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FORMATION AND INHIBITION OF HETEROCYCLIC AROMATIC AMINES IN FRIED GROUND BEEF PATTIES

presented by

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has been accepted towards fulfillment
of the requirements for

Master's degree in Food Science

J. Ian Gray
Major professor

Date 8-03-95

FORMATION AND INHIBITION OF HETEROCYCLIC AROMATIC AMINES IN FRIED GROUND BEEF PATTIES

By

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ABSTRACT

FORMATION AND INHIBITION OF HETEROCYCLIC AROMATIC AMINES IN FRIED GROUND BEEF PATTIES

By

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This study was designed to determine the effect of vitamin E and oleoresin rosemary on heterocyclic aromatic amine formation in fried ground beef patties. Patties were fried at three temperatures (175°C, 200°C, 225°C) for 6 and 10 minutes per side to determine the conditions for optimum heterocyclic aromatic amine (HAA) formation. HAAs were isolated by solid phase extraction and quantitated by high performance liquid chromatography. Greatest concentrations were generated when patties were fried at 225°C for 10 min/side ---- 16.9 ng/g raw meat PhIP [2-amino-1-methyl-6-phenylimidazo (4,5-b) pyridine] and 3.1 ng/g raw meat MeIQx [2-amino-3,8-dimethylimidazo (4,5-f) quinoxaline].

Vitamin E, when used at two concentrations (1 and 10% based on fat content) and added directly to the ground beef pattie, reduced PhIP concentrations by approximately 72%. Smaller but more variable reductions were achieved for MeIQx. Oleoresin rosemary also successfully reduced HAA formation, although not to the same extent as vitamin E.

The application of vitamin E (1% based on fat content) to the surface of the pattie provided comparable inhibition of HAA formation. The concentrations of five HAAs studied were all significantly reduced ($p<0.006$). The average reductions ranged from 49% to 77%.

To my husband, Zoltan, and my daughter, Krisztina,
for their enduring love, patience and kindness.

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INTRODUCTION

A series of mutagenic and carcinogenic heterocyclic aromatic amines have been found in meat and fish cooked at temperatures over 150°C. 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), 4,8-DiMeIQx (2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline), and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. Many of these compounds are multi-potential carcinogens in rodent bioassays (Sugimura and Wakabayashi, 1990; Ohgaki et al., 1991), and one of the most abundant heterocyclic aromatic amines, PhIP, has been shown to induce colon and mammary carcinomas in rats (Ito et al., 1991; Ochiai et al., 1991).

Epidemiological studies suggest that the consumption of well-done red meat is associated with a high risk of colon and other cancers (Norrel et al., 1986; Schiffman and Felton, 1990; Steineck et al., 1990; Willett et al., 1990). Recent estimates of potential human cancer potency are consistent with an upper-bound cancer risk between 10^{-3} and 10^{-4} for an average lifetime cooked-beef intake of 3.3 g/kg/day (or approximately 0.5 lb/day) (Bogen, 1994).

Precursors of these mutagenic/carcinogenic compounds in beef are creatine/creatinine, amino acids and sugars (Jagerstad et al., 1983a). However, heterocyclic aromatic amines can also be formed in dry-heated mixtures of amino acids and creatine (Yoshida et al., 1984; Taylor et al., 1987; Knize et al., 1988a; Overvik et al., 1989; Felton and Knize, 1990). Cooking temperature and time are also important factors in the formation of these mutagens (Commoner et al., 1978a; Bjeldanes et al., 1983; Miller and Buchanan, 1983; Overvik et al., 1984; Knize et al., 1985, 1994; Reutersward et al., 1987a,b; Nielsen et al., 1988).

The formation of heterocyclic aromatic amines has been suggested to follow the Maillard reaction (Spingarn and Garvie, 1979; Shibamoto et al., 1981; Wei, 1981; Powrie et al., 1982) through vinylpyrazine, vinylpyridine and aldehyde formation (Jagerstad et al., 1983b). However, the formation of the free radical, N,N'-disubstituted pyrazine cation, by early carbon fragmentation prior to the Amadori product was demonstrated by Namiki and Hayashi (1981). Thus, another route for the formation of heterocyclic aromatic amines was suggested to proceed via a free radical process (Pearson et al., 1992), for which further support was provided by Milic et al. (1993).

Reports of synthetic phenolic antioxidants inhibiting the formation of these compounds lend credence to this theory (Wang et al., 1982; Barnes et al., 1983; Chen et al., 1992; Faulkner, 1994). Inhibition of the formation of IQ-like compounds by vitamin E was demonstrated by Chen et al. (1992). However, the reported concentrations of the mutagenic compounds were 1000-fold higher than those cited by other researchers (Felton et al., 1986a,b; Sugimura et al.,1988; Thiebaud et al., 1994). Thus, the quantitative data of Chen et al. (1992) are suspect as no confirmatory studies were carried out. Recently, Faulkner (1994) confirmed the inhibition of PhIP formation by vitamin E using both the *Salmonella typimurium* overall mutagenicity test and an analytical procedure to quantitate the extent of inhibition. This analytical procedure is a challenging one which is reflected in the fact that reported heterocyclic aromatic amine concentrations in beef are based on recoveries of standards compounds that range from as low as 5% to as high as 85% (Jackson et al., 1994; Johansson and Jagerstad, 1994; Knize et al., 1994; Thiebaud et al.,1994). Thus, some modification of the method is necessary to obtain reproducible recoveries of the heterocyclic aromatic amines in cooked meats.

The objectives of this study are:

(1) To optimize the extraction and analysis of heterocyclic aromatic amines in fried ground beef patties using the Standard Addition Quantitation procedure developed by Gross and Gruter (1992).

(2) To compare the formation of heterocyclic aromatic amines in ground beef patties fried at various time/temperature combinations.

(3) To evaluate the inhibition of heterocyclic aromatic amine formation by natural phenolic antioxidants

(a) by the direct addition of vitamin E and oleoresin rosemary to the beef pattie before frying;

(b) through surface application of vitamin E to the beef pattie before frying.

LITERATURE REVIEW

Formation of heterocyclic aromatic amines

Types of mutagenic compounds found in fried ground beef

Several mutagenic/carcinogenic substances are produced or introduced into foods during their cooking, processing, and storage. Among the first reported were the polycyclic aromatic hydrocarbons, including benzo[α]pyrene, formed by the pyrolysis of fat which dripped onto the heated coals during the barbecuing of meats (Lijinsky and Shubik, 1964). Another group of compounds was reported by Japanese investigators, who found that heating proteins or amino acids to high temperatures (>300°C) produced several potent mutagens. These were called 'pyrolytic mutagens' (Matsumoto et al., 1977; Nagao et al., 1977). Cooking meat at lower temperatures produces another group of mutagenic compounds (Commoner et al., 1978a; Dolara et al., 1979), often referred to as 'thermic mutagens'. Several have been identified in cooked meat, fish and food grade beef extracts (Table 1).

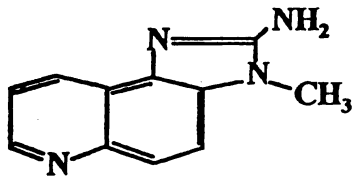
Thermic mutagens are heterocyclic aromatic amines and are often called aminoimidazoazaarenes. They can be broken down into four categories : quinolines, quinoxalines, pyridines, and furopyridines (Skog, 1993). The chemical structures of the principal mutagens in cooked foods are shown in Figure 1.

Heterocyclic aromatic amines in fried ground beef

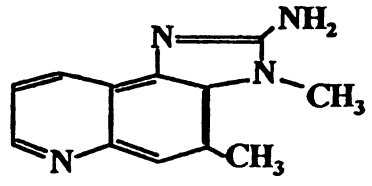
QUINOLINES:

The heterocyclic aromatic amines, 2-amino-3-methylimidazo[4,5-f]-quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-f]-quinoline (MeIQ), were isolated from the crust of broiled sardines (Kasai et al., 1980a,b; 1981a). Early studies reported the presence of IQ in fried ground beef at concentrations ranging

Quinolines

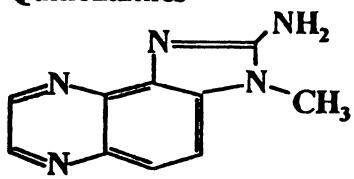


IQ

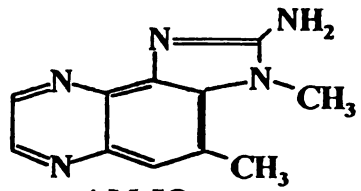


MeIQ

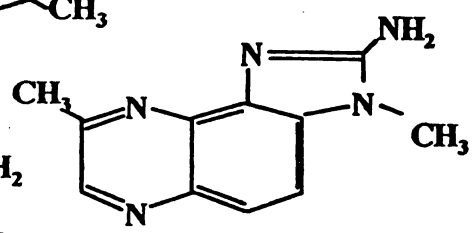
Quinoxalines



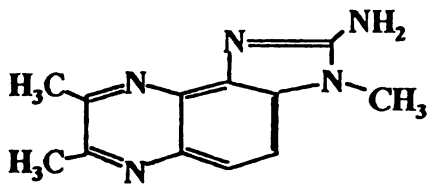
IQ_X



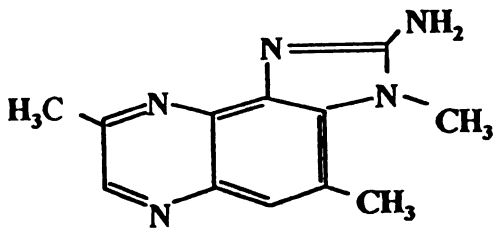
4-$MeIQ_X$



$MeIQ_X$

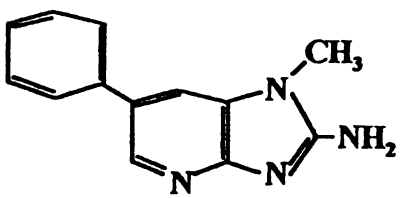


7,8-Di$MeIQ_x$

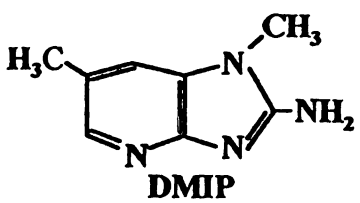


4,8-Di$MeIQ_x$

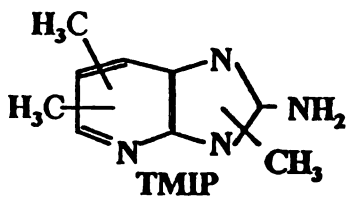
Pyridines



PhIP

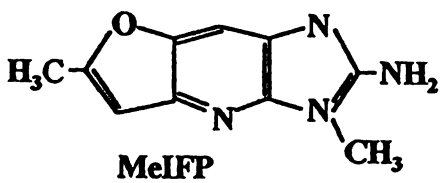


DMIP



TMIP

Furopyridines



MeIFP

Figure 1. Chemical structures of the principal mutagens in cooked foods (Skog, 1993).

from 0 to 20 ng/g (Barnes et al., 1983; Felton et al., 1984; Turesky et al., 1988). Reported concentrations for MeIQ are lower (Felton et al., 1986a; Yamaizumi et al., 1986; Gross et al., 1993).

QUINOXALINES:

The first quinoxaline to be identified in fried ground beef was 2-amino-3,8-dimethylimidazo[4,5-f]-quinoxaline (MeIQx) (Kasai et al., 1981b), followed by 2-amino-3,7,8-trimethylimidazo[4,5-f]-quinoxaline (7,8-DiMeIQx) (Negishi et al., 1984a), and 2-amino-3,4,8-trimethylimidazo[4,5-f]-quinoxaline (4,8-DiMeIQx) (Grivas et al., 1985). MeIQx has been identified in fried ground beef at concentrations ranging from non-detectable to 12.3 ng/g (Kasai et al., 1981b; Hargraves and Pariza, 1983; Felton et al., 1984, 1986a; 1992; Wakabayashi et al., 1986; Murray et al., 1988; Sugimura et al., 1988; Turesky et al.,1988, 1989; Knize et al., 1994). The other two quinoxalines, 4,8-DiMeIQx and 7,8-DiMeIQx, are both present in fried ground beef, but in relatively small concentrations: 0 to 3.9 ng/g (Felton et al., 1986a; 1992; Turesky et al., 1988; Murray et al, 1988; Sugimura et al., 1988; Knize et al., 1994).

PYRIDINE:

2-Amino-1-methyl-6-phenylimidazo[4,5-f]-pyridine (PhIP), first isolated from the crust of fried ground beef by Felton et al. (1986b), is the most abundant heterocyclic aromatic amine in cooked meat, with concentrations ranging from 0 to 67.5 ng/g (Felton et al. 1986b; 1992; Gross et al., 1989; Gross, 1990; Hayatsu et al., 1991, Knize et al., 1994; Thiebaud et al., 1994). Two other pyridines, 2-amino-n,n,n-trimethylimidazopyridine (TMIP) and 2-amino-1,6-dimethylimidazopyridine (DMIP) have also been identified in fried meat (Becher et al., 1988; 1989; Felton et al., 1984).

Table 1. Heterocyclic aromatic amine content of cooked foods

Food type	Mutagen	Amount[1]	Temp[2]	Time[3]	Weight[4]	Ref[5]
BEEF						
Fried	MeIQx	0.5			0	43
	4,8-DiMeIQx	3.9	275	15	0	38
	8-MeIQx	12.3	200	15	0	38
	8-MeIQx	4.0	150	6	0	25
	IQ	1.9	200	15	0	38
BOUILLON						
Heated	4,8-DiMeIQx	0.3			0	33
	8-MeIQx	0.6			0	33
	PhIP	0.3			0	33
EXTRACT						
Boiled	4,8-DiMeIQx	28.0			1	14
	4,8-DiMeIQx	0.0-3.7			1	39
	4,8-DiMeIQx	0.0-4.4			1	9
	4,8-DiMeIQx	0.0			1	33
	4,8-DiMeIQx	2.5-4.9			1	10
	7,8-DiMeIQx	0.0			1	39
	8-MeIQx	28.0			1	14
	8-MeIQx	3.1			1	41
	8-MeIQx	20.5			1	38
	8-MeIQx	8.5-30.0			1	9
	8-MeIQx	23.0-69.0			1	40
	8-MeIQx	0.0-44.0			1	10
	8-MeIQx	0.6			1	33
	8-MeIQx	3.1			1	43
	8-MeIQx	11.7-36.4			1	39
	AαC	0.0			1	10
	IQ	0.0			1	41
	IQ	0.0			1	14
	IQ	0.5			1	36
	IQ	4.8-6.2			1	40
	IQ	0.0-6.2			1	39
	IQ	0.0-8.0			1	9
	PhIP	3.62			1	15
	PhIP	0.0			1	9
	PhIP	0.0			1	19

Food type	Mutagen	Amount[1]	Temp[2]	Time[3]	Weight[4]	Ref[5]
	PhIP	0.0			1	33
	4'-OH-PhIP	21.0	15	60	1	27
EXTRACT						
with creatine						
Heated	4-CH_2OH-8-Mex	6.2	121	60	1	24
	4-CH_2OH-8-Mex	6.7	160	300	1	24
	4-CH_2OH-8-Mex	7.2	200	300	1	24
FLAVOR						
	4,8-DiMeIQx	0.0			1	16
	4,8-DiMeIQx	0.0			1	16
	MeIQx	0.0-12.5			1	16
	MeIQx	0.0-4.4			1	16
Roasted	4,8-DiMeIQx	0.0			1	16
	MeIQx	0.0-4.4			1	16
Grilled	4,8-DiMeIQx	0.0			1	16
	MeIQx	0.0			1	16
GROUND						
Charbroiled	4,8-DiMeIQx	0.2		6	1	17
	4,8-DiMeIQx	0.1		10	1	17
	MeIQ	0.0		6	1	17
	MeIQ	0.4		10	1	17
	MeIQx	1.0		6	1	17
	MeIQx	0.4		10	1	17
	IQ	0.0		6	1	17
	IQ	0.1		10	1	17
	PhIP	0.0		6	1	17
	PhIP	0.0		10	1	17
Broiled	IQ	0.5			1	44
Grilled	AαC	0.0	270	3	1	12
	AαC	0.0	270	5	1	12
	AαC	0.0	270	7	1	12
	4,8-DiMeIQx	0.0	270	3	1	12
	4,8-DiMeIQx	0.0	270	5	1	12
	4,8-DiMeIQx	0.0	270	7	1	12
	MeIQx	0.8	270	3	1	12
	MeIQx	2.0	270	5	1	12
	MeIQx	0.0	270	7	1	12
	PhIP	0.7	270	3	1	12
	PhIP	1.4-4.8	270	5	1	12

