150 °C. The most common HAAs identified in fried ground beef are: 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), DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine).

HAAs have been divided into two categories (pyrolytic and thermic) based upon the temperatures at which they form. Pyrolytic mutagens are formed at temperatures above 300 °C and are characterized by a pyridine ring with an amino group attached (Skog, 1993). Thermic mutagens, also called aminoimidazoazaarenes, are formed at temperatures below 300 °C. These compounds can be subdivided into four categories: quinolines, quinoxalines, pyridines, and furopyridines (Skog, 1993).

In long term feeding studies of rats and mice, HAAs have caused tumors in the liver, urinary bladder, small and large intestines, skin, oral cavity, mammary gland, prostate, forestomach, lung, colon, and lymphoid tissue (Wakabayashi and Sugimura, 1998). Epidemiological studies also suggest that dietary intake puts humans at risk for developing cancer. Felton et al. (1997) estimated that the risk of developing cancer may range from 1 per 10,000 to 1 in 50, depending upon the amount of well-done muscle meats a person ingests and the person's genetic susceptibility.

The risk to individuals from consumption of HAAs forms the basis for studies on the mechanism of HAA formation, which would lead to interventions that minimize dietary consumption of HAAs. Based on studies in model systems, three naturally occurring compounds present in meat (creatine, amino acids, and hexoses) were suggested by Jagerstad et al. (1983a) to be precursors in formation of the imidazoquinoline or imidazoquinoxaline mutagens. Heating of creatine and certain amino acids without the presence of sugars has also produced HAAs, although the presence of substoichiometric amounts of reducing sugars promote HAA formation. Jagerstad et al. (1983b) proposed that IQ compounds are formed via the Maillard reaction through vinylpyrazine, vinylpyridine and aldehyde formation. Namiki and Hayashi (1981) demonstrated the formation of a free radical, N, N'-disubstituted pyrazine cation, by early carbon fragmentation prior to formation of the Amadori product.

Britt et al. (1998) found that the addition of tart cherry tissue (11.5% w/w) to ground beef patties prior to cooking significantly reduced the oxidation of lipids in both cooked and uncooked hamburger patties. The antioxidant effect of tart cherries on reducing lipid oxidation, coupled with previous studies suggesting that antioxidants inhibit HAA formation (Barnes et al., 1983; Chen, 1988; Weisburger et al., 1994; Kato et al., 1996; Oguri et al., 1998), led them to conduct studies on the effects of cherries on HAA formation. They found that the addition of whole cherry tissue (11.5% w/w) to ground beef patties prior to cooking resulted in a substantial reduction of HAA formation. The overall inhibition of HAA formation was 78.5% for Montmorency cherries. Wang et al. (1999c) found that the antioxidant activities of anthocyanins from Montmorency and Balaton tart cherries were comparable to those of butylated hydroxyanisole (BHA),

butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ). Polyphenols such as 7-dimethoxy-5,8,4-trihydroxyflavone and quercetin 3-rhamnoside and other flavonoids isolated from extracts of tart cherries have also been shown to be good antioxidants (Wang et al., 1999b). **Therefore, we hypothesize that the strong antioxidant activity of anthocyanins plays a key role in inhibiting the formation of HAAs in ground beef patties.**

Although Britt et al. (1998) established that the addition of whole cherry tissue (11.5% w/w) to ground beef patties prior to cooking inhibits HAA formation, they did not investigate other levels of cherry addition or establish overall mutagenicity of the cooked product. Therefore, our **first objective** was to investigate the formation of HAAs in fried ground beef patties cooked at two temperatures, to establish a dose–response relationship of added cherry tissue to ground beef patties, and to determine overall mutagenicity of these ground beef patties. After completion of our first objective, our results and Britt et al. (1998) findings coupled with preliminary data (Gomaa, unpublished data) suggested that anthocyanins were the primary inhibitory factors, acting as antioxidants. To test this hypothesis, our **second objective** was to evaluate the effectiveness of pure anthocyanins in a model system and in meat. After completion of the second objective and disproving our original hypothesis, we formed a new hypothesis that other compounds in cherries, acting as antioxidants, were responsible for inhibiting HAA formation by scavenging free radicals in the Maillard reaction pathway. Thus, our **third objective** was to determine, by sequential solvent extraction of whole cherries, which compound or group of compounds was responsible for the inhibition of HAA formation in ground beef patties.

LITERATURE REVIEW

HETEROCYCLIC AROMATIC AMINES IN COOKED FOODS

Categories. Heterocyclic aromatic amines (HAAs) have been divided into two categories (pyrolytic and thermic) based upon the temperatures at which they form. Pyrolytic mutagens are formed at temperatures above 300°C and are characterized by an amino group attached to a pyridine ring (Skog, 1993). Pyrolytic mutagens are not commonly found in the Western diet because the high temperatures required for their formation are seldom used in cooking or processing. Thermic mutagens, also called aminoimidazoazaarenes, are characterized by an amino group attached to an imidazole ring and are formed at temperatures below 300°C. These compounds can be subdivided into four categories: quinolines, quinoxalines, pyridines, and furopyridines (Skog, 1993). The most common HAAs found in food are: 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), DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine). The structures of these HAAs are illustrated in Figure 1.

Quinolines. The HAAs 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) were first isolated and identified in broiled sardines (Kasai et al., 1980). These compounds were subsequently identified in various food products including fried ground beef (Barnes et al., 1983; Felton et al.,

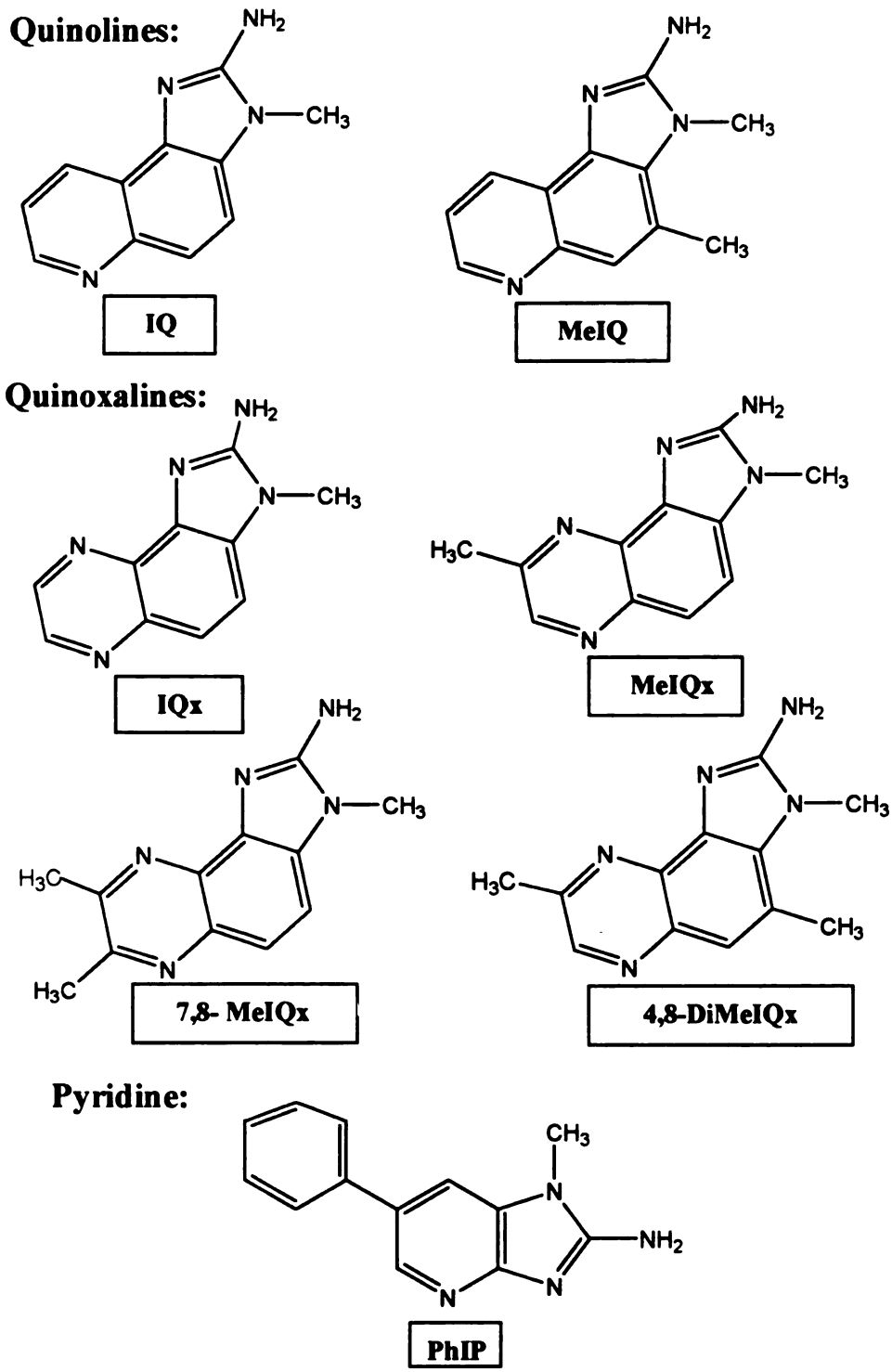


Figure 1. Chemical structures of commonly formed HAAs in cooked foods (Skog, 1993).

1984;), broiled ground beef (Sugimura et al., 1988), charbroiled ground beef (Johansson and Jagerstad et al., 1994), various fishes (Johansson and Jagerstad et al., 1994; Yamaizumi et al., 1986; Zhang et al., 1988), and fried pork (Skog et al., 1995; Vahl et al., 1988). The concentration of IQ in fried ground beef has been reported as ranging from 0.5 to 20 ng/g (Barnes et al., 1983; Felton et al., 1986; Turesky et al., 1988), while only trace amounts of MeIQ have been reported (Felton et al., 1986; Yamaizumi et al., 1986).

Quinoxalines. Three distinct quinoxaline compounds have been identified in food. The first two quinoxalines, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx) were isolated from fried ground beef by Negishi et al. (1984). Grivas et al. (1985) isolated the third quinoxaline, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) in fried ground beef. MeIQx and 4,8-DiMeIQx have subsequently been found in a variety of foods including fried bacon, fried and barbecued chicken, fish, fried turkey, fried reindeer, and fried meatballs (Gross et al. 1993; Murray et al., 1993; Murkovic et al., 1997; Sinha et al., 1995; Skog et al., 1995). Concentrations of MeIQx in fried ground beef range from < 0.1 to 16.4 ng/g (Knize et al., 1997; Skog et al., 1995; Felton et al., 1994; Thiebaud et al., 1995). The highest concentration of MeIQx (45ng/g) in a meat product was found in fried pork bacon (Thiebaud et al., 1995). Concentrations of 4,8-DiMeIQx in fried ground beef range from < 0.1 to 4.5 ng/g (Thiebaud et al., 1995; Skog et al., 1995). 7,8-DiMeIQx has been found in fried ground beef (0.7 ng/g) (Turesky et al., 1988) and roasted eel (5.3 ng/g) (Lee and Tsai, 1991).

MeIQx and 4,8-DiMeIQx have been produced in several model systems containing creatinine, one sugar (glucose, fructose or ribose) and one amino acid

(alanine, glycine, lysine, phenylalanine, or threonine). In contrast, 7,8-DiMeIQx has only been isolated in model systems containing glucose and glycine (Johansson and Jagerstad, 1993; Neigishi et al., 1984; Skog and Jagerstad, 1990).

Pyridines. The most abundant HAA formed in cooked meat is 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP). This compound was first isolated from the crust of fried ground beef by Felton et al. (1986). PhIP has been identified in a variety of foods including bacon, ground beef, steak, chicken, meatballs, and turkey (Gross et al.,1993; Felton et al., 1994; Skog et al., 1995; Sinha et al., 1995; Murkovic et al., 1997). The concentrations of PhIP in fried ground beef range from < 0.1 to 68.0 ng/g (Knize et al., 1997; Thiebaud et al., 1994). In comparison to other foods, barbecued chicken yielded the highest concentrations of PhIP with levels ranging from 27 – 480 ng/g (Sinha et al., 1995).

PhIP has also been produced in many model systems containing creatine, glucose, and several amino acids. A model system with and without glucose plus creatine, phenylalanine yields large quantities of PhIP (Felton and Knize, 1990; Overvik et al., 1989; Skog and Jagerstad, 1991). Overvik et al. (1989) found that using leucine as the amine source in a model system instead of an aromatic amino acid yielded only small quantities PhIP.

PhIP has not only been isolated from muscle foods and model systems containing creatine, amino acids, and sugars, but also from other items including: diesel exhaust, cigarette smoke, beer, and wine. Manabe et al. (1993) reported the concentrations of

PhIP in beer and wine to be 14.1 ng/l and 30.4 ng/l, respectively. The concentration of PhIP in cigarette smoke was 16.4 ng/cig (Manabe et al., 1991).

Furopyridines. Several unidentified mutagenic HAAs are present in cooked food products (Skog et al., 1992b). Two mutagenic compounds with molecular weights (MW) of 202 and 216 have been isolated from fried meat emulsion, fried minced beef, and fried minced pork (Becher et al., 1988; Felton et al., 1986; Gry et al., 1986). Knize et al. (1990) identified a methylimidazo-furopyridine containing oxygen (MW 202) from a mixture of fried minced meat, creatine, and milk. Another new HAA, 2-amino-6-(4-hydroxyphenyl)-1-methylimidazo[4,5-*b*]-pyridine (4-OH-PhIP) was identified in broiled or fried beef (Kurosaka et al., 1992; Reistad et al., 1997) and in a model system containing glucose, tyrosine, and creatine (Wakabayashi et al., 1995). Recently, the structure of methylimidazo-furopyridine (MW 202) was determined to be 2-amino-(1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*])pyridine (IFP) and found in a model system containing a mixture of amino acids, creatine, and glucose (Pais et al. 2000). Analysis of well-done meats purchased from restaurants showed that IFP levels range from < 0.1 to 46 ng/g.

MECHANISM OF HETEROCYCLIC AROMATIC AMINE FORMATION

Quinolines and Quinoxalines. The mechanism by which HAAs form is not completely understood, but model system studies have provided clues. It is generally accepted that HAAs form through intermediates of the Maillard or nonenzymatic browning reaction (Spingarn and Garvie, 1979; Powrie et al., 1981; Wei et al., 1981).

Creatine/creatinine and amino acids are necessary precursors in the formation of HAAs. Reducing sugars enhance the production of HAAs by promoting the formation of intermediates like pyrazines, pyridines, and aldehydes (Jagerstad et al., 1983a).

In the Maillard reaction, reducing sugars and primary amino groups from proteins, amino acids or peptides combine to form a glycosylamine, which then undergoes an Amadori rearrangement to yield a 1-amino-2-keto sugar (Hodge, 1953). This sugar derivative undergoes a series of reactions to form a variety of compounds (aldehydes, ketones, and melanoidin pigments) via either of two pathways (methyl α-dicarbonyl or 3-deoxyhexosone). Pyrazines and pyridines can be produced by the Strecker degradation pathway in which the α-dicarbonyls from the Maillard reaction react with amino acids. This is a key reaction in the production of flavors (Rizzi, 1994).

Jagerstad et al. (1983a) proposed a hypothesis for the mechanism of HAA formation. Pyridines and pyrazines, formed via the Maillard reaction and Strecker degradation react with an aldehyde to form the quinoline or quinoxaline portion of the HAA, respectively. Creatine, when heated, is dehydrated and cyclized to form creatinine which condenses with the aldehyde to form the IQ or IQx-type HAA (Figure 2). Jagerstad et al. (1983b) provided support for this hypothesis by using a model system consisting of creatinine, glucose, and glycine or alanine dissolved in diethylene glycol/ water (86/14). After boiling under reflux at 130 °C for 2 hrs, MeIQx and 7,8- DiMeIQx were identified. These products showed high mutagenic activity in the Ames assay, whereas heating the reactants two by two produced products with only weak mutagenic activity. Mutagenic activity increased 50% when exogenous pyridines or pyrazine were

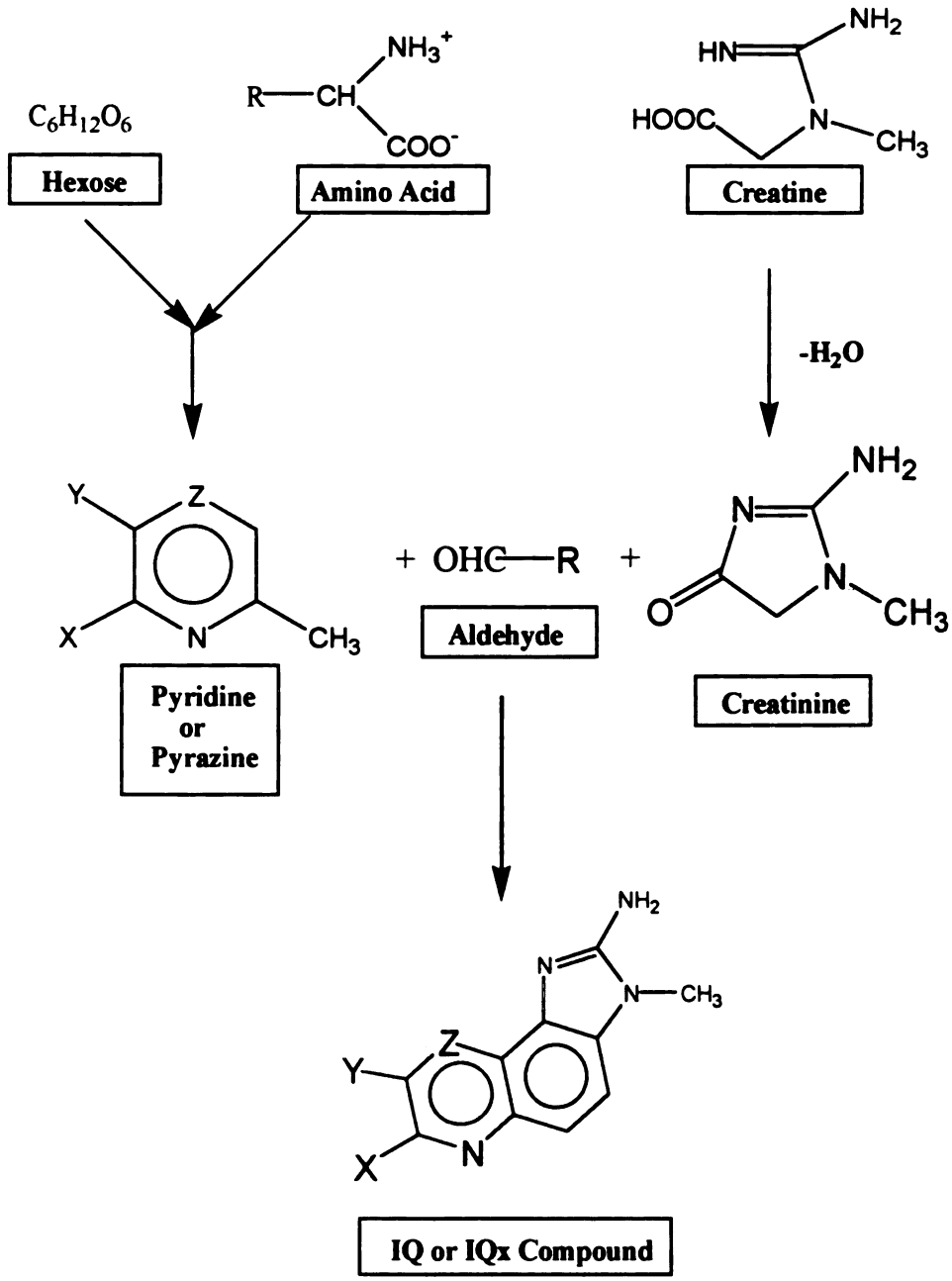


Figure 2. Theoretical reaction pathway for formation of IQ and IQx compounds. R, X, and Y may be H or CH_3: Z may be CH or N (Jagerstad et al. 1983a)

added to the reaction mixture. The theory of Jagerstad et al. (1983a) assumes that condensation occurs between pyridines or pyrazines and aldehydes, followed by ring closure with creatinine. Lee et al. (1994) gave supporting evidence by showing that IQ was formed in a model system containing 2-methylpyridine, creatinine, and various aldehydes as reactants.