Food type	Mutagen	Amount[1]	Temp[2]	Time[3]	Weight[4]	Ref[5]
	PhIP	0.0	270	7	1	12
Fried	AαC	21.0	277	6	1	37
	DiMeIQx	4.5	277	6	1	37
	4,8-DiMeIQx	0.5	300	6	0	7
	4,8-DiMeIQx	0.0	275	5	0	39
	4,8-DiMeIQx	0.0	275	10	0	39
	4,8-DiMeIQx	3.9	275	15	0	39
	4,8-DiMeIQx	0.5-1.2	200		2	32
	4,8-DiMeIQx	0.0	250	10	0	9
	4,8-DiMeIQx	0.12			0	35
	4,8-DiMeIQx	0.3			0	25
	4,8-DiMeIQx	0.0-0.28			2	5
	4,8-DiMeIQx	0.54	250	12	1	25
	4,8-DiMeIQx	0.0	150	2	0	26
	4,8-DiMeIQx	0.0	150	4	0	26
	4,8-DiMeIQx	0.1	150	6	0	26
	4,8-DiMeIQx	0.7	150	10	0	26
	4,8-DiMeIQx	0.0	190	2	0	26
	4,8-DiMeIQx	0.10	190	4	0	26
	4,8-DiMeIQx	0.55	190	6	0	26
	4,8-DiMeIQx	2.6	190	10	0	26
	4,8-DiMeIQx	0.0	230	2	0	26
	4,8-DiMeIQx	0.15	230	4	0	26
	4,8-DiMeIQx	0.25	230	6	0	26
	4,8-DiMeIQx	9.35	230	10	0	26
	4,8-DiMeIQx	0.7	225	6	1	29
	4.8-DiMeIQx	3.1			1	25
	7,8-DiMeIQx	0.0	275	5	0	39
	7,8-DiMeIQx	0.0	275	10	0	39
	7,8-DiMeIQx	0.7	275	15	0	39
	MeIQ	0.0	300	5.5	0	4
	4-MeIQ	0.1	300	6	0	7
	MeIQx	1.0	250	6	1	6
	MeIQx	16.4	277	6	1	37
	MeIQx	1.0	300	5.5	0	4
	MeIQx	0.0			0	19
	MeIQx	0.0-0.68			2	5
	MeIQx	0.3			0	43
	MeIQx	1.3-2.4	200		2	32

Food type	Mutagen	Amount[1]	Temp[2]	Time[3]	Weight[4]	Ref[5]
	MeIQx	2.7	275	5	0	39
	MeIQx	4.2	275	10	0	39
	MeIQx	12.3	275	15	0	39
	MeIQx	0.5-1.5			0	40
	8-MeIQx	0.1			0	6
	8-MeIQx	0.45	190		0	14
	8-MeIQx	1.1	250	10	1	9
	8-MeIQx	1.0	300	6	0	7
	8-MeIQx	0.64			0	35
	8-MeIQx	0.8			1	25
	8-MeIQx	2.95	250	6	1	25
	8-MeIQx	0.0	150	2	0	26
	8-MeIQx	0.0	150	4	0	26
	8-MeIQx	0.15	150	6	0	26
	8-MeIQx	2.7	150	10	0	26
	8-MeIQx	0.1	190	2	0	26
	8-MeIQx	0.25	190	4	0	26
	8-MeIQx	1.3	190	6	0	26
	8-MeIQx	5.1	190	10	0	26
	8-MeIQx	0.0	230	2	0	26
	8-MeIQx	0.4	230	4	0	26
	8-MeIQx	1.1	230	6	0	26
	8-MeIQx	8.0	230	10	0	26
	8-MeIQx	2.2	225	6	1	29
	8-MeIQx	10.8			1	25
	IQ	0.5-20.0	240	5	0	1
	IQ	0.02	250	6	1	6
	IQ	0.5			2	44
	IQ	0.0	192		0	18
	IQ	0.3	275	5	0	39
	IQ	0.3	275	10	0	39
	IQ	1.9	275	15	0	39
	IQ	0.02	300	5.5	1	4
	IQ	0.0	150	2	0	26
	IQ	0.0	150	4	0	26
	IQ	0.1	150	6	0	26
	IQ	1.5	150	10	0	26
	IQ	0.1	190	2	0	26
	IQ	0.1	190	4	0	26

Food type	Mutagen	Amount[1]	Temp[2]	Time[3]	Weight[4]	Ref[5]
	IQ	0.45	190	6	0	26
	IQ	0.82	190	10	0	26
	IQ	0.0	230	2	0	26
	IQ	0.15	230	4	0	26
	IQ	0.25	230	6	0	26
	IQ	1.8	230	10	0	26
	IQ	0.0	250	10	1	9
	PhIP	15.0	300	5.5	1	4
	PhIP	67.5	277	6	1	37
	PhIP	1.2	250	10	1	9
	PhIP	5.0			1	25
	PhIP	0.56			1	15
	PhIP	0.0	150	2	0	26
	PhIP	0.0	150	4	0	26
	PhIP	0.25	150	6	0	26
	PhIP	0.9	150	10	0	26
	PhIP	0.0	190	2	0	26
	PhIP	0.15	190	4	0	26
	PhIP	1.9	190	6	0	26
	PhIP	6.0	190	10	0	26
	PhIP	0.55	230	2	0	26
	PhIP	1.35	230	4	0	26
	PhIP	4.1	230	6	0	26
	PhIP	21.5	230	10	0	26
	PhIP	16.4	225	6	1	29
	PhIP	21.8			1	25
	TMIP	0.5	300	6	0	7
	Trp-P-1	0.0	300	6	0	8
	Trp-P-1	0.19			0	35
	Trp-P-2	0.0	200		0	31
	Trp-P-2	0.21			0	35
STEAK						
Broiled or fried	4,8-DiMeIQx	1.3	190	3	1	10
	4,8-DiMeIQx	2.0	190	6.5	1	10
	4,8-DiMeIQx	0.1	225	6	1	33
	8-MeIQx	2.11			0	35
	8-MeIQx	5.1	190	3	1	10
	8-MeIQx	8.3	190	6.5	1	10
	8-MeIQx	0.5	225	6	1	33

Food type	Mutagen	Amount[1]	Temp[2]	Time[3]	Weight[4]	Ref[5]
	AαC	1.2			0	35
	AαC	3.2	190	3	1	10
	AαC	8.9	190	6.5	1	10
	Glu-P-1	0.0			0	18
	Glu-P-2	0.0			0	45
	IQ	0.19			0	35
	PhIP	15.7			1	15
	PhIP	23.5	190	3	1	10
	PhIP	48.5	190	6.5	1	10
	PhIP	0.6	225	6	1	33
	Trp-P-1	53.0			0	45
	Trp-P-1	0.21			0	35
	Trp-P-2	0.25			0	35
BONITO						
Grilled	4,8-DiMeIQx	5.4	220	15	1	22
	8-MeIQx	5.2	220	15	1	22
Grilled, dried	8-MeIQx	2.5			0	22
CHICKEN						
Charbroiled	4,8-DiMeIQx	0.1			1	33
	8-MeIQx	0.3			1	33
Broiled	4,8-DiMeIQx	0.81			1	35
	MeIQx	2.1				44
	8-MeIQx	2.33			1	35
	AαC	0.21			1	35
	PhIP	38.1			1	15
	Trp-P-1	0.12			1	35
	Trp-P-2	0.18			1	35
Fried	Trp-P-1	0.0	300	6	0	8
CONSOMME'						
Heated	4,8-DiMeIQx	0.0			0	33
	8-MeIQx	0.1			0	33
	PhIP	0.0			0	33
EEL, roasted						
Fried, canned	7,8-DiMeIQx	5.3	180	4	1	28
	8-MeIQx	1.1	180	4	1	28
FALUN SAUSAGE						

Food type	Mutagen	Amount[1]	Temp[2]	Time[3]	Weight[4]	Ref[5]
Boiled, smoked	4,8-DiMeIQx	0.0	160	2.5	1	17
	MeIQ	0.0	160	2.5	1	17
	MeIQx	0.6	160	2.5	1	17
	IQ	0.3	160	2.5	1	17
	PhIP	0.0	160	2.5	1	17
FISH						
FLOUNDER						
	4,8-DiMeIQx	0.6			1	17
Smoked	MeIQ	0.3			1	17
	MeIQx	0.0-2.9			1	17
	IQ	0.7			1	17
	PhIP	0.0			1	17
HERRING						
Fried	4,8-DiMeIQx	0.3			1	17
	MeIQ	0.1			1	17
	MeIQx	0.6			1	17
	IQ	0.2			1	17
	PhIP	0.0			1	17
POLLACK						
Fried	4-MeIQ	0.03	260	8	1	46
	4,8-DiMeIQx	0.1	260	8	1	46
	8-MeIQx	6.44	260	8	1	46
	IQ	0.16	260	8	1	46
	PhIP	69.2	260	8	1	46
SALMON						
Baked	8-MeIQx	0.0	200	20	1	11
	8-MeIQx	4.6	200	30	1	11
	8-MeIQx	3.1	200	40	1	11
	AαC	0.0	200	20	1	11
	AαC	0.0	200	30	1	11
	AαC	0.0	200	40	1	11
	PhIP	0.0	200	20	1	11
	PhIP	18.0	200	30	1	11
	PhIP	5.9	200	40	1	11
Broiled, flesh	4-MeIQ	0.6-2.8			1	44
Broiled, skin	4-MeIQ	1.1-1.7			1	44
Broiled	4-MeIQ	0.1-0.9			1	3
	MeIQ	1.4-5.0			1	11
Broiled, flesh	IQ	0.3-1.8			1	44

Food type	Mutagen	Amount[1]	Temp[2]	Time[3]	Weight[4]	Ref[5]
Broiled, skin	IQ	1.1-1.7			1	44
Broiled	IQ	0.2-0.4			1	3
	PhIP	1.7-23.0			1	11
Charbroiled	8-MeIQx	0.0	270	4	1	11
	8-MeIQx	0.0	270	6	1	11
	8-MeIQx	0.0	270	9	1	11
	8-MeIQx	0.0	270	12	1	11
	AαC	2.8	270	4	1	11
	AαC	6.9	270	6	1	11
	AαC	73.0	270	9	1	11
	AαC	109.0	270	12	1	11
	PhIP	2.0	270	4	1	11
	PhIP	6.2	270	6	1	11
	PhIP	69.0	270	9	1	11
	PhIP	73.0	270	12	1	11
Cooked	4,8-DiMeIQx	0.2	150	9	1	17
	MeIQ	1.0-1.6	150	9	1	17
	MeIQx	0.6	150	9	1	17
	IQ	0.6	150	9	1	17
	PhIP	2.7-3.3	150	9	1	17
Fried	8-MeIQx	1.4	200	3	1	11
	8-MeIQx	5.0	200	6	1	11
	8-MeIQx	4.7	200	9	1	11
	8-MeIQx	3.7	200	12	1	11
	AαC	0.0	200	3	1	11
	AαC	4.6	200	6	1	11
	AαC	8.0	200	9	1	11
	AαC	9.0	200	12	1	11
	PhIP	1.7	200	3	1	11
	PhIP	23.0	200	6	1	11
	PhIP	14.0	200	9	1	11
	PhIP	17.0	200	12	1	11
Smoked	4,8-DiMeIQx	0.0			0	17
	MeIQ	0.0			0	17
	MeIQx	1.2-1.4			0	17
	IQ	0.3			0	17
	PhIP	0.0			0	17
SARDINE						
Broiled	4-MeIQ	16.6			1	45

Food type	Mutagen	Amount[1]	Temp[2]	Time[3]	Weight[4]	Ref[5]
	4-MeIQ	20.0			0	20
	8-MeIQx	0.0			2	45
	Glu-P-1	0.0			1	45
	IQ	20.0			0	19
	IQ	4.9			1	44
	IQ	20.0			0	20
	Phe-P-1	8.6			1	45
	Trp-P-1	13.3			1	45
	Trp-P-2	13.1			1	45
UNSPECIFIED						
Fried	Trp-P-2	0.0	200		1	31
Heated	4,8-DiMeIQx	5.4			1	23
	MeIQx	5.2			1	23
Smoked, dried	4,8-DiMeIQx	0.08			1	21
	MeIQx	0.8			1	21
LAMB, mutton						
Broiled	4,8-DiMeIQx	0.67			1	35
	8-MeIQx	1.01			1	35
	AαC	2.5			1	35
	AMαC	0.19			1	35
	PhIP	42.5			1	15
	Trp-P-2	0.15			1	35
MEATBALLS						
Fried	4,8-DiMeIQx	0.2			1	17
	MeIQ	0.3			1	17
	MeIQx	0.7			1	17
	IQ	0.2			1	17
	PhIP	0.6			1	17
MEAT EXTRACT						
Boiled	4,8-DiMeIQx	2.9-3.6			1	34
	8-MeIQx	6.2-28.3			1	34
	IQ	1.9-4.8			1	34
PORK						
Charbroiled	4,8-DiMeIQx	0.1			0	33
	8-MeIQx	0.4			0	33
	PhIP	4.2			0	33
Fried	Trp-P-1	0.0	300	6	0	8
BACON						

Food type	Mutagen	Amount[1]	Temp[2]	Time[3]	Weight[4]	Ref[5]
Fried	MeIQx	0.9-18.0		12-16	1	12
	4,8-DiMeIQx	0.0-1.		12-16	1	12
	PhIP	0.0-53.0		12-16	1	12
	AαC	0.0		12-16	1	12
Fried, moderate	4,8-DiMeIQx	1.7-5.1	150	2.5	1	17
Fried, well-done	4,8-DiMeIQx	1.0	150	5	1	17
Fried, moderate	MeIQ	0.0	150	2.5	1	17
Fried, well-done	MeIQ	1.4-2.0	150	5	1	17
Fried, moderate	MeIQx	0.0-5.8	150	2.5	1	17
Fried, well-done	MeIQx	1.4-3.6	150	5	1	17
Fried, moderate	IQ	2.3-5.3	150	2.5	1	17
Fried, well-done	IQ	9.5-11.5	150	5	1	17
Fried, moderate	PhIP	0.2	150	2.5	1	17
Fried, well-done	PhIP	1.0	150	5	1	17
BACON, fatty						
Fried	4,8-DiMeIQx	0.3	225	6	1	33
	8-MeIQx	1.2	225	6	1	33
	PhIP	2.7	225	6	1	33
BACON, lean						
Fried	4,8-DiMeIQx	0.2	225	6	1	33
	8-MeIQ	0.9	225	6	1	33
	PhIP	1.6	225	6	1	33
GROUND						
Fried	4,8-DiMeIQx	0.6	250	5	0	42
	4,8-DiMeIQx	0.24	180		1	2
	4,8-DiMeIQx	0.0			0	13
	4-MeIQ	0.0			0	13
	4-MeIQ	0.02	250	5	0	42
	4-MeIQx	0.1	250	5	0	42
	4-MeIQx	1.4	250	5	0	42
	4-MeIQx	0.4	180		1	2
	4-MeIQx	0.0			0	13
	IQ	0.04	250	5	0	42
	IQ	0.01	180		1	2
	IQ	0.0			0	13
	PhIP	4.5	250	5	0	42
	PhIP	1.7	180		1	2
	PhIP	0.0			0	13
GROUND, gravy						

Food type	Mutagen	Amount[1]	Temp[2]	Time[3]	Weight[4]	Ref[5]
Fried	4,8-DiMeIQx	0.9	250		1	2
	8-MeIQx	1.5	250		1	2
	IQ	0.04	250		1	2
	PhIP	10.0	250		1	2
SAUSAGE						
Fried	4,8-DiMeIQx	0.2	160	6	1	17
	MeIQ	0.2	160	6	1	17
	MeIQx	0.7	160	6	1	17
	IQ	0.1	160	6	1	17
	PhIP	0.1	160	6	1	17
	Trp-P-1	0.0	300	6	0	8

AαC = 2-amino-9H-pyrido[2,3-b]indole; AMαC = 2-amino-3-methyl-9H-pyrido[2,3-b]indole; 4-OH-PhIP = 2-amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-b]pyridine; 4-CH_2OH-8-Mex = 2-amino-4-hydroxy-methyl-3,8-dimethylimdazo[4,5-f] quinoxaline; Trp-P-1 = 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole; Trp-P-2 = 3-amino-1-methyl-5H-pyrido[4,3-b]indole; Glu-P-1 = 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole; Glu-P-2 = 2-aminodipyrido[1,2-a:3',2'-d]imidazole.

[1] Amount of mutagen formed (ng/g)

[2] Temperature of frying (°C)

[3] Cooking time (minutes per side), except for sausage (total frying time)

[4] Basis for heterocyclic amine concentration. 0=cooked weight of food, 1=uncooked weight, 2=unspecified

[5] References:

1- Barnes et al., 1983
2- Dragsted, 1992
3- Edmonds et al., 1986
4- Felton et al., 1986a
5- Felton et al., 1992
6- Felton et al., 1984
7- Felton et al., 1986b
8- Felton et al., 1987
9- Gross et al., 1989
10- Gross, 1990
11- Gross and Gruter, 1992
12- Gross et al., 1993
13- Gry et al., 1986

14- Hargraves and Pariza, 1983
15- Hayatsu et al., 1991
16- Jackson et al., 1994
17- Johansson et al., 1994
18- Kasai et al., 1981b
19- Kasai et al., 1981a
20- Kasai et al., 1980a
21- Kato et al., 1986
22- Kikugawa et al., 1986
23- Kikugawa and Kato, 1987
24- Kim et al., 1994
25- Knize, personal communication
26- Knize et al., 1994
27- Kurosaka et al., 1992
28- Lee and Tsai, 1991
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31- Murray et al., 1987
32- Murray et al., 1988
33- Murray et al., 1993
34- Schuirmann and Eichner, 1991
35- Sugimura et al., 1988
36- Taylor et al., 1985
37- Thiebaud et al., 1994
38- Turesky et al., 1983
39- Turesky et al., 1988
40- Turesky et al., 1989
41- Takahashi et al., 1985
42- Vahl et al., 1988
43- Wakabayashi et al., 1986
44- Yamaizumi et al., 1986
45- Yamaizumi et al., 1980
46- Zhang et al., 1988

FUROPYRIDINES:

Because of the complexity of the interactions occurring in cooked meats, it is not surprising that new heterocyclic aromatic amines are still being identified. A methylimidazofuropyrine (MeIFP), with a molecular weight of 202, was isolated from fried ground beef with added milk and creatinine (Felton et al., 1986a;

Becher et al., 1988). Evidence suggests that this mutagen is related to the food mutagen with a molecular weight 216, a amino-dimethylimidazofuropyridine (Knize et al., 1990).