Namiki and Hayashi (1981, 1983) investigated the formation of novel free radical products in the early stages of the Maillard reaction. They reported the formation of the N,N'-disubstituted pyrazine cation by early carbon fragmentation prior to the Amadori product. The radical products were assumed to form by the condensation of two molecules of the two-carbon enaminol compounds, which are formed either directly from the Schiff base products (route B) or indirectly through the reaction of glycolaldehyde with amino compounds (route A) (Figure 3). They also found that C-2 and C-3 fragments were produced prior to the Amadori rearrangement by a reverse-aldol reaction of glycosylamine, producing glycolaldehyde alkylimines. These compounds can then be oxidized to form glyoxal monoalkylimines, which produced less free radicals and reacted more slowly than the glycolaldehyde system (Namiki and Hayashi, 1981; 1983).

Nyhammer (1986) proposed an alternative reaction route that included the same precursors as Jagerstad et al. (1983a), but creatine condenses with an aldehyde and then reacts with a pyridine or pyrazine to yield either an imidazoquinoline or an imidazoquinoxaline. Milic et al. (1993) performed further studies using electron spin resonance studies on HAA formation which confirmed the presence of pyrazine/pyridine free radical cations as possible precursors in HAA formation.

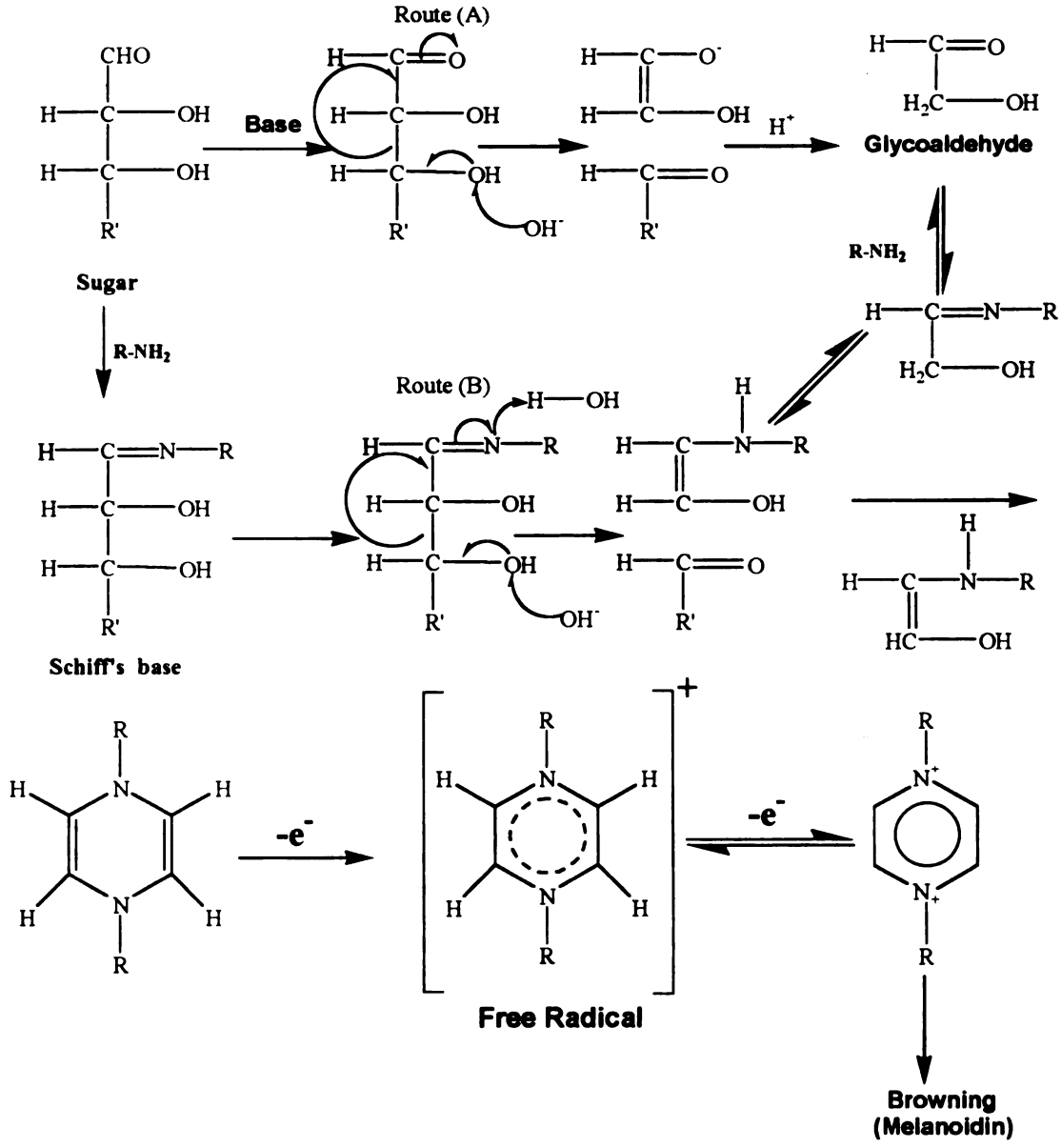


Figure 3. A suggested pathway of browning in Maillard reaction through a free radical intermediate (Namiki and Hayashi, 1981).

Arvidsson et at. (1997) developed a more complex model system to evaluate the kinetics of IQ and IQx compound formation. Their system contained not only glucose and creatinine as precursors, but also all of the amino acids found in bovine meat. The relative ratio of each compound in the model system was adjusted to that of meat, whereas the total concentration of each compound was four-fold greater than that of the meat system. A first-order reaction model and the Eyring equation were fitted to the formation of IQ compounds to obtain rate constants and temperature dependence, respectively. The activation entropy was calculated and found to be negative, indicating that IQ compounds are formed by a bimolecular reaction. They suggested that the rate-limiting step was the reaction between creatinine-aldehyde condensation product and pyrazine.

To further evaluate the kinetics of IQ compound formation, Arvidsson et al. (1999) used a model system containing concentrated meat juice prepared from bovine roast beef. This study verified their earlier findings, which suggested the rate limiting steps in the formation of IQ compounds were bimolecular and followed pseudo-first order kinetics.

There are two proposed reaction pathways for the formation of quinoline and quinoxaline compounds, but evidence is lacking to support one reaction pathway over the other. The two pathways both require the same precursors (creatine, amino acids, and hexoses) and involve the Maillard reaction. However, the two pathways form different intermediate products prior to forming the quinoline or quinoxaline compounds. More research on the intermediate products is required to clarify differences that exist between these two reaction pathways.

PhIP. Felton et al. (1986) first isolated and identified PhIP from the crust of fried ground beef patties. The reaction mechanism by which PhIP is formed has not been determined, but the essential role of various precursors has been demonstrated using model systems. PhIP was produced in a model system containing phenylalanine, creatine and glucose by Shioya et al. (1987). Felton and Knize (1991) demonstrated the importance of phenylalanine and creatine as precursors to the formation of PhIP by dry heating a mixture of ^{13}C-labelled phenylalanine and creatine. PhIP has also been produced from three other amino acids (tyrosine, leucine, and isoleucine) heated separately with creatinine in a model system (Johansson et al. 1995; Manabe et al. 1992; Overvik et al. 1989).

The model system previously described in the quinoline section above was also used to evaluate the formation of PhIP. Arvidsson et al. (1997) calculated the activation entropy and concluded the rate limiting step for PhIP formation was a mono-molecular reaction (activation entropy equals near zero values), whereas the rate limiting step for formation of IQ compounds was a bimolecular reaction (activation entropy equals negative values).

In a subsequent study, Arvidsson et al. (1999) used a concentrated meat juice model system, described previously, to study the kinetics of PhIP formation. In comparison of their two model system studies, they found that formation of PhIP was 8-fold higher in the meat juice model system than the other model system containing only amino acids, glucose, and creatine. They re-evaluated the reactants in relation to creatinine content and showed that PhIP formation was still 4-fold higher, while IQ compounds formed at the same amounts in both model systems.

Arvidsson et al. (1999) concluded that there are some differences between their two studies, but no single factor was responsible for the increased formation of PhIP in the meat juice model system. The amounts of phenylalanine, tyrosine, and isoleucine were lower in the meat juice than in the other model system, indicating the meat juice may contain other precursors or modifying factors that contribute to PhIP formation. This latter study showed low activation enthalpies and negative activation entropies, which indicated a bimolecular rate-limiting step. Thus, the model system consisting of amino acids, glucose, and creatinine was inadequate to characterize the kinetics of formation of PhIP.

MUTAGENICITY OF HETEROCYCLIC AROMATIC AMINES

The Ames *Salmonella typhimurium* mutagenicity assay has been used to test the mutagenicity of HAAs and other representative carcinogens (Table 1). *S. typhimurium* strain TA 98, a detector of frameshift mutations, and strain TA 100, a detector of basepair change mutations are the most common strains used in the assay. HAAs are more likely to produce frameshift mutations and therefore display more mutagenicity towards TA 98. HAAs can also cause mutations and induce chromosomal change in cultured mammalian cells (Nakayasu et al. 1983; Thompson et al 1983). The mutagenic potential of HAAs is obvious upon comparison with other well-known carcinogens. For example, aflatoxin B1 shows an activity of 6,000 rev/ug whereas MeIQ shows an activity of 661,000 rev/ug. Unlike IQ, MeIQ, MeIQx, and DiMeIQx, PhIP displays only weak mutagenic activity in the Ames *Salmonella typhimurium* Assay. However, PhIP which is found in the highest abundance in cooked foods, is more effective in inducing DNA damage in cultured

Table 1. Mutagenicity of HAAs and typical carcinogens in *Salmonella typhimurium* (Sugimura and Sato, 1982; Sugimura et al., 1988)

Compound	Revertants/ug	
	TA 98	TA 100
IQ	433,000	7,000
MeIQ	661,000	30,000
IQx	75,000	1,500
MeIQx	145,000	14,000
4,8-DiMeIQx	183,000	8,000
7,8-DiMeIQx	163,000	9,900
PhIP	1,800	120
Aflatoxin B1	6,000	28,000
Benzo[a]pyrene	320	660

mammalian cells than MeIQx and DiMeIQx (Munro et al., 1993; Lynch et al., 1992; Thompson et al., 1987).

HAAs are metabolically activated by cytochrome P450IA1 and IA2 enzymes located in the liver. HAAs undergo N-hydroxylation by cytochrome P450, followed by esterification to an acetyl or sulfate moiety (Okamoto et al., 1981; Paterson and Chipman, 1987; Synderwine et al., 1987). Turteltaub et al (1990) found that PhIP is metabolically activated by cytochromes P450IA1 and IA2 enzymes. Cytochrome P450IA2 is also the enzyme responsible for the metabolic activation of MeIQ, MeIQx and 4,8-DiMeIQx (Aoyama et al., 1990; Butler et al. 1989). In humans, HAAs initially undergo N-hydroxylation by cytochrome P4501A2 (Turesky et al., 1991). This N-hydroxy arylamine metabolite is O-acetylated in the liver or another target organ by the

polymorphic N-acetyltransferase (NAT2) to form an arylamine-DNA adduct (Sinha et al., 1994).

Compounds that display high mutagenicity in the Ames Assay may or may not be carcinogens. Carcinogenicity must be determined using cell culture or animal studies. Several studies have found tumors in rats and mice induced by IQ, MeIQ, MeIQx, or PhIP. These tumors were found in the liver, forestomach, small and large intestine, mammary gland, skin, lymphoid tissue and colon (Ohgaki et al., 1984; Kato et al., 1988; Esumi et al., 1989; Takayama et al. 1989; Ohgaki et al., 1991). PhIP induces colon cancer in male rats and mammary gland cancers in female rats (Ito et al., 1991.)

RISK EVALUATION OF HETEROCYLIC AROMATIC AMINES

Many animal studies have found that HAAs induce tumors in the large intestine, mammary glands, prostate, liver, forestomach, small intestine, urinary bladder, blood vessels, and skin (Wakabayashi et al., 1993). Correlation between ingestion of cooked food and risk of colon cancer has been reported (Schiffman and Felton, 1990). Therefore, ingestion of these mutagenic compounds needs to be accurately assessed in relation to cancer risk.

The human diet is a complex mixture of foods that contain harmful and protective components that interact with each other. Common cooking procedures of protein-rich food (meat and fish) include: broiling, frying, roasting, and flame grilling which increase the formation of food mutagens including HAAs. Risk assessment for the human population consuming these compounds requires the integration and knowledge of dosimetry, metabolism, carcinogenic potency, and epidemiology (Felton et al., 1997).

The daily intake of HAAs or total daily mutagen intake is low and may range from 5 to 10 *u*g per serving for well done meat (Skog, 1993). Layton et al. (1995) evaluated the ingestion of dietary HAAs and their overall effect on colorectal cancer in the United States. Based on their assessment, the cumulative lifetime risk of developing colorectal cancer was 4.5%, based on a 75-year lifespan. Thus, at most, the ingestion of HAAs would cause 0.25% of all colorectal cancers. Felton et al. (1997) concluded the magnitude of cancer risk was difficult to calculate, but most likely ranged from 1 per 10,000 for the average person to greater than 1 in 50 for individuals ingesting large amounts of well-done muscle meats. Careful interpretation of these risk assessments is advised based upon the generalizations and assumptions (genetic differences, preparation and type of food, daily exposure, etc.) used in these studies.

A recent study focussed on well-done meat intake and breast cancer risk among postmenopausal women was based on individual N-acetyltransferase-2 activity. Polymorphic N-acetyltransferase-2 catalyzes the activation of HAAs via O-acetyltransferase, suggesting that N-acetyltransferase-2 genotypes with high O-acetyltransferase activity (rapid/intermediate acetylator phenotype) may increase the risk of breast cancer in women who consume well-done meat (Deitz et al., 2000). Individuals were subdivided into rapid, intermediate, and slow acetylator phenotypes (Hein et al., 2000). A significant dose response relationship was observed between breast cancer risk and consumption of well-done meat among women with rapid/intermediate N-acetyltransferase-2 genotype that was not evident among women with slow acetylator genotype. Consumption of well-done meat among women with the rapid/intermediate N-acetyltransferase-2 genotype increases breast cancer risk nearly 8-fold when compared to

those who consumed medium or rare meats (Deitz et al., 2000). Marchand et al. (2001) found that exposure to HAAs through consumption of well-done meat increases the risk of colorectal cancer, particularly in individuals who are genetically susceptible (as determined by rapid phenotype of N-acetyltransferase-2).

FACTORS THAT AFFECT THE YIELD OF HETEROCYCLIC AROMATIC AMINES

Time and Temperature. Several researchers have investigated the effects of cooking times and cooking temperatures on the formation of HAAs and mutagenic compounds and found that cooking time and temperature are among the most important factors influencing the formation of HAAs. Skog et al. (1995) cooked a variety of meat products (pork chops, pork belly, bacon, minute steak, sirloin steak, and ground beef) at different temperatures (150, 175, 200, and 225 °C). They observed increased formation of MeIQx, DiMeIQx, and PhIP as the cooking temperature increased. The greatest yield of HAAs was observed at the highest cooking temperature.

Knize et al. (1985) compared ground beef patties cooked at 200 and 300 °C and found that total mutagenic activity in *S. typhimurium* TA 1538 (a frameshift mutation strain) was approximately four times higher in the patties cooked at 300 °C than the patties cooked at 200 °C. Knize et al. (1994) studied the effects of cooking time and temperature on the formation of HAAs in fried beef patties. They fried beef patties at three temperatures (150, 190, and 230 °C) and five cooking times (2, 4, 6, 8, and 10 min/side). They observed an increase in HAA formation as the cooking time and temperature increased. The most abundant HAA produced in fried beef patties was PhIP.

Dramatic increases in HAAs were observed when cooking time was increased from six to ten minutes per side. Increased formation of HAAs with increased cooking temperatures and times was also observed by Balogh et al. (2000).

Fats. Fat has been shown to have both physical and chemical effects on the formation of mutagens. Fat can aid in efficient heat transfer during cooking, dilute mutagen precursors, and serve as a source of antioxidants which may decrease the formation of HAAs.

Knize et al. (1985) examined the role of fat content in mutagen production and specifically its effect on the formation of IQ. They used ground beef patties with three fat contents (8%, 15%, and 30%) and fried patties at two temperatures (180 °C and 240 °C). They found that increasing fat content from 8 to 15% enhanced mutagen activity at both temperatures. Increasing fat content from 15 to 30% decreased mutagen activity by 33%, but had little effect on IQ formation. They suggested that the low mutagenic activity at the low fat content (8%) might be due to the decreased solubility of the mutagen precursors or poor heat transfer in the meat. The slight decrease at the highest fat content was assumed to be the dilution of the mutagen precursors by the increased fat presence in the meat. Holtz et al. (1985) showed oven-baking meat loaves with high fat content took less time to reach an internal temperature of 70 °C than loaves containing less fat, indicating fat as a more efficient heat transfer agent.