A hydroxy derivative of PhIP, 2-amino-1-methyl-6-(4-hydroxyphenyl) imidazo[4,5-b]pyridine, was detected in broiled beef by Kurosaka et al. (1992), at concentrations similar to those for PhIP. Recently, a compound similar to 4'-OH-PhIP, containing an exocyclic oxygen atom, was identified as 2-amino-4-hydroxy-methyl-3,8-dimethylimidazo[4,5-f]quinoxaline (4-CH_2OH-8-MeIQx) in a beef extract (Kim et al., 1994).

Mutagenicity of heterocyclic aromatic amines

Heterocyclic aromatic amines are highly active in the Ames *Salmonella* system (Wakabayashi et al., 1992), in the DNA repair test of Williams, and in almost all tests measuring genotoxicity (Yoshimi et al., 1988). The heterocyclic aromatic amines have specific mutagenic activities toward *Salmonella typhimurium* TA98 and TA100. The mutagenicity of heterocyclic aromatic amines and other typical carcinogens is listed in Table 2.

Heterocyclic amines are metabolically activated by cytochrome P450. Metabolic activation of these compounds, in general, involves N-hydroxylation, followed by esterification to an acetyl or sulfate moiety (Okamoto et al., 1981; Saito et al., 1985; Paterson and Chipman, 1987; Snyderwine et al., 1987).

Bioassays have revealed them to be toxic and carcinogenic for several specific target organs, including the liver, urinary bladder, pancreas, intestinal tract, colon and mammary gland (Ohgaki et al., 1984; 1986; 1987; 1991; Kato et al., 1988; 1989; Ito et al., 1991; Snyderwine et al., 1993).

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

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

Mechanism(s) of heterocyclic aromatic amine formation

REACTANTS:

The mechanism by which heterocyclic aromatic amines are formed during the cooking of meats has not been fully elucidated. It was demonstrated by

Yoshida and Okamoto (1980a,b,c) and Yoshida and Fukuhara (1982) that dry heating creatinine with either glucose, fatty acids or various amino acids produced high mutagenic activity, and suggested that these reactants were possible precursors of heterocyclic aromatic amines. Bjeldanes et al. (1982a) reported a positive mutagenic response of the Ames *Salmonella* assay for extracts of cooked beef, pork, ham, bacon, lamb, chicken, fish and eggs. However, other foods with high protein content, e.g., tofu, milk, cheese, shrimp and organ meats, showed very low or negligible mutagen formation (Bjeldanes et al., 1982b). Jagerstad et al. (1983a) proposed that three naturally occurring substances in meat, creatine/creatinine, free amino acids and sugars, were the precursors of the imidazoquinoline- and imidazoquinoxaline-type mutagens. It was later demonstrated that chicken and beef contain the same heterocyclic aromatic amines in similar proportions as does fried ground fish, although in smaller amounts. This suggested that heterocyclic aromatic amines in cooked muscle foods all have similar precursors (Knize et al., 1988b; Felton and Knize, 1991).

Supporting evidence for creatine/creatinine involvement in the formation of heterocyclic aromatic amines is the low or nonexisting mutagenic activity in foods high in protein but lacking in creatine, e.g., liver and kidney (Reutersward et al., 1987b; Felton and Knize, 1990). Jagerstad et al. (1983a) demonstrated a significant increase in the mutagenic activity of beef when creatine was spread over the surface before frying. Mutagenic activity was detected also in shrimp when treated with creatine before heating (Miller, 1985). Other investigators have demonstrated the importance of creatine/creatinine in the formation of mutagenic activity (Nes, 1986; Becher et al., 1988; Knize et al., 1988a; Overvik et al., 1989; Felton and Knize, 1991).

In addition to creatine, free amino acids and dipeptides play an important role in the formation of heterocyclic aromatic amines. Mutagenic activity was first

reported in protein-rich foods, but when beef extracts were subjected to enzymatic proteolysis before boiling, increased mutagenic activity was observed (Taylor et al., 1984; 1985). These results indicate that amino acids and not proteins participate in the formation of heterocyclic aromatic amines.

When amino acids were dry heated with creatine at 200°C for 1 hr, mutagenic activity was detected (Overvik et al., 1989). Many studies have indicated that a single amino acid can produce several food mutagens in model reactions, and under similar conditions a specific heterocyclic aromatic amine can be produced from several amino acids (Table 3).

The involvement of sugars in heterocyclic aromatic amine formation was proposed by Jagerstad et al. (1983a). However, their role in the formation of these compounds remains unclear. Model systems with creatine/creatinine, amino acids and various sugars, have shown that sugars have a substantial impact on the formation of mutagens (Muramatsu and Matsushima, 1985; Skog and Jagerstad, 1990; 1991; Manabe et al., 1992). Many of the mutagens have been identified in reaction systems without sugars (Knize et al., 1988a; Overvik et al., 1989).

REACTION ROUTE:

Several investigators have proposed the Maillard reaction to be important in the formation of heterocyclic aromatic amines, but without a specified reaction route (Spingarn and Garvie, 1979; Shibamoto et al., 1981; Wei, 1981; Powrie et al., 1982). In the Maillard reaction, reducing sugars and amino groups from either amino acids, peptides or proteins combine to form a glycosylamine, which undergoes an Amadori rearrangement to yield a 1-amino-2-keto sugar (Hodge, 1953). This sugar may then be broken down into 2- and 3- carbon fragments by two pathways (3-deoxyhexosone and methyl α-dicarbonyl routes), leading to the formation of a variety of compounds such as aldehydes, ketones and

Table 3. Heterocyclic aromatic amines produced in model systems from creatin(in)e and amino acids, with or without sugar (Skog, 1993).

Compound	Yield[1]	Amino acids	Sugar	Heating conditions	Reference
IQ	0.4	pro	-	Dry	Yoshida et al., 1984
	1.0	gly	fru	DEG-H_2O	Grivas et al., 1986
	3.0	phe	-	Dry	Felton and Knize, 1990
	13.5	phe	glu	Dry	Felton and Knize, 1990
	3.7	ser	-	Dry	Knize et al., 1988a
MeIQ	nd	ala	fru	DEG-H_2O	Grivas et al., 1985
IQx	2.7	ser	-	Dry	Knize et al., 1988a
	nd	gly	glu	H_2O	Skog and Johansson,unpublished,1993
	nd	thr	glu	H_2O	Skog and Jagerstad, 1993
MeIQx	4.4	gly	glu	DEG-H_2O	Jagerstad et al., 1984
	0.9	ala	glu	DEG-H_2O	Muramatsu and Matsushima, 1985
	1.8	ala	rib	DEG-H_2O	Muramatsu and Matsushima, 1985
	4.2	lys	rib	DEG-H_2O	Muramatsu and Matsushima, 1985
	nd	thr	glu	DEG-H_2O	Negishi et al., 1985
	6-7	gly	fru	DEG-H_2O	Grivas et al., 1986
	nd	ser	-	Dry	Overvik et al., 1989
	nd	ala	-	Dry	Overvik et al., 1989
	nd	tyr	-	Dry	Overvik et al., 1989
	4.0	gly	glu	DEG-H_2O	Skog and Jagerstad, 1990
	nd	phe	glu	DEG-H_2O	Skog and Jagerstad, 1991
	10.0	ala, thr	glu	DEG-H_2O	Skog et al., 1992a
	8.8-17.9	gly	glu	H_2O	Johansson et al., 1993
	7.0-10.0	gly	glu	H_2O	Skog and Jagerstad, 1993
	9.0	thr	glu	H_2O	Skog and Jagerstad, 1993
4,8-DiMeIQx	nd	thr	glu	DEG-H_2O	Negishi et al., 1984a; 1985
	1.9-2.6	ala	fru	DEG-H_2O	Grivas et al., 1985
	4.2	ala	glu	DEG-H_2O	Muramatsu and Matsushima, 1985
	1.5	ala	rib	DEG-H_2O	Muramatsu and Matsushima, 1985
	26.1	lys	rib	DEG-H_2O	Muramatsu and Matsushima, 1985
	nd	gly	glu	DEG-H_2O	Skog and Jagerstad, 1990
	nd	phe	glu	DEG-H_2O	Skog and Jagerstad, 1991
	36.0	ala, thr	glu	DEG-H_2O	Skog et al., 1992a
	30.0	thr	glu	H_2O	Skog and Jagerstad, 1993
	nd	gly	glu	H_2O	Johansson et al., 1993
7,8-DiMeIQx	1.1	gly	glu	DEG-H_2O	Negishi et al., 1984b
	nd	gly	glu	DEG-H_2O	Skog and Jagerstad, 1990
	nd	gly	glu	H_2O	Johansson and Jagerstad, unpublished data, 1993

4,7,8-TriMeIQx	6.0	ala, thr	glu	DEG-H_2O	Skog et al., 1992a
PhIP	3.6	phe	glu	DEG-H_2O	Shioya et al., 1987
	735.0	phe	-	Dry	Felton and Knize, 1990
	560.0	phe	glu	Dry	Felton and Knize, 1990
	nd	phe	-	Dry	Overvik et al., 1989
	nd	leu	-	Dry	Overvik et al., 1989
	20.9	phe	glu	DEG-H_2O	Skog and Jagerstad, 1991
	6.4	phe	-	DEG-H_2O	Skog and Jagerstad, 1991
	< 0.058	phe	glu	DEG-H_2O	Manabe et al., 1992

[1]Yield in nmol/mmol creatin(in)e
Dry = dry hheating at 180°C or 200°C for 1 hr
DEG-H_2O = reflux boiling in diethylene glycol/water
H_2O = heated in water in closed metal tubes at 180°C for 10 or 30 min
Amino acids: pro = proline; gly = glycine; phe = phenylalanine; ser = serine; ala = alanine; thr = threonine; lys = lysine; tyr = tyrosine; leu = leucine
Sugars: fru = fructose; glu = glucose; rib = ribose
nd = not determined

melanoidin pigments. Pyrazines and pyridines can be produced from the interaction of the α-dicarbonyls from the Maillard reaction with amino acids, the so-called Strecker degradation.

The mechanism proposed by Hodge (1953) for the early stages of the Maillard reaction, identifying the Amadori rearrangement as a key step, was questioned by Namiki and Hayashi (1981). They reported the formation of the N,N'-disubstituted pyrazine cation by early carbon fragmentation prior to the Amadori product. They demonstrated that the radical products are formed by the condensation of two molecules of the two-carbon enaminol compounds which might be formed either directly from Schiff base products or indirectly through the reaction of glycolaldehyde with amino compounds (Fig.2). Thus, C2 and C3 fragments are produced prior to the Amadori rearrangement by a reverse-aldol reaction of the glycosylamine, forming glycolaldehyde alkylimines. These compounds could then be oxidized to form glyoxal monoalkylimines, which produced less free radicals and reacted more slowly than the glycolaldehyde

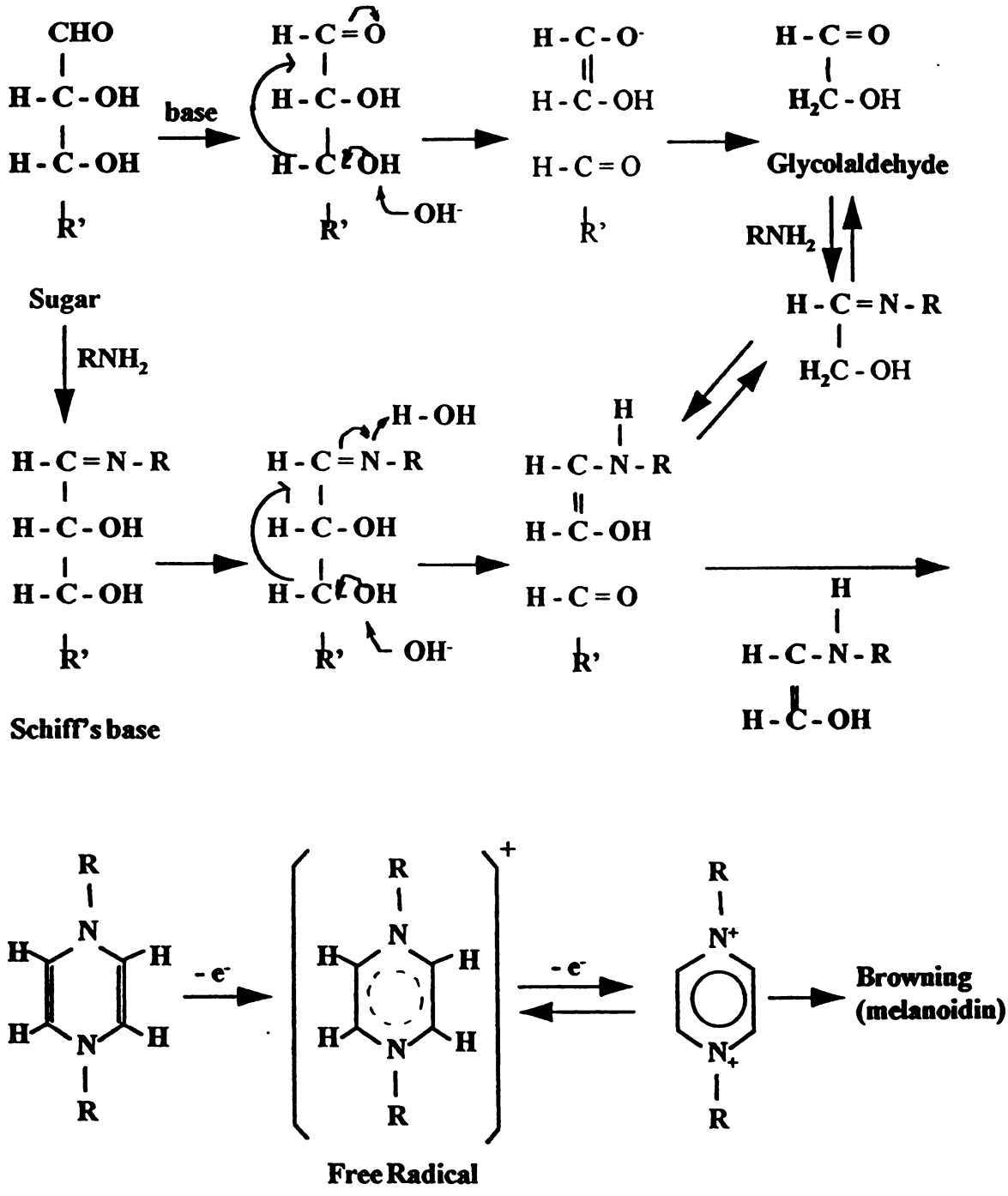

Figure 2. A possible pathway of browning in the Maillard reaction through a novel free radical (Namiki and Hayashi, 1981).

system (Namiki and Hayashi, 1981; Namiki et al.,1983). Glycolaldehyde is very effective in facilitating rapid and extensive radical formation compared to glyoxal.

In a later study, Nyhammer (1986) proposed that heterocyclic aromatic amines are formed by an aldol-type condensation between an aldehyde and a pyridine or pyrazine molecule, followed by the cyclic addition of creatine to yield either an imidazoquinoline or an imidazoquinoxaline.

Support for the theory of free radical involvement in heterocyclic aromatic amine formation was provided by Milic et al.(1993). Glucose, aminobutyric acids, and 2,3-diamino-1,4-naphthohydroquinone were heated in a model system and pyridine free radicals were detected by electron spin resonance. When only glucose and aminobutyric acids were heated, the formation of pyridine free radicals occurred. To further establish the reaction pathway, 2,5-dimethylpyrazine was heated with creatinine and acetaldehyde, DiMeIQx formation was observed. When 2-methylpyridine was heated instead of 2,5-dimethylpyrazine, MeIQ was formed. The extracts were analyzed by high performance liquid chromatography, direct probe mass spectrometry and nuclear magnetic resonance.