The effects of glycerol, various fatty acids (stearic, oleic, linoleic, and linolenic acids) and oils (corn and olive) on the formation of MeIQx in a model system containing glycine, creatinine, and glucose were studied by Johannson et al. (1993). They found that

the addition of these lipids to the model system heated for 10 or 30 min. did not affect the species of the food mutagens formed, but did affect the yield of MeIQx. The heating of the model system with or without the various fatty acids and oils for the first ten minutes, produced similar amounts of MeIQx. The addition of corn or olive oils heated for 30 minutes almost doubled the amount of MeIQx formed, as compared to the amount formed without corn or olive oil present. These results had large variations which made it difficult to make any definitive conclusions about fatty acids and oils on the formation of food mutagens, but these results suggest that in the presence of certain fats cooked over long periods of time that food mutagen formation can increase.

Johannson and Jagerstad (1993) investigated the effects of oxidized deep-frying fat and iron on the formation of food mutagens in a model system. They found that the oxidation status of the fat added to the model system had little effect on the formation of MeIQx, but the fat content significantly enhanced mutagen formation after 30 minutes of heating. They also observed significant increases in MeIQx formation when iron was added to the model system, suggesting that lipids enhance the formation of IQ compounds both by physical and chemical means.

The complex role of fats on HAA formation has been demonstrated in both meat and model system studies. These studies have shown physical and chemical effects of fat on the formation of HAAs. Increasing fat content may dilute the mutagen precursors, aid in the transfer of heat or provide more abundant antioxidants (depending upon the type of fat used) that decrease the formation of HAAs. These effects are evident in both the meat and model systems.

Carbohydrates. The role of sugar in the formation of HAAs is not completely understood, but many studies have provided insight. Skog and Jagerstad (1990) found that glucose at half the molar concentrations of creatine and glycine in a model system produces the highest concentrations of HAAs. Increasing the concentration of glucose to an equimolar ratio with the other two precursors significantly reduces the mutagenicity in the Ames assay and the formation of HAA compounds. Similar results were obtained when glucose was substituted with other sugars (fructose, lactose, and sucrose). The removal of glucose from the model system results in loss of mutagenicity, suggesting that sugar is needed in the formation of HAAs. Sucrose induces a much higher mutagenicity over a wide range of concentrations than the other sugars (glucose, fructose or lactose). Therefore, the inhibitory effect of excess sucrose (≥ equimolar with other reactants) does not inhibit mutagenicity as effectively as the other sugars.

Skog and Jagerstad (1991) investigated the effect of glucose on the formation of PhIP in a model system containing a mixture of phenylalanine, creatinine, and glucose at different ratios. Several mutagens were produced when glucose was half the molar ratio of the other two precursors. When phenylalanine and creatinine were heated without glucose, only low levels of PhIP were produced as a single mutagen. Glucose heated at equimolar ratios or higher resulted in a significant decrease of mutagens.

Skog and Jagerstad (1993) demonstrated that glucose is a precursor in the formation of some HAAs. They heated a mixture of creatinine, threonine, and ^{14}C-labelled glucose. These radioactive carbons originating from glucose were found to be present in MeIQx, 4,8 DiMeIQx, and IQx, providing further evidence that glucose is an important precursor in the formation of imidazoquinoxalines.

The effect of carbohydrates on the formation of HAAs in fried beef patties was investigated by Skog et al. (1992a). Beef patties were mixed with different carbohydrates prior to frying. Glucose, lactose, or powdered milk were individually added at levels of 1, 2, and 4%. All treatments showed significant decreases in mutagenicity. Glucose (4%) or lactose (4%) added to the beef patties with or without golden bread crumbs prior to frying showed the most pronounced inhibitory effect (76–79%) on mutagenicity. The addition of powdered milk containing 4% lactose inhibited mutagenic activity by only 66%. The inhibitory effects of glucose and lactose were more pronounced than the inhibition from the powder milk. Powdered milk and golden bread crumbs have a large water-binding capacity, resulting in less weight loss during frying, which would allow more water to be retained in the patty and less evaporation, resulting in more energy for heating the crust. However, the addition of starch to the patties only slightly inhibited mutagen formation when compared to the patties containing only glucose or lactose, suggesting that inhibition was due to the reactivity of the glucose or lactose.

Although the inhibitory effect of excess sugars (≥ equimolar with other reactants) in meat and model systems has been demonstrated, the mechanism is unknown. Several studies have suggested that with increasing concentration of reducing sugars, the Maillard reaction may favor the formation of inhibitory Maillard reaction products (Skog and Jagerstad, 1990; Skog and Jagerstad, 1991; Skog et al., 1992a;). Therefore, reducing sugars may act as competitive inhibitors for HAA formation. Skog and Jagerstad (1990) suggested that some Maillard reaction products may react with creatine or creatinine, thus competing with HAA formation. They found that the addition of the Maillard reaction

product (5-hydroxymethyl-2-furfural) to a model system resulted in an inhibitory effect on the formation of mutagens.

INHIBITION OF HETEROCYCLIC AROMATIC AMINE FORMATION

Microwave pretreatment. Felton et al. (1994) investigated the effects of microwave pretreatment of meat prior to frying. Beef patties were cooked in a microwave oven for different amounts of time (0, 1.0, 1.5, 2, and 3 min) prior to frying at 200 °C or 250 °C for six minutes per side. They demonstrated that HAA precursors (creatine, creatinine, amino acids, and sugar), water, and fat decreased by 30%, resulting in an overall mutagenic decrease of 95%. There are two possible explanations for these results. First, assuming second-order reaction kinetics, if two reactants are reduced by 30%, the product formation would be reduced by 50%. If three reactants are reduced by 30%, the product formation would be reduced by 70 – 80%. The second possibility is that the remaining precursors may not be available as reactants, due to water loss during microwaving, thus preventing the diffusion of small molecule precursors to the meat surface where temperatures are highest.

Addition of antioxidants. The effect of antioxidants on formation of HAAs was first observed by Wang et al. (1982). They found that the addition of butylated hydroxyanisole (BHA) prior to frying significantly reduced mutagenicity. Barnes et al. (1983) found that the addition of BHA to beef prior to cooking inhibited the formation of IQ by 40%. Chen (1988) found that the addition of butylhydroquinone (TBHQ), n-propyl gallate (PG) and BHA all inhibited the formation of IQ, MeIQx, and 4, 8-

DiMeIQx in ground beef. However, the antioxidant butylated hydroxytoluene (BHT) enhanced the formation of 4, 8-DiMeIQx. Pearson et al. (1992) proposed that BHT may act as an alkylating agent and increase the formation of the precursors for 4, 8-DiMeIQx. They suggested that the methyl group attached at the para-position of BHT may react with the dialkylpyrazine free radical and creatinine to become the methyl group at the 8-position, thereby forming 4, 8-DiMeIQx.

Weisburger et al. (1994) studied the effects of tea and tea polyphenols on the formation of HAAs. A model system containing 1 mmol creatinine, 1 mmol phenylalanine, and 0.5 mmol glucose in 3.3 ml of diethylene glycol-water mixture was used to study the effects of green tea, black tea, theaflavine gallate, and epigallocatechin gallate on the formation of MeIQx and PhIP. They showed a significant decrease of MeIQx and PhIP when theaflavine gallate and epigallocatechin gallate were added to the model system. They also showed a significant decrease of PhIP when green and black tea were added to the model system. These studies suggest that tea polyphenols act as competitive traps for Maillard reaction intermediates and the antioxidant attributes may affect the production of Maillard intermediates, but the underlying mechanism still needs to be explored. These studies found that addition of tea polyphenols to meat prior to cooking may be a practical means of blocking the formation of HAAs.

Kato et al. (1996) found that the phenolic antioxidants BHA, PG, sesamol, esculetin, and epigallocatechin gallate (EGCG) prevent formation of HAAs in a model system containing glucose, glycine, and creatinine. The formation of HAAs was inhibited in bonito meat using EGCG and green tea extract. They demonstrated the formation of an unstable pyrazine cation radical in the model system and showed

inhibition by adding BHA, sesamol and EGCG. They suggested that the phenolic antioxidants effectively scavenge the unstable free radical Maillard intermediates and prevent formation of HAAs.

Johansson and Jagerstad (1996) used a model system consisting of creatinine, glycine, glucose and corn oil to investigate the effect of antioxidants on the formation of HAAs. They added α-tocopherol at five concentrations (10, 100, 200, 1000, 10000 ppm), ascorbic acid at three concentrations (10, 100, 1000 ppm), and TBHQ at two concentrations (100 and 1000 ppm). All three antioxidants failed to inhibit the formation of MeIQx at all levels, but ascorbic acid did inhibit MeIQx formation by 84% at 1000 ppm. Another model system containing creatinine, glycine, glucose, corn oil and iron sulphate (pro-oxidant) was used to study the effect of synthetic antioxidants (BHA, BHT, and PG) on the formation of MeIQx. These antioxidants did not show any inhibition of MeIQx formation. The inability of these antioxidants to inhibit MeIQx formation may be due to the presence of iron sulphate. Another possibility is that the concentration of antioxidants used may have a pro-oxidative effect in this model system, since antioxidants are known to exert both anti- and pro-oxidative effects depending upon the concentration.

Oguri et al. (1998) investigated the effect of eight different antioxidants on the formation of MeIQx in a model system consisting of creatinine, glucose, and glycine dissolved in diethylene glycol/water (86/14) and heated at 128 °C for 2 hrs. Syringic acid, ferulic acid, quercetin, luteolin, caffeic acid, epigallocatechin gallate, ellagic acid, and green tea catechins inhibited MeIQx formation by 78,57,54,45,40,35,30, and 21

percent, respectively. These antioxidants showed a significant decrease in mutagenic activity ranging from 29 to 91 percent.

Murkovic et al. (1998) studied the effects of antioxidant spices on the formation of HAAs in fried beef. They found significant inhibition of HAA formation using rosemary, thyme, sage, and garlic. Balogh et al. (2000) achieved significant reduction in formation of HAAs using Vitamin E prior to frying ground beef patties. They also inhibited the formation of PhIP by 44 % using oleoresin rosemary.

Marination. The effects of marinating meat prior to cooking have recently been investigated and found to be effective at inhibiting the formation of HAAs. Marinating chicken breasts in a mixture of brown sugar, olive oil, cider vinegar, garlic, mustard, lemon juice, and salt for four hours prior to grilling decreased PhIP concentration by 92-99%. The reduction in PhIP varied with the time of grilling. Salmon et al. (1997) concluded that marinating chicken coupled with twenty minutes or less of cooking time would prevent formation of high levels of HAAs. Nerurkar et al. (1999) studied the effects of marinating beef steaks with two different marinades. Beef steaks marinated in teriyaki sauce (16 – 20 hr.) and grilled for ten and fifteen minutes show decreased MeIQx by 44% and 66%, respectively. Levels of PhIP also decreased by 45% and 67% under the same conditions. Similar decreases in MeIQx and PhIP were observed when beef steaks were marinated in turmeric-garlic sauce. Mutagenic activities of unmarinated and marinated beef steaks, as measured by the Ames assay, parallel the differences observed in HAA levels. These marination studies show good potential for the reduction of HAA formation, but these studies failed to elucidate which ingredient or ingredients are

responsible for inhibiting HAA formation. They only suggest that ingredients within the marinate (soy, garlic, sugar, etc.) may be responsible for inhibiting HAA formation and do not suggest possible mechanisms by which HAA formation is inhibited.

Addition of tart cherries. Britt et al. (1998) found that the addition of tart cherry tissue (11.5% w/w) to ground beef patties prior to cooking significantly reduces the oxidation of lipids in both cooked and uncooked hamburger patties. The antioxidant effect of tart cherries together with previous studies suggesting that antioxidants inhibit HAA formation led to the hypothesis that cherries would inhibit formation of HAAs. Addition of whole cherry tissue (11.5% w/w) to ground beef patties prior to cooking resulted in a significant reduction of HAA formation. Specifically, IQ, MeIQ, MeIQx, DiMeIQx, and PhIP were decreased 72.1, 50, 62, 81, and 92.7%, respectively. The overall inhibition of HAA formation was 78.5% for Montmorency cherries. These studies concluded that the major inhibitory effect of cherry tissue is due to some component or components present in tart cherries that show high antioxidant activity. The anthocyanins were strong candidates for the inhibitory action of cherries based on their antioxidant activity.

Wang et al. (1999c) investigated the antioxidant activity of anthocyanins isolated from Montmorency and Balaton™ cherries using a fluorescence-based liposome assay. They found that the antioxidant activity of anthocyanins were comparable to those of butylated hydroxyanisole, butylated hydroxytoluene, and tert-butylhydroquinone. The natural abundance of anthocyanins in cherries, together with the strong antioxidant

activity of anthocyanins, suggests that they may play a role in the reduction of HAA formation by cherries.

CHAPTER ONE

THE INHIBITION OF HETEROCYCLIC AROMATIC AMINE FORMATION IN GROUND BEEF PATTIES USING TART CHERRIES (*PRUNUS CERASUS*)

ABSTRACT

The effects of temperature and tart cherry tissue concentration on heterocyclic aromatic amine (HAA) formation in fried ground beef patties were investigated. Tart cherry tissue was added to ground beef patties at three levels (4.0, 7.5 and 11.5% w/w); the patties were fried at two temperatures (190 and 225 °C) for 10min/side. HAAs were isolated by solid phase extraction and quantified by HPLC. Salmonella strain TA98 with metabolic activation was used to test for mutagenicity of the cooked beef. Ground beef patties containing 11.5% tart cherries and fried at 190 °C showed decreased mutagenicity by 69%, and showed reductions in 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) (53.0%) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine (PhIP) (87%). The addition of 4.0% and 7.5% tart cherries also showed significant reductions in PhIP. Overall, mutagenicity decreased by 69%. Patties fried at 225 °C with the addition of 4.0, 7.5, and 11.5% (w/w) tart cherries showed no difference in total mutagenicity, but showed reductions in PhIP formation, 50, 71, and 85%, respectively.

INTRODUCTION

Over the past twenty years, a group of mutagenic and/or carcinogenic heterocyclic aromatic amines (HAAs) has been found on the charred surfaces of meat and fish. The most common heterocyclic aromatic amines identified in fried ground beef are: 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), DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine). Other HAAs have been isolated and identified in meat and model systems; altogether, this group comprises sixteen mutagenic HAAs and two comutagenic HAAs (Pais et al., 1999).

Epidemiological studies suggest that humans are continuously exposed to HAAs in their diet, but the level of exposure is small. The daily intake of MeIQx and PhIP is estimated to be 0.5 –1.8 and 0.1 – 13.8 μg, respectively (Wakabayashi et al., 1993). Heterocyclic aromatic amines are mutagenic in the Ames Assay, with specific activities of IQ, MeIQ, MeIQx, DiMeIQx, and PhIP ranging from 120 to 661,000 revertants/μg (Wakabayashi and Sugimura, 1998). The risk of developing cancer from ingesting HAAs is difficult to calculate, but it may range from 1 per 10,000 to 1 in 50 depending upon the amount of well-done muscle meats ingested and the genetic susceptibility of the person (Felton et al., 1997).

The formation of HAAs can be significantly reduced by the addition of various compounds to meats and model systems. Weisburger et al. (1994) showed a decrease of PhIP when green and black tea were added to a model system. They showed decreases of MeIQx and PhIP when the tea flavonoids theaflavine gallate and epigallocatechin

gallate were added to the model system. Chen (1988) added synthetic antioxidants BHA, PG, and TBHQ to raw meat and found significant reductions in HAAs. Balogh et al. (2000) found that addition of vitamin E to ground beef patties inhibited PhIP formation by 72%.

Recently, Britt et al. (1998) found that addition of whole tart cherry tissue (11.5% w/w) to ground beef patties prior to cooking resulted in a substantial reduction of HAA formation. Specifically, IQ, MeIQ, MeIQx, DiMeIQx, and PhIP were decreased 72.1, 50, 62, 81, and 92.7%, respectively. The overall inhibition of HAA formation was 78.5%. These experiments were conducted using only one concentration of cherry tissue in ground beef. The relationship of cherry tissue concentration to HAA inhibition is unknown. It is possible that a similar degree of inhibition could be achieved using lower cherry tissue concentrations. In addition, although specific HAAs were inhibited, it is possible that other mutagens could be formed by cooking cherry compounds in the meat.

The objectives of this study were to investigate the formation of HAAs in fried ground beef patties containing cherries cooked at two temperatures, to establish a dose–response relationship of added cherry tissue to ground beef patties, and to determine overall mutagenicity of these ground beef patties.

MATERIALS AND METHODS

Reagents. The HAA standards (MeIQx, 4, 8-DiMeIQx, and PhIP) were purchased from Toronto Research Chemicals (Toronto, Canada). The HAA standard (FEMA - Flavor and Extracts Manufacturer's Association) were gifts from Dr. Mark Knize, Lawrence Livermore National laboratory, Livermore, CA. The FEMA standard contained IQ, MeIQ, MeIQx, DiMeIQs, and PhIP, each at 5 ng/µl. A polysulfonic acid (PRS) Bond Elute columns (500 mg), C-18 cartridges (100 mg), and Hydromatrix were purchased from Varian, Inc. (Harbor City, CA). Extrelut – 20 columns were obtained from E. M. Separations Technology (Gibbstown, NJ). All other chemicals were of analytical grade and were obtained from Fisher Scientific (Fair Lawn, NJ).

Materials. Fresh ground beef was obtained from Michigan State University Meat Laboratory. Quick-frozen, pitted Montmorency tart cherries were obtained from Cherry Marketing Institute, Inc. (Dewitt, MI). The cherries were placed in freezer bags, flushed with nitrogen, and stored (- 20 °C) until ready to use. The fat content of the meat (15 ± 1%) was determined by the method of Folch et al. (1957).

Preparation of Ground Beef Patties. Patties were prepared from ground beef (15 ± 1% fat) as follows: control patties (no cherries added) and patties containing three levels of tart Montmorency cherries (4.0, 7.5, and 11.5% w/w). Cherries were pulverized through an Urshel comitrol (Urshel Laboratories, Inc., Valparaiso, IN) using a 3-K-030240-V head. Patties were prepared by mixing the pulverized cherry tissue with the ground beef in a Keebler mixer (Keebler Inc., Chicago, IL). Patties were formed by

weighing 100 g of meat in a Petri dish (9 cm x 1.5 cm) to ensure patty uniformity. Patties were then frozen and stored at -20 °C until ready to use.