A possible reaction route for the formation of heterocyclic aromatic amines via the Maillard reaction was proposed by Jagerstad et al.(1983b). They postulated that creatine formed the amino-imidazo ring of the heterocyclic aromatic amine molecule by cyclization and water elimination to creatinine, a reaction that takes place when the temperature is raised above 100°C. The imidazo ring is common to the heterocyclic aromatic amines produced during normal cooking. The other two precursors, sugar and amino acids, were suggested to react following the Maillard reaction, and produce typical Maillard reaction products such as vinylpyrazines, vinylpyridines, and aldehydes (Fig.3). Thus, the quinoline or quinoxaline portion of the heterocyclic aromatic amine compound was assumed to arise from these

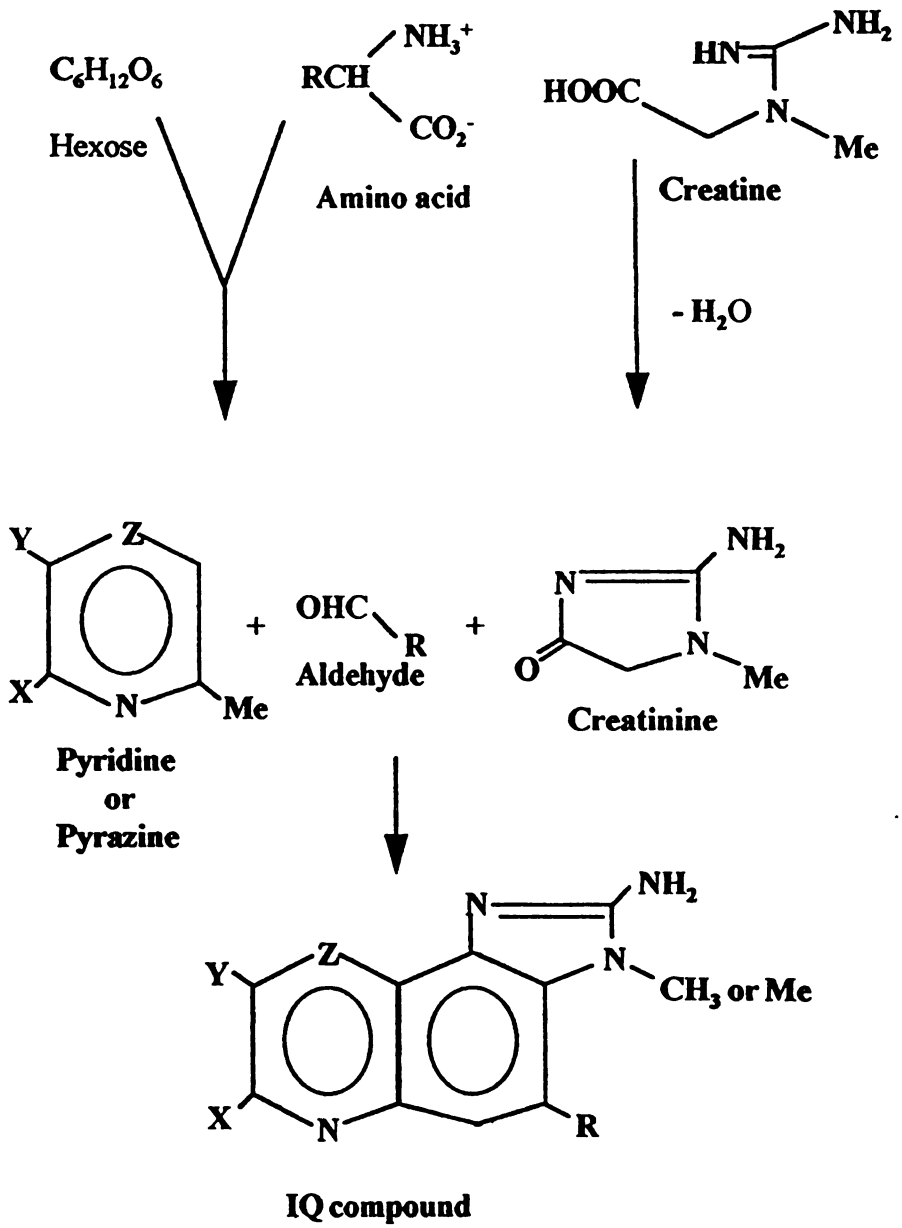

Figure 3. Postulated reaction route for formation of IQ compounds R, X, and Y may be H or Me; Z may be CH or N (Jagerstad et al. 1983a).

latter compounds by aldol condensation. This hypothesis was verified using a model system where creatin(in)e, glycine or alanine, and glucose, dissolved in diethylene glycol containing 14% water, were boiled under reflux at 130°C for 2 hr. The mixture showed high mutagenic activity, whereas heating the reactants two by two produced only weak, if any, mutagenic activity. The addition of synthetic pyridines or pyrazines to the reaction mixture increased the mutagenic activity by about 50% (Jagerstad et al., 1983a).

According to the hypothesis of Jagerstad et al. (1983b), the aldol condensation first occurred between vinylpyrazines or vinylpyridines and aldehydes, followed by ring closure with creatinine. However, a series of tests involving the addition of selected alkylpyridines and/or pyrazines, including 2-methylpyridine or 2-vinylpyridine, to mixtures containing creatinine failed to yield any mutagen formation (Jones and Weisburger, unpublished data, 1988). Another way of formation was proposed by Nyhammar (1986) who assumed that the condensation first occurred between aldehydes and creatinine, which then condensed with a vinylpyrazine or vinylpyridine (Fig.4). Support for the latter reaction route was provided when Jones and Weisburger (1989) reported the formation of different IQ-like mutagenic products through reactions between creatinine and different aldehydes. Thus, aldehydes may be involved in the formation of heterocyclic aromatic amines through a series of chemical reactions involving specific reagents and creatinine.

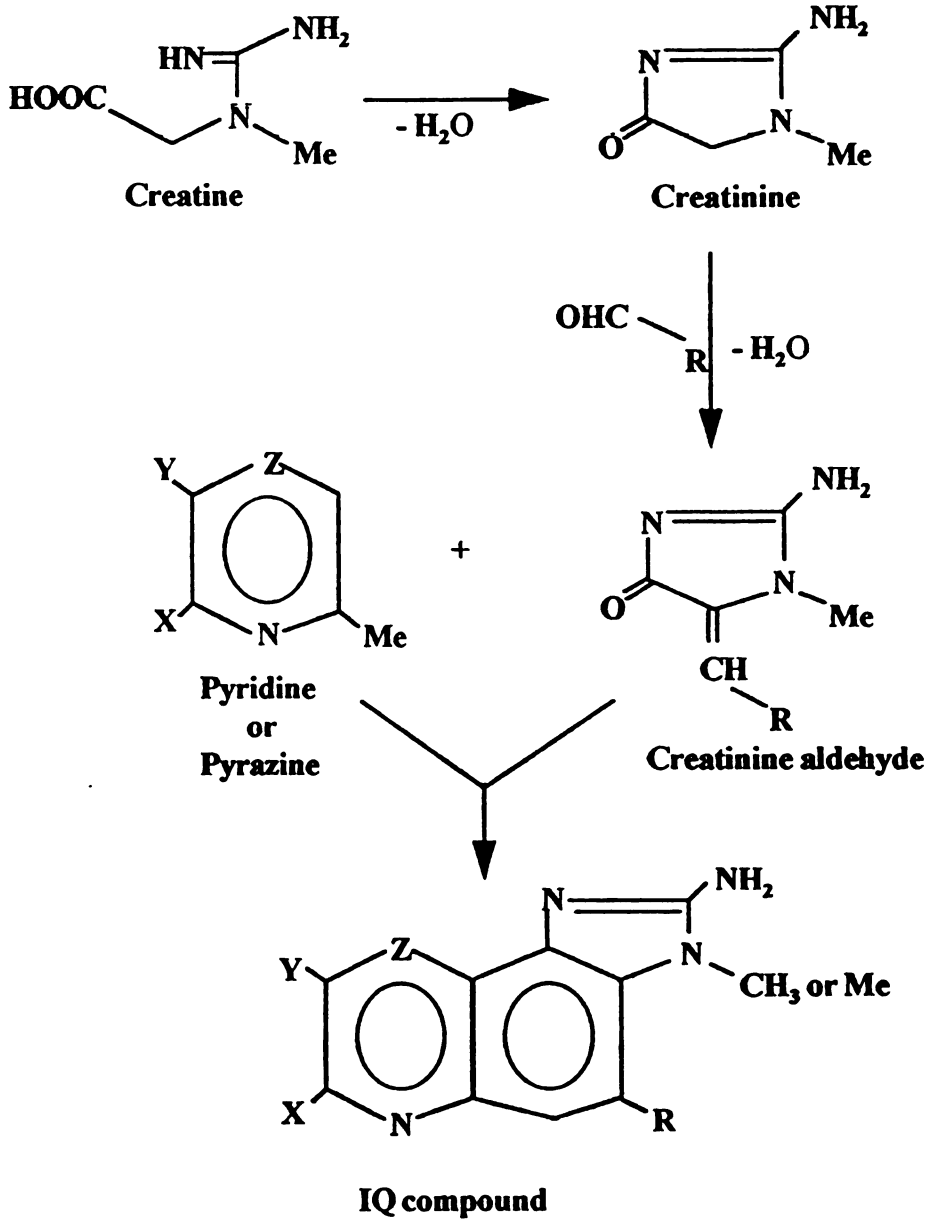

Figure 4. Alternative route for formation of IQ compounds R, X, and Y may be H or Me; Z may be CH or N (Nyhammar, 1986).

Factors influencing heterocyclic aromatic amine formation

Fat

Many studies have investigated the influence of the fat content of the product on mutagen formation (Spingarn et al., 1981; Barnes and Weisburger, 1983; 1984; Bjeldanes et al., 1983; Knize et al., 1985; Chen, 1988), although it may be difficult to distinguish between the physical and chemical effects of fat. These studies revealed that beef patties containing about 15% fat produced the highest mutagenic activity upon frying because heat penetration is increased. When the fat content was increased to above 15%, mutagenicity was slightly reduced. It was proposed that this could be due to diluting the precursors of the mutagens by the fat in the meat (Knize et al., 1985).

The influence of different frying fats on the mutagenic activity of pork fried at 200°C for 10.5 minutes was also studied (Nilsson et al., 1986; Overvik et al., 1987). Results showed that there were no differences in mutagenic activity produced by different frying fats, but there was higher mutagenic activity than when frying took place without fat. This was probably due to the higher temperature generated at the meat surface when a frying fat was used.

The effects of edible oils and fatty acids on the formation of mutagenic heterocyclic amines was also studied by Johansson et al. (1993). When corn oil or olive oil was added to model systems containing creatinine, glycine and glucose dissolved in water and heated to 180°C for 30 minutes, the yield of MeIQx was almost doubled relative to the yield without fat. However, this increase was not observed when a fatty acid or glycerol was added to the model system. This may be due to the participation of the lipids in the Maillard reaction. Lipids are known to enhance the production of pyrazines and other products in the Maillard reaction (Watanabe and Sato, 1971a,b; Buttery et al., 1977; Parihar et al., 1981; Kawamura, 1983; Arnoldi et al., 1987; 1990). It was also proposed that aldehydes

are formed more rapidly if fat is present in the reaction mixture (Arnoldi et al., 1987). Lipids can also produce carbonyl compounds upon autoxidation, which can react with amino compounds (Kawamura, 1983). Furthermore, lipid hydroperoxides can decompose to form aldehydes, which can react with amino acids and give Schiff base adducts (Gardner, 1979). However, the addition of oxidized linoleic acid or linolenic acid, to the model system (creatinine, glycine, and glucose) produced about the same amount of MeIQx as a mixture with no fat added when heated for 30 min at 180°C (Johansson et al., 1993). The degree of oxidation of the fat had small effects on the formation of MeIQx, no consistent trend was demonstrated between the yield of MeIQx and the degree of oxidation (Johansson and Jagerstad, 1993). When oxidized oleic acid was added to the model system, a decrease in the amount of MeIQx was observed (Johansson et al., 1993).

In a later study, Johansson and Jagerstad (1994) observed that the type of frying fat had a significant influence on the heterocyclic aromatic amine content in the pan residue. For example, the pan residue from beefburgers fried in butter contained higher amounts of heterocyclic aromatic amines than the pan residue from beefburgers fried in oil. This might be due to the inhibiting effects of different antioxidants present in the frying oils.

Creatine/creatinine

Investigators have reported the absence, or only very low levels, of mutagenic activity in liver, kidney, cheese, tofu, beans and shrimp when cooked at normal cooking temperatures (Felton and Knize, 1990). However, in other protein-rich foods such as beef, pork, lamb, chicken and fish, the mutagenic activity was considerably higher (Bjeldanes et al., 1982a, b). The absence of mutagenic activity in non-muscle foods and shrimp (invertebrates) can be explained by the lack of creatine. Creatine, in the form of creatine phosphate, is an energy reserve only in

vertebrates (Sulser, 1978), and is transformed into free creatine within 24 hr after slaughter (Fabiansson and Reutersward, 1985).

It has been established that mutagenic activity in beef is increased if a solution of creatine is spread over the surface before frying (Jagerstad et al., 1983a). To facilitate the identification of heterocyclic aromatic amines, several investigators have added creatine to different meat products before frying to enhance their formation (Nes, 1986; Becher et al., 1988; Overvik et al., 1989, Felton and Knize, 1991). These investigations suggest that the content of creatine in meat products is rate-limiting for the formation of heterocyclic aromatic amines.

It has been shown that creatine is converted to creatinine during cooking (Lempert, 1959) and that the proportion of creatinine in the crusts and pan residues increases with increasing temperature (Reutersward et al., 1987a). When beef joints were roasted in an oven at 115°C-245°C, the concentration of creatine and creatinine on a dry matter basis was considerably higher in the pan residues than in the crust, indicating a loss from the meat to the pan (Reutersward et al., 1987a). Results of a later study indicated that there is a transportation of creatin(in)e to the crust as well as a leakage to the pan (Skog et al., 1992b). As a result of this leakage, the mutagenicity of the pan residues can be as high as that of the crust of the meat (Felton et al., 1981; Overvik et al., 1987; Berg et al., 1988, 1990; Knize et al., 1988a; Johansson and Jagerstad, 1994).

It was suggested by Jagerstad et al.(1983a) that creatine and/or creatinine are essential precursors in the formation of heterocyclic aromatic amines, thus confirming the observations of previous investigators (Reutersward et al., 1987a, b; Overvik et al., 1989) that the mutagenic activity of cooked foods is related to its creatine/creatinine content. Vikse and Joner (1993) reported that the correlation between creatine/creatinine and mutagenicity was observed only when a creatinase treatment was applied. The average decrease in creatine concentration was 65%,

and this resulted in a decrease of approximately 73% in the mutagenic response from the meat extract. However, the differences in the normal creatine and creatinine contents of meat (from 16 different animal species) did not explain the varying mutagenic activity in the extracts of fried meat; thus, the relationship between them is not a simple one. The results of a study by Jackson et al. (1994) also indicate the lack of a direct relationship between the creatine/creatinine content and mutagenic activity in beef flavors. However, flavor extracts containing heterocyclic aromatic amines also had high mutagenic activity and high levels of creatine and creatinine.

Further studies are needed to establish the relationships between creatine and creatinine, mutagenic activity and heterocyclic aromatic amine formation in meat products.

Amino acids and dipeptides

Mutagenic activity was first reported in protein-rich foods (Commoner et al., 1978a). However, when proteins instead of amino acids were used in model systems, no mutagenic activity was detected (Jagerstad et al., 1983a). When beef extracts were subjected to enzymatic proteolysis before boiling, increased mutagenic activity was observed (Taylor et al., 1984; 1985). These results indicate the participation of amino acids and not proteins in the mutagen-forming reactions. When creatine and glucose were refluxed with amino acids at 128°C for 2 hr, most of the latter compounds produced mutagenic activity (Jagerstad et al., 1983b). When several amino acids were dry heated with creatine at 200°C for 1 hr, mutagenic activity was also detected (Overvik et al., 1989). As indicated previously, a specific heterocyclic aromatic amine can be produced from several different amino acids, while a single amino acid can produce several of these mutagenic compounds.

The importance of free amino acids in heterocyclic aromatic amine formation has also been shown in meat systems. It was demonstrated by Overvik et al. (1989) that the addition of 15 amino acids to pork before frying enhanced mutagenic activity by 1.5 to 43 times. Ashoor et al. (1980) reported that only proline, when added to ground beef, increased the mutagenic activity. On the other hand, no significant mutagenicity was detected after frying kidney or liver, although these two organs contain about twice the amount of free amino acids as does muscle meat (Reutersward et al., 1987a). However, as pointed out previously, the creatine content of organ meats is very small, which would explain the low mutagenicity in fried organ meats.

When the dipeptide, carnosine, was refluxed with creatinine and glucose, at 128°C for 2 hr, mutagenic activity similar to that produced from free amino acids was developed (Reutersward et al., 1987b; Overvik et al., 1989). These observations demonstrate the importance of not only amino acids but also dipeptides in the production of mutagenic activity and perhaps in the formation of heterocyclic aromatic amines.

Sugars

The role of sugars in the formation of heterocyclic aromatic amines is not clear. Different model systems with creatin(in)e, amino acids and sugars (glucose, fructose, ribose, galactose, arabinose, erythrose) have demonstrated the importance of sugars in the formation of heterocyclic aromatic amines (Muramatsu and Matsushima, 1985; Skog and Jagerstad, 1990; 1991; Manabe et al., 1992). Although not obligatory when added to model systems containing amino acids and creatinine, the quantities of heterocyclic aromatic amines are increased and different products are formed when sugars are involved. For example, when phenylalanine was heated with creatine at 180°C for 10 minutes, PhIP was detected as a single mutagen, but when glucose was added, the amount of PhIP

increased three-fold and at least two other mutagens (MeIQx and 4,8-DiMeIQx) were formed (Skog and Jagerstad, 1991). When sugars other than glucose were heated with phenylalanine and creatinine, PhIP formation was again observed. Erythrose was the most active sugar in this reaction (Manabe et al., 1992).

These studies suggest that there might be two pathways for the formation of heterocyclic aromatic amines:

(1) with sugars, via the interaction of Maillard reaction products with creatinine (Jagerstad et al., 1983a; Nyhammar, 1986; Pearson et al., 1992); and

(2) without sugars, via the reaction of creatinine with breakdown products of amino acids (Felton et al., 1986a).

The role of sugars in the formation of heterocyclic aromatic amines is complex. Model system studies show that mutagens can be formed by dry heating creatinine with different amino acids without sugars. For example, dry heating of phenylalanine with creatinine produced PhIP (Overvik et al., 1989; Felton and Knize, 1990; Skog and Jagerstad, 1991); dry-heating either serine, alanine or tyrozine with creatinine produced MeIQx (Overvik et al., 1989). However, the incorporation of the carbon label into MeIQx when [U-^{14}C]-labeled glucose, glycine and creatinine were heated together, suggests that glucose is a precursor in the formation of heterocyclic aromatic amines (Skog et al., 1992b).

Cooking time and temperature

The effect of temperature on mutagen formation in cooked ground beef was first described by Commoner et al.(1978b). A number of investigators have subsequently shown that mutagen production increases with the temperature of cooking (Spingarn and Weisburger, 1979; Hatch et al., 1982; Bjeldanes et al., 1983; Chen, 1988; Knize et al., 1994). Cooking methods that employ higher

heating temperatures generally induce greater heterocyclic aromatic amine formation than low temperature methods (Murray et al., 1993; Knize et al., 1994).

Several researchers have observed that there is a progressive increase in the mutagenic activity of cooked products with increasing cooking time (Commoner et al., 1978a; Bjeldanes et al., 1983; Miller and Buchanan, 1983; Overvik et al., 1984; Knize et al., 1985; Knize et al., 1994). However, there is a lag period of 2 to 4 minutes during the frying of ground beef patties when no mutagenicity is observed. This is the time required for the crust surface to reach a temperature above 100°C.

Results published by Knize et al. (1994) show the increase in heterocyclic aromatic amine formation in ground beef patties with increasing time and/or temperature of frying. There was no mutagen formation at 150°C after 2 or 4 minutes of frying, and PhIP and DiMeIQx were not detected in patties fried at 190°C for 2 minutes.

At each temperature/time combination of frying, PhIP was present in the highest concentration, demonstrating again that it is the most abundant heterocyclic aromatic amine in fried ground beef. Although the concentrations of PhIP in the beef patties fried at 230°C for 10 minutes, were approximately 10 times greater than those of MeIQx, PhIP took a longer time to form. A longer time and a higher temperature are necessary to produce the initial 20% of the PhIP formed in fried beef patties compared to MeIQx.

To study the rate of PhIP formation, a model system study was conducted by Knize et al. (1994), and compared to the production of the same compound in the fried ground beef. Results of the heating of 0.05M phenylalanine and 0.05M creatinine in 80% diethylene glycol, at 150°C or 200°C for 10 minutes, suggest that the rate of formation of PhIP in meat and in the model systems is similar. PhIP

formation in the simple model system was analogous to that in the more complex ground beef system.

Inhibition of mutagen formation

Sugars and other carbohydrates

Sugars are naturally occurring substances in meat systems and have a substantial impact on the formation of heterocyclic aromatic amines. Studies by Taylor et al. (1986) revealed that when glucose was added to beef-stock supernatant at a concentration four times greater than that of creatine, mutagenic activity was decreased. A more comprehensive study on the effect of sugar on heterocyclic aromatic amine formation was carried out by Skog and Jagerstad (1990). They demonstrated that excess amounts of sugar in model systems inhibited the formation of heterocyclic aromatic amines. When sugars were present in equimolar or greater amounts than the creatin(in)e concentration, the formation of mutagens was almost completely inhibited. When the glucose concentration was about half the molar concentration of creatine, the mutagenicity was the highest. The mechanism behind the inhibitory effect is not known, but there was a decrease in the recovery of creatine and creatinine as the glucose concentration increased. This phenomenon was observed only when amino acids were present in the reaction mixture, indicating a reaction between Maillard reaction products such as 5-hydroxymethyl-2-furfural (HMF) and creatinine. The creatinine would be less available to form heterocyclic aromatic amines (Skog and Jagerstad, 1990).

The inhibitory effect of sugars added in excess has also been studied in meat systems. When different carbohydrates were added to beef patties before frying, the mutagenic activity of the crust was dependent on the type of the carbohydrate added. Inhibition (from 40% to 70%) of the mutagenic activity was demonstrated with glucose and pure lactose or lactose from milk powder added at

concentrations up to 4%. The greatest inhibitory effect was achieved by golden breadcrumbs when added in combination with glucose or lactose. Furthermore, the mutagenic activity decreased when a mixture of potato starch and glucose was added to the beef patties (Skog et al., 1992b). After frying, a major portion (90%) of the initial concentrations of creatine and creatinine was still present in the crust of the beef patties. This demonstrated again that the role of creatine/creatinine as a rate limiting factor in the formation of heterocyclic aromatic amines is not fully understood.

Soy protein concentrate

The prevention of mutagen formation in fried beef patties by the addition of soy protein concentrates was reported by Wang et al. (1982). The mutagenicity of the control beef patties (i.e., without added soy protein concentrate) was over 25,000 revertants per 50 g beef. When soy protein concentrate was added at a level of 24%, total inhibition was achieved. These results clearly showed that the reduction of mutagenicity by soy protein concentrates occurs during the cooking process, as separately cooked patties when mixed with soy protein concentrates, did not have reduced mutagenicity. Overall mutagenicity of the fried ground beef was determined by the Ames assay using *Salmonella typhimurium* TA98 and the rat liver microsomal fraction for metabolic activation (Ames et al., 1975).

Most of the reduction in mutagenicity was attributed to volumetric effects such as through the reduction of interactions among the beef components, and by the reduction of the amount of beef that came into contact with the heating surface. However, some consideration was given to chlorogenic acid which is a naturally occurring polyphenolic antioxidant in soy protein concentrate (Smith and Circle, 1978; Pratt and Birac, 1979; Rappaport et al., 1979) in soy protein concentrates. Wang et al. (1982) demonstrated that this compound, when added directly to

ground beef patties, succesfully inhibited the development of mutagenicity in the fried beef.

Defatted glandless cottonseed flour

Defatted glandless cottonseed flour added at a level of 5% (w/w) to ground beef before frying also reduced the mutagencity of the cooked meat (Rhee et al., 1987). The magnitude of the reduction in mutagenicity tended to be much greater than the meat dilution effect by the glandless cottonseed flour. Glandless cottonseed ingredients are effective naturally-occurring antioxidants and have been shown to retard lipid oxidation in various soy and meat products (Rhee et al., 1981; Ziprin et al., 1981). Flavones (mainly quercetin derivatives) are the major flavonoids present in cottonseed. However, the investigators did not clearly define whether the reduction in mutagenicity was a result of volumetric effects or through the antioxidant properties of glandless cottonseed components.

Synthetic antioxidants

The effects of synthetic phenolic antioxidants on mutagen formation in cooked ground beef was first described by Wang et al. (1982). They showed that butylated hydroxyanisole (BHA) successfully reduced the mutagenic activity of fried ground beef patties. BHA also reduced the mutagenicity when it was directly added to the testing mixture of beef extract, S-9 mix and the bacterial culture in the Ames bioassay.

The inhibitory effects of BHA, propyl gallate and tertiary butylhydroquinone were more intensively studied by Chen et al. (1992). These antioxidants reduced the overall concentrations of IQ-like compounds (IQ, MeIQx and DiMeIQx) in fried ground beef by approximately 80-90% when added at 0.1% of the fat. At this low level of addition, the volumetric effect was insignificant.

More recently, Faulkner (1994) reported a reduction of mutagen formation in fried ground beef on adding BHA to the patties before frying. When BHA

(0.1% based on the fat content) was added to ground beef, a significant ($p<0.005$) reduction in the mutagenicity, from 7000 revertants/100g raw meat revertants to 2800 revertants/100g raw meat, was achieved. The PhIP concentrations were reduced from 2.4 ng/g to 1.2 ng/g.

Vitamin E

Much research is currently focused on the use of naturally occurring ingredients as antioxidants because of growing concerns about the safety of synthetic antioxidants and a general consumer perception that natural is better (Gray and Crackel, 1992). Vitamin E is an effective monophenolic antioxidant in lipids and lipid-containing foods because it effectively scavenges peroxy radicals (Niki, 1987).

The inhibitory effects of vitamin E on mutagen formation in fried ground beef were studied by Chen et al. (1992). When vitamin E (1% based on the fat content) was added to ground beef patties, the concentration of IQ-like compounds (IQ, MeIQx and DiMeIQx) was reduced by 50%. The reported concentrations of the mutagenic compounds were 1000-fold higher than those published by other researchers (Felton et al., 1986a,b; Sugimura et al., 1988; Thiebaud et al., 1994). Therefore, the quantitative data of Chen et al. (1992) are questionsble as no confirmatory studies were carried out. However, these data did establish for the first time the inhibitory effects of vitamin E on the formation of heterocyclic aromatic amines.

In a recent study, Faulkner (1994) confirmed the inhibition of PhIP formation by vitamin E (1% based on fat content). PhIP concentrations in ground beef patties were reduced by 80% by adding vitamin E (1% based on fat content) to the ground beef before frying. The overall mutagenicity was reduced by 70%, from 7000 revertants/100 g raw meat to 1900 revertants/100 g raw meat. This was the first reported study of the simultaneous use of both the *Salmonella*

typhimurium overall mutagenicity test and an analytical procedure to quantitate the extent of inhibition of specific heterocyclic aromatic amines.

Tea phenolics

Tea polyphenolic compounds, particularly epigallocatechin gallate, epicatechin gallate and epigallocatechin, have been established as potent antioxidants (Sorata et al., 1984; Chen et al., 1990; Sichel et al., 1991; Ho et al., 1992; Terao et al., 1994). Namiki and Osawa (1986) evaluated the antioxidant activities of different polyphenols including α-tocopherol, propyl gallate, catechin, epicatechin, gallocatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate, in rabbit blood cells subjected to peroxidation. Peroxide generation was suppressed to the same extent by α-tocopherol, propyl gallate, epicatechin gallate and epigallocatechin gallate.

The effect of black tea (BT) and green tea (GT) and the polyphenols, theaflavine gallate (TFG) and epigallocatechin gallate (EGCG), on the formation of heterocyclic aromatic amines was studied by Weisburger et al. (1994). They demonstrated substantial reduction in heated model systems containing creatinine, glycine and glucose. Black tea had a substantial effect in lowering the formation of both MeIQx and PhIP (from 9350 to 7340 revertants/plate and from 6530 to 2070 revertants/plate, respectively). The effect of green tea in reducing the formation of mutagenic compounds was effective only for PhIP (from 6530 to 2180 revertants/plate). When EGCG was added to the model system, there was an 80% reduction in the mutagenic activity for both MeIQx and PhIP. Considerable inhibition of MeIQx and PhIP formation (83% and 74%) was achieved when TFG was applied. In a separate experiment, in which the mutagenicity was lowered to 38% by EGCG, HPLC analysis showed a 15% decrease in PhIP formation.

The relationship between antioxidant activity and antimutagenicity of green tea, pouchong tea, oolong tea and black tea was investigated by Yen and Chen

(1995). The antimutagenic effect of tea extracts on IQ toward *Salmonella typhimurium* TA98 and TA100 was correlated with their reducing power and scavenging effect on the hydroxyl radical. All tea extracts exhibited antioxidant activity and reducing power. The antioxidant effect of tea extracts was well correlated to their antimutagenicity in some cases, but varied with the mutagen and antioxidative properties.

Flavone

Flavone is a naturally occuring flavonoid that is present in edible and medicinal plants (Brown, 1980). The inhibitory effects of flavone on the formation of heterocyclic aromatic amines was studied by Lee et al. (1992) in a glycine, creatine and glucose model system. Results showed a decrease in the mutagenic activity of MeIQx and 7,8-DiMeIQx by 31.1% and 27.8%, respectively. The total mutagenic activity of the heated glycine/creatine/glucose mixture was decreased by the addition of flavone in a dose-related response relationship.

Quantitation of mutagens

The extraction/detection of heterocyclic aromatic amines has been a challenging undertaking because of the small concentrations (low ng/g levels) of these compounds, the diversity of the different mutagens formed under same reaction conditions, and the complexity of the food samples to be analyzed.

In the early 1980's, two procedures were used to extract organic material from cooked and uncooked meat samples. First, an extraction procedure described by Commoner et al. (1978a) used dilute acid and ammonium sulfate to precipitate proteins, followed by pH adjustment and solvent extraction of the organic constituents. The second procedure used acetone to extract the organic constituents directly from the cooked protein foods. These organic extracts were then separated into basic, neutral, and acidic fractions (Felton et al., 1981).

A method which utilized Amberlite XAD-2 resin to isolate mutagenic activity from an initial aqueous acid extract of fried beef was developed by Bjeldanes et al. (1982c). However, thin layer chromatographic profiles of the mutagenic extracts isolated by this method indicated poor recoveries for the different mutagens.

An improved method for the isolation and characterization of new mutagens from fried ground beef was described by Felton et al. (1984). Mutagens were separated by aqueous/acid extraction from the beef, XAD adsorption, acid/neutral/base-liquid/liquid extraction, preparative reverse phase HPLC, normal phase HPLC, and analytical reverse phase HPLC. The identification was carried out by low and high resolution mass spectrometry, ultraviolet absorption spectroscopy and nitrite sensitivity assays. The mutagenicity of each fraction was monitored by the *Salmonella* assay described by Ames et al. (1975).

Simple methods for quantifying mutagenic heterocyclic aromatic amines in food products were developed by Gross (1990). The solid-phase extraction procedure included a copper phthalocyanine (CPC) tandem extraction, and a propylsulfonyl silica gel (PRS) tandem extraction, both followed by further clean-up. The method was improved by Gross and Gruter (1992) to allow the purification of the entire range of heterocyclic aromatic amines. This procedure involved tandem extraction with diatomaceous earth and an ion exchange resin (PRS), followed by clean-up with a C18 column, and subsequent separation and identification of the heterocyclic aromatic amines on HPLC using a photodiode array UV detection system. The advantage of this solid-phase extraction procedure is a simpler and more rapid sample preparation prior to chromatographic analysis.

Peak confirmation is a crucial problem when working with such low levels of heterocyclic aromatic amines since co-elution with other compounds can occur. Thus, HPLC retention times alone do not provide unequivocal identification of

these compounds. Some researchers have combined HPLC directly with mass spectrometry (MS) to identify heterocyclic aromatic amines. Yamaizumi et al. (1986) identified MeIQ and IQ in broiled salmon by using HPLC - thermospray mass spectrometry. Data show that MeIQx, IQ, and DiMeIQx were identified in beef extracts and fried beef by using HPLC-MS analysis at concentrations ranging from 0.3 to 52 ng/g (Turesky et al., 1988).

The most sensitive approach for heterocyclic aromatic amine analysis is that devised by Murray et al. (1988). Using cooked meat samples spiked with heavy-isotope-labeled standards, samples were dissolved in dilute hydrochloric acid, washed with dichloromethane and then extracted into ethyl acetate. Dried extracts were derivatized with 3,5-bis-trifluoromethylbenzyl bromide and analyzed by gas chromatography - electron-capture negative ion chemical ionization mass spectrometry. Lean minced beef patties were found to contain 1.0 to 2.4 ng/g MeIQx and 0.5 to 1.2 ng/g DiMeIQx.

MATERIALS AND METHODS

Materials

Freshly ground beef (85% lean) was obtained from local supermarkets and used within one hour of purchase, or stored at -20°C until required for frying. Samples of meat were taken randomly for fat and moisture determinations. Vitamin E (dl-α-tocopherol) was purchased from Sigma Chemical Company (St Louis, MO), while oleoresin rosemary was donated by Kalsec Inc. (Kalamazoo, MI). Extrelut-20 columns were obtained from EM Separations (Gibbstown, NJ). Bond-Elut PRS (500 mg) and C18 (100 mg) cartridges were purchased from Varian, Inc. (Harbor City, CA). All solvents were HPLC or glass-distilled reagent grade. The heterocyclic aromatic amine standards (IQ, MeIQ, MeIQx, 4,8-DiMeIQx and PhiP) were obtained from Toronto Research Chemicals (Toronto, Canada). The heterocyclic aromatic amine standard (FEMA - Flavour and Extracts Manufacturers' Association) and the internal standard, caffeine, were kind gifts from Dr. Mark Knize, Lawrence Livermore National Laboratory, University of California, CA. The FEMA standard contained IQ, MeIQ, MeIQx, 4,8-DiMeIQx and PhIP, each at 0.5 ng/μl.

Methods

Moisture and fat determination

Moisture and fat contents were determined by the AOAC official methods (AOAC, 1992).

Influence of temperature and time of frying on heterocyclic aromatic amine formation in ground beef patties

Frying of ground beef patties:

A teflon-coated electric frying pan without a lid was used to fry the ground beef patties. The temperature control of the frying pan was set at 175°C or 200°C and the surface temperature was measured by a thermocouple (Pacific Transducer Co., Los Angeles, CA) during the frying. Before frying, the frying pan was preheated to the selected temperature. Ground beef purchased from three different sources (sold as 85% lean beef) contained different levels of fat (14.8%, 22.7% and 17.5%). Patties weighing 100 g were formed in a petri dish (9 cm dia. x 1.5 cm thickness), and fried for 6 or 10 minutes on each side at each of the two temperatures. Two ground beef patties were fried in the frying pan, and blended together in a Waring laboratory blender (Dinamics Co. of America, New Hartford, CT) at 70 rpm for 2 minutes. Two samples were taken from the blended patties and subsequently analyzed for heterocyclic aromatic amine content.