Cooking Preparation. Patties were thawed overnight in a cooler at 4 °C. Patties were removed from the cooler and placed at room temperature for twenty minutes prior to frying. Patties were fried in an electric, Teflon-coated pan at two temperatures: 190 °C and 225 °C. The frying temperature was measured using a surface temperature thermometer (Pacific Transducer Corp., Los Angeles, CA). Each treatment consisted of frying two patties for ten minutes per side, for a total cooking time of 20 min. After frying, the patties were cooled to 25 °C and homogenized in a Waring blender. The homogenized patties were placed in polystyrene bags and stored at -20 °C until time of extraction.

Sample Extraction. The procedure used for HAA extraction was adopted from Gross and Gruter (1992). Meat samples were extracted by blending 25 g of cooked meat from two patties with 75 g 1 N NaOH and subsequently divided into four 16-g aliquots and transferred into 250 ml beakers. To determine extraction recoveries for each HAA and aid in their identification, two of the four samples were spiked with 250 ng of each HAA (IQ, MeIQ, MeIQx, DiMeIQx, and PhIP) dissolved in 50 µl methanol. Each sample was mixed with Hydromatrix (Varian, Inc) and packed into an Extrelut 20 column. A propylsulfonic acid silica (PRS) column was attached in tandem to each Extrelut 20 column. The HAAs were extracted with dichloromethane/toluene (95:5) until 40 ml of solvent passed through the PRS column. The PRS column was detached and

transferred to a Visiprep vacuum (Supelco), and dried for 15 min. The PRS columns were connected to a peristaltic pump and washed sequentially with 6 ml 0.1 N HCl, 15 ml 0.1 N HCl/methanol (6:4), and 2 ml of water. Bond Elute C18 cartridges were attached to the PRS columns and rinsed with 20 ml of ammonium acetate buffer solution (0.5 M, pH 8.0) to transfer the HAAs. The C18 cartridges were dried for 30 minutes using the Visiprep vacuum (Supelco). The HAAs were eluted with 1.0 ml of methanol/ammonia (9:1). The solvent was evaporated to dryness in a 40 °C water bath using a stream of nitrogen, and the dried compounds were refrigerated until required for HPLC analysis.

HAA Quantification. The dried samples were redissolved in methanol containing 50ng/μl caffeine (internal standard). HPLC separation was carried out on a TSK-gel ODS80-TM column (4.6 mm i.d. x 25 cm, Tosoh Haas, Montgomeryville, PA) attached to a precolumn (Supelguard LC-8-DB, Supelco, Bellefonte, PA). The flow rate of the mobile phase was 1 ml/min. The initial ratio of acetonitrile/buffer (triethylamine phosphate, 0.01 M, pH 3.2) was 8:92, and was increased to 17:83 during the first 10 min. Acetonitrile concentration was increased to 25:75 in the next 10 min and to 55:45 in the following 10 min. Over the next 5 min, the acetonitrile/buffer ratio was increased to 80:20 to elute all other compounds. The ratio was then returned to the original 8:92 for 10 min to allow the column to re-equilibrate prior to the next injection. Samples were analyzed on a Waters HPLC system (Millipore, Milford, MA) with a photodiode array detector (model 996) and a scanning fluorescence detector (model 474). The sample peak areas were integrated using the Millennium 2000 Chromatography Manager (Millipore, Milford, MA).

The peaks from the spiked samples were used to identify the peaks in the unspiked samples by comparing retention times. Also, UV spectral characteristics of the HPLC peaks in each sample were compared to a spectral library of HAA standards using the Millennium software. For each set of experiments, before HPLC separation of sample extracts, four aliquots (25, 30, and 35 µl) of two HAA standards (containing 0.5 ng/µl of each compound), the internal standard caffeine (5 ng/µl), and HAA standard FEMA (5 ng/µl) were injected. Linear regression (ng compound vs. peak area) was performed for each HAA in each mixture. A correlation coefficient of 0.97 or greater for laboratory mixtures of HAAs was acceptable, and 0.99 or greater for the FEMA internal standards. All HAA peak areas were corrected using the internal standard regression line and expressed as ng/g of meat. Extraction efficiencies and quantification of HAAs were determined using the standard addition method of Gross and Gruter (1992). Each data point consisted of four subsamples: two spiked and two unspiked. The average area of the spiked samples minus the average area of the unspiked samples allowed comparison with the regression line for the standard mixture. Individual data points were determined and then corrected for extraction efficiency, or percent yield. The average of the unspiked samples was used to determine the concentrations of each HAA formed. The linear regression slope for FEMA was used to determine the exact amount of each HAA present in each sample.

Mutagen Extraction and Assay. The procedure used for mutagen extraction followed the procedure described above for "sample extraction" up through the elution of the PRS column. The compounds absorbed to the PRS column were eluted with 1.5 ml

methanol/ammonia (9:1) into a small microvial, dried under nitrogen, and stored at -20 °C until plating.

The mutagenicity was determined by dissolving samples in DMSO and testing by the method of Maron and Ames (1983) using the *Salmonella typhimurium* TA 98 (Molecular Toxicology Inc., Boone, NC). Aroclor 1254-induced rat liver S-9 mixture was used for metabolic activation; 0.5 ml S9 mix containing 10% Aroclor 1254-induced rat liver (Molecular Toxicology Inc.) was added per plate. 2- aminoanthracene was used as a positive control and DMSO was used as a negative control (spontaneous reverent colonies) for S. *typhimurium* TA 98. Mutagenic activity was calculated from the linear portion of the dose - response curve using the method of Moore and Felton (1983). A minimum of four dose response points from duplicate platings was used and the linear portions of the curves were used to calculate the revertants/g of cooked beef patties.

Statistical Analyses. All results were analyzed by Sigma Stat 2.0 (Jandel Corp., San Rafael, CA). One-way analysis of variance (ANOVA) was performed. Appropriate comparisons were made using Student-Newman-Kreuls test for one-way ANOVA analysis.

RESULTS AND DISCUSSION

The recoveries of HAAs present in the cooked ground beef with and without added tart cherry tissue ranged from 26 to 72%. The average recoveries of spiked samples were 48 ± 6, 30 ± 4, and 44 ± 8% for MeIQx, DiMeIQx, and PhIP, respectively. These results are comparable to those of other workers who reported similar recoveries ranging from 32 to 98% (Salmon et al., 1997; Balogh et al., 2000).

To simulate household cooking practices, ground beef patties were fried at 190 °C with and without tart cherry tissue. This procedure yielded two HAAs above the limit of detectability (0.1 ng/g): MeIQx and PhIP. As shown in Figure 1 and Table 1, MeIQx was more abundant and yielded a total of 8.91 ng/g ± 1.8 of meat in the control. The addition of tart cherry tissue to ground beef at the 11.5% level decreased the formation of MeIQx by 53% ($P<0.05$). Lower levels of cherries in ground beef did not result in significant inhibition at 190 °C. A significant reduction in the formation of PhIP was found at all three treatment levels (4.0, 7.5 and 11.5% w/w), which yielded inhibitions of 34.5, 72.5 and 86.9%, respectively.

Three HAAs, MeIQx, DiMeIQx, and PhIP were found above the limit of detectability when ground beef patties were fried at 225 °C (to increase yield of HAAs) with and without cherry tissue addition. As expected, the higher temperature produced a greater quantity of HAAs. These results were consistent with the study of Arvidsson et al. (1997) who showed that MeIQx formed at the highest rates at 225 °C followed by 7,8-DiMeIQx and 4,8-DiMeIQx. Adding tart cherry tissue (4.0, 7.5, and 11.5% w/w) to ground beef patties significantly reduced the formation of PhIP. The inhibition of PhIP formation ranged from 50.8 to 85.3% (Figure 2 and Table 2). MeIQx and DiMeIQx did

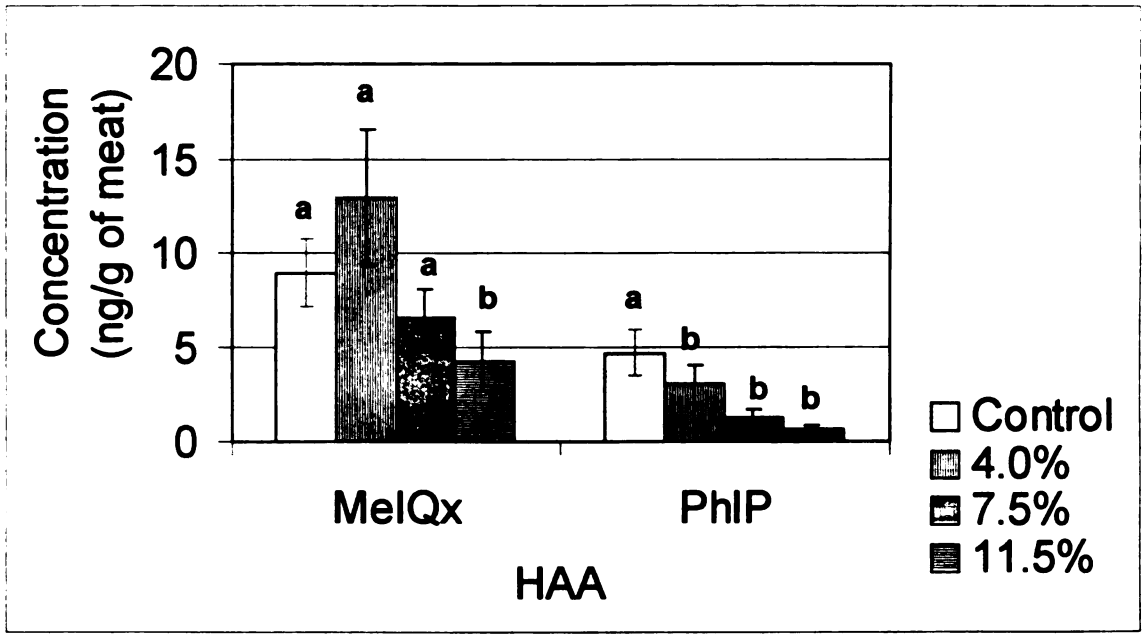


Figure 1. Effect of Cherry Tissue Addition on Heterocyclic Aromatic Amine Content of Ground Beef Patties Fried at 190 °C

Table 1. Effect of Cherry Tissue Addition on Heterocyclic Aromatic Amine Content of Ground Beef Patties Fried at 190 °C

Sample	MeIQx (ng/g)	% Inhibition	PhIP (ng/g)	% Inhibition
Control	8.91 ± 1.8 [a]	-	4.66 ± 1.2 [a]	-
4.0%	12.97 ± 3.6 [a]	NSD	3.05 ± 1.0 [b]	34.5
7.5%	6.51 ± 1.6 [a]	NSD	1.29 ± 0.4 [b]	72.3
11.5%	4.19 ± 1.6 [b]	53.0	0.61 ± 0.2 [b]	86.9

Data represent the mean and standard deviation of five analyses per treatment (n=5).
Values are expressed on a cooked ground beef basis.
Means in the same columns bearing different superscripts are significantly different ($p<0.05$).
NSD- No significant difference

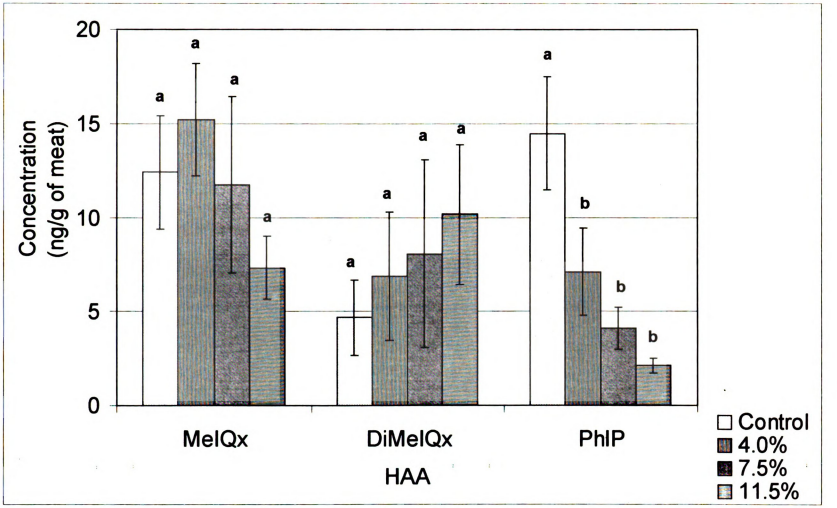


Figure 2. Heterocyclic Aromatic Amine Content of Ground Beef Patties Fried at 225 °C

Table 2. Heterocyclic Aromatic Amine Content of Ground Beef Patties Fried at 225 °C

Sample	MeIQx (ng/g)	% Inhibition	DiMeIQx (ng/g)	% Inhibition	PhIP (ng/g)	% Inhibition
Control	12.41 ± 3.0 [a]	-	4.67 ± 2.0 [a]	-	14.48 ± 3.0 [a]	-
4.0%	15.19 ± 3.0 [a]	NSD	6.88 ± 3.4 [a]	NSD	7.12 ± 2.3 [b]	50.8
7.5%	11.73 ± 4.7 [a]	NSD	8.07 ± 5.0 [a]	NSD	4.11 ± 1.1 [b]	71.6
11.5%	7.33 ± 1.7 [a]	NSD	10.18 ± 3.7 [a]	NSD	2.13 ± 0.4 [b]	85.3

Data represent the mean and standard deviation of five analyses per treatment (n=5).

Values are expressed on a cooked ground beef basis.

Means in the same columns bearing different superscripts are significantly different ($p < 0.05$).

NSD- No significant difference

not dramatically change with the addition of tart cherry tissue. However, MeIQx tended to decrease whereas DiMeIQx tended to increase with the addition of tart cherry tissue. These results contrast with those of Britt et al. (1998), who reported sizeable decreases in the formation of IQ, MeIQ, MeIQx, DiMeIQx, and PhIP with the addition of 11.5% (w/w) cherry tissue. Moreover, they detected levels the presence of IQ and MeIQ in control samples; these HAAs also decreased with addition of cherry tissue. Differences that exist between these results and Britt et al. (1998) results may be due to differences in meat composition, especially with respect to levels of HAA precursors or variation in cherry composition due to ripeness, age, location, and seasonal variation. Inherent temperature fluctuations in frying pans resulting in slightly different heating conditions may also play a role.

The cooking temperature plays a key role in the formation and the amount of HAAs produced. Murkovic and Pfannhauser (2000) reported dramatic increases in HAA formation as cooking temperature and time increased. Knize et al. (1994) evaluated three frying temperatures and found that patties fried at higher temperatures yielded greater amounts of HAAs. They showed higher amounts of PhIP and MeIQx formation and lesser amounts of DiMeIQx formation. Skog et al. (1995) fried an array of meat products at temperatures ranging from 150 °C to 225 °C and reported the highest levels of HAA formation were found at the highest temperatures. Our results demonstrated similar trends in that ground beef patties fried at a lower temperature (190 °C) produced detectable levels of only two HAAs, while patties fried at a higher temperature (225 °C) produced significantly higher levels of three HAAs.

Although, these results clearly show decreases in formation of major HAA species, it is also important to independently establish reduction of overall mutagenicity of the cooked product. It is also possible that the presence of cherry tissue could lead to an increase in the formation of other mutagenic compounds. Therefore, the mutagenic activities of cooked ground beef patties with and without cherry tissue were evaluated by the Ames assay. *S. typhimurium* TA98, was used because HAAs are generally more likely to induce a frameshift mutation than a basepair mutation (Wakabayashi and Sugimura, 1998). Ground beef patties fried at 190 °C with the addition of tart cherry tissue at the 7.5 and 11.5% treatment levels had reduced mutagenicity, with inhibition of 41.7 and 68.8%, respectively (Figure 3 and Table 3). These findings were consistent with the levels of individual HAAs presented in Table 1. The ground beef patties fried at 225 °C showed a trend toward decreased mutagenic activity, but these differences were not significant. Although the level of PhIP formation was inhibited by as much as 85.3% in the 11.5% treatment level, the amount of DiMeIQx tended to increase. Sugimura and Sato (1982) and Sugimura et al. (1988) reported that DiMeIQx is a much more potent mutagen than PhIP; DiMeIQx produced 183,000 revertants/ug, whereas PhIP produced only 1800 revertants/ug. Since DiMeIQx is over 100 times more mutagenic than PhIP, even a 7-fold reduction in PhIP (Table 2) is likely masked by small changes in DiMeIQx. Therefore, our mutagenicity results are consistent with measured HAA levels.

Inhibition by various means of HAA formation in meats and simple model systems (consisting of two or three reactants) has been well documented. Marination of meat prior to cooking has been effective at decreasing the formation of HAAs. Nerurkar et al. (1999) observed inhibition of the formation of MeIQx and PhIP in beef steaks. A

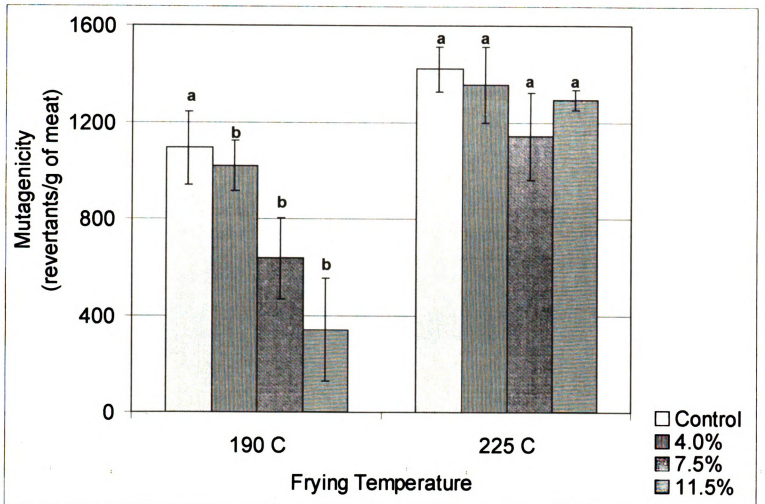


Figure 3. Mutagenic Activities of Ground Beef Patties with Differing Cherry Content Fried at 190 °C and 225 °C

Table 3. Mutagenic Activities of Ground Beef Patties with Differing Cherry Content Fried at 190 °C and 225 °C

Sample	Frying Temp. 190 °C Mutagenicity (Revertants/g of meat)	% Inhibition	Frying Temp. 225 °C Mutagenicity (Revertants/g of meat)	% Inhibition
Control	1094 ± 93 [a]	-	1421 ± 151 [a]	-
4.0%	1019 ± 157 [a]	NSD	1356 ± 103 [a]	NSD
7.5%	638 ± 181 [b]	41.7	1144 ± 167 [a]	NSD
11.5%	342 ± 41 [b]	68.8	1294 ± 213 [a]	NSD

Data represent the mean and standard deviation of three analyses per treatment (n=3).