The beef patties fried at 225°C were prepared from 15% fat ground beef obtained from a local supermarket (only one source). Two ground beef patties, weighing 100 g each and formed in a petri dish, were fried for 6 and 10 minutes per side, then blended and combined. Heterocyclic aromatic amine concentrations at 225°C were based on three replications. During the study, four sub-samples were taken and analyzed for each replicated experiment.

Statistical analysis:

Statistical analysis of the concentrations of heterocyclic aromatic amines in meat fried at 175°C and 200°C for 6 or 10 minutes was based on two replications and three different sources of meat. Two ground beef patties were fried for each experimental replication under similar conditions. Extraction, analysis and quantitation were carried out in duplicate for each replicated experiment, with four

sub-samples analyzed. The results were analyzed by a statistical computer program (MSTAT-C) developed at Michigan State University (Department of Crop and Soil Sciences) by a three-way analysis of variance (ANOVA), and f-values were calculated (manually) for specific comparisons between mean values for the different sources of meat.

To compare the heterocyclic aromatic amine concentrations at all three temperatures on an equal fat basis (~15%), meat from only one source was compared. Thus, concentrations from one replication (at 175°C and 200°C) were compared to results from three experimental replications (at 225°C). Manually calculated t-values (Gill, 1988) were compared to determine significant differences between mean values of heterocyclic aromatic amine concentrations.

Inhibition of heterocyclic aromatic amine formation in fried ground beef patties by direct addition of phenolic antioxidants

Frying of ground beef patties:

Vitamin E (1% or 10% based on fat content) and oleoresin rosemary (1% or 10%) were dissolved in 1 ml corn oil and added directly as separate treatments to the ground beef patties two hours prior to frying. Ground beef patties mixed with 1 ml corn oil served as the control. Patties weighing 100 g were formed in a petri dish (9 cm dia. x 1.5 cm thickness), and fried for 10 minutes per side at 225°C in a teflon-coated electric frying pan. Two patties were fried for each replication, and three experimental replicates were analyzed for each treatment.

Statistical analysis:

Statistical analysis of the heterocyclic aromatic amine concentrations in the fried ground beef patties was based on three replicates for all five treatments. All treatments were from the same source of meat and fried under the same conditions. For each replicate, four sub-samples were analyzed (two for concentration and two

for recovery). Therefore, the mean value contains two data points. The results were analyzed by a statistical computer program (MSTAT-C) by a one-way analysis of variance (ANOVA). The Bonferoni's t test was used to determine the significance of the treatment compared to the control, the effect of the different treatments, and the interaction between them (Steel and Torrie, 1980).

Inhibition of heterocyclic aromatic amine formation in fried ground beef patties through surface application of vitamin E

Frying of ground beef patties:

Vitamin E (1% based on the fat content) was dissolved in 1 ml corn oil and spread on the surface of ground beef patties 30 minutes before frying. As a control, 1 ml corn oil was applied to the surface of the patties. The patties were fried for 10 minutes at 225°C in a teflon-coated frying pan. Seven experimental replications were analyzed for both treatments for heterocyclic aromatic amine concentration.

Statistical analysis:

All analyses were performed with seven experimental replications for both control and antioxidant treatments. Only one source of meat was used to form the ground beef patties. Two fried patties were combined for each treatment and four sub-samples analyzed. The results were analyzed by the Student's t test to determine the significance of the vitamin E treatment compared to the control.

To analyze statistically the heterocyclic aromatic amine concentrations in fried patties prepared from ground beef (fresh and after two months of freezer storage), t-values were calculated (manually) and compared. These manual calculations were necessary as there were only three replications for the fresh (unfrozen) meat compared to seven experimental replications for meat which had been frozen for two months.

Analysis of heterocyclic aromatic amines in fried ground beef patties (Figure 5)

A fried ground beef sample (25 g) was weighed into a glass beaker and homogenized thoroughly with 75 g 1N NaOH in a Ultra Turrax (Tekmar Co., Cincinnati, OH) high speed mixer for 1 to 2 minutes. Four 16 g aliquots of the homogenized mixture (equal to 4 g meat) were removed and two were spiked with a mixture of the heterocyclic aromatic amines in 50 µl methanol (i.e., 250 ng of each heterocyclic aromatic amine). Each sample was mixed thoroughly with one package of Extrelut diatomaceous earth to give a free-flowing homogenous, lump-free powder.

Extrelut cartridge assembly:

A small paper filter was placed in the bottom tip of the column and the column body was inserted. The bottom of the column was then covered with a small amount of diatomaceous earth and the Extrelut mix added. A large paper filter was subsequently placed over the sample.

Extrelut - PRS tandem (propylsulfonic acid silica):

The PRS cartridges were filled with dichloromethane:toluene (95:5 v/v) and a slight positive pressure was applied until the solvent passed through the cartridge (Figure 6). The cartridge was filled again with the dichloromethane:toluene solvent system, and a needle was assembled at the end of the cartridge for flow reduction. The Extrelut cartridges were filled with the same solvent, when it passed to the bottom of the cartridge, the PRS and Extrelut cartridges were coupled. A 40 ml aliquot of the dichloromethane:toluene solvent mixture was allowed to flow through the tandem columns. The extraction was stopped by separating the Extrelut column from the PRS column. The dichloromethane:toluene was discarded and needles were removed from the PRS cartridges.

Removal of interferences from the PRS cartridge:

PRS cartridges were transferred to a Visiprep vacuum (Supelco), and the cartridges were dried for 10-15 minutes under maximum vacuum. The PRS cartridges were then connected to a peristaltic pump and successively rinsed at about 1.5-2.0 ml/min with 6 ml of 0.1 M hydrochloric acid, 15 ml of methanol: 0.1M hydrochloric acid (4:6) , and 2 ml of water.

Transfer of heterocyclic aromatic amines from PRS to C18:

The C18 cartridges were slowly (by gravity) rinsed with 2 ml of MeOH , followed by 5 ml of water, and kept wet until used. The C18 and PRS cartridges were then connected. To transfer the heterocyclic aromatic amines from the PRS column to the C18 column, a volume of 20 ml of 0.5N ammonium acetate buffer (pH 8.0) was pumped through each tandem at a flow of approximately 1.5-2.0 ml/min. The PRS cartridges were then discarded, and the C18 columns were rinsed with 1 ml of water.

Elution of heterocyclic aromatic amines from the C18 cartridges:

The C18 cartridges were dried completely by applying vacuum (Visiprep) for 30 minutes. The heterocyclic aromatic amines were eluted slowly from the C18 cartridges with 1.0 ml of methanol:ammonia (9:1) by applying gentle overpressure through a plastic syringe directly into the microvials. The solvent was evaporated in a 40°C water bath using a stream of nitrogen, and the samples were refrigerated until required for HPLC analyses.

Antioxidant treatments mixed in 1 ml corn oil, added to 100 g of ground beef; patties formed in a petri dish (9 cm dia. x 1.5 cm thickness)

⇓

Fry two patties for each replicate, and grind the patties together in a blender

⇓

Add 75 g 1N NaOH to 25 g fried beef sample and homogenize

⇓

Divide into 4x16g samples, two of which are spiked with 50μl of standard mixture (250 ng/each compound)

⇓

Mix 16g sample with Extrelut-20, fill column with mixture, attach to PRS column containing 2 ml dichloromethane:toluene (95:5)

⇓

Rinse columns with dichloromethane:toluene, collect 40 ml of solvent mixture, detach Extrelut column, and dry PRS under vacuum (10-15 minutes)

⇓

Add 6 ml of 0.1M HCl to PRS followed by 15 ml MeOH:0.1M HCl (4:6), and then 2 ml water

⇓

Condition C18 column with 1 ml of MeOH followed by 10 ml of water

⇓

Attach PRS column to C18, rinse with 20 ml of ammonium acetate (pH 8.0), discard PRS , and rinse C18 with 2 ml of water

⇓

Dry C18 column by applying high vacuum for 25-30 minutes

⇓

Elute heterocyclic aromatic amines with 1.0 ml of MeOH:cc. ammonia (9:1) by applying gentle overpressure through a plastic syringe

⇓

Evaporate solvent to dryness under nitrogen in a 40°C water bath and refrigerate until injection

Figure 5. Extraction of heterocyclic aromatic amines in fried ground beef patties following the method of Gross and Gruter (1992).

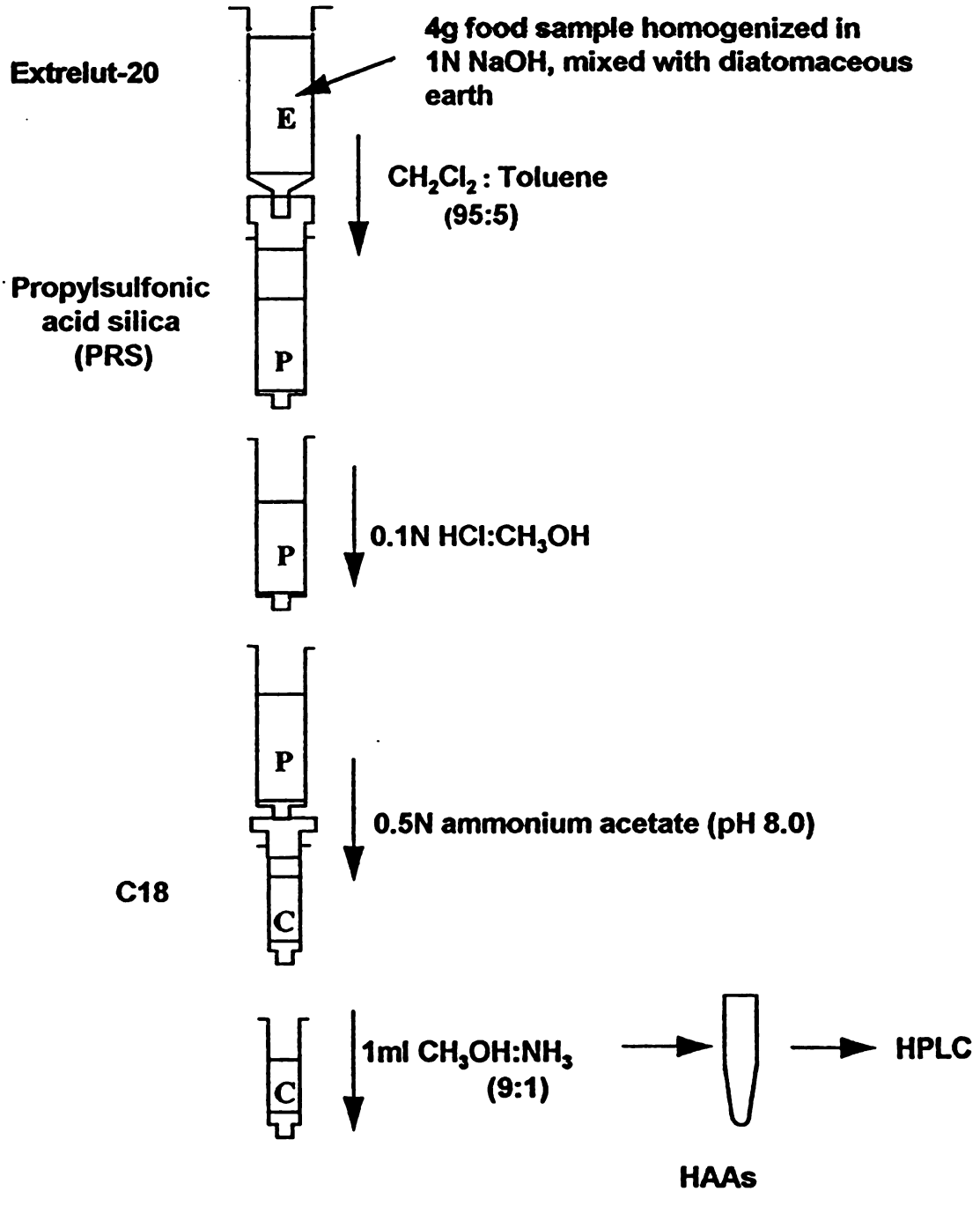

Figure 6. Solid phase extraction to detect heterocyclic aromatic amines from food systems.

Quantitative determinations of heterocyclic aromatic amines

To measure extraction efficiency and to quantitate the heterocyclic aromatic amines in the fried patties, the standard addition method of Gross and Gruter (1992) was used. Quadruplicate determinations (four sub-samples) were carried out with two unspiked samples and two samples spiked with 50 μl of the heterocyclic aromatic amine standard mixture. The extraction efficiencies were thus calculated for each analyte as the slope of the linear regression line: added analyte concentration versus measured analyte concentration. Quantitative measures of heterocyclic aromatic amines were corrected for incomplete analyte recovery.

High performance liquid chromatographic (HPLC) analysis of heterocyclic aromatic amines

Calibration:

Five aliquots (5, 10, 15, 20, and 25 μl) of the two standard heterocyclic aromatic amine mixtures (containing 0.5 ng/μl and 5 ng/μl of each compound) and the caffeine internal standard (5 ng/μl caffeine) were injected prior to the analysis of the sample extracts. The response linearity of the HPLC was checked by performing a linear regression calculation: μl standard solution injected versus peak area. A positive slope with a correlation coefficient of 0.99 was obtained in each case.

Analysis:

Beef pattie extracts were redissolved in 50μl methanol (containing 5 ng/ μl caffeine) and an aliquot of 20μl was injected through a Rheodyne 7125 (50 μl loop) injector (Rheodyne Inc., Cotati, CA) into a Waters 510 HPLC, equipped with a Waters 991M photodiode array UV detector (Millipore, Milford, MA). The heterocyclic aromatic amines were detected as follows: 254 nm for IQ and MeIQ,

262 nm for MeIQx and 4,8-DiMeIQx, and 316 nm for PhIP. In order to improve peak shape, triethylamine (1.4 ml/liter) was added to HPLC grade water. The mobile phase was vacuum filtered through a 0.45 μm membrane and adjusted to pH 3.2 with dilute phosphoric acid (above pH 3.2, Glu-P-1 and MeIQ co-elute). Acetonitrile was used as the second mobile phase. A reversed phase silica HPLC column (TSK-Gel ODS-80TM column; 4.6 mm ID x 25 cm; Tosoh Haas; Montgomeryville, PA) protected by a Supelguard LC-8-DB (Supelco) precolumn was used to separate the heterocyclic aromatic amines. The particle size of the column packing material was 5 μm in diameter. The mobile phase flow rate was set at 1 ml/minute. The acetonitrile concentration in the mobile phase was increased from 8% to 17% during the first 10 minutes and then to 25% in another 10 minutes. Percent acetonitrile was then increased to a concentration of 55% in 10 minutes. By this time, all heterocyclic aromatic amines were eluted from the column. Over a 5-minute period, the percent of acetonitrile was increased to its maximum concentration of 80%, and this was necessary to clean the column of other unwanted compounds. At the end of the 45 minute elution period, the acetonitrile was decreased to its original concentration of 8% and the column was allowed to equilibrate for 10-15 minutes before the next injection. All separations were carried out at ambient temperature.

RESULTS AND DISCUSSION

Optimization of the extraction/quantitation procedures for heterocyclic aromatic amines

The extraction and quantitation of hetrocyclic aromatic amines in fried ground beef patties was achieved using the Standard Addition Quantitation procedure developed by Gross and Gruter (1992). This procedure is a challenging one which is reflected in the fact that reported heterocyclic aromatic amine concentrations in beef are based on recoveries of heterocyclic aromatic amine standards that range from as low as 5% to as high as 85% (Jackson et al., 1994; Johansson and Jagerstad, 1994; Knize et al., 1994; Thiebaud et al., 1994). Thus, the first priority of this study was to optimize the extraction procedure to obtain consistent recoveries of the heterocyclic aromatic amines and to reduce the standard deviations of the data obtained. To accomplish this, several short studies were performed to identify critical steps in the extraction process which impacted recoveries, and to devise ways to overcome these challenges.

Results of these preliminary investigations indicated that it was essential to optimize the size of the meat sample for a number of reasons:

(a) to obtain a powder-like blend upon combining the fried ground beef with the Extrelut refill diatomaceous earth, and to charge the Extrelut-20® extraction column with an appropriately-sized meat sample.

(b) to be able to extract sufficient heterocyclic aromatic amines to enable their detection by the HPLC procedure employed; and

(c) to avoid overloading the PRS and/or C18 columns in subsequent phases of the extraction procedure.

These studies revealed that for maximum recoveries of the heterocyclic aromatic amines in fried beef patties, the optimum weight of the meat sample should be

approximately 4 g. When larger amounts of ground beef were used, recoveries of PhIP were greatly reduced, generally below 10%. It was also observed that the recovery of each heterocyclic aromatic amine was improved by 5-10% when the C18 column was completely free of solvent before using. This was achieved by applying vacuum to the column for approximately 25-30 minutes, after rinsing the column with solvent.

A review of reported heterocyclic aromatic amine concentrations in meat products using the Gross and Gruter (1992) procedure reveals that smaller recoveries are generally obtained for PhIP than for any of the other heterocyclic aromatic amines. Thus, more attention was directed toward improving its recovery in this study, including an investigation of various solvent systems used to elute the heterocyclic aromatic amines from the Extrelut-20® extraction columns. It was determined that the dichloromethane /toluene (95:5) solvent system (Knize, personal communication) increased the recovery of PhIP by 5% relative to that achieved by dichloromethane alone.