Means in the same columns bearing different superscripts are significantly different ($p<0.05$)

NSD- No significant difference

teriyaki sauce marinade inhibited PhIP formation by 67% and MeIQx formation by 60%. Lower levels of PhIP and MeIQx were also observed in beef steaks marinated in turmeric-garlic sauce. Mutagenic activities of unmarinated and marinated beef steaks, as measured by the Ames assay, were consistent with the differences observed in HAA levels. Salmon et al. (1997) marinated chicken breasts in a mixture of brown sugar, olive oil, cider vinegar, garlic, mustard, lemon juice, and salt for four hours prior to grilling and found that PhIP concentration decreased by 92-99%. The reduction in PhIP varied directly with the length of time of grilling. The mechanism by which marinating meat inhibits the formation of HAAs has not been elucidated, but they concluded that marinating chicken, coupled with twenty minutes or less of cooking time, would prevent formation of high levels HAAs.

Another effective way of reducing HAA formation is by reducing the amount of precursors available in the meat system. Felton et al. (1994) pretreated beef patties in a microwave oven prior to frying. Beef patties were cooked in a microwave oven for 0, 1., 1.5, 2, and 3 min prior to frying at 200 and 250 °C. They demonstrated that HAA precursors (creatine, creatinine, amino acids, and sugar), water, and fat decreased by 30%, resulting in an overall mutagenicity decrease of 95%.

The addition of antioxidants to meat prior to cooking has shown to be effective at inhibiting the formation of HAAs. Kato et al. (1996) suggested that phenolic antioxidants effectively scavenge the unstable free radical Maillard intermediates, thus preventing the formation of HAAs. Weisburger et al. (1994) showed inhibition of PhIP in model systems containing glucose, creatinine, and phenylalanine using green and black tea and individual polyphenols derived from tea. Oguri et al. (1998) studied the effect of

eight different antioxidants on the formation of MeIQx in a model system. Syringic acid, ferulic acid, quercetin, luteolin, caffeic acid, epigallocatechin gallate, ellagic acid, and green tea catechins inhibited MeIQx formation by 78,57,54,45,40,35,30,21 percent, respectively. These antioxidants showed a significant decrease in mutagenic activity ranging from 29 to 91%. Balogh et al. (2000) demonstrated the inhibitory effects of vitamin E and oleoresin rosemary on HAA formation in ground beef patties. Vitamin E was effective at inhibiting PhIP formation by as much as 72%.

Tsai et al. (1996) studied the effect of six naturally occurring organosulfur compounds, which are found in garlic, on mutagen formation in boiled pork juice. They showed that diallyl disulfide and dipropyl disulfide had the greatest inhibitory effect on mutagen formation.

All of these studies have shown inhibition of HAA formation by using many different means. Our results with addition of cherry tissue to ground beef provide another means of inhibiting the formation of HAAs. It may be possible that antioxidants present in cherries are responsible for inhibiting HAA formation. Still, many questions remain in our results and many of those listed above as to the mechanism by which HAAs are inhibited.

This study clearly demonstrated a dose – response associated with the addition of tart cherry tissue to ground beef patties. The formation of PhIP was incrementally reduced by the addition of each level of tart cherry tissue (4.0, 7.5 and 11.5%). The formation of MeIQx at the lower frying temperature (190 °C) was significantly inhibited at the highest treatment level (11.5%). The addition of tart cherry tissue at 11.5% yielded the greatest inhibitory effect and the least mutagenic activity. The amounts of HAAs

formed at the higher temperature were significantly greater than those formed at the lower frying temperature. Further research is in progress to better understand which compound or compounds present in tart cherry tissue are responsible for inhibition of HAA formation.

CHAPTER TWO

THE EFFECT OF HETEROCYCLIC AROMATIC AMINE FORMATION USING TART CHERRY ANTHOCYANINS IN A MODEL SYSTEM AND IN FRIED GROUND BEEF PATTIES

ABSTRACT

Previous studies showed that tart cherry tissue added to ground beef at 11.5% (w/w) resulted in inhibition of HAA formation. Preliminary experiments suggested that cherry anthocyanins may be the inhibiting factor in tart cherries. To test this hypothesis, the effects of tart cherry anthocyanins on heterocyclic aromatic amine (HAA) formation in fried ground beef patties and in a model system were investigated. The model system contained a mixture of three reactants (0.2 mmol phenylalanine, 0.2 mmol creatinine, and 0.1 mmol glucose). A mixture of cherry anthocyanins (15 mg) was added to the model system, which was then heated at 180 °C for 30 min. Cherry anthocyanins were also added to ground beef patties at a dose equivalent to 11.5% (w/w) cherry tissue, which was subsequently fried at 225 °C for twenty minutes. No significant inhibition of HAA formation was observed in the model system or in ground beef patties with the addition of cherry anthocyanins.

INTRODUCTION

Heterocyclic aromatic amines (HAAs) are mutagenic compounds that form on the surfaces of muscle foods. The most common HAAs identified in fried ground beef are: 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), DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine). HAAs have shown potent mutagenic activity in the Ames Salmonella Assay, with activities ranging from 120 to 661,000 revertants/ug (Wakabayashi and Sugimura, 1998).

Factors that determine the formation of HAAs in foods include precursor concentrations (free amino acids, creatine and sugars), pH, fat content, and cooking time and temperature (Skog et al., 1998). Model system studies have shown that amino acids, creatine, and glucose are the minimal precursors necessary to produce significant quantities of HAAs. These simplified model systems have enabled researchers to propose partial mechanisms by which HAAs form during the cooking of muscle foods. Jagerstad et al. (1983a) proposed that the amino-imidazo portion of an HAA is formed by water elimination and cyclization of creatine followed by aldol condensation with the pyridines or pyrazines formed via the Maillard reaction. Another alternative potential reaction involves the formation of a free radical, N,N-disubstituted pyrazine cation, by early carbon fragmentation prior to formation of the Amadori product (Namiki and Hayashi, 1981).

The fact that addition of antioxidants to muscle foods prior to cooking results in decreased HAA formation lends support for the free-radical theory. Chen (1988) showed

inhibition of HAA formation by adding synthetic antioxidants butylated hydroxyanisole (BHA), propyl gallate (PG), and tert-butylhydroquinone (TBHQ) individually to ground beef patties. Kato et al. (1996) found that the phenolic antioxidants BHA, PG, sesamol, esculetin, and epigallocatechin gallate (EGCG) prevents formation of HAAs in model system containing glucose, glycine, and creatinine. Balogh et al. (2000) added vitamin E to ground beef patties and showed inhibition of PhIP formation by 72% in the cooked product.

As discussed in Chapter 1, there was an inverse dose-response relationship of tart cherry tissue to HAA formation when cherries were added to ground beef patties at various treatment levels (4.0, 7.5 and 11.5%). Britt et al. (1998) also reported reductions in lipid oxidation as well as HAA formation with the addition of 11.5% (w/w) cherry tissue to ground beef patties. Wang et al. (1999b) analyzed extracts from tart cherries and showed that extracts containing cherry anthocyanins had high antioxidant activity. Therefore, we hypothesized that anthocyanins, present in tart cherries, are responsible for inhibiting the formation of HAAs in ground beef patties containing 11.5% (w/w) cherry tissue.

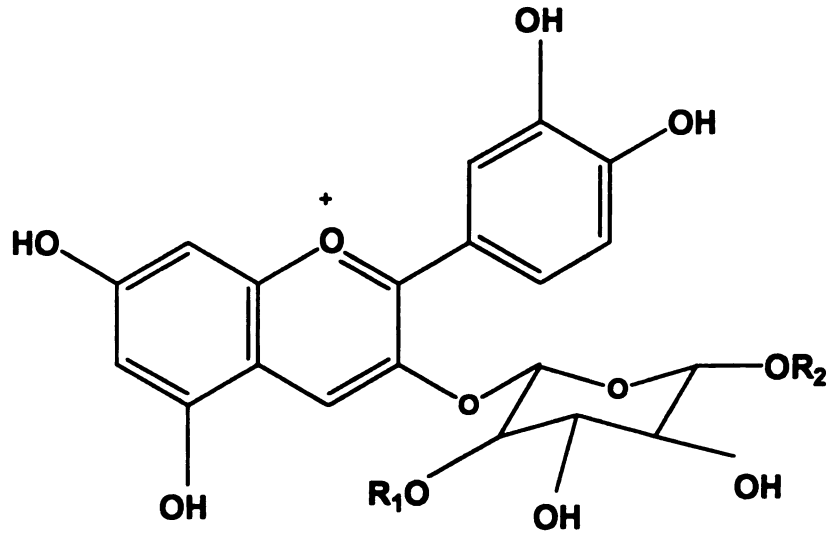
The objective for this study was to investigate the effects of cherry anthocyanins in ground beef patties and in a model system.

MATERIALS AND METHODS

Materials and Reagents. The HAA standards (IQx, MeIQx, 4, 8-DiMeIQx, and PhIP) were purchased from Toronto Research Chemicals (Toronto, Canada). The HAA standard (FEMA - Flavor and Extracts Manufacturer's Association) was a gift from Dr. Mark Knize, Lawrence Livermore National laboratory, Livermore, CA. The FEMA standard contained IQ, MeIQ, MeIQx, DiMeIQs, and PhIP, each at 5 ng/μl. Polysulfonic acid (PRS) Bond-Elute columns (500 mg), C-18 cartridges (100 mg), and Hydromatrix were purchased from Varian, Inc. (Harbor City, CA). Extrelut – 20 columns were obtained from E. M. Separations Technology (Gibbstown, NJ). All other chemicals were of analytical grade and were obtained from Fisher Scientific (Fair Lawn, NJ).

Threaded, stainless steel test tubes with self-sealing stainless steel caps, and a total capacity of 2.3 ml, were manufactured by the Machine Shop within the Engineering Research Complex at Michigan State University. The heating module was a Reacti-Therm III, model 18835 (Pierce Co., IL).

Fresh ground beef was obtained from the Meat Laboratory at Michigan State University. The fat content of the meat was determined by the method of Folch et al. (1957). Cyanidin and a mixture of pure cherry anthocyanins **1** [3-cyanidin 2"-O-β-D-glucopyranosyl-6"-O-α-L-rhamnopyranosyl-β-D-glucopyranoside] (65%), **2** [3-cyanidin 6"-O-α-L-rhamnopyranosyl-β-D- glucopyranoside] (30%), and **3** [3-cyanidin O-β-D-glucopyranoside] (5%), were obtained from the Bioactive Natural Products and Phytoceuticals, Department of Horticulture and National Food Safety and Toxicology Center at Michigan State University (Figure 1).



1 (65 %) R_1= glucose, R_2=rhamnose
2 (30 %) R_1= H, R_2=rhamnose
3 (5 %) R_1= H, R_2=H

Figure 1. Anthocyanins **1-3** from Tart Cherries

Preparation and Heating of Reactants in a Model System. The control model system contained a mixture of three reactants (0.2 mmol phenylalanine, 0.2 mmol creatinine, and 0.1 mmol glucose) in 1.5 ml of water at pH 5.5. The mixtures of reactants and test compounds (cyanidin or anthocyanins) were added directly to the stainless steel test tubes which were then tightly sealed with Teflon-wrapped caps. Two separate test tubes were heated for each replication. Silicon oil (0.5 ml) was placed inside each cavity of the heating block to facilitate efficient heat transfer from the heating block to the individual test tubes. The module was heated in a fume hood until the temperature reached 210°C. Test tubes were placed in the heating block and allowed to heat for 30 min at 180 °C. Upon completion of heating, the test tubes were immediately cooled in an

ice bath for 30 min. The contents of each test tube were transferred to a microvial (1.5 ml) and stored in a cooler (4 °C) until extraction.

Preparation of Ground Beef Patties. Anthocyanins (11 mg) were added to each individual ground beef patty on a weight basis equivalent to that of 11.5% (w/w) fresh whole cherries. Beef patties were prepared by weighing 100 g of ground beef in a Petri dish (9 cm x 1.5 cm) to ensure patty uniformity. Patties were mixed thoroughly by hand and incubated at 4 °C for 12 h until time of frying.

Cooking Preparation. Patties were removed from the cooler (4 °C) and placed at room temperature for 20 min prior to frying. Patties were fried in an electric, Teflon-coated pan at 225 °C. The frying temperature was measured using a surface temperature thermometer (Pacific Transducer Corp., Los Angeles, CA). Each treatment consisted of frying two patties for 10 min per side, total cooking time of 20 min. After frying, the patties were cooled to 25 °C and homogenized in a Waring blender. The homogenized patties were placed in polystyrene bags and stored at -20 °C until time of extraction.

Sample Extraction for Model System. The micro-vials were removed from the cooler (4 °C) and the contents of each of the two micro-vials were combined with 57 ml (5 N NaOH) and stirred with a spatula. Ten-milliliter aliquots were removed from the mixture and placed in four 250 ml beakers. The remaining mixture was placed in the cooler (4 °C) and saved for any additional analyses. Two of the aliquots were spiked with 1 μg each of IQx, MeIQx, and PhIP dissolved in 50 μl of methanol to determine

extraction recoveries. The remaining extraction, purification, and quantification procedures are described below.

Sample Extraction for Ground Beef Patties. The procedure used for HAA extraction was adopted from Gross and Gruter (1992). Meat samples were extracted by blending 25 g of cooked meat from two patties with 75 g 1 N NaOH and subsequently divided into four 16-g aliquots and transferred into 250 ml beakers. To determine extraction recoveries for each HAA and aid in their identification, two of the four samples were spiked with 250 ng of each HAA (IQ, MeIQ, MeIQx, DiMeIQx, and PhIP) dissolved in 50 µl methanol. Each sample was mixed with Hydromatrix (Varian, Inc) and packed into an Extrelut 20 column. A propylsulfonic acid silica (PRS) column was attached in tandem to each Extrelut 20 column. The HAAs were extracted with dichloromethane/toluene (95:5) until 40 ml of solvent passed through the PRS column. The PRS column was detached and transferred to a Visiprep vacuum (Supelco), and dried for 15 min. The PRS columns were connected to a peristaltic pump and washed sequentially with 6 ml 0.1 N HCl, 15 ml 0.1 N HCl/methanol (6:4), and 2 ml of water. Bond Elute C18 cartridges were attached to the PRS columns and rinsed with 20 ml of ammonium acetate buffer solution (0.5 M, pH 8.0) to transfer the HAAs. The C18 cartridges were dried for 30 min using the Visiprep vacuum (Supelco). The HAAs were eluted with 1 ml of methanol/ammonia (9:1). The solvent was evaporated to dryness in (/Ua 40 °C water bath using a stream of nitrogen, and the dried compounds were refrigerated until required for HPLC analysis.

HAA Quantification. The dried samples were redissolved in methanol containing 50ng/μl caffeine (internal standard). HPLC separation was carried out on a TSK-gel ODS80-TM column (4.6 mm i.d. x 25 cm, Tosoh Haas, Montgomeryville, PA) attached to a precolumn (Supelguard LC-8-DB, Supelco, Bellefonte, PA). The flow rate of the mobile phase was 1 ml/min. The initial ratio of acetonitrile/buffer (triethylamine phosphate, 0.01 M, pH 3.2) was 8:92, and was increased to 17:83 during the first 10 min. Acetonitrile concentration was increased to 25:75 in the next 10 min and to 55:45 in the following 10 min. Over the next 5 min, the acetonitrile/buffer ratio was increased to 80:20 to elute all other compounds. The ratio was then returned to the original 8:92 for 10 min to allow the column to re-equilibrate prior to the next injection. Samples were analyzed on a Waters HPLC system (Millipore, Milford, MA) with a photodiode array detector (model 996) and a scanning fluorescence detector (model 474). The sample peak areas were integrated using the Millennium 2000 Chromatography Manager (Millipore, Milford, MA).

The peaks from the spiked samples were used to identify the peaks in the unspiked samples by comparing retention times. Also, UV spectral characteristics of the HPLC peaks in each sample were compared to a spectral library of HAA standards using the Millennium software. For each set of experiments, before HPLC separation of sample extracts, four aliquots (25, 30, and 35 μl) of two HAA standards (containing 0.5 ng/μl of each compound), the internal standard caffeine (5 ng/μl), and HAA standard FEMA (5 ng/μl) were injected. Linear regression (ng compound vs. peak area) was performed for each HAA in each mixture. A correlation coefficient of 0.97 or greater for laboratory mixtures of HAAs was acceptable, and 0.99 or greater for the FEMA internal

standards. All HAA peak areas were corrected using the internal standard regression line and expressed as ng/g of meat. Extraction efficiencies and quantification of HAAs were determined using the standard addition method of Gross and Gruter (1992). Each data point consisted of four subsamples: two spiked and two unspiked. The average area of the spiked samples minus the average area of the unspiked samples allowed comparison with the regression line for the standard mixture. Individual data points were determined and then corrected for extraction efficiency, or percent yield. The average of the unspiked samples was used to determine the concentrations of each HAA formed. The linear regression slope for FEMA was used to determine the exact amount of each HAA present in each sample.

Statistical Analyses. All results were analyzed by Sigma Stat 2.0 (Jandel Corp., San Rafael, CA). One-way analysis of variance (ANOVA) was performed. Appropriate comparisons were made using Student-Newman-Kreuls test for one-way ANOVA analysis.

RESULTS AND DISCUSSION

Three HAAs were found in the model system containing phenylalanine, creatinine, and glucose: IQx, MeIQx, and PhIP (Table 1). The average recoveries ranged from 44 – 78%, 52 – 75%, and 40 – 65 % for IQx, MeIQx, and PhIP, respectively.

The effect of cyanidin, the aglycon of anthocyanin, was tested in the model system at two concentrations (0.03 and 0.1 mmol). There was no inhibition of HAA formation using either concentration of cyanidin. A mixture of pure cherry anthocyanins 1–3 was added to the model system (15 mg) to investigate the effects of HAA formation. The addition of anthocyanins did not inhibit the formation of HAAs.