When these changes were introduced into the extraction procedure, the recoveries of the heterocyclic aromatic amines matched the upper range of recoveries published in the literature (Johansson and Jagerstad, 1994; Knize et al., 1994; Thiebaud et al., 1994). Table 4 shows the range of recoveries obtained for each compound. Recovery experiments involved the addition of 250 ng each of IQ, MeIQ, MeIQx, 4,8-DiMeIQx and PhIP to two of four samples of fried ground beef before packing the Extrelut-20 cartridge. In this way, peak confirmation was more accurate as half of the samples had peaks with retention times identical to those of the added standard heterocyclic aromatic amines.

This complex three-step solid phase extraction and clean-up procedure is necessary because the UV absorption maxima for imidazoquinolines and imidazoquinoxalines are located at wavelengths around 260 nm where many other

aromatic compounds absorb light (Gross and Gruter, 1992). This means that a sample must be as completely free of interfering compounds as possible to allow the quantification of heterocyclic aromatic amines at the very low concentrations (ng/g) that these are present in fried beef patties. To accomplish this, one has to be very precise when using this procedure. Peak confirmation is a crucial problem when working with such concentrations since co-elution with other co-extracted compounds can occur. To confirm the identity of the heterocyclic aromatic amines, a derivatization procedure developed in our laboratory by Faulkner (1994) is routinely used. This involves derivatization of the heterocyclic aromatic amines to their mono-pentafluoropropionic derivatives, followed by gas chromatographic - mass spectrometric confirmation. In addition, because all of the heterocyclic aromatic amines have characteristic ultraviolet (UV) spectra and high extinction coefficients, a photodiode array UV detection system essentially prevented false peak identification. The use of fluorescence detection also assisted in the confirmation process because of the strong signal generated for PhIP.

Table 4. Percent recoveries of heterocyclic aromatic amines from ground beef patties using solid phase extraction[1]

Compound	IQ	MeIQ	MeIQx	4,8-DiMeIQx	PhIP
Percent recovery	58.2-84.8	51.7-78.2	66.7-88.5	72.1-92.4	32.1-61.9

[1]Range of recoveries are based on twelve sample analyses

Influence of temperature and time of frying on heterocyclic aromatic amine formation in ground beef patties

In order to optimize the formation of heterocyclic aromatic amines in the fried patties prepared from three sources of ground beef, various time/temperature combinations were investigated. This initially involved frying the ground beef patties at two different temperatures (175°C and 200°C-measured by a thermocouple on the surface of the frying pan during the frying of the patties) for 6 and 10 min/side. The purchased ground beef was advertised as containing 15 % fat. This was desirable for the study as it has been reported previously that maximum heterocyclic aromatic amine formation in fried patties occurs at this level of fat (Spingarn et al., 1981; Holtz et al., 1985; Knize et al., 1985; Nilsson et al., 1986; Overvik et al., 1987). However, analyses of the three sources of ground beef revealed fat contents of 14.8%, 22.7%, and 17.5%.

Results show that the formation of the mutagenic compounds was dependent on the time and temperature of cooking (Table 5). PhIP concentrations in fried beef patties increased significantly with time ($p<0.1$) and temperature ($p<0.05$) of cooking. PhIP is the most abundant heterocyclic aromatic amine found in cooked meat with reported concentrations ranging from 0 to 67.5 ng/g fried beef (Thiebaud et al., 1994). Although the concentrations of PhIP in the fried patties were approximately 6 times greater than those of MeIQx under these frying conditions, PhIP took a longer time to form (e.g., at 175°C, 6 min: 0.3 ng/g fresh meat MeIQx and 0.4 ng/g fresh meat PhIP; at 10 min: 0.4 ng/g fresh meat MeIQx and 2.6 ng/g fresh meat PhIP). The same phenomenon was reported by Knize et al. (1994), who indicated that a longer time and a higher temperature are necessary to produce the initial 20% of the PhIP formed in fried beef patties compared to MeIQx.

Table 5. Heterocyclic aromatic amine contentrations in ground beef patties (ng/g cooked meat) fried using different time / temperature combinations[1,2,3]

Time / Temperature	IQ	MeIQ	MeIQx	4,8-DiMeIQx	PhIP
175°C					
6 min	0.3±0.3[a]	0.1±0.1[a]	0.3±0.0[e]	0.3±0.2[a]	0.4±0.1[e]
10 min	0.6±0.2[a]	0.4±0.3[b]	0.4±0.1[f]	0.4±0.1[b]	2.6±0.9[f]
200°C					
6 min	0.8±0.2[b]	0.3±0.1[c]	0.7±0.2[g]	0.5±0.1[c]	1.9±0.6[g]
10 min	1.3±1.0[b]	0.7±0.4[d]	1.4±0.9[h]	1.3±1.2[d]	8.2±5.4[h]

[1] Values are expressed on a raw ground beef basis. Values are based on measured cooking losses for individual patties.
[2] Each value represents the mean of two samples per source of ground beef in which duplicate analyses have been averaged ± standard deviation, i.e., six samples in all.
[3] Mean values in columns with different superscripts are significantly different at $p<0.1$.

After completion of this study, a frying pan was purchased that permitted the use of a higher frying temperature (>200°C). Thus, time/temperature combinations of 6 and 10 minutes at 225°C were investigated. Three experimental replications were performed and results are presented in Table 6. Heterocyclic aromatic amine data presented for 175°C and 200°C are those values pertaining to ground beef patties from the ground beef source containing 14.8% fat. Because of the reported influence of fat concentration on heterocyclic aromatic amine formation, it was desirable to compare the heterocyclic aromatic amine concentrations at all temperatures on an equal fat basis, recognizing that other intrinsic variables (e.g., creatine/creatinine contents and sugar) may also play a major role in heterocyclic aromatic amine formation in meat. Statistical analyses

indicated that the concentrations of MeIQx and PhIP were significantly ($p<0.05$) influenced by the temperature of cooking, while the concentrations of IQ, MeIQ, and PhIP were only significant at $p<0.1$ level. The difficulty in establishing the significance of the data was attributed to the high standard deviations in the results. These data represent heterocyclic aromatic amine formation in one source of meat containing 15% fat. Heterocyclic aromatic amine concentrations at 175°C and 200°C are based on one replication, while those at 225°C are the mean value of three replications. However, each replication involves two sub-samples which provide two data sets per replication

Table 6. Heterocyclic aromatic amine concentrations in ground beef patties using different time / temperature combinations of frying[1,2,3]

Time / Temperature	IQ	MeIQ	MeIQx	4,8-DiMeIQx	PhIP
6 min					
175°C	0.4±0.1^{a}	0.1±0.0^{c}	0.3±0.2^{a}	0.5±0.0^{c}	0.5±0.2^{a}
200°C	1.0±0.1^{a,b}	0.3±0.0^{c,d}	0.9±0.3^{a,b}	0.5±0.1^{c,d}	2.3±0.3^{a,b}
225°C	1.6±1.2^{b}	1.2±1.9^{d}	1.9±0.7^{b}	1.7±1.0^{d}	7.2±3.9^{b}
10 min					
175°C	0.7±0.5^{e}	0.2±0.2^{e}	0.5±0.0^{g}	0.5±0.0^{e}	3.6±1.3^{g}
200°C	2.4±0.5^{e,f}	1.2±0.7^{e,f}	2.3±0.1^{g,h}	2.5±0.0^{e,f}	14.0±1.6^{g,h}
225°C	2.8±1.9^{f}	1.9±1.9^{f}	3.1±0.9^{h}	2.6±2.4^{f}	16.9±7.0^{h}

[1]Mean values for similar frying times in columns with different superscripts are significantly different at $p<0.1$, and for MeIQx and PhIP at $p<0.05$.
[2]Ground beef patties contain 15% fat.
[3]Values are expressed on a raw ground beef basis. Values are based on measured cooking losses for individual patties.

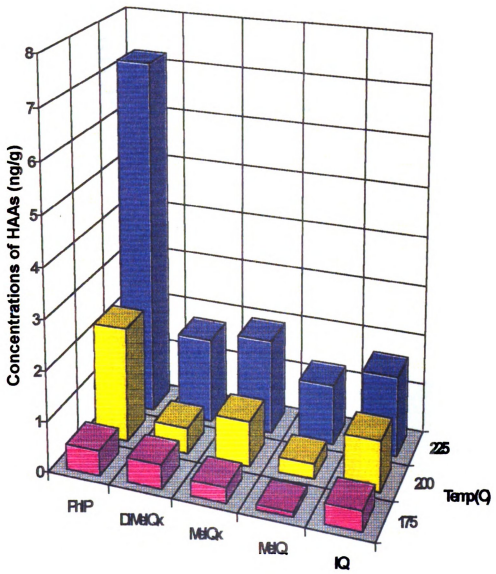

Figure 7. Formation of heterocyclic aromatic amines in ground beef patties fried for 6 minutes at three temperatures.

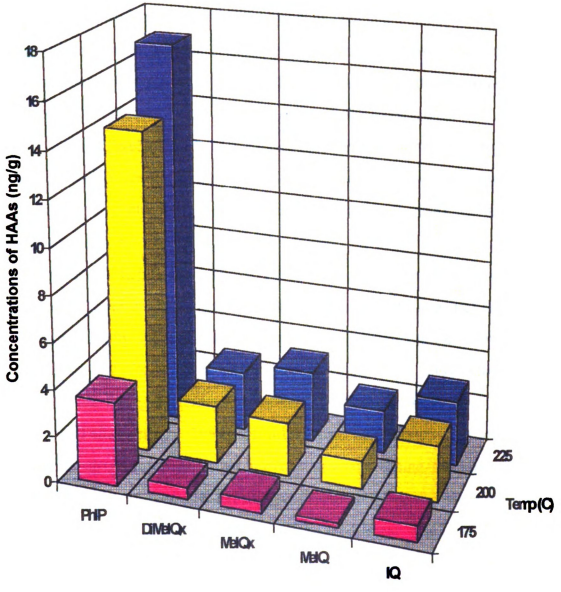

Figure 8. Formation of heterocyclic aromatic amines in ground beef patties fried for 10 minutes at three temperatures.

Even by analyzing four sub-samples from each replicate, it is hard to overcome the problem of variation of data. Because of this, there are few published reports that include an exhaustive statistical treatment of data.

The concentrations of the five heterocyclic aromatic amines detected in the fried ground beef patties fall within the range of concentrations reported by other investigators (Barnes et al., 1983; Turesky et al., 1988; Lynch et al., 1992; Knize et al., 1994; Thiebaud et al., 1994). Reported concentrations for IQ, MeIQ, MeIQx and DiMeIQx are generally smaller then those concentrations reported for PhIP (Felton et al., 1984; Felton et al., 1986a; Sugimura et al., 1988; Gross, 1990; Knize et al., 1994).

Many studies have addressed the effects of the method of cooking on heterocyclic aromatic amine formation in meats. Mutagenic compounds are formed rapidly when meat is cooked by frying, and more slowly by broiling (Spingarn and Weisburger, 1979). Recent studies also show that microwaving meat before frying decreases substantially the formation of heterocyclic aromatic amines (Felton et al., 1994). Besides cooking methods, the most important physical variables affecting heterocyclic aromatic amine formation are cooking time and temperature. Several researchers have observed that there is a progressive increase in the mutagenic activity of cooked meat products with increasing cooking time (Commoner et al., 1978a; Bjeldanes et al., 1983; Miller and Buchanan, 1983; Overvik et al., 1984; Knize et al., 1985; Reutersward et al., 1987a, b; Nielsen et al., 1988). Often there is a lag period of 2 to 4 minutes when no mutagenicity is observed. This is related to the time required for the crust surface to reach a temperature above 100°C. However, there is no pan temperature at which mutagenic activity in meat is not developed (Skog, 1993).

Inhibition of heterocyclic aromatic amine formation in fried ground beef patties by direct addition of phenolic antioxidants

When vitamin E (dl-α-tocopherol) and oleoresin rosemary were added directly to the ground beef before frying, reduction in heterocyclic aromatic amine formation was observed (Table 7, Figure 9). Control patties in all cases contained the greatest concentrations of heterocyclic aromatic amines.

Statistical analyses indicated that concentrations of IQ, MeIQx, and PhIP were significantly different ($p<0.05$) between the different treatments, while the concentration of DiMeIQx was only significant at $p<0.1$ level. Of the four treatments investigated, i.e., two levels of both vitamin E and oleoresin rosemary, the greatest inhibition of heterocyclic aromatic amine formation was achieved with vitamin E when added to ground beef at the 1% level (fat basis). Inhibition was significant ($p<0.05$) for IQ, MeIQ, MeIQx, DiMeIQx and PhIP. Inhibition achieved by the other three treatments (10% vitamin E, 1% oleoresin rosemary, 10% oleoresin rosemary) was only significant ($p<0.05$) for IQ, and PhIP. Statistical analyses also revealed a significant ($p<0.05$) treatment effect (vitamin E vs. oleoresin rosemary) for PhIP formation. No treatment or dose effect was significant for the other heterocyclic aromatic amines. With respect to IQ and PhIP, both levels of antioxidants provided similar degrees of inhibition. However, with oleoresin rosemary, the overall inhibition was a little smaller than that achieved by vitamin E. Both vitamin E and oleoresin rosemary inhibited the formation of 4,8-DiMeIQx to the same extent. Total inhibition of mutagen formation by vitamin E was not achieved as was reported by Chen et al.(1992).

The reduction of mutagen formation in fried ground beef by the addition of vitamin E and BHA before frying was studied previously by Chen et al. (1992). When BHA (1% based on the fat content) was added to ground beef, the concentration of IQ-like compounds was reduced from 7316 ng/g meat to 3244

Table 7. Inhibition of heterocyclic aromatic amine formation in fried beef patties by the direct addition of antioxidants[1,2,3,4,5]

Treatment	Control	Vitamin E 1%	Vitamin E 10%	Oleoresin Rosemary 1%	Oleoresin Rosemary 10%
IQ					
Concentration	2.8±1.8[a]	0.4±0.1[b]	0.3±0.3[b]	0.8±0.3[b]	0.8±0.6[b]
Percent inhibition	0	85.7	89.3	71.4	71.4
MeIQ					
Concentration	1.8±0.8[a]	0.4±0.2[b]	0.6±0.3	0.4±0.4[b]	0.9±0.9
Percent inhibition	0	77.8	66.7	77.8	50.0
MeIQx					
Concentration	3.0±0.9[c]	1.4±0.9[d]	2.0±0.4	2.0±0.4	2.7±0.1
Percent inhibition	0	53.3	33.3	33.3	10.0*
4,8-DiMeIQx					
Concentration	2.5±2.3[a]	0.5±0.2[b]	0.7±0.2	0.6±0.3	0.7±0.0
Percent inhibition	0	80.0	72.0	77.0	72.0
PhIP					
Concentration	16.4±6.8[c]	4.7±2.5[d]	4.2±1.9[d]	8.9±2.9[e]	8.9±6.0[e]
Percent inhibition	0	71.3	74.4	45.7	45.7

[1] Antioxidant concentrations are based on the fat (15%) content of the beef patties.
[2] Concentrations are expressed as ng heterocyclic aromatic amine/g fresh ground beef. Values are based on measured cooking losses for individual patties.
[3] Each value represents the mean of duplicate analyses for three samples ± standard deviation.
[4] Means in rows with different superscripts are significantly different from the control at $p<0.05$.
[5] Patties were fried at 225°C, for 10 minutes per side.
* This value represents the mean of three samples; data points from two samples (one per sample) were not included because of interfering peaks from the oleoresin rosemary.

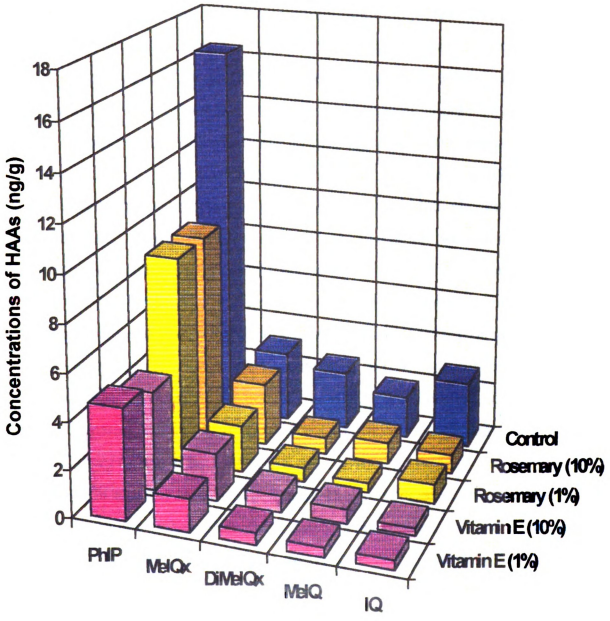

Figure 9. Inhibition of heterocyclic aromatic amine formation in fried beef patties by the direct addition of antioxidants.

ng/g meat, a reduction of 56%. Likewise, the addition of vitamin E (1% fat basis) reduced mutagen formation (IQ, MeIQx and DiMeIQx) by 50%. The concentrations of heterocyclic aromatic amines reported by Chen et al. (1992) were 1000-fold higher than those reported by other researchers (Felton et al., 1986a,b; Sugimura et al., 1988; Knize et al., 1994; Thiebaud et al., 1994). This may be due to the fact that Chen et al. (1992) used the older analytical procedure of Felton et al. (1981) which did not provide as effective sample clean-up as the three-step solid phase extraction procedure of Gross and Gruter (1992). In addition, the more sensitive fluorescent and photodiode array UV detection systems for proper identification of these compounds were not available at this time. This could explain why Chen et al. (1992) did not report any PhIP, even though this is the most abundant of the heterocyclic aromatic amines in fried beef. It had been established earlier by Felton et al. (1986b) that ground beef, when fried for 9 minutes per side at 215°C, contained considerable concentrations of PhIP. The quantitative data of Chen et al. (1992) are therefore questionable as no confirmatory studies were carried out. However, these data did establish the inhibitory effects of both vitamin E and BHA.