Wang et al. (1999c) showed that the three cherry anthocyanins as well as the aglycon cyanidin displayed antioxidant activities which compared favorably to synthetic antioxidants BHA and BHT and showed superior antioxidant activity to α-tocopherol. Preliminary findings suggested that a methanol extract of whole cherries, containing anthocyanins, showed inhibition of HAAs in ground beef patties when added prior to frying (E. Gomaa, unpublished).

The lack of effect of cyanidin or anthocyanins in the model system may be explained by inherent deficiency of any model system to adequately simulate conditions for HAA formation in meat. Therefore, anthocyanins were tested in a meat system. The effect on HAA formation by the addition of anthocyanins (11 mg/patty) to ground beef patties (fat content 15 % ± 1) prior to frying was investigated (Table 2). Anthocyanins were added to ground beef patties at a dose equivalent to 11.5 % (w/w) fresh cherries and the patties were fried at 225 °C. There was no significant inhibition of HAA formation using anthocyanins.

Table 1. Effect of Cyanidin and Cherry Anthocyanins on the Formation of Heterocyclic Aromatic Amines in a Model System Containing Phenylalanine, Glucose, and Creatinine

Sample	IQx (nmol/mmol of creatinine)	MeIQx (nmol/mmol of creatinine)	PhIP (nmol/mmol of creatinine)
Control	0.74 ± 0.13 [a]	0.05 ± 0.01 [a]	0.86 ± 0.73 [a]
0.03 mmol Cyanidin	1.14 ± 0.86 [a]	0.04 ± 0.01 [a]	0.95 ± 0.65 [a]
0.1 mmol Cyanidin	2.34 ± 1.62 [a]	0.08 ± 0.01 [a]	0.71 ± 0.15 [a]
15 mg Anthocyanins	1.47 ± 0.35 [a]	0.12 ± 0.02 [a]	0.69 ± 0.15 [a]

Data represent the mean and standard deviation of three analyses per treatment (n=3).

Means in the same columns bearing different superscripts are significantly different ($p < 0.05$).

Table 2. Heterocyclic Aromatic Amine Content of Ground Beef Patties Containing Anthocyanins (11 mg/patty)

Sample	MeIQx (ng/g)	DiMeIQx (ng/g)	PhIP (ng/g)
Control	6.29 ± 1.27 [a]	1.12 ± 0.05 [a]	10.55 ± 1.85 [a]
Anthocyanins	6.02 ± 0.50 [a]	1.33 ± 0.14 [a]	10.48 ± 1.30 [a]

Data represent the mean and standard deviation of three analyses per treatment (n=3).
Values are expressed on a cooked ground beef basis
Means in the same columns bearing different superscripts are significantly different ($p < 0.05$).

It is easy to dismiss the failure of the anthocyanins to inhibit HAA formation in the model system because of an inherent deficiency of the model system to effectively simulate that of a meat product. However, the lack of inhibition when anthocyanins were added to meat at levels comparable to that of fresh whole cherries suggests that other compounds in cherries are responsible for the inhibitory effect. Moreover, although anthocyanins have been shown to be effective antioxidants (Wang et al. 1999c), these results indicate that anthocyanins are not present at sufficiently high levels to be effective. Alternatively, they could act in concert with other compounds present in cherries to inhibit HAA formation. Finally, it is possible that non-antioxidant compounds are responsible for inhibition.

These findings disprove our hypothesis that anthocyanins are responsible for inhibiting the formation of HAAs when tart cherries are added to ground beef patties. Further work is needed to isolate which compound or compounds in tart cherries are responsible for inhibiting the formation of HAAs.

CHAPTER THREE

THE INHIBITION OF HETEROCYCLIC AROMATIC AMINE FORMATION IN FRIED GROUND BEEF PATTIES USING EXTRACTS AND ISOLATED FRACTIONS FROM TART CHERRIES (PRUNUS CERASUS)

ABSTRACT

Lyophilized Montmorency tart cherries were sequentially extracted with solvents to determine which component or group of components in cherry tissue was responsible for inhibiting the formation of heterocyclic aromatic amines (HAAs). Individual fractions extracted from cherries were added at a dose equivalent to 11.5 % (w/w) cherry tissue to ground beef patties which were then fried at 225 °C. HAAs were isolated from cooked patties by solid phase extraction and quantified by HPLC. The methanol extract inhibited the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine (PhIP) by 62%. Purification of the methanol extract yielded a fraction containing three sugars (glucose, fructose, and maltose), which inhibited PhIP formation by 49%. Further studies showed no significant difference between using a combination of three sugars or using glucose alone at the same concentration. These results suggest that the inhibition of HAA formation by whole cherry fruits is mostly attributable to the sugar fraction.

Analysis of several meat samples for concentration of precursor compounds glucose and creatine indicated considerable variation. Instead of promoting HAA formation in these samples as predicted by various models, glucose was inhibitory in meat at all levels tested. Several possible mechanisms for inhibition by glucose are discussed.

INTRODUCTION

Heterocyclic aromatic amines (HAAs) are formed during the cooking of meat and fish products. The most common HAAs identified in fried ground beef are: 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), DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine).

In long term feeding studies of rats and mice, HAAs have caused tumors in the liver, urinary bladder, small and large intestines, skin, oral cavity, mammary gland, prostate, forestomach, lung, colon, and lymphoid tissue (Wakabayashi and Sugimura, 1998). Epidemiological studies suggest that humans who consume muscle foods are continuously exposed to HAAs in their diet. A recent study focussed on intake of well-done meat and breast cancer risk among postmenopausal women based on individual N-acetyltransferase-2 activity. They concluded that consumption of well-done meat among women with the rapid/intermediate N-acetyltransferase-2 genotype increased breast cancer risk nearly 8-fold when compared to those who consumed medium or rare meats (Deitz et al., 2000). For this reason, it is of great interest to define mechanism by which HAA formation can be inhibited during the cooking of meats.

Three naturally occurring compounds present in meat (creatine, amino acids, and hexoses) were suggested by Jagerstad et al. (1983a) to be precursors of the imidazoquinoline or imidazoquinoxaline mutagens. Reaction of creatine with certain amino acids without the presence of sugars has also produced HAAs. Jagerstad et al. (1983b) proposed a pathway for formation of IQ compounds via the Maillard reaction

through vinylpyrazine, vinylpyridine and aldehyde formation. Namiki and Hayashi (1981) demonstrated the formation of a free radical, N, N'-disubstituted pyrazine cation, by early carbon fragmentation prior to formation of the Amadori product.

The formation of HAAs can be significantly reduced by the addition of various compounds with antioxidant potential to meat and model systems, thus supporting the theory that a free radical mechanism maybe involved in HAA formation. Weisburger et al. (1994) showed a significant decrease of PhIP when green or black tea was added to a model system. They showed decreases of MeIQx and PhIP when the tea flavonoids theaflavine gallate and epigallocatechin gallate were added to the model system; both of these compounds are potent antioxidants. Chen (1988) showed inhibition of HAA formation by adding synthetic antioxidants BHA, PG, and TBHQ individually to ground beef patties. Kato et al. (1996) found that the phenolic antioxidants BHA, PG, sesamol, esculetin, and epigallocatechin gallate prevents formation of HAAs in model system containing glucose, glycine, and creatinine. Balogh et al. (2000) added vitamin E to ground beef patties and showed inhibition of PhIP formation by 72% in the cooked product.

Our previous studies found significant reductions in MeIQx, PhIP, and mutagenicity when ground beef patties containing 11.5% (w/w) tart cherries were fried at 190 °C. When patties containing 4.0, 7.5, or 11.5% (w/w) tart cherries were fried at 225 °C there were reductions in PhIP formation of 50.8, 71.6, and 85.3%, respectively (Chapter 1).

One of the dominant antioxidants present in cherries in the group of anthocyanin compounds which was shown by Wang et al. (1999c) to have antioxidant activity

comparable to compounds such as BHT and TBHQ. However, we have found that addition of anthocyanins to a model system and to meat at levels comparable to that of 11.5 % (w/w) cherry tissue failed to inhibit HAA formation.

The objective of this study was to test sequential solvent extracts of cherries for HAA-inhibitory activity. Inhibitory extracts were then further purified to determine which compound or group of compounds in tart cherries is responsible for the inhibition of HAA formation in fried ground beef patties.

MATERIALS AND METHODS

Reagents. The HAA standards (MeIQx, 4, 8-DiMeIQx, and PhIP) were purchased from Toronto Research Chemicals (Toronto, Canada). The HAA standard (FEMA - Flavor and Extracts Manufacturer's Association) was a gift from Dr. Mark Knize, Lawrence Livermore National laboratory, Livermore, CA. The FEMA standard contained IQ, MeIQ, MeIQx, DiMeIQs, and PhIP, each at 5 ng/μl. Polysulfonic acid (PRS) Bond Elute columns (500 mg), C-18 cartridges (100 mg), and Hydromatrix were purchased from Varian, Inc. (Harbor City, CA). Extrelut – 20 columns were obtained from E. M. Separations Technology (Gibbstown, NJ). All other chemicals were of analytical grade and were obtained from Fisher Scientific (Fair Lawn, NJ).

Materials. Fresh ground beef was obtained from Michigan State University Meat Laboratory. Quick-frozen, pitted Montmorency tart cherries were obtained from Cherry Marketing Institute, Inc (Dewitt, MI). The cherries were placed in freezer bags, flushed with nitrogen, and stored (-20 °C) until ready to use. The fat content of the meat was determined by the method of Folch et al. (1957).

Extraction of Tart Cherries. Whole Montmorency cherries were pulverized through an Urshel comitrol (Urshel Laboratories, Inc., Valparaiso, IN) using a 3-K-030240-V head and lyophilized. The dried tart cherries (202 g) were extracted sequentially with hexane, ethyl acetate and methanol (500 ml x 3) and the solvents were evaporated under reduced pressure at 40 °C using a rotavap (Buchi, Switzerland) to yield

crude extracts of 0.21, 3.4, and 98.5 g, respectively. The scheme used to extract cherries and fractionate extracts is shown in Figure 1.

The methanol extract was applied to an XAD-2 column (100 g, Amberlite resin, mesh size 20-50, Sigma Chemical Co., St. Louis, MO), which was prepared as described by Chandra et al. (1993). The sugars and acids were eluted together from the column with distilled water and the aqueous eluate was lyophilized (sugar/acid extract). The adsorbed compounds on the resin were then eluted with methanol. The red methanolic solution was concentrated at 40 °C using a rotavap (Buchi, Switzerland), and then lyophilized to yield the polar polyphenolic fraction.

The sugar/acid extract was applied to an ion-exchange column (Basic, Amberjet 4200 (Cl) ion-exchange resin, Aldrich Chemical Co.). To separate sugars from acids, the resin was prepared by washing sequentially with distilled water (250 ml x 3) and methanol (250 ml x 3). The resin was soaked in methanol for one hour, dried, and washed with distilled water (250 ml x 3). The resin was soaked overnight in distilled water and packed into a column. The sugar/acid fraction was dissolved in distilled water, applied to the column, and equilibrated for 30 min. The sugars were eluted with distilled water (3 volumes) and lyophilized to yield the sugar fraction. The adsorbed acids were then sequentially eluted with 3 N ammonium hydroxide (1 volume).

Preparation of Cherry Fractions and Ground Beef Patties. The ethyl acetate extract, methanol extract, sugar/acid fraction, and polar polyphenolic fraction were redissolved in methanol. The sugar fraction was redissolved in distilled water. Pure malic acid, the dominant acid present in cherries, was used instead of the acid fraction

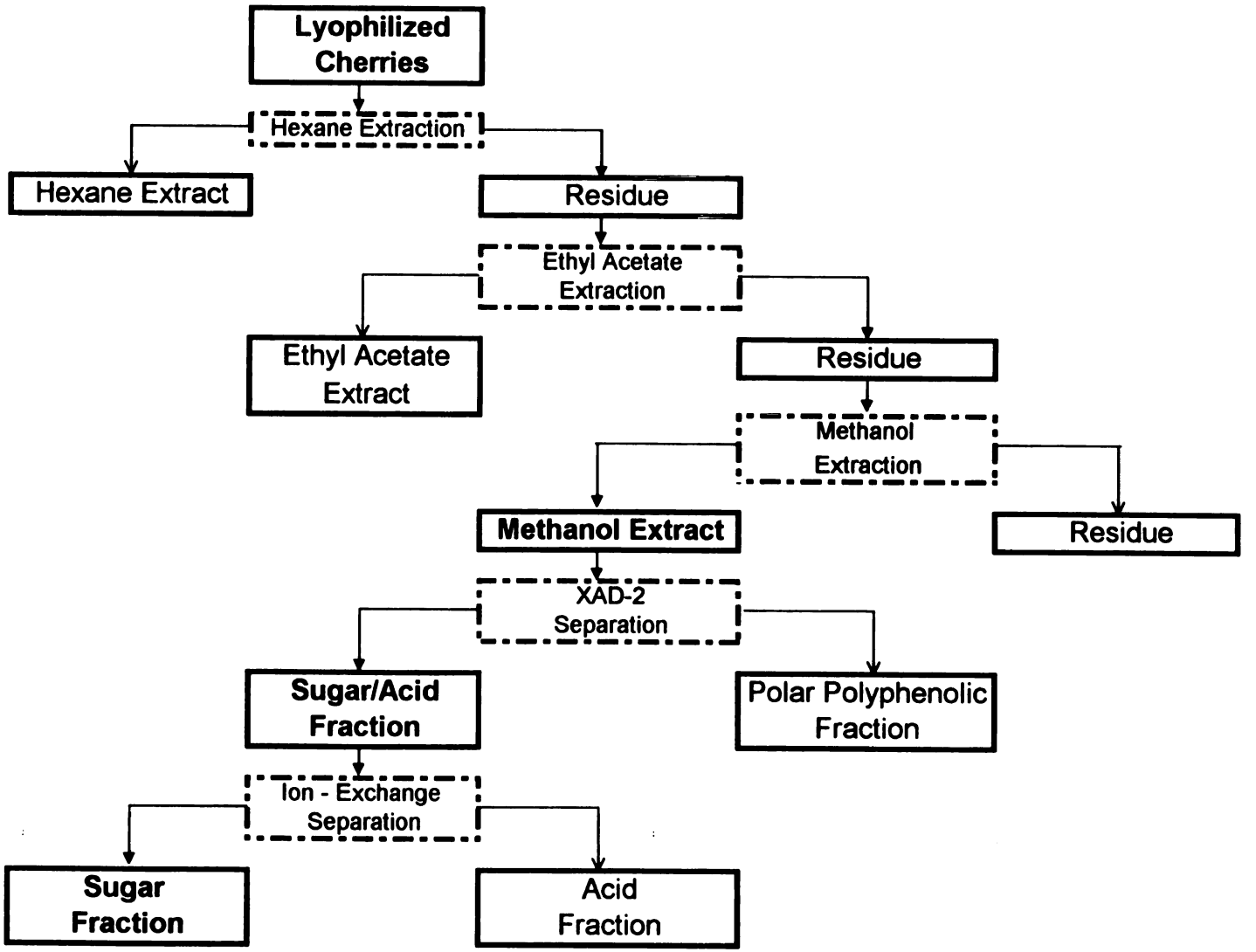


Figure 1 Sequential Extraction and Fractionation of Whole Cherries

because of the presence of excessive salts derived from elution of the ion-exchange column. Each of these extracts and fractions were added as a treatment directly to an individual ground beef patty on a weight basis equivalent to 11.5% (w/w) fresh cherries. Control patties contained equal amounts of methanol as the treatments. Patties were formed by weighing 100 g of ground beef in a Petri dish (9 cm x 1.5 cm) to ensure patty uniformity. Patties were mixed thoroughly by hand and incubated at 4 °C for 12 h until time of frying.

Preparation of Ground Beef Patties for Sugar and Gluconic Acid Studies. Stock solutions of sugars and gluconic acid were prepared by dissolving in water. Sugar or gluconic acid solutions were added to each individual ground beef patty on a weight basis equivalent to 11.5% (w/w) fresh cherries. Control patties contained equal amounts of distilled water to that of the treatments. Patties were formed by weighing 100 g of ground beef in a Petri dish (9 cm x 1.5 cm) to ensure patty uniformity. Patties were mixed thoroughly by hand and incubated at 4 °C for 12 h until time of frying.

Cooking Preparation. Patties were removed from the cooler (4 °C) and placed at room temperature for 20 min prior to frying. Patties were fried in an electric, Teflon-coated pan at 225 °C. The frying temperature was measured using a surface temperature thermometer (Pacific Transducer Corp., Los Angeles, CA). Each treatment consisted of frying two patties for 10 min per side, total cooking time of 20 min. After frying, the patties were cooled to 25 °C and homogenized in a Waring blender. The homogenized patties were placed in polystyrene bags and stored at -20 °C until time of extraction.

Sample Extraction. The procedure used for HAA extraction was adopted from Gross and Gruter (1992). Meat samples were extracted by blending 25 g of cooked meat from two patties with 75 g 1 N NaOH and subsequently divided into four 16-g aliquots and transferred into 250 ml beakers. To determine extraction recoveries for each HAA and aid in their identification, two of the four samples were spiked with 250 ng of each HAA (IQ, MeIQ, MeIQx, DiMeIQx, and PhIP) dissolved in 50 µl methanol. Each sample was mixed with Hydromatrix (Varian, Inc) and packed into an Extrelut 20 column. A propylsulfonic acid silica (PRS) column was attached in tandem to each Extrelut 20 column. The HAAs were extracted with dichloromethane/toluene (95:5) until 40 ml of solvent passed through the PRS column. The PRS column was detached and transferred to a Visiprep vacuum (Supelco), and dried for 15 min. The PRS columns were connected to a peristaltic pump and washed sequentially with 6 ml 0.1 N HCl, 15 ml 0.1 N HCl/methanol (6:4), and 2 ml of water. Bond Elute C18 cartridges were attached to the PRS columns and rinsed with 20 ml of ammonium acetate buffer solution (0.5 M, pH 8.0) to transfer the HAAs. The C18 cartridges were dried for 30 minutes using the Visiprep vacuum (Supelco). The HAAs were eluted with 1.0 ml of methanol/ammonia (9:1). The solvent was evaporated to dryness in a 40 °C water bath using a stream of nitrogen, and the dried compounds were refrigerated until required for HPLC analysis.

HAA Quantification. The dried samples were redissolved in methanol containing 50ng/µl caffeine (internal standard). HPLC separation was carried out on a TSK-gel ODS80-TM column (4.6 mm i.d. x 25 cm, Tosoh Haas, Montgomeryville, PA) attached to a precolumn (Supelguard LC-8-DB, Supelco, Bellefonte, PA). The flow rate

of the mobile phase was 1 ml/min. The initial ratio of acetonitrile/buffer (triethylamine phosphate, 0.01 M, pH 3.2) was 8:92, and was increased to 17:83 during the first 10 min. Acetonitrile concentration was increased to 25:75 in the next 10 min and to 55:45 in the following 10 min. Over the next 5 min, the acetonitrile/buffer ratio was increased to 80:20 to elute all other compounds. The ratio was then returned to the original 8:92 for 10 min to allow the column to re-equilibrate prior to the next injection. Samples were analyzed on a Waters HPLC system (Millipore, Milford, MA) with a photodiode array detector (model 996) and a scanning fluorescence detector (model 474). The sample peak areas were integrated using the Millennium 2000 Chromatography Manager (Millipore, Milford, MA).

The peaks from the spiked samples were used to identify the peaks in the unspiked samples by comparing retention times. Also, UV spectral characteristics of the HPLC peaks in each sample were compared to a spectral library of HAA standards using the Millennium software. For each set of experiments, before HPLC separation of sample extracts, four aliquots (25, 30, and 35 µl) of two HAA standards (containing 0.5 ng/µl of each compound), the internal standard caffeine (5 ng/µl), and HAA standard FEMA (5 ng/µl) were injected. Linear regression (ng compound vs. peak area) was performed for each HAA in each mixture. A correlation coefficient of 0.97 or greater for laboratory mixtures of HAAs was acceptable, and 0.99 or greater for the FEMA internal standards. All HAA peak areas were corrected using the internal standard regression line and expressed as ng/g of meat. Extraction efficiencies and quantification of HAAs were determined using the standard addition method of Gross and Gruter (1992). Each data point consisted of four subsamples: two spiked and two unspiked. The average area of

the spiked samples minus the average area of the unspiked samples allowed comparison with the regression line for the standard mixture. Individual data points were determined and then corrected for extraction efficiency, or percent yield. The average of the unspiked samples was used to determine the concentrations of each HAA formed. The linear regression slope for FEMA was used to determine the exact amount of each HAA present in each sample.

Sugar and Creatine Analyses. Covance Laboratories (Madison, WI) determined the identity and quantity of each sugar in the sugar fraction extracted from tart cherries. Creatine was extracted from raw meat and quantified using the method of Khan and Cowen (1977). Ten grams of raw meat were homogenized with 50 ml of trichloroacetic acid (10% w/v) and centrifuged for 20 min @ 3000 g. The supernatant was collected and the residue was washed twice with 50 ml of trichloroacetic acid (10% w/v). The supernatant and washings were combined, filtered, and adjusted to 200 ml with trichloroacetic acid (10% w/v) and allowed to stand at room temperature for 2 h. Creatine content was analyzed using 1 ml of sample extract, 5 ml of NaOH-Na_2CO_3 solution (60 g of NaOH + 160 g of Na_2CO_3/l.), 3 ml of α-naphthol solution (freshly prepared, 1% w/v, in NaOH-Na_2CO_3 solution), 2 ml of diacetyl solution (freshly prepared, 0.1% v/v, in water), and diluted with water to a total volume of 25 ml. This test solution along with appropriate blanks and standards were allowed to stand at room temperature for 20 min and read using a spectrophotometer (Varian Cary3 UV- Visible Spectrophotometer) set at 520 nm. Glucose content of raw meat was determined using

the extraction method of creatine followed by adjustment of the extract to pH 7.0 and analysis for glucose content using a glucose assay kit (Sigma Kit GAHK–20).

Statistical Analyses. All results were analyzed by Sigma Stat 2.0 (Jandel Corp., San Rafael, CA). One-way analysis of variance (ANOVA) was performed. Appropriate comparisons were made using Student-Newman-Kreuls test for one-way ANOVA analysis.

RESULTS AND DISCUSSION

The recoveries of HAAs present in the cooked ground beef with and without treatments ranged from 36 to 88%. The average recoveries of spiked samples were 69 ± 8, 76 ± 8, and 56 ± 7% for MeIQx, DiMeIQx, and PhIP, respectively. These results are comparable to those of other studies who reported similar recoveries ranging from 35 to 85% (Knize et al., 1994; Balogh et al., 2000).

Dried tart cherries were sequentially extracted with solvents to yield three main extracts (hexane, ethyl acetate, and methanol). The hexane extract did not yield significant quantities for testing, therefore it was not used. The ethyl acetate extract, which contains primarily non-polar flavonoids (Wang et al., 1999a), was tested in ground beef patties and did not show any inhibition of HAAs. The methanol extract, which contains predominantly polar flavonoids, sugars and anthocyanins (Wang et al., 1999b), inhibited the formation of PhIP by 62.5% (Figure 2 and Table 1). The other HAAs were not affected by the addition of the methanol extract to ground beef patties.

The methanol extract was applied to the XAD-2 column, which yielded two fractions (sugar/acid fraction and polar polyphenolic fraction). The polar polyphenolic fraction, which contains mainly anthocyanins and polar flavonoids, has been shown to contain compounds with high levels of antioxidant activity (Wang et al., 1999b). As reported in Chapter 2, anthocyanins did not inhibit the formation of HAAs and the polar polyphenolic fraction also failed to inhibit HAA formation. The sugar/acid fraction, which is composed of mainly glucose, fructose, and malic acid, inhibited PhIP formation in ground beef patties by 58.6%. The other HAAs were not affected by the addition of the sugar/acid fraction to ground beef patties (Figure 3 and Table 1). These results are

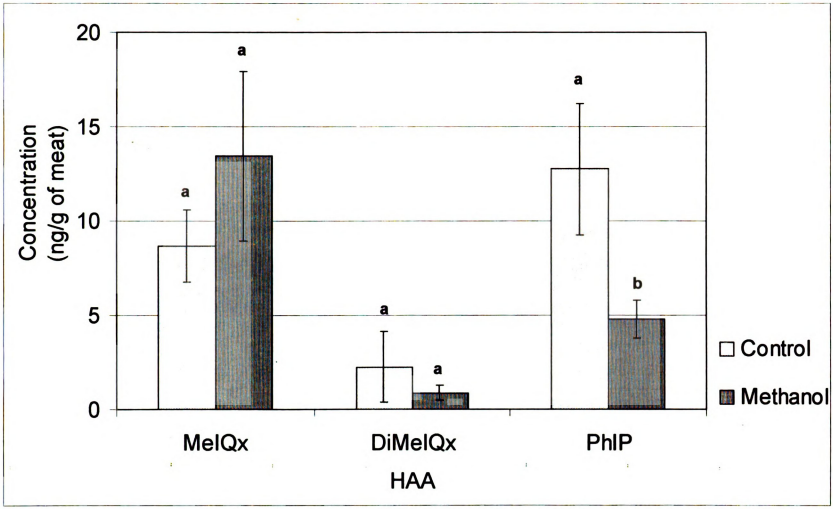


Figure 2. Inhibition of Heterocyclic Aromatic Amine Formation Using a Methanol Extract Obtained from Tart Cherry

Table 1. Heterocyclic Aromatic Amine Content of Ground Beef Patties Containing Fractions from Cherry Extracts

Sample	MeIQx (ng/g)	% Inhibition	DiMeIQx (ng/g)	% Inhibition	PhIP (ng/g)	% Inhibition
Control	8.7 ± 1.9 [a]	-	2.3 ± 1.9 [a]	-	12.8 ± 3.5 [a]	-
MeOH	13.4 ± 4.5 [a]	NSD	0.9 ± 0.4 [a]	NSD	4.8 ± 1.0 [b]	62.5
Sugar/Acid	4.7 ± 0.6 [a]	NSD	2.3 ± 0.2 [a]	NSD	5.3 ± 1.1 [b]	58.6
Sugar	5.6 ± 2.5 [a]	NSD	1.9 ± 1.4 [a]	NSD	6.5 ± 2.2 [b]	49.2

Data represent the mean and standard deviation of five analyses per treatment (n=5).

Values are expressed on a cooked ground beef basis.

Means in the same columns bearing different superscripts are significantly different (p<0 0.05).

NSD- No significant difference

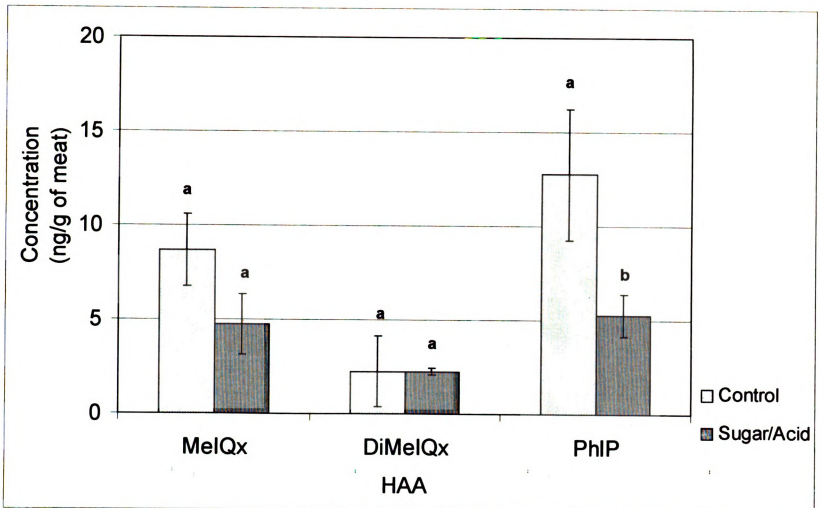


Figure 3. Inhibition of Heterocyclic Aromatic Amine Formation Using a Sugar/Acid Fraction Obtained from Tart Cherry

similar to those reported in Chapter 1, which showed the lack of inhibition of either MeIQx or DiMeIQx and inhibition of PhIP formation at 11.5% (w/w) (85.3%).

The sugar/acid fraction was applied to an ion-exchange column to separate sugars from acids. The sugar fraction consisted of glucose (26.3% w/v), fructose (15.9% w/v), and maltose (0.33% w/v). The sugar fraction was added to the ground beef patties, at a dose equivalent level of 11.5% (w/w) of fresh cherries, and inhibited PhIP formation by 49.2%. There is no significant difference between the inhibition of PhIP formation in the methanol extract, sugar/acid fraction or the sugar fraction, suggesting that sugars alone inhibit PhIP formation. The other HAAs were not affected by the addition of the sugar fraction to the ground beef patties (Figure 4 and Table 1). The acid fraction, which consists of predominately malic acid (Krishna and Markakis, 1965), was added to ground beef patties at a level consistent with that of 11.5% (w/w) fresh cherry (2.94 mg/patty) and did not show any inhibition of HAA formation.

The next study was designed to verify the finding that sugars alone were responsible for the inhibition of PhIP formation. A stock solution of three sugars (26.3% glucose, 15.9% fructose, and 0.33% maltose) was prepared and added, at a dose equivalent level of 11.5% (w/w) of fresh cherries, to ground beef patties. Glucose alone was added (134 mg/patty) to ground beef patties to investigate if there was a difference in the type of sugar used and if there was synergy among the three sugars. The mixture of sugars inhibited the formation of PhIP and DiMeIQx by 69.2 and 62.5%, respectively (Figure 5 and Table 2). By comparison, glucose alone inhibited the formation of PhIP and DiMeIQx by 67.4 and 50.0%, respectively. These findings confirmed our previous

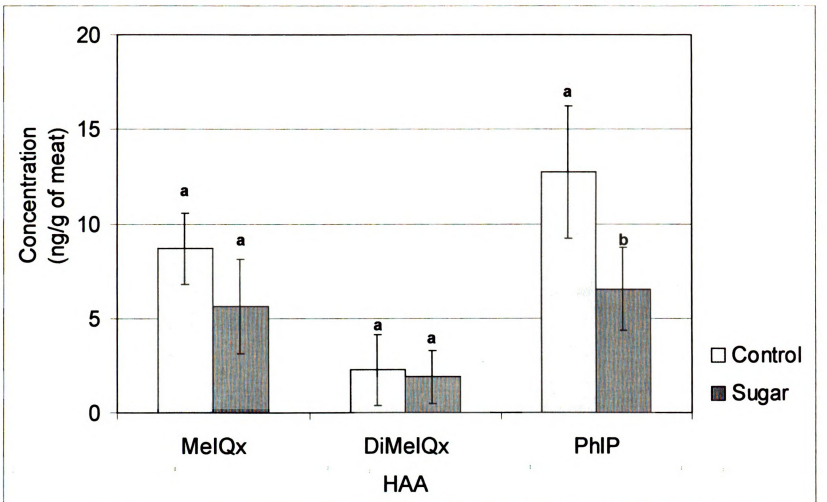


Figure 4. Inhibition of Heterocyclic Aromatic Amine Formation Using a Sugar Fraction Obtained from Tart Cherry

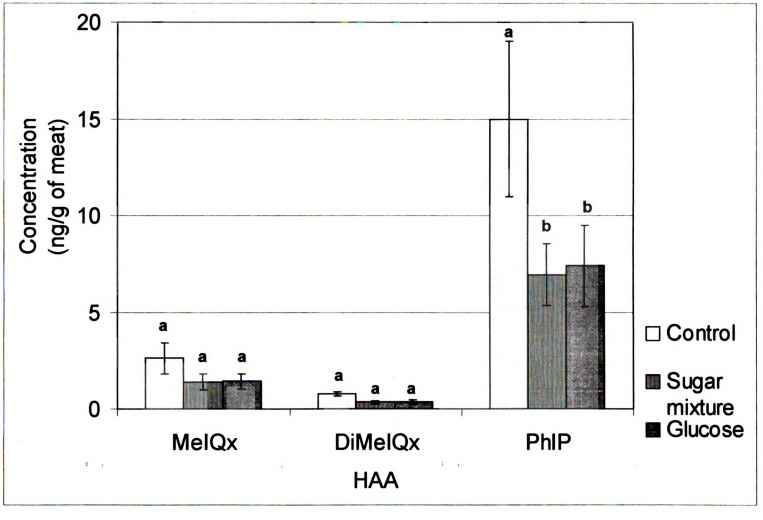


Figure 5. Inhibition of Heterocyclic Aromatic Amine Formation Using a Mixture of Sugars and Glucose Alone (134mg/patty)

Table 2. Heterocyclic Aromatic Amine Content of Fried Ground Beef Patties Containing Sugars (134 mg/patty)*

Sample	MeIQx (ng/g)	% Inhibition	DiMeIQx (ng/g)	% Inhibition	PhIP (ng/g)	% Inhibition
Control	2.6 ± 0.8 [a]	-	0.8 ± 0.1 [a]	-	22.7 ± 5.0 [a]	-
Gluc,Fruct,Malt**	1.4 ± 0.4 [a]	NSD	0.3 ± 0.1 [b]	62.5	7.0 ± 1.6 [b]	69.2
Glucose	1.4 ± 0.4 [a]	NSD	0.4 ± 0.1 [b]	50.0	7.40 ± 2.1 [b]	67.4

Data represent the mean and standard deviation of five analyses per treatment (n=5).
Values are expressed on a cooked ground beef basis.
Means in the same columns bearing different superscripts are significantly different ($p < 0.05$).
NSD- No significant difference
*-Sugar content is based on that of pitted cherry fruit
**-Glucose (82.8 mg), fructose (50.2 mg), maltose (1.0 mg)

results (Table 1) and showed no significant difference among the sugars used to inhibit HAA formation. Similarly, Skog and Jagerstad (1990) using a model system observed no significant difference in inhibition of mutagenicity among glucose, fructose or lactose treatments. In contrast, they found that sucrose induced a much higher mutagenicity over a broad range of concentrations.

As discussed in Chapter 1, there was an inverse dose-response relationship of tart cherry tissue to HAA formation when cherries were added to ground beef patties at various treatment levels (4.0, 7.5 and 11.5% w/w). To further investigate the role of cherry sugars in inhibiting HAA formation, glucose was added to ground beef patties at 88.5 mg/patty and 134 mg/patty, which correspond to the levels of sugars in whole tart cherry tissue added at 7.5 and 11.5% treatment levels. The addition of glucose (134 mg/patty) inhibited the formation of PhIP by 42.9%, whereas the lower glucose level had no significant effect on HAA formation (Table 3). These results are consistent with the dose-dependence of whole cherries on HAA formation (Chapter 1, Table 2).

With the inhibitory effect of glucose on HAA formation established, we hypothesized that the reducing group in glucose was the reactive moiety. To test this hypothesis, glucose (a reducing sugar) or gluconic acid (an oxidized form of glucose) was added to ground beef patties at 134 mg/patty. After cooking, extraction, and analysis, glucose was shown to inhibit the formation of PhIP (Tables 2 & 3), whereas an equivalent amount of gluconic acid had no effect on HAA formation (Table 3). These findings strongly suggest that the reducing group is the reactive moiety on glucose, which is responsible for glucose inhibition of PhIP formation.

Table 3. Heterocyclic Aromatic Amine Content of Fried Ground Beef Patties Containing Glucose or Gluconic Acid

Sample	MeIQx (ng/g)	% Inhibition	DiMeIQx (ng/g)	% Inhibition	PhIP (ng/g)	% Inhibition
Control	3.1 ± 0.3 [a]	-	1.0 ± 0.2 [a]	-	16.1 ± 2.4 [a]	-
Gluconic acid 134 mg	3.2 ± 0.7 [a]	NSD	1.2 ± 0.5 [a]	NSD	15.9 ± 3.9 [a]	NSD
Glucose 88.5 mg	3.6 ± 0.6 [a]	NSD	0.7 ± 0.3 [a]	NSD	14.6 ± 2.1 [a]	NSD
Glucose 134 mg	2.9 ± 0.6 [a]	NSD	0.9 ± 0.4 [a]	NSD	9.2 ± 2.7 [b]	42.9

Data represent the mean and standard deviation of five analyses per treatment (n=5).
Values are expressed on a cooked ground beef basis.
Means in the same columns bearing different superscripts are significantly different ($p < 0.05$).
NSD- No significant difference

Studies conducted in model systems suggested that there is a biphasic relationship between HAA formation and the ratio of reducing sugar to creatine (Skog and Jagerstad, 1990). Glucose added at half the molar concentration of creatine and glycine in a model system produced the greatest mutagenicity. When glucose was added at amounts less than or more than half the molar concentration of the other two reactants, a substantial decrease in mutagenicity was observed. Reacting glycine and creatine together without glucose yielded no mutagenicity. When glucose was present at half the molar amount of creatine, almost 90% of creatinine was recovered after being heated, indicating that only a small amount of creatine is needed to form HAA compounds. Adding a molar excess of glucose to the model system resulted in a decreased recovery of unreacted creatinine.