In a recent study, Faulkner (1994) also reported considerable inhibition of PhIP formation when BHA (0.1% based on fat content) and vitamin E (1% based on the content) were added to ground beef patties before frying. PhIP concentrations were reduced by 50% (from 2.4±0.15 ng/g to 1.2±0.16 ng/g) by BHA, while an 80% reduction was achieved with vitamin E. The overall mutagenicity was reduced by 70% by vitamin E (7000 revertants to 1900 revertants/100 g raw meat), and by 60% by BHA (2800 revertants/100g raw meat). Faulkner (1994) was the first to report the use of both an analytical procedure and the *Salmonella typhimurium* overall mutagenicity assessment to monitor inhibition. This combination is desirable when evaluating the ability of natural

antioxidants to inhibit heterocyclic aromatic amine formation as there is a possibility that other mutagenic compounds may be introduced into the ground beef through the interaction of the potential inhibitors and meat component(s), or by the thermal breakdown of the inhibitor itself.

The prevention of heterocyclic aromatic amine formation by natural components has also been demonstrated by Weisburger et al. (1994). They studied the effect of black and green teas and the polyphenols, theaflavine gallate and epigallcatechin gallate, on the formation of MeIQx and PhIP in model systems containing creatinine, glycine and glucose, and creatinine, glycine and phenylalanine. Green and black teas and the individual polyphenols substantially reduced the formation of PhIP (from 6500 revertants to 2100 revertants), but had little impact on the formation of MeIQx.

There are no published reports on the inhibition of heterocyclic aromatic amine formation by spice extracts. The results presented here do indicate the potential of rosemary extracts (and possibly other spice extracts as a result of their having similar phenolic components) as inhibitors of mutagen formation in meats. The data obtained in this study warrant some discussion. For example, the inhibition of MeIQx formation with oleoresin rosemary when used at the 10% level, seems questionably low and is probably an inaccurate result because of a potential interfering compound. During the HPLC quantitation step, the MeIQx peak had a similar retention time as that of an unknown peak (overlapping peaks), thus making it difficult to quantitate accurately the concentration of MeIQx. The origin of this extraneous peak was not established, although it could be a component of oleoresin rosemary or its breakdown product.

It was also observed that both levels of added oleoresin rosemary produced a smaller inhibition of PhIP formation (~46%) than their vitamin E counterparts (~ 72%). These smaller inhibitions could be due to the relatively lower

concentrations of antioxidant compounds in the oleoresin rosemary. The oleoresin rosemary used in this study contained approximately 5% phenolic compounds (Kalsec Inc., personal communication). Such compounds include carnosic acid, carnosol, rosmanol, rosmariquinone and rosmaridiphenol, all of which are potent antioxidants (Chipault et al., 1952; Nakatani and Inatani, 1983, 1984; Nozaki, 1989; Schuler, 1990). Therefore, because these compounds function in the same antioxidant manner as vitamin E, it would be interesting to compare them to α-tocopherol as inhibitors of heterocyclic aromatic amine formation in fried ground beef patties on an equimolar basis.

Inhibition of heterocyclic aromatic amine formation in fried ground beef patties through surface application of vitamin E

The application of vitamin E (1% level based on the fat content) to the surface of the ground beef pattie provided excellent inhibition of heterocyclic aromatic amine formation. The concentrations of the five heterocyclic aromatic amines studied were all significantly reduced ($p<0.006$). The average reductions ranged from 49% for MeIQx to 77% for MeIQ (Table 8). Based on previous experiments and consultation with a biometrician, seven experimental replications were performed in order that a suitable statistical treatment could be applied to the data. The level of significance obtained ($p<0.006$) is due in part to the number of replications performed. Large standard deviations create difficulties with statistical analyses, it is important to increase the number of experimental replications to overcome them. As indicated previously, published data on heterocyclic aromatic amine formation in meat products have rarely been subjected to any statistical treatments. For example, Knize et al. (1994) recently showed the effect of temperature and time of frying on heterocyclic aromatic amine formation; however, their data were not statistically analyzed. Other studies discuss the

inhibition of heterocyclic aromatic amine formation in model systems using tea phenolics, but again without any statistical treatment of the heterocyclic aromatic amine data (Weisburger et al., 1994).

Table 8. Formation and inhibition of heterocyclic aromatic amines in fried ground beef patties following surface application of vitamin E[1,2,3,4]

Compounds / Treatment	IQ	MeIQ	MeIQx	4,8-DiMeIQx	PhIP
Control	8.5±2.0[a]	6.4±2.5[c]	3.7±0.2[e]	3.1±1.8[g]	27.9±10.6[i]
Vitamin E (1%)	3.1±2.8[b]	1.5±1.6[d]	1.9±0.6[f]	0.8±0.4[h]	6.9±3.0[k]
Inhibition (%)	63.5	76.6	48.7	74.2	75.3

[1] Concentrations are expressed in ng/g fresh ground beef basis. Values are based on measured cooking losses for individual patties.
[2] Each value represents the mean of seven replicated samples in which duplicate analyses were averaged ± standard deviation.
[3] Means in columns with different superscripts are significantly different at $p<0.006$.
[4] Vitamin E added is based on the fat (15%) content of ground beef patties.

The heterocyclic aromatic amine information presented here has clearly established that vitamin E is an effective inhibitor of heterocyclic aromatic amine formation in ground beef patties, regardless of mode of application. The degree of inhibition achieved by surface application of vitamin E immediately before frying demonstrates again that the formation of heterocyclic amines is primarily a surface phenomenon. During frying, the high temperature of the outer layer of the meat, in combination with a decreased moisture content, causes chemical and physical

changes that form the crust. Inside the crust, a zone of water evaporation moves inward, while moisture, released through protein denaturation and shrinkage, moves outward. Water and water-soluble precursors (sugars, amino acids and creatine/creatinine) move to the surface of the patties, thereby providing optimum conditions for heterocyclic aromatic amine formation (Jagerstad et al., 1983a; Skog, 1993).

The meat used in the study of surface application of vitamin E came from the same source of ground beef that was used in the initial experiments, except that it was stored in a freezer (-20°C) for 2 months. Freezing the ground beef before forming the patties gave interesting results with respect to heterocyclic aromatic amine formation. There was an apparent increase in the concentrations of all five heterocyclic aromatic amines (Table 9). Statistical analysis of the data revealed that only the IQ and MeIQ concentrations were significantly ($p<0.05$) increased. Thus, the question: why were the heterocyclic aromatic amine concentrations greater in the patties prepared from the ground beef that had been frozen for two months than in patties from the freshly ground beef when other conditions were similar, i.e. same frying pan, similar cooking times and temperatures, and same extraction procedure.

Table 9. Concentrations of heterocyclic aromatic amines (ng/g fresh beef) in fried patties prepared from fresh ground beef and from the same beef that had been frozen for two months[1]

Storage (months)	IQ	MeIQ	MeIQx	4,8-DiMeIQx	PhIP
0	2.8±1.8[a]	1.8±0.8[c]	3.0±0.9	2.5±2.3	16.4±6.8
2	8.5±2.0[b]	6.4±2.5[d]	3.7±0.2	3.1±1.8	27.9±10.6

[1] Means in columns with different superscripts are significantly different at $p<0.05$

It was observed by Gray and Collins (1977) that storage of pork at 2°C for 28 days resulted in the development of free proline. This means that during the storage of ground beef there could be an increase in the levels of free amino acids and dipeptides due to proteolytic reactions. It is also possible that lipid oxidation may play a role in heterocyclic aromatic amine formation. During freezing, lipid oxidation is slowed down, but it is not stopped. Lipid free radicals are soluble in the fat fraction and are stable at low temperatures, thus allowing them to diffuse longer distances and to spread the reaction (Kanner, 1994). Another possible explanation is the fact that the freeze-thaw process alters the concentrations of natural components in meat.

This study was designed initially to evaluate the effect of surface application of vitamin E on heterocyclic aromatic amine formation . It was not designed to evaluate the impact of freezing and subsequent storage of ground beef on heterocyclic aromatic amine formation. The increase is an interesting observation, and obviously a more detailed study needs to be done to confirm this apparent increase.

Further studies are needed to establish whether lower levels of these antioxidants can effectively reduce heterocyclic aromatic amine formation in fried ground beef patties. Since surface application of vitamin E effectively inhibited the formation of the five heterocyclic aromatic amines evaluated, it is important to study the effect of smaller levels of this antioxidant (e.g., 0.05%, 0.1%, 0.5%, fat basis). Alternative modes of application of this vitamin could also be addressed, such as through the use of a spray similar to the one used to prevent sticking of food products in the pan during frying.

It is also important to relate the inhibition of heterocyclic aromatic amine formation to overall reduction of mutagenicity as determined by the *Salmonella typhimurium* mutagenicity assay (Ames et al., 1975). This is essential as the toxicology of antioxidant degradation products is still not clear (Shahidi and Wanasundara, 1992). The phenolic compounds added to meat may themselves be converted to mutagenic species during the cooking process.

Mechanisms of heterocyclic aromatic amine formation and inhibition

The fact that phenolic antioxidants minimize heterocyclic aromatic amine formation in fried ground beef patties raises questions about how they exercise this inhibitory effect. The mechanism(s) by which antioxidants inhibit mutagen formation has not been fully elucidated. One possible mechanism is that phenolic antioxidants function as free radical scavengers and act in the early stages of the Maillard reaction prior to the Amadori rearrangement Nyhammar (1986) proposed that pyrazine and pyridine radicals are intermediates in the formation of heterocyclic aromatic amines. Namiki and Hayashi (1981) demonstrated that free radicals, probably N,N'-disubstituted pyrazine cation radical products, are produced through sugar fragmentation in the early stages of the Maillard reaction. They demonstrated that the radical products are formed by the condensation of two molecules of the two-carbon enaminol compounds which might be formed either directly from Schiff base products or indirectly through the reaction of glycolaldehyde with amino compounds. Both involve the cleavage of the C-2-C-3 bond of the sugar molecule. Pearson et al. (1992) suggested that the free radical scavenger-type antioxidants may stabilize the sugar fragment or else react with the free radicals formed by the Maillard reaction (either with the alkylpyridine free radicals or dialkylpyrazine free radicals).

The theories presented are based on the premise that sugars, amino acids and creatine/creatinine are all involved in hetereocyclic aromatic amine formation. However, it is clearly demonstrated that heterocyclic aromatic amines can be formed in dry-heated mixtures of amino acids and creatine (Yoshida et al., 1984; Taylor et al., 1987; Knize et al., 1988b; Overvik et al., 1989; Felton and Knize, 1990). It has also been reported by Wang and Odell (1973) that dry heating of specific amino acids (notably amino-hydroxy compounds) without sugar produced several derivatives of pyrazine. They demonstrated that the participation of sugars was obviously favorable to but not necessary for their formation.

It is important to note that PhIP can be formed in dry heated systems from the direct reaction between creatine and phenylalanine or creatine and leucine. Specific carbon and nitrogen labeling studies indicate that all the carbon atoms, as well as the nitrogen atoms in the ring structure of PhIP, can be derived from these compounds (Felton and Knize, 1990). However, it has been reported by Wang and Odell (1973) that no pyrazines were found when leucine or phenylalanine were dry-heated individually without sugars. These reports raise the following questions:

(a) Does the formation of PhIP follow the same route as those for imidazoquinolines and imidazoquinoxalines (i.e., through the formation of pyrazine or pyrazinium ion)?,

(b) would phenolic antioxidants function under these conditions (i.e., in the absence of sugars) to inhibit the formation of IQ, MeIQ, MeIQx, DiMeIQx, and PhIP?

Another possible mechanism by which antioxidants inhibit the formation of heterocyclic aromatic amines might be through their interference of the conversion of creatine to creatinine during heating. It is well known that creatine/creatinine is the major precursor of heterocyclic aromatic amines in meats (Jagerstad et al.,

1983a). The imidazole moiety of these compounds is believed to originate from creatine which, during cooking, is converted to creatinine. Thus, antioxidants might interfere or compete with this reaction route that usually results in the formation of mutagenic heterocyclic aromatic amines. For example, sugar at a certain concentration interferes with interconversion of creatine to creatinine (Skog et al, 1992b). It remains to be determined whether antioxidants at the levels used in this study would function in a similar manner.

More studies are needed to further address the inhibition of heterocyclic aromatic amine formation in fried meats by vitamin E, oleoresin rosemary and other natural antioxidants as these compounds are extremely mutagenic/carcinogenic (Sugimura et al., 1988). The use of naturally occurring compounds to reduce risks associated with food is viewed positively by the consumer.

SUMMARY AND CONCLUSIONS

Results of this study clearly demonstrate that the formation of heterocyclic aromatic amines in fried ground beef patties depends on the time and temperature of cooking. Greater concentrations of heterocyclic aromatic amines are formed with longer cooking times and higher cooking temperatures.

To evaluate the effects of different phenolic antioxidants on heterocyclic aromatic amine formation in fried ground beef, vitamin E and oleoresin rosemary were added at two different concentrations to the beef patties before frying. When vitamin E and oleoresin rosemary were added directly to the ground beef before frying, reduction in heterocyclic aromatic amine formation was observed. Control patties in all cases contained the greatest concentrations of heterocyclic aromatic amines. However, it was also observed that the addition of oleoresin rosemary resulted in a smaller inhibition of PhIP formation than was achieved with vitamin E.

When vitamin E was applied to the surface of the ground beef pattie heterocyclic aromatic amine formation was again reduced. The inhibition achieved in this way was significant ($p<0.007$) for all five heterocyclic aromatic amines studied. The results of this study indicate the potential of vitamin E and rosemary extracts as inhibitors of mutagen formation in meats.

FUTURE RESEARCH

This study focused on the inhibitory action of two phenolic antioxidants on the formation of heterocyclic aromatic amines in fried ground beef patties. Both vitamin E and oleoresin rosemary inhibited the formation of the five heterocyclic aromatic amines studied. The antioxidant effects of α-tocopherol and oleoresin rosemary in meats are well established (reviewed by Gray and Crackel, 1992). Recently, the synergistic effect of these two antioxidants in fish has been demonstrated by Fang and Wada (1993). Whether this enhanced antioxidant activity would translate into a greater inhibition of heterocyclic aromatic amine formation in fried ground beef remains to be determined.

The reduction of mutagen formation in fried ground beef by the addition of food additives such as nitrites, sulfiting agents, citrates, and polyphosphates was studied by Chen et al. (1992). However, the potential synergistic effects of these compounds with phenolic antioxidants requires more research. In addition, the Chen et al. (1992) data need to be reconfirmed because of the questionable concentrations of heterocyclic aromatic amines reported by these researchers.

Based on their antioxidant effects in meat systems, other natural components should be investigated as to their potential to inhibit heterocyclic aromatic amine formation. Examples would include

- Maillard reaction products - while their antioxidant activity is well established (Bailey, 1987), the addition of Maillard reaction products to beef patties is of considerable interest as some of these products are thought

to be involved in the formation of the 2-aminoimidazo -type mutagens (Jagerstad et al., 1983a).

- Spice extracts - because of the potent antioxidant activity of rosemary, sage and other spices (Loliger, 1983), their potential inhibition of mutagen formation in meats requires investigation.
- Carnosine - a naturally occurring skeletal muscle dipeptide that has been reported to be more effective than α-tocopherol, BHA and sodiumtripolyphosphate in controlling lipid oxidation in meats (Decker and Crum, 1991). The use of this compound as a potential inhibitor of heterocyclic aromatic amines requires some investigation as dipeptides, like amino acids, can produce heterocyclic aromatic amines under the appropriate reaction conditions (Skog, 1993).
- Tea and tea polyphenols - these compounds can inhibit heterocyclic aromatic amine formation in model systems, but no studies have been carried out in ground beef systems.
- Cherry and grape extracts - the antioxidant properties of these fruit extracts have been established by Liu et al. (1995) and Frankel et al. (1993). Again, their potential as heterocyclic aromatic amine inhibitors has not been evaluated.

Although high levels of vitamin E and oleoresin rosemary (1 and 10% based on fat level) did inhibit heterocyclic aromatic amine formation in fried beef patties, further studies are needed to establish whether lower levels of these and other antioxidants can effectively reduce mutagen formation. Clinical studies indicate that intakes of <720mg/day vitamin E do not have any adverse effects in man (WHO, 1987), but excessive supplements of vitamin E are potentially toxic. It is also important to develop better ways of applying these antioxidants to beef

patties such as through a spray. Antioxidants added to the meat in this way would facilitate the application of very low levels (e.g., 0.5, 0.1, 0.05%).

The application of both analytical procedures and the *Salmonella typhimurium* overall mutagenicity test (Ames et al., 1975) to monitor inhibition is necessary. This is important because of the possible toxicity of antioxidant degradation products (Shahidi and Wanasundara, 1992). The natural compounds added to meat systems may be converted to mutagenic species during cooking.

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