Skog and Jagerstad (1991) studied the effect of glucose on the formation of PhIP in a model system and found that PhIP could be formed by reacting phenylalanine and creatine alone. However, the formation of PhIP was significantly enhanced when glucose was added at half the molar ratio of creatine. They also found that the formation of PhIP was inhibited when the glucose concentration was greater than or less than half the molar ratio of creatine. These studies indicate that the role of sugar in the formation of HAAs is biphasic. Sugar can enhance or inhibit the formation of HAAs depending upon the proportion of the reactants.

It was subsequently shown by Laser Reutersward et al. (1987) and Skog et al. (1992a) that the creatine to glucose ratio in meat was approximately 2:1, which the model system studies suggested is the optimum ratio for HAA formation. To compare our results with the literature values we analyzed the creatine and glucose content from three sources of ground beef. The results were highly variable (Table 4). The ratio of creatine

Table 4. Analysis of Fat, Creatine, and Glucose in Raw Ground Beef Samples

Ground Beef	Fat Content (%)	Creatine (mg/100 g)	Glucose (mg/100 g)	Creatine/Glucose (molar ratio)	Addition of Glucose (134 mg/100g Patty) Glucose (mg/100 g)	Addition of Glucose (134 mg/100g Patty) Creatine/Glucose (molar ratio)
# 1	14.0	456.0	20.2	31.1	154.2	4.0
# 2	13.5	510.0	85.8	8.2	219.8	3.2
# 3	19.7	350.0	31.8	15.1	165.8	2.9

Data represent the mean of three analyses per treatment (n=3).
Values are expressed on a raw ground beef basis.

to glucose ranged from 8.2 to 31.1, and differs substantially from that of Laser Reutersward et al. (1987) and Skog et al. (1992a). The major sugars found in post mortem beef muscles are glucose, fructose, and ribose. The total carbohydrates in beef yield about 48 mg/100 g of tissue, and 95 % of that is glucose (Macy et al., 1964).

Since the composition of ground beef varied from source to source, especially with respect to levels of HAA precursors, the two additional meat sources (#2 and #3) were used to assess the formation of PhIP when glucose was added to ground beef patties. The addition of glucose at a concentration of 134 mg/100g to ground beef patties decreased the creatine : glucose ratio to a range of 2.9 to 4.0. This ratio is nearer the optimum 2:1 ratio for HAA formation than the meat samples without added glucose. Based on model system studies we should have observed an increase in HAA formation by the addition of glucose to ground beef patties according to Jagerstad et al. (1983a), who found that addition of glucose to glucose-deficient beef resulted in a two- to three-fold increase in mutagen formation. However, the addition of glucose at 134 mg/patty to meat sources #2 and #3 inhibited PhIP formation by 53.9 and 65.5%, respectively (Figure 6 and Table 5). These results indicate that model system results must be interpreted with caution.

Skog et al. (1992a) studied the inhibitory effect of carbohydrates on mutagen formation in fried beef patties. They observed the greatest inhibitory effect of mutagen formation when glucose and lactose were used in the fried beef patties. Glucose and lactose were used at concentrations of 1.0, 2.0, and 4.0%. This resulted in creatine : glucose molar ratios of 1:2, 1:4, and 1:7, respectively. When the ratio of creatine : glucose was 1:4, the mutagenic activity was inhibited by 66%. Our studies found

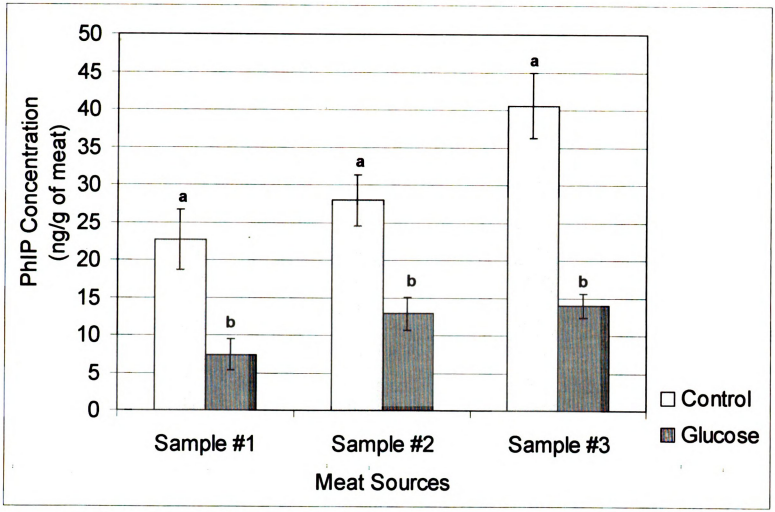


Figure 6. Inhibition of PhIP Formation Using Glucose (134mg/patty) on Three Meat Sources

Table 5. Heterocyclic Aromatic Amine Content of Fried Ground Beef Patties (three meat sources) Containing Glucose (134 mg/patty)

Sample	MeIQx (ng/g)	% Inhibition	DiMeIQx (ng/g)	% Inhibition	PhIP (ng/g)	% Inhibition
Control #1	2.6 ± 0.8 [a]	-	0.8 ± 0.1 [a]	-	22.7 ± 5.0 [a]	-
Glucose	1.4 ± 0.4 [a]	NSD	0.4 ± 0.1 [b]	50.0	7.40 ± 2.1 [b]	67.4
Control #2	4.1 ± 0.6 [a]	-	2.2 ± 0.6 [a]	-	28.0 ± 3.4 [a]	-
Glucose	3.8 ± 0.8 [a]	NSD	1.8 ± 0.4 [a]	NSD	12.9 ± 2.2 [b]	53.9
Control #3	N/A	-	1.10 ± 0.1 [a]	-	40.6 ± 4.3 [a]	-
Glucose	N/A	-	1.40 ± 0.6 [a]	NSD	14.0 ± 1.6 [b]	65.5

Data represent the mean and standard deviation of five analyses per treatment (n=5).
Values are expressed on a cooked ground beef basis.
Means in the same columns bearing different superscripts are significantly different ($p < 0.05$).
NSD-No significant difference

significant decreases in PhIP formation when glucose was added to ground beef patties at only 0.134%. The addition of glucose to three sources of ground beef patties resulted in a creatine : glucose ratio of 4.0:1, 3.2:1, and 2.9:1 and a total inhibition of PhIP formation by 67.4, 53.9, and 65.5%, respectively.

The inhibitory role of sugars as constituents of food ingredients was also observed in a study in which onions added to ground beef patties decreased mutagenicity by as much as 20% (Kato et al., 1998). Onion extracts reduced mutagenicity by as much as 40%. Analysis of components of onion which were responsible for mutagen inhibition, indicated that sugars were exclusively responsible for this effect. Although studies conducted by Tsai et al. (1996) found that organosulfur compounds diallyl disulfide (DAD) and dipropyl disulfide (DPD) added to boiled pork juice reduced overall mutagenicity by 80 to 98%, these compounds apparently had no effect at the levels found in onion. They observed a significant decrease (40%) in mutagenicity when they added 0.2 g of glucose to 30 g of ground beef, which is equivalent to adding 666 mg of glucose to 100 g of ground beef. They also found that glucose was more effective than corn starch at inhibiting mutagen formation, which is consistent with Skog et al. (1992a).

The addition of glucose at 0.66% to ground beef is the lowest amount reported in literature to date that results in significant reductions in mutagenicity. Our studies have shown repeatedly, using different meat sources, that the addition of glucose to ground beef patties at 1/5 of this level (0.134%) is effective at inhibiting the formation of PhIP.

The paradoxical ability of sugar to act as a promoter and inhibitor of HAAs has been extensively studied and is still not clear. Skog and Jagerstad (1990; 1991) have investigated the effects of sugars in model systems and proposed that Maillard reaction

products may block the formation of imidazoquinoxalines by reacting with creatine and that the Maillard reaction may favor the formation of products, which would compete with the formation of HAAs in the presence of an excess amount of sugar. The inhibitory effects of excess sugars on the formation of IQ type compounds and PhIP are most likely different.

The addition of glucose to ground beef patties changed the creatine : glucose ratio from 31:1 to 4.0:1 and inhibited the formation of PhIP, while MeIQx and DiMeIQx were not affected. The mechanism by which glucose inhibited the formation of PhIP is unknown. The mechanism by which quinolines and quinoxalines are formed has only been hypothesized and many questions still remain. However, the formation of PhIP is less clear than the formation of IQ-type compounds. Model studies have shown repeatedly that formation of PhIP is greatest at a creatine : glucose ratio of 2:1, but these model studies contain only the simplest reaction precursors and do not approach the complexity of the meat matrix.

We offer three possible explanations for the effect of glucose on PhIP formation. 1) It is possible that the addition of glucose to the ground beef patties alters the reaction route to form other Maillard reaction products, hypothesized by Skog and Jagerstad (1990; 1991). They gave support for this hypothesis by showing that glucose at half the molar concentration of creatine resulted in recovery of 90% of creatinine. A lowered recovery of unreacted creatinine was shown with increasing glucose concentrations. Additional support was shown by decreased recovery of unreacted creatinine and overall mutagenicity decrease after the addition of a Maillard reaction product (5-hydroxymethyl-2-furfural). 2) The additional glucose may react with specific amino

acids (phenylalanine, luecine, isoleucine, and tyrosine) resulting in less amino acids available to form PhIP. Support is provided by Arvidsson et al. (1997) who demonstrated that in a model system that all of the glucose had reacted after the first 2.5 minutes, while about 75% of amino acids were reacted after 10 minutes. They also found the activation energy for formation of PhIP was higher than other HAAs tested. Knize et al. (1994) demonstrated the formation of PhIP takes a longer time to make the first 20% of the quantity measured than MeIQx formation. 3) The additional glucose may alter part of the Maillard reaction that decreases the formation of pyrazines and in turn decreases the formation of PhIP. Support for this theory is provided by Koehler and Odell (1970) who found that the addition of glucose (3 to 1 molar ratio to other reactants) in a sugar-amino acid model system decreased the yield of 2-methylpyrazine by ten-fold and dimethylpyrazine by 125-fold.

Considering the abundance of antioxidants present in tart cherries, we expected that fractions high in antioxidant activity would have inhibited the formation of HAAs. Wang et al. (1999c) found that anthocyanins and cyanidin were comparable to the antioxidant activities of tert-butylhydroquinone and butylated hydroxytoluene and superior to vitamin E. Polyphenols such as 7-dimethoxy-5,8,4-trihydroxyflavone and quercetin 3-rhamnoside isolated from the methanol extract of tart cherries have been shown to be potent antioxidants (Wang et al., 1999b). Other flavonoids isolated from extracts of tart cherries have also shown high antioxidant activity, yet none of these compounds present in these cherry extracts have inhibited the formation of HAAs. It is possible that these compounds are not present in sufficient quantity to be effective inhibitors of HAAs.

These studies have systematically isolated fractions from tart cherries and identified fractions that inhibited formation of HAAs. Our results clearly demonstrated that sugars present in tart cherries are responsible for inhibiting the formation of PhIP. The addition of glucose at levels based on their concentration in fresh cherries to ground beef patties prior to cooking significantly reduced the formation of PhIP. The mechanism by which glucose inhibited the formation of PhIP is unclear, but these studies have established effective levels of glucose addition that are far lower than other documented studies.

CHAPTER FOUR

SUMMARY AND CONCLUSIONS

A series of studies were conducted to investigate the effects of tart cherry tissue on HAA formation in ground beef patties. The main focus of these studies was to establish a dose-dependence of cherry tissue addition and determine which compounds in tart cherries were responsible for the inhibition of HAA formation.

The results of these studies have shown that tart cherry addition to ground beef patties has inhibited the formation of HAAs, decreased mutagenicity and established a dose-dependence relationship. These studies have also shown that anthocyanins have no effect on HAA formation in a model system or ground beef patties when added at an equivalent dose of 11.5% (w/w) of fresh cherry.

The sequential extraction of lyophilized cherries and fractionation of these extracts enabled us to determine that sugars (glucose, fructose and maltose) in tart cherries were responsible for inhibiting PhIP formation. Further studies showed that the addition of glucose at levels based on their concentration in fresh cherry reduced PhIP formation in ground beef patties with activity comparable to that of whole cherry tissue. These studies have established effective levels of glucose addition that are far lower than other documented studies.

CHAPTER FIVE

FUTURE RESEARCH

The effect of whole tart cherries on HAA formation and overall mutagenicity in fried ground beef patties was investigated. A sequential extraction of tart cherries revealed that sugars present in tart cherries were responsible for inhibiting the formation of HAAs. These results indicate that the addition of tart cherries or glucose prior to cooking may be an alternative approach to reduce the formation of HAAs. These findings raise questions for future research to address.

1. We showed that the addition of tart cherries to ground beef patties fried at 220 °C basically inhibited PhIP formation. However, previous results by Britt et al. (1998) showed decreased formation of five HAAs. The differences that exist between these studies should be further addressed.

2. Several flavonoid and phenolic compounds did not inhibit HAA formation at levels consistent with their weight in fresh cherries. These compounds show good antioxidant activity, yet fail to inhibit HAAs. This may be due to low levels of these compounds and require higher levels to inhibit HAA formation. Further investigation is needed.

3. The role of anthocyanins in inhibiting HAA formation in fried ground beef patties and a model system was investigated. Although anthocyanins failed to inhibit HAA

formation, an additional study to determine if other levels of anthocyanins inhibit HAA formation should be conducted.

4. After sequential extraction of whole tart cherries, we showed that sugars were responsible for inhibiting the formation of PhIP. Further studies found that glucose at 0.134% inhibited PhIP formation in ground beef patties. Additional research should be carried out to determine if glucose at higher treatment levels inhibits PhIP formation and determine if additional levels of glucose affect other HAAs.

5. Additional studies should focus on the mechanism of PhIP formation and how various sugar concentrations inhibit and promote PhIP formation.

APPENDIX

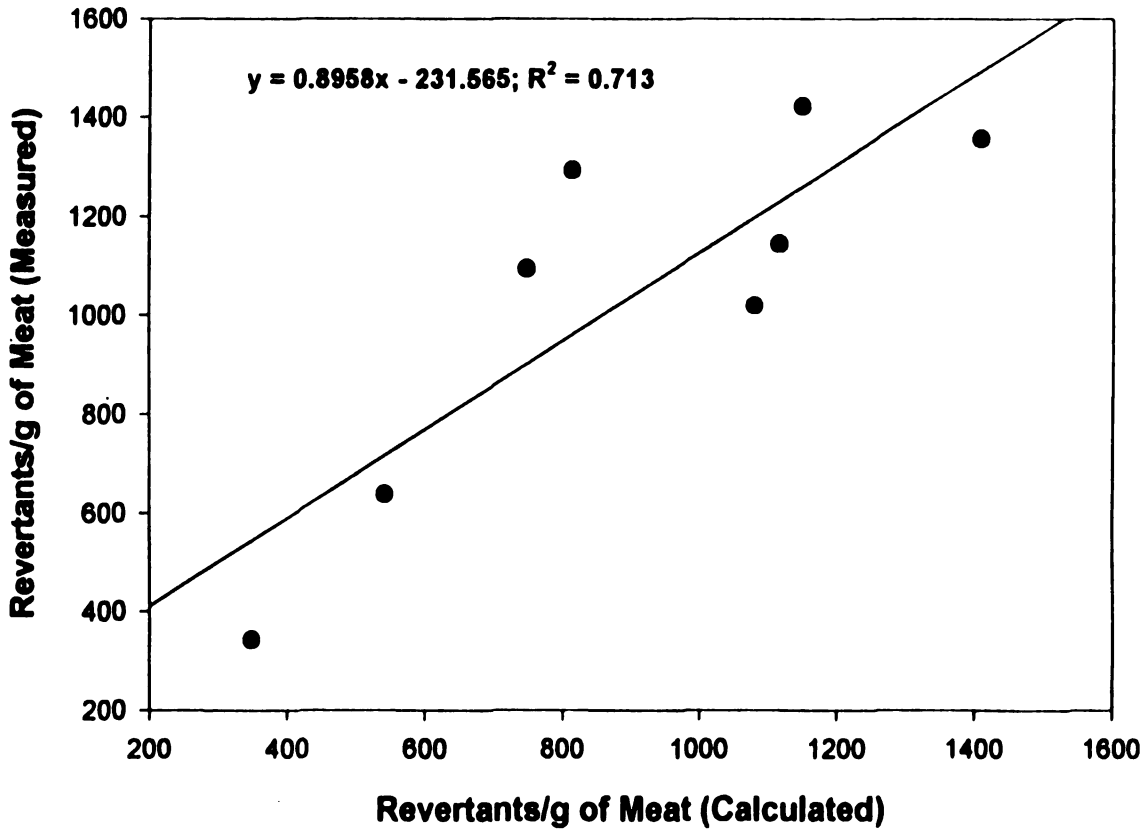


Figure 1. Plot of mutagenic activity quantitated by the *S. typhimurium* TA 98 assay and mutagenic activity calculated from the heterocyclic aromatic amine concentrations in fried beef patties as determined by HPLC analyses. Each data point is the mean of five replicated experiments

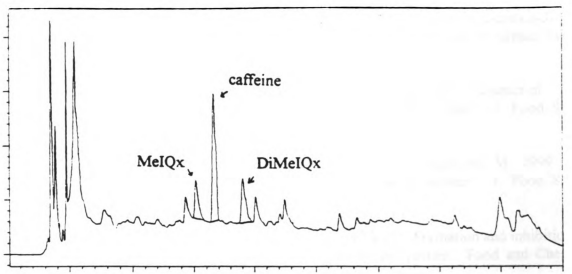


Figure 2. A Profile of a Typical HPLC Chromatogram Illustrating the Elution of MeIQx, caffeine (internal std.), and DiMeIQx Using a Photodiode Array Detector Set at 254 nm

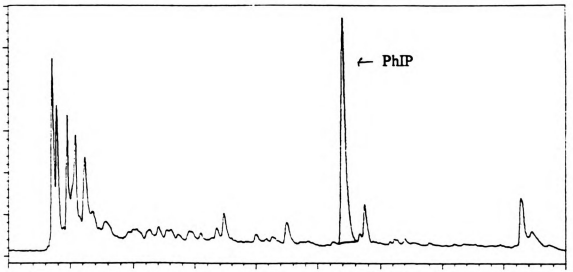


Figure 3. A Profile of a Typical HPLC Chromatogram Illustrating the Elution of PhIP using a Fluorescence Detector with Excitation Set at 330 nm and Emission at 375 nm

